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Biodegradation and toxicity of waste from anaerobic fermentation of stillage

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Several studies have been carried out to produce biofuels by anaerobic digestion of stillage from ethanol fermentation. This type of process, known as ABE Fermentation, generates by-products such as acetone, butanol and ethanol. This fermentation can use pure or mixed cultures of anaerobic bacteria, mainly of the genus *Clostridium*. In this context, there is the need for deeper studies for proper disposal of the residue resulting from Anaerobic Fermentation of ethanolic stillage, hereinafter referred to as AF stillage (stillage from Anaerobic Fermentation), obtained after this fermentation. Thus, the aim of this study was to evaluate the biodegradation and toxicity of stillage from Anaerobic Fermentation. The biodegradation of AF stillage in soil samples in the presence and absence of commercial inoculum was evaluated using a respirometric method described by Bartha & Pramer, according to the standard NBR 14283 (ABNT, 1999). The production of CO_2 was evaluated for 57 days. Additionally, quantification of microorganisms was carried out at the beginning and end of the experiment. Toxicity tests were performed with the microcrustacean *Daphnia similis* using raw AF stillage and leachate from the respirometry test, according to the standard NBR 12713 (ABNT, 2009). The results of the respirometry test showed that, after 13 days of incubation, the biodegradation efficiency of all samples was above 30%. Using the Friedman statistical test, results showed that adding the inoculum caused no statistically significant difference in the biodegradation of AF stillage. The acute toxicity tests were performed on raw AF stillage and on the leachates showed that toxicity was removed after biodegradation in soil at all concentrations used. Thus, discharge of AF stillage into soil is an alternative viable disposal.

Key words: Biodegradation, stillage, anaerobic fermentation, toxicity.

INTRODUCTION

Taking into account that ethanol production in Brazil in 2015/2016 was 30.5 billion liters, the production of ethanol stillage was 305 billion liters. Ethanol stillage is characterized as a distillery effluent having a high polluting potential approximately a hundred times more polluting than domestic sewage (CONAB, 2016; Lelis Neto, 2008). Its chemical composition vary depending on the water content, sugarcane characteristics and

fermentation, and distillation processes that were used. In general, stillage presents increased turbidity and low pH, with high levels of organic matter, potassium and calcium, and moderate amounts of nitrogen and phosphorus (Ferraz et al., 1986; Rodella et al., 1983; Tauk, 1987; Gómez and Rodriguez, 2000). According to Fuess and Garcia (2014), currently, almost the whole volume of stillage generated in Brazilian distilleries is directed to the fertigation of sugarcane fields, due to its fertilizer character. In reasonable amounts, fertigation has positive effects on agricultural productivity, acting as a partial or total replacement of mineral fertilizers, especially potassium. However, long-term intensive application of stillage can increase soil salinity (Corazza, 1999), affect sugarcane quality, and also contaminate groundwater (Gonçalves and Silva, 2000). According to Freire and Cortez (2000), stillage can present high levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD), reaching values up to 210.000 and 100.000 $mg \cdot L^{-1}$, respectively.

According to Green (2011), acetone-butanol-ethanol (ABE) fermentation has been recently reestablished in China. ABE fermentation is one of the oldest micro-biological processes for the commercial production of solvents (Qureshi and Ezeji, 2008). In this type of fermentation, bacteria of the genus, *Clostridium* are used to convert sugars into solvents, and fermentation takes place in two steps. The first step (acidogenesis) is a growth phase in which acetic and butyric acids are rapidly produced, lowering the pH. The second step (solventogenesis) is characterized by acid reassimilation for solvent production. Fermentation also produces carbon dioxide and hydrogen (Green, 2011). The average ABE fermentation time is 36 to 72 h, when approximately 15 to 20 g/L of solvents accumulate, depending on the culture and raw materials used. One of the greatest technological challenges is associated with the low yield of solvents. Choosing microorganisms appropriately is crucial for successful ABE fermentation and for the production of target solvents. Pure cultures (whether natural or mutant) are the most widely used for many scientific studies, since they present a rate of conversion of glucose into solvents within the maximum tolerance limits due to microbial inhibition caused by solvents (Liu and Qureshi, 2009). However, mixed cultures have proven to be more advantageous when complex substrates are used, promoting their degradation by forming integrated communities, in which some species act in a balanced way, or even symbiotically (Santos, 2015).

Although, Brazil finds itself in an advantageous position, especially when it comes to ethanol from sugarcane, the country needs to consolidate its position in the world energy market in a sustainable way, taking into account environmental, economic and social issues (Santos et al., 2011). According to Mello et al. (2007), in the coming years, Brazil will maintain its leading position in the production of ethanol as a result of the natural characteristics of its territory, its large agricultural and industrial experience in the sugar and alcohol sector, and the recent development of biodiesel agribusiness. The expansion of biofuels in Brazil offers great environmental challenges and opportunities, especially for one of the main sources of pollutants from plants, namely stillage. Since there are limits to the use of stillage in fertigation, one of the alternatives is its biodigestion through ABE fermentation.

Improvements in the refining process over time will enable the use of more economical sources of raw materials, such as lignocellulosic biomass and even sugars currently used in ethanol plants. In the latter, only minor modifications would be needed, since bacteria of the genus *Clostridium* are well adapted to utilize sugars derived from cellulosic materials. The production of low cost butanol using lignocellulosic biomass, such as agricultural and forestry waste from biorefineries, has been regarded as a necessary change in order to improve process economy.

Solutions for the stillage generated during the production of ethanol from conventional and cellulosic raw materials were discussed by Wilkie et al. (2000), who supported anaerobic digestion of stillage followed by fertigation. Corazza (1999) described alternative technology that allows stillage to be recycled in the fermentation process, treated in anaerobic reactors, or used in yeast production, animal feed, and even in construction. In this context, reuse of ethanol stillage has been considered as an alternative for the production of biofuels or their byproducts.

Ahn et al. (2011) investigated fermentation for butanol production by *Clostridium pasteurianum* DSM 525 using stillage containing glycerol as a substrate. Glycerol is a byproduct of ethanol fermentation and its concentration in thin stillage ranges from 5.1 to 24.6 g/L (Dowd et al., 1994; Gonzalez et al., 2010; Kim et al., 2008). *C. pasteurianum* DSM 525 is capable of producing butanol using glycerol as the sole source of carbon and energy (Biebl, 2009). Other organic compounds present in thin stillage are lactic and acetic acids, which are mainly produced by bacterial contamination in the ethanol fermentation process (Skinner and Leathers, 2004). The authors found that *C. pasteurianum* DSM 525 was produced from 6.2 to 7.2 g/L of butanol, using glycerol present in stillage as the main source of carbon. The lactic acid in stillage acted as a buffering agent, maintaining the pH of the medium in the range of 5.7 to

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Table 1. Physicochemical composition of the AF stillage.

Parameter	Result
pH	7.21
Electrical conductivity (mS/cma)	18.96
Biochemical oxygen demand (BOD) (mg/L)	654
Chemical oxygen demand (COD) (mg/L)	17616
Total organic carbon (TOC) (%)	0.82
Total nitrogen (Kjeldahl) (mg N/L)	0.3
Dissolved oxygen (mg O_2/L)	5.03
Total solids (mg/L)	29288
Fixed solids (mg/L)	15048
Volatile solids (mg/L)	14240
Ashes (%)	1.54
Humidity (%)	97.05
Sulfate (mg SO_4^{-2}/L)	1525
Sulfide (mg S^{-2}/L)	<0.002
Manganese (mg Mn/L)	0.958
Calcium (mg Ca/L)	254
Magnesium (mg Mg/L)	86
Sodium (mg Na/L)	4823
Potassium (mg K/L)	1827
Total phosphate (mg PO_4/L)	156

6.1. The results of the study showed the economic viability of butanol production using stillage as a substrate, with the potential to provide all the nutrients that are necessary for fermentation.

In the context of anaerobic digestion of stillage aiming to obtain by-products (ABE fermentation), deeper studies focusing on the final residue obtained after fermentation (AF stillage) are necessary. After removal of the bacterial biomass and separation of the solvents generated in the process, AF stillage is disposed of. Thus, it is appropriate to discuss solutions for proper disposal of AF stillage. Among them, fertigation is a viable alternative. Nonetheless, studies on biodegradation/bioremediation and toxicity are needed for AF stillage correct disposal on soil.

Bioremediation is the use of living organisms, especially microorganisms, to degrade environmental contaminants to less toxic products. The bioremediation process can occur *in situ*, where microorganisms originate from a contaminated area, or *ex situ*, where microorganisms are isolated and transferred to the contaminated site. Contaminating compounds are transformed through the metabolic activity of microorganisms (Vidali, 2001). Microbial diversity in soil plays a key role in this ecosystem. Several scientific papers have demonstrated the role of soil bacteria and fungi in the bioremediation of contaminated areas or even industrial waste. Bio-augmentation involves using microbial consortia capable of degrading the target pollutant. In intrinsic or natural bioremediation, the microorganisms used are autochthonous, that is, native to the site, without any interference of active remediation technologies (Bento et al., 2003).

The respirometric method by Bartha and Pramer (1965) is a technique that is used to determine the biodegradation of organic matter in soil by measuring the amount of CO_2 released into the system. A respirometer acts as a closed system consisting of two interconnected chambers, in which biodegradation of waste and removal of the CO_2 generated during the process take place. This method assumes a correlation between CO_2 production and biodegradation of organic matter (sample) in soil. The efficiency of sample degradation is determined by the amount of CO_2 produced, which allows determining waste stabilization time at different application rates.

Ecotoxicological tests with standard organisms have been used to estimate the toxic effects of certain compounds, providing data for risk assessments in aquatic environments. Among these organisms, micro-crustaceans of the genus *Daphnia* spp. stand out not only because they are easy to grow in a well-controlled laboratory, but also because of their population homogeneity, resulting from parthenogenetic reproduction (Nour et al., 2014).

The aims of this work were: (1) to study the application of AF stillage in soil through respirometry; (2) to carry out microbial quantification at the beginning and end of the test; and (3) to perform toxicity tests using *Daphnia similis.*

MATERIALS AND METHODS

Anaerobic fermentation (AF) stillage

The AF stillage is the residue or stillage generated by anaerobic fermentation of ethanol stillage. It was obtained from the Sanitation Laboratory of the Faculty of Agricultural Engineering (FEAGRI/ UNICAMP) and resulted from acetone-butanol-ethanol (ABE) fermentation intended for the production of alcohols and organic acids. For this fermentation, synthetic culture medium was used (Monot et al., 1982 modified by Santos, 2015), supplemented with ethanol stillage and molasses, as well as 1 N NaOH for pH control.

The AF stillage was centrifuged to remove the bacterial biomass. Afterwards, the supernatant recovered was subjected to rotoevaporation in a Marconi rotary evaporator (MA-120) to eliminate the solvents produced during fermentation (acetone, butanol and ethanol). The pressure necessary for the evaporation of the three solvents in a water bath at a temperature between 45 and 50°C (over 404 mm Hg) was taken into account. The AF stillage was then heated at 80°C for 15 min.

The physicochemical properties of the stillage used in this study was characterized according to APHA (1998, 2012) and are shown in Table 1.

Soil sample

The soil used in the respirometry test was collected according to the technical rule L.6.245 from Cetesb (1984), in the geographic coordinates: Latitude -22.39624915/Longitude -47.5431633, localized in the municipality of Rio Claro, in the State of São Paulo,

Table 2. Physical characteristics of the control soil.

Parameter	Grain size distribution (%)					
	Sand				Class	Sub-class
	Thick	Fine	Clay	Loam		
Soil	24.4	11.9	25.9	37.8	Clay	Clay

Table 3. Chemical characteristics of control soil (macro and micronutrientes).

Parameter	Macronutrient (mmolc/dm^3) IFSA							V	Relation	
	K	Ca	Mg	H^+Al	Al	SB	CTC	%	Ca/Mg	Mg/K
Soil	4.9	43	15	34	1	63	97	64.9	2.86	3.06
	Micronutrient (mg/dm^3)							pH	OM	P res
	S	Na	Fe	Mn	Cu	Zn	B	$CaCl_2$	g/dm^3	g/dm^3
Soil	8	3	19	26.2	1.4	1.9	0.18	5.5	34	3,0

P, K, Ca, Mg, exchange resin anionic + cationic; Fe, Mn, Ca, Zn: Extractor DTPA-TEA; B, Barium chloride extractor 0.125%; Soil sample was analyzed by Inst. Campineiro de Análise de Solo e Adubo Ltda. (ICASA).

Brazil. These samples were taken from the soil superficial layer of non-contaminated places. Tables 2 and 3 show the physico-chemical characteristics of soil.

Biodegradation test

The biodegradation tests, performed according to the standard NBR 14283 (ABNT, 1999), was used to evaluate changes in CO_2 production in the soil containing AF stillage. Three concentrations of AF stillage in soil (3, 6 and 12%) in the presence and absence of 0.2% commercial inoculum (VCTEC/ByoAct), consisting of a pool of microorganisms isolated from soil, were evaluated. Each treatment included three replicates, according to the protocol; soil; soil + 3% AF stillage; soil + 6% AF stillage; soil + 12% AF stillage; soil + 0.2% inoculum; soil + 0.2% inoculum + 3% AF stillage: soil + 0.2% inoculum + 6% AF stillage; soil + 0.2% inoculum + 12% AF stillage. The respirometers were incubated at 28±2 °C and measurements of the produced CO_2 were performed daily for the first 7 days and at longer intervals for 57 days.

From determination of the amount of biodegradable carbon during the test, the efficiency of biodegradation (EB) was calculated by the equation:

$$EB\% = \frac{\text{Total amount of biodegradable carbon (µmol)}}{\text{Amount of initial organic carbon in soil (µmol)}} \times 100$$

Microbiological analysis of biodegradation test

Heterotrophic bacteria and fungi in the respirometer samples were quantified at the beginning and end of the test. The quantification was performed by plating on Plate Count Agar (PCA) containing actidione (5 ppm) and Sabouraud broth containing chloramphenicol (400 ppm), respectively. Bacterial counts were carried using pour plate technique, and plates were incubated at 35°C for 48 h. Counts for yeasts and molds were performed by spread-plating, and plates were incubated for 5 days at 28°C. The results are given in CFU/g of dry soil.

The same procedure for microbial quantification was carried out for the commercial inoculum and AF stillage. The results obtained are expressed as CFU/ml.

Toxicity tests

Leachates from the samples in the respirometers were prepared at the beginning and end of the test, at a concentration of 10% in distilled water. The solutions were stirred in a shaker for 30 min and then decanted for 7 days, at room temperature. The supernatant was used in acute toxicity tests with the microcrustacean *D. similis* according to the standard NBR 12713 (ABNT, 2009), as well as raw AF stillage.

Statistical analysis

The Friedman test (Zar, 1999), which is a nonparametric test, was used to analyze the results from the respirometric biodegradation test and the quantification of microorganisms. The level of significance was 5%. The results of the toxicity tests with *D. similis* were calculated from statistical analysis carried out by the Trimmed Spearman-Karber method using the software JSPEAR (Hamilton et al., 1977).

RESULTS AND DISCUSSION

Biodegradation test

The occurrence of biodegradation was observed in all respirometers, which was evidenced by the production of CO_2. It was observed that microbial activity was not affected by the addition of AF stillage, since the amount of cumulative CO_2 increased as the concentration of

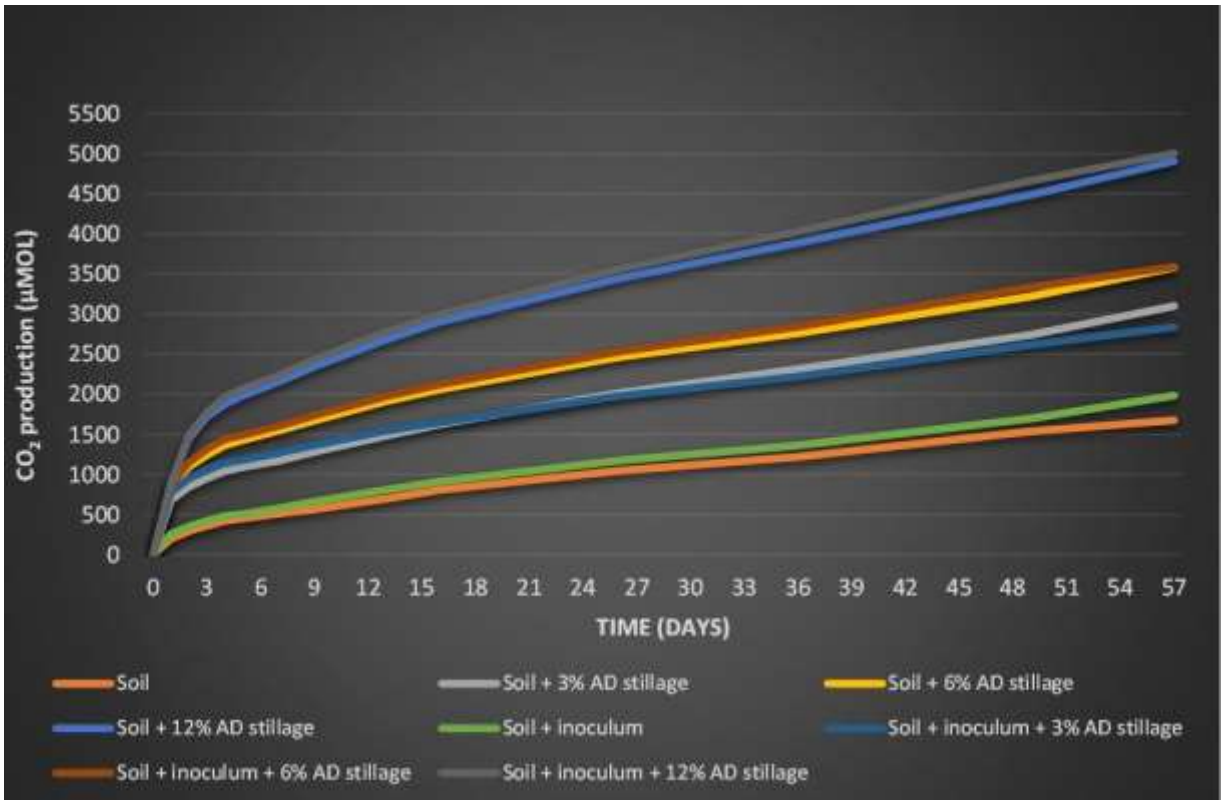

Figure 1. Cumulative production of CO_2 in different treatments in the respirometry test with AF stillage.

residue (AF stillage) increased (Figure 1). The statistical analysis of the data on the daily production of CO_2 (µmol) using the Friedman test revealed that adding the inoculum did not cause significant differences (with a significance level of 5%) as compared to control soil and AF stillage at the concentrations of 6 and 12% (P-values of the probability tests: p=0.13, 0.78 and 0.98, respectively). There was statistical difference only between soil with 3% AF stillage and the same sample supplemented with inoculum (p=0.047).

Almeida et al. (2013) also found similar results in a respirometry test, in which a commercial inoculum was added to help with the biodegradation of ethanol stillage. The results for clayey soil showed that the curves of CO_2 production were very similar in the presence and absence of inoculum. The authors concluded that the addition of the inoculum did not affect stillage biodegradation positively.

Adding the three concentrations of AF stillage to the soil had a significant effect (p<0.0001) with respect to the daily production of CO_2 (µmol); also, adding 6 and 12% AF stillage to the soil containing inoculum had significant effect (p=0.0039 and p<0.0001, respectively). There was no difference between adding AF stillage to the soil at the concentrations of 3 and 6% (p=0.47), but both were different from 12% AF stillage (p=0.003 and 0.0041, respectively). For the soils supplemented with inoculum, the three concentrations of AF stillage were different from each other (p=0.0154, p<0.0001 and p=0.0015, respectively, for comparisons between 3 and 6; 3 and 12; and 6 and 12%).

Efficiency of biodegradation

Table 4 shows that after 13 days, all the samples presented biodegradation efficiency above 30%. This result is significant, since there is a Brazilian technical standard that establishes a minimum biodegradation efficiency of 30% for waste disposal on soil (NBR 14283) (ABNT,1999). At the end of 57 days, the highest efficiency was observed for the sample containing soil with 3% AF stillage (92.64%), followed by sample containing soil + inoculum + 3% AF stillage (75.52%). In Figure 1, the same samples presented the lowest amount of cumulative CO_2; however, their efficiency at the start of incubation was above 30%. The samples containing soil + 6% AF stillage reached this value on the 3rd and 4th day in the presence and absence of inoculum, respectively. The samples containing soil + 12% AF stillage reached this value on the 9th and 13th day in the presence and absence of inoculum, respectively. Although its addition did not lead to statistically significant differences, samples with inoculum reached 30%

Table 4. Efficiency of biodegradation of AF stillage in the respirometry test for 57 days.

Day	Samples					
	Soil + 3% AF stillage	Soil + 6% AF stillage	Soil + 12% AF stillage	Soil + inoculum + 3% AF stillage	Soil + inoculum + 6% AF stillage	Soil + inoculum + 12% AF stillage
0	0.00	0.00	0.00	0.00	0.00	0.00
1	31.32	21.44	11.42	37.46	21.70	11.18
2	35.88	26.14	18.80	42.81	27.91	19.61
3	38.46	28.76	22.09	45.82	31.13	22.94
4	40.30	30.64	23.65	47.21	33.20	25.12
5	41.59	31.75	24.89	47.42	33.80	26.31
7	42.69	33.60	26.73	49.41	35.52	28.07
9	46.61	35.98	28.94	51.99	38.09	30.42
13	50.95	39.53	32.25	55.18	41.48	33.89
16	53.15	40.77	34.07	54.69	42.70	35.58
26	63.09	46.11	39.00	60.62	48.34	40.89
36	71.18	50.16	43.38	65.27	52.82	45.73
49	78.20	54.66	47.95	70.31	58.43	50.81
57	92.64	61.95	52.54	75.52	62.27	54.12

Table 5. Initial and final counts of heterotrophic bacteria and fungi from the respirometry test, expressed in colony forming units per gram of soil (CFU/g).

Sample	Bacteria (10^5 CFU/g)		Fungi (10^2 CFU/g)	
	Initial	Final	Initial	Final
Soil	3.4	23.5	4.7	36.0
Soil + 3% AF stillage	35.7	26.5	7.9	33.0
Soil + 6% AF stillage	67.5	44.5	11.1	31.5
Soil + 12% AF stillage	132.4	45.0	17.6	38.0
Soil + inoculum	153.4	11.0	7.7	49.0
Soil + inoculum + 3% AF stillage	185.7	39.5	10.9	41.0
Soil + inoculum + 6% AF stillage	217.9	18.5	14.1	25.5
Soil + inoculum + 12% AF stillage	282.4	109.5	20.6	33.0

biodegradation efficiency in a shorter time.

Microbiological analysis of biodegradation test

Table 5 indicates that the initial bacterial and fungal counts in the control soil were 3.4×10^5 and 4.7×10^2 CFU/g, respectively, while addition of the inoculum increased these values. In the inoculum, the load of bacteria was 1.5×10^7 CFU/ml, and the concentration of fungi was 3×10^2 CFU/ml.

The number of microorganisms was also proportionally higher with the addition of 3, 6 and 12% AF stillage, in which the number of bacteria was 1.08×10^8 and that of fungi was 1.08×10^4 CFU/ml. There were some variations in the final quantification. At the end of the test, the overall number of bacteria was lower than in the initial plating. This was probably due to the intense metabolic activity of microorganisms during the test, with consequent depletion of the nutrients present. On the other hand, there was an increase in the fungal count (CFU/g) at the end of the experiment. It is worth noting that fungi were present in a smaller amount at the beginning of the test. The Friedman test showed significant differences between the initial and final number of microorganisms, both for bacteria and fungi ($p<0.05$).

Toxicity tests

The acute toxicity test on *D. similis* was performed according to NBR 12713 method (ABNT, 2009). The results showed acute toxicity, with an EC_{50} of 9.10% and approximately 11 toxic units (TU) for the raw AF stillage studied (Table 6). This result indicates that AF stillage at a concentration of 9.10% is potentially toxic to aquatic organisms. The same test was carried out with leachate

Table 6. Results of the acute toxicity test on *D. similis* for raw AF stillage.

EC(50) (%)	Toxic units (TU)
9.10	10.99

EC(50), Median effective concentration = sample concentration that causes acute effects (mortality or immobility) to 50% of the organisms within a given period of exposure under the test conditions. TU = toxic units = 100/EC(50).

Table 7. Results of the acute toxicity test on *D. similis* performed with leachate using respirometry test.

Sample	Toxicity (toxic units)	
	Initial	Final
Soil	NT	NT
Soil + 3% AF stillage	1.0	NT
Soil + 6% AF stillage	1.2	NT
Soil + 12% AF stillage	NT	NT
Soil + inoculum	NT	NT
Soil + inoculum + 3% AF stillage	1.2	NT
Soil + inoculum + 6% AF stillage	1.7	NT
Soil + inoculum + 12% AF stillage	NT	NT

NT = non-toxic.

from the samples in the respirometers, at the beginning and end of the test, for both experiments.

In Table 7, it is shown that leachate from the controls used in the respirometry test showed no acute toxicity to *D. similis* (soil and soil + inoculum), at the beginning and end of the test. In the beginning of the respirometry test, changing AF stillage addition from 3 to 6% increased the toxicity of the samples, both in the presence and absence of inoculum, which can be verified by the increase of UT values. However, samples containing AF stillage 12% showed no toxicity, in the presence and absence of inoculum.

One possible explanation would be that the leachates were decanted for 7 days at room temperature, and during that time, existing microorganisms in AF stillage were able to biodegrade compounds that could be toxic to *D. similis*. Thus, the supernatant of these leachates showed no toxicity since the amount of microorganisms present in the soil after addition of 12% AF stillage was higher than that present in the other concentrations (Table 5).

At the end of the respirometry test, in which AF stillage underwent biodegradation, the leachate samples showed no toxicity. These results are similar to those obtained by Quiterio (2013) who found that raw ethanol stillage was extremely toxic to *D. similis*. However, after biodegradation in soil quantified with the respirometric technique, toxicity was removed.

Conclusion

In the respirometry test, it was observed that addition of the commercial inoculum caused no statistically significant difference in the biodegradation of AF stillage. In the absence of inoculum, the two lowest concentrations were not different from each other; there were differences only with the addition of AF stillage at a concentration of 12%. This shows that it may be necessary to use the minimum concentration for the biodegradation process to be more efficient, since the minimum efficiency of 30% was reached on the 13th day when 12% AF stillage was used, while the same efficiency was reached on the 1st day when 3% AF stillage was used.

The acute toxicity tests showed that raw AF stillage was extremely toxic to *D. similis.* However, after the biodegradation process, toxicity was removed. Thus, disposal of AF stillage on soil becomes a viable option, since it did not show potential toxicity to aquatic organisms in the case of groundwater contamination and it presented a minimal biodegradation efficiency of 30%, as recommended by the Brazilian technical standard for waste disposal on soil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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