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Antimicrobial and Antibiofilm Activity of Essential Oil of *Lippiagracilis* Schauer on *Clostridium Bifermentans* and Fungal-containing Biofilms

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ABSTRACT

In oil industry microbiologically influenced corrosion plays a key role since it costs a lot of money yearly. This kind of corrosion is mainly induced by the microbial biofilms occurring on the metal surface and their metabolites that modify the electrochemical conditions from metal-solution interface. This study focused on the evaluation of the antimicrobial activity of essential oil of *Lippiagracilis* Schauer over *Clostridium bifermentans* isolated from ballast of ship transporter of crude oil as well as against fungi occurring on microbial biofilms. Additionally, it was evaluated the influence of the essential oil on the corrosion of AISI 1020 carbon steel by electrochemical and gravimetric techniques. A minimum inhibitory concentration of the 20.0 $\mu g \cdot L^{-1}$ was obtained for the essential oil over the *C. bifermentans* that was the same used for investigating the biocide activity against fungal biofilms for different contact time. Results showed that colony former unit (CFU) for fungi reduced to zero after 120 minutes exposition to the essential oil. Also, the essential oil of *L. gracilis* Schauer showed a quite good potential to control effectively the growth of *C. bifermentans*. Electrochemical polarization and gravimetry assays showed that essential oil of *L. gracilis* Schauer at concentration of 60 $\mu g \cdot L^{-1}$ was efficient to inhibit the corrosion of AISI 1020 carbon steel. *L. gracilis* Schauer essential oil acted as a powerful biocide.

Keywords: Microbiologically Influenced Corrosion; Essential Oil; *Lippiagracilis* Schauer; Biocide; Biofilm

1. Introduction

In oil industry microbiologically influenced corrosion (MIC) plays a key role since it costs a lot of money yearly accounting for up to 20% of total corrosion. The financial loss in the USA can achieve values as high as 60 billion dollars per year[1].There is no official data about the financial loss caused by MIC in Brazil on the oil and gas industry, however it certainly plays a role since this country shows climatic conditions favorable to development of biofilms on the surface of materials immersed in natural water, not necessarily marine water[2]. However, it estimates that Brazilian financial loss can accounts for approximately 10 billion dollars per year, mainly due to oil companies[3]. MICon the oil industry can affect many active-

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ties such as extraction, processing, distribution as well as storage of oil.

It is known that many different types of microorganisms can be involved on the MIC process mainly those in the planktonic or growing in sessile microbial communities called biofilms. The bacteria *C. bifermentans*stands for favoring the corrosion by itself or for making part of biofilms associated or not to others microorganisms such as fungi, for instance. Therefore, *C. bifermentans* has been associated to MICon production and industrial treatment of water, also in pipeline of fields of oil and gas[4-7] *C. bifermentans*is an aerobic facultative, motile, Gram-positive bacterium and can be isolated and grown on solid nutrient medium[8]. Bacterium belonging to the *Clostridium* genus is able to produce solvents and organic acids. These acids can then hasten the corrosion process and this fact can be associated with favoring corrosion by the species belonging to this genus[9]. Additionally, fungi occurring on biofilms can produce these organic acids then causing problems to the oil and gas industry[10]. Some species of fungi can be easily isolated from pipeline being these species *Hormoconis*, *Aspergillus*, *Fusarium* and *Trichosporon* the most present.

Chemicals (organic or inorganic) used in order to reduce or eliminate the microorganisms in industrial systems are called as biocides. They have been widely used in the oil industry to protect pipeline during oil process steps such as, for instance, on drilling fluids in production water and so on in order to control the planktonic microorganisms and, mainly, those building biofilms.

Nowadays the biocides most used to control of *C. bifermentans* and fungi, as well as the consequent corrosion caused by these microorganisms, are the hydrogen peroxide, quaternary ammonium compounds and glutaraldehyde[4]. However, many factors can interfere on action of these biocides as, for instance, the endospores production, inactivation mechanism of these substances and the development of resistance due to mutations[11]. Another negative point of using chemical as biocides is its toxicity to the environment, since it usually has lower degradability rate then increasing the residence time in the environment[12].

In this context, the use of natural products as an alternative to the chemical biocides is gathering attention due economic and environmental reasons. They show interesting properties as sustainability, lower cost and are environmentally-friend. The genus *Lippia*is known to show anti-bacterial activity. *Lippiagracilis* Schauer is a native bush from semiarid area of the Northeast region in Brazil, mainly at Pernambuco and Rio Grande do Norte States[13,14]. This specie has fruits with seeds that rarely germinate and small and aromatic leaves rich in essential oils. The *L. Gracilis* Schauer essential oil is rich in substances such as thymol, carvacrol, p-cymene and γ-terpinene that give to the oil powerful anti-microbial activities. Brazilian Healthy Ministry through the Research Program on Medicinal Plant cites *L. gracilis* Schaueras one of the most importantplants[15]. Many studies report the anti-microbial activity of the essential oil from *L. gracilis* Schauer[2,16]. However, to the best of our knowledge there is no report dealing with evaluation of this oil aiming to control MIC.

In this context, this study investigated the anti-microbial potential of the essential oil from *L. gracilis* Schauer over *C. bifermentans*, isolated from ballast of ship transporter of crude oil and against fungi occurring on microbial biofilms. Additionally, the influence of the essential oil on the corrosion of AISI 1020 carbon steel by electrochemical and gravimetric techniques was also investigated.

2. Material and methods

2.1. Harvest of *L. gracilis* Schauer and essential oil extraction

The harvest of *L. gracilis* Schauer was carried out in the city of Mossoró (5° 11’ 17” South and 37° 20’ 39” North) at semiarid region of Rio Grande do Norte State - Brazil. Taxonomic identification was performed comparing harvested samples to one present as an exsiccatae at herbarium from Federal Rural University of Pernambuco (UFRPE/Brazil) also a taxonomickey has been performed.

The essential oil was extracted using the hydro-distillation method by using *Clevenger* system adapted to a glass ballon-flask. The yield was calculated by the difference between the total oil quantity obtained and

the total mass of dried leaves used during extraction.

2.2. Chemical characterization of the *L. gracilis* Schauer essential oil

Samples of *L. gracilis* Schauer essential oil were assayed by Gas chromatography-mass spectrometry (GC-MS) in order to identify its components. Firstly, a search on the CG-MS library was performed in order to compare to the retention index of those obtained by the co-injection of the oil together the linear hydrocarbons (C_{11}-C_{24}) calculated according to the Van &Kratz equation[17]. Followed, the results were compared to others CG-MS libraries as well as with other studies on the literature. Also, a qualitative assay was performed by using the Hewllet-Packard 5890 SERIES II CG chromatographer coupled to a flame ionization detector (FID) and a capillary silica column (J&W Scientific DB-5). Column temperature was adjusted to 35 ºC and hydrogen (H_2) gas was used as carrier. The oil assay was performed using the Hewllet-Packard CG/MS (CG: 5890 SERIES II/CG-MS: MSD 5971) system coupled to the same column and temperature above mentioned. However, the H_2 gas was changed to Helium (He) having a flow-rate of 1.0 mL/min.

2.3. Isolation and identification of *C. bifermentans*

The isolation of *C. bifermentans* was performed in laminar flow hood from to tak microbial biofilms obtained using crude oil samples of ballast of ship transporter donated by Center for Research and Development Leopoldo Américo Miguez de Melo (CENPES – PETROBRAS - Rio de Janeiro, Brazil). The biocupons (1020 AISI carbon steel) were inserted in a dynamic system made from acrylic with forced water circulation (flowrate: 500.0 mL/h) for obtaining the total microbial biofilms. Before insertion in the dynamic system, the biocoupons were treated using sonication (UltraCleaner 1600A®-Skymen) using dichloroethylene (by 15 minutes) followed by washing with etanol, acetone and deionized water in order to remove residues andimpurities. After seven days in the dynamic system the biocoupon pickling was performed. The microorganisms existing at the biofilms for every biocoupon were kept separately on Petri plates after pickling. It was used five biocoupons in this assay.

After pickling the microorganisms existing on the Petri plate were transferred to approximately 10 mL added to rounded bottom-flasks containing the cultivation medium specific to total aerobic bacteria: Nutrient broth (10.0 g/L of *Lab-Lemco Powder*, 10.0 g/L of peptone and 5.0g/L of NaCl) and incubated in BOD at 30 ºC for 48 hours. Followed, samples were transferred to plates of Petri containing the Agar nutrient medium for selecting the pure colonies. Molecular identification of bacteria was carried out after 48 h of cultivation growth. In this case, gene sequencing of 16S rRNA subunit was performed and further compared to the DNA and proteins sequencing in data bank, *Genbank*[18]. Briefly, the DNA extraction during the bacterial cultivation was performed and visualized through 1.0 % agarose gel electrophoresis and marked by SYBR Safe® (Thermo Fischer Scientific). After DNA extraction the 16S rRNA (1500 pb) was amplified by PCR using universal *primers* for bacterium, SAdir (5'-AGAGTTTGATCATGGCTCAGA-3') and S17rev (5'-GTTACCTTGTTACGACTT-3'). The amplification reactions were carried out using the GeneAmp PCR System 9700® thermocycler (*Applied Biosystems*). The PCR products were purified using the UltraClean® PCR Clean-up® kit (MOBio). After purification, quantification and purity were estimated by optic density using NanoDrop® ND-1000 UV-Vis spectrophotometer (*Thermo Scientific*). PCR products were sent to sequencing at Human Genomic Center at São Paulo University (USP-Brazil) using the MEGABACE 1000 automated sequencer. The sequencing result was assayed using the *Chromas Lite* software version 2.01 (*Technelysium*) and compared to those existing in the *Genbank*. The sequence similarity was performed using the BLASTn (*Basic Alignment Search Tool*) software.

2.4. Activity of *L. gracilis* Schauer essential oil over *C. bifermentans*

In order to determine the minimum inhibitory concentration of *L. gracilis* Schauer essential oil different concentrations were added to the cultivation medium. Next, samples of the *C. bifermentans* obtained

from biofilms were added to the solid medium on the Petri plates and incubated in Biochemical Oxygen Demand (BOD) equipment at 30 ˚C for 24 hours. The following concentrations were assayed 0.5; 1.0; 10.0; 30.0; 60.0and 90.0 µg with four repetitions. A blank was used as control and results were obtained after 24hours.

2.5. Activity of *L. gracilis* Schauer essential oil in fungal biofilms

To obtain the fungal-containing biofilms in order to assay the biocide activity of the *L. gracilis* Schauer the experiments were carried out in dynamic system with forced water circulation following the same methodology as shown for *C. bifermentans*.

The coupons were withdrawn after seven days in which the total biofilms were fully built. The coupons were fixed to a nylon wire and submitted to different essential oil concentrations using *Erlemeyer* flasks capped with cotton. The flasks were incubated on a reciprocating shaker at 25 ºC and 50 rpm during 0, 60, 90 and 120 minutes. In this case, an essential oil concentration of the 20.0 $\mu g \cdot L^{-1}$ was added to 250 mL of salt solution (0.85 g/L) together 200.0 µL of Tween (80% v/v). Sterile water was used as negative control and 200.0 µL of Tween (80% v/v) as positive one. After the contact time with the *L. gracilis* Schauer essential oil every coupon was scraped separately for quantifying the microbial density, in terms of colony forming unity (CFU) using a medium specific to fungi. The colony forming unit (CFU) was obtained from dilution of 10 mL of total biofilms, thus from every dilution samples of 0.1 mL were added directly to the *Saborround* medium and spread with the aid of a Drigalski holder. The plates were stored in BOD at 30 ºC for 48 hours. Experiments were carried out in triplicate.

3. Corrosion experiments

3.1. Electrochemical assays

The corrosion experiments were carried out using an electrochemical cell containing NaCl (0.5M) as the corrosive medium and three electrodes. The copper electrode was used as the working electrode, it is highlight that the Ag/AgCl electrode was used as the reference electrode, and the platinum electrode was used as counter-electrode. The inhibition corrosion efficiency on AISI 1020 carbon steel due to the *L. gracilis* Schauer essential oil microemulsion was assayed by the linear potential dynamic polarization (LPP) and electrochemical impedance spectroscopy (EIS) techniques by means of the potentiostat/gavalnostat Autolab® model PG STATE 204 (Metrohm) coupled to NOVA software version 1.11. The essential oil concentration assayed were 0.0, 20.0, 40.0 and 60.0 $\mu g \cdot L^{-1}$.

The polarization curves were obtained by scanning the potential below and above 300 mV in relation to the open circuit potential using a rate of 1.0 mV/s by 180 minutes of immersion. The assays for EIS were carried out using the open circuit potential at 25 ºC at a frequency ranging from 10000 Hz to 6 MHZ. The TAFEL straight extrapolation method was used to obtain the curves and the corrosion inhibition efficiency (IE) according to equation (1):

$$\mathrm{IE}(\%) = \frac{Icorr - Icorr(inh)}{Icorr} \quad (1)$$

where I_{corr} and $I_{corr(inh)}$ are the AISI 1020 carbon steel currents in the absence and presence of each essential oil concentration, respectively.

3.2. Gravimetric analysis

Biocoupons (AISI 1020 carbon steel) were used to investigate the mass loss after contact with each essential oil concentration by gravimetry for the contact time previously described. The specimens were withdrawn from the dynamic system every four days thus after pickling of microbial biofilms they were washed using acetone and water followed by drying with hot air. The mass loss was calculated according to equation

(2):

$$CR = 87.6\frac{W}{dAt} \quad (2)$$

where CR is the corrosion rate, W is the mass loss (mg), d is the biocoupon density (g/cm^3), A is the biocoupon exposed area (cm^2) and *t* is the exposure time (h).

The AISI 1020 carbon steel density was 7.86 g/cm^3 according to NACE-TM (2000)[19]. The corrosion rates were calculated in mm/year. The test was performed in triplicate for each experiment at room temperature, and the observed differences were found to be lower than 5%. An analysis of variance was carried out using the different microemulsion contact time. The positive control used only the biocoupon and distilled water, while a 0.5 M NaCl solution was used as the negative control.

4. Statistical analysis

Tukey test at 5% confidence level was used to determine the inhibition rate of the essential oil over *C. bifermentans*. For inhibition in the biofilms it was used a fully casualised delineation in which it was assayed the contact time of the biofilms to *L. gracilis* Schauer essential oil with three repetitions for each treatment. For analysis the data were transformed to log (x+1) and submitted to analysis of variance and mean separate by Tukey test. Regression curves were obtained from the raw data mean, in this case the contact time was the independent variable. Exponential, logarithm, quadratic and polynomial model were selected based in the coefficient of determination (R^2) as well as the mean squared residue (MQR). The statistical analysis was performed using the software for variance analysis system (SISVAR 5.6).

5. Results and discussion

5.1. *L. gracilis* Schauer essential oil chemical characterization and yield

Substance	*R.I	Total (%)
Hexenol<(4Z)->	871	1.46
Thujene<α->	924	1.80
Pinene<α->	932	0.44
Pinene<β->	974	0.40
Myrcene	988	4.29
Terpinene<α->	1014	1.68
Cymene<O->	1022	4.50
Sylvestrene	1025	0.46
Ocymene<(E)-β->	1044	0.33
Terpinene<γ->	1054	14.97
Sabinene Hidrate <cis->	1065	0.45
Terpineolene	1086	1.14
Epoxymyrcene<6,7->	1090	0.14
Linalool	1095	0.70
Menthatriene	1108	0.03
Terpinen-4-ol	1174	4.67
Thymol methylether	1232	0.59
Carvacrolmethylether	1241	0.56
Carvacrol	1298	49.51
Thymolacetate	1349	0.01
Carvacrolacetate	1370	0.14
Total Chemical Classes		
Benzenoids	-	55.90
Monoterpenoids	-	33.80
Sesquiterpenoids	-	10.20
Total		99.90

*R.I.: Retention index

Table 1. Essential oil of *Lippia gracilis* Schauer chemical characterization.

The essential oil of *L. gracilis* Schauer resulting from the extraction process showed a higher concentra-

tion of the bioactive substance carvacrol (approximately 50%). However, it was observed a quite lower thymol quantity as shown in **Table 1**.

It is important to highlight that oil extraction yield was of 1.74%. The quantitative fluctuations of the majority essential oil components occurred mainly due to genetic as well as environmental conditions of the plant cultivation. It is known that others factors such as the harvest time, season of year, climate, soil as well as sample quantity and life cycle in which the plant is submitted besides the harvesting local can affect directly the chemical composition and the raw yield of the oil[20,21]. This ranging on the chemical composition can also happen in the same locality despite having relatively similar environmental conditions[22]. Another important factor that plays an important role in the bioactive compounds produced by the plants is the parasite attack, since the production of these substances works as a defense mechanism to the plant, then helping in the inhibition attack as well as in the infection process by pathogens[22].

5.2. Biomolecular identification of the bacteria

A molecular identification of bacteria obtained from biofilmwas carried out after 48 h of cultivation growth. In this case, gene sequencing of 16S rRNA subunit was assayed and further compared to the DNA and proteins sequencing data bank, *Genbank*. The PCR product fragments of 1320 bp (**Figure 1**) showed 99.9% similarity to the bacteria *Clostridium bifermentans*, according to *Genbank*. It is important to highlight that this bacteria is common in oil industry. There are in the literature some studies that used a quite similar methodology and that identified the genus *Clostridium* associated tomicrobial corrosion in pipeline on fields of oil and gas. Jan-Roblero *et al*.[5] used molecular assay to phylogenetic identification of bacterium in the pipeline of gas during oil production in Mexico and detected the presence of *Clostridium* causing corrosion in association with other bacterium on a biofilm. They reported that the presence of genus *Clostridium* is favored by the ideal conditions generated by the *Enterobacteria* also is quite common *Clostridium* being associated with sulfate-reducing bacteria (SRB) in biofilm. Khelifi *et al*.[6] showed this bacterium is predominant, including *C. bifermentans*, in water of industrial treatment also associated to SRB. Recently, Biswas *et al*.[23] reported this genus presence in fungal biofilms built in treated water pipeline. It is highlighted that this bacterium favors biocorrosion mainly for producing sulfide and hydrogen sulphide through the cystein and sulphate-rich compounds fermentation, respectively[14,24]. Specifically on carbon steel *C. bifermentans* causes *pitting* and associated others bacteria it potentiates this action[25].

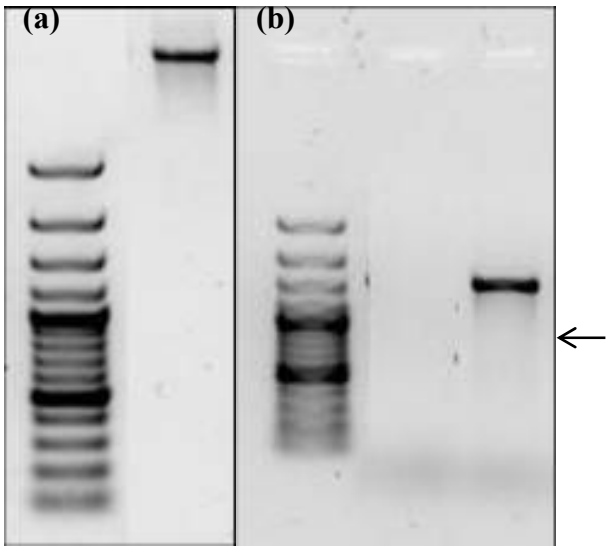

Figure 1. Electrophoresis of the products obtained using the SYBR stainin 1% agarose gel. (**a**) bacterial DNA genomic: lane 1, mark of 80 bp and 2 DNA extracted from the bacteria. (**b**) Amplificatilon by primers standard SAdir and S17rev from subunity 16S gene by PCR-GeneAmp: lane 1, molecular mark; lane 2 blank and 3 bacteria sample with 99.9% similarity to Clostridium bifermentans according to GenBank.

5.3. Essential oil minimum inhibitory concentration (MIC) over *C. bifermentans*

An evaluation of the MIC for *L. gracilis* Schauer essential oil was carried out during incubation in Petri plates at 30 °C for 24 hours. It was observed that samples in which the essential oil of *L. gracilis* Schauer was lower or equal to 10.0 $\mu g \cdot L^{-1}$. They were inefficient over the bacteria growth. However, concentrations of 30.0, 60.0 and 90.0 $\mu g \cdot L^{-1}$ inhibited completely the bacteria growth (**Table 2**). Albuquerque *et al.*[26] assayed the influence of essential oil of *L. gracilis* Schauer up to 400 $\mu g \cdot L^{-1}$ over the *Bacilluspumilus* growth. This bacterium is also associated to biofilms causing biocorrosion. However, they did not observe any effect over the *B. pumilus* growth for this range of concentration. In order to define the MIC the concentration range it was reduced from 15.0 to 30.0 $\mu g \cdot L^{-1}$ (15.0, 20.0, 25.0 and 30.0 $\mu g \cdot L^{-1}$). It was observed that 20 and 25 $\mu g \cdot L^{-1}$ allowed the inhibition of the bacteria growth *in vitro* then the former was taken as the MIC (**Table 3**). Albuquerque *et al.*[26] investigated the effect of the essential oil of *L. gracilis* Schauer over several microorganisms and reported a MIC of the 420 $\mu g \cdot L^{-1}$. It is known that each oil concentration acts in a different way over the microorganisms then showing or not inhibition concerning the cell growth. Also, characteristics such as type of microorganism as well as oil chemical composition play an important role on the MIC. As shown by the results of oil chemical characterization, despite the lower thymol concentration there was a complete inhibition of *C. bifermentans*. Additionally, it is possible the synergism occurrence between these two molecules, thymol and carvacrol, then favoring the antimicrobial activity of the oil. Others substances such as *p*-cymeneandγ-terpinenecoupled to carvacrol could strengthen the antimicrobial activity of the essential oils[25]. Therefore, it is possible that in this study the presence of these others compounds strengthened the antimicrobial activity of the essential oil of *L. gracilis* Schauer over *C. bifermentans*, then inhibiting totally the *in vitro* cell growth.

Essential oil concentration ($\mu g \cdot L^{-1}$)	Inhibition zone of *C. bifermentans* (mm)	Inhibition (%)
0.0	0	0
0.5	0	0
1.0	0	0
5.0	0	0
10.0	0	0
30.0	89 ± 0.9^a	100.0
60.0	89 ± 0.9^a	100.0
90.0	89 ± 0.9^a	100.0

Table 2. MIC of essential oil of *L. gracilis* Schauer for the higher range concentration experiment. Tukey test ($p<0.05$).

Essential oil concentration ($\mu g/L^{-1}$)	Inhibition zone of *C. bifermentans* (mm)	Inhibition (%)
0.0	0.0	0.0
15.0	0.0	0.0
20.0	89 ± 0.9^a	100.0
25.0	89 ± 0.9^a	100.0

Table 3. MIC for essential oil of *L. gracilis* Schauer using the lower range concentration, aMIC of 20.0 $\mu g \cdot L^{-1}$ was considered. Tukey test ($p<0.05$).

5.4. Antifungal activity of essential oil of *L. gracilis* Schauer

The essential oil of *L. gracilis* Schauer showed *in vitro* different behaviors ($p<0.05$) concerned to the contact time of the fungal biofilms. The results showed that there was significant difference of the essential oil of *L. gracilis* Schauer over the biolfilms ($p<0.05$) for the four contact times (0, 60, 90 and 120 minutes) assayed. In this case, a variation coefficient (VC) of 26.65 was observed. Also, the decreased of the CFU concerned to the contact time of essential oil of *L. gracilis* Schauer fitted to a polynomial model in which a time of 120 minutes was determined as the needed time to inhibits completely the fungal growth as shown in **Figure 2**.

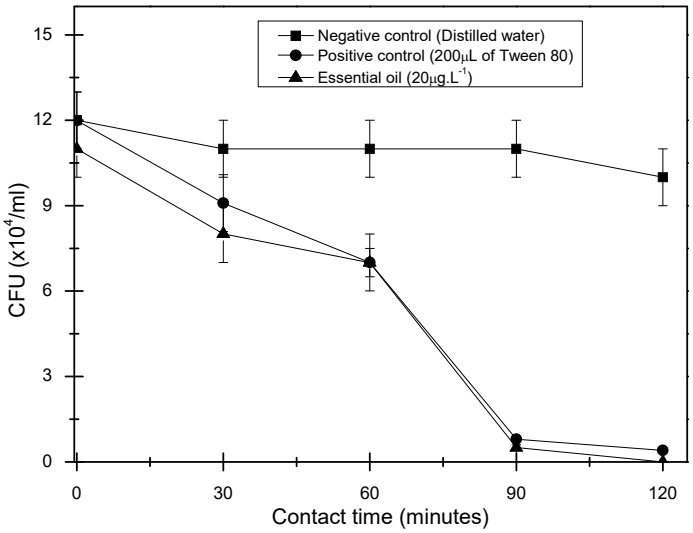

Figure 2. Effect of the *L. gracilis* Schauer essential oil contact time over the fungal biofilm using an oil concentration of 20.0 $\mu g \cdot L^{-1}$. Negative control: distilled water. Positive control: 200 μL of Tween (80%). The means were obtained in triplicate.

Currently, it has been observed the occurrence of new strategies for MIC control since this is a problem that affects different industrial sectors and increases year to year. Also, the use of biocides leads to a higher toxicity potential that brings to a non-favorable option sometimes. The natural products use to carry out the task is playing role mainly for environmental as well as economic reasons despite is not a new practice. Some studies have shown the efficiency of these natural products in the biofilms control, mainly the bacterial one[27-29]. Pioneer studies using plant extracts were carried out primary to act of the fungal biofilms, for instance, the use of aqueous extract of *Alliumsativum* and *A. cepa*inhibited by full the growth on different fungal types[28,30]. Others natural products such those form algae and cyanobacteria were assayed in order to control the biofouling and biocorrosion showing good results over the fungal growth inhibition[31,32].

The antimicrobial activity of the essential oil of *L. gracilis* Schauer shown in this study is probably due to the presence of carvacrolit has been cited in the literature that it can act over pathogenic as well as non-pathogenic fungals. The main mechanism of this substance action as well as of thymol over fungal is based on the mitochondrial membrane depolarization caused by the reduction of the cellular membrane potential then affecting the pH, the ATP synthase, the calcium cycle and other canals. Therefore, the occurrence of a conidial germination reduction leads to the fungi death[19].

In the present study, the essential oil of *L. gracilis* Schauer appeared as a powerful antifungal biocide even at lower concentration, then confirming its fungicide power. The use of this substance can represent as an alternative to control efficiently the biocorrosion on oil and others industries. It has some advantages since is environmentally-friend, obtained from a renewable source and shows lower toxicity.

5.5. Corrosion experiments

5.5.1. Electrochemical assays

The essential oil of the *L. gracilis* Schauer did not show activity compared to the control when the EIS analysis was performed on the AISI 1020 carbon steel at room temperature. According to the Nyquist model none assay was able to form prominent capacitive arcs, reducing the current intensity. All gave lower activities than the control (NaCl 0.5M) as seen in **Figure 3**. The concentrations of the 20.0 and 40.0 $\mu g \cdot L^{-1}$ showed better performance than the higher concentration assayed (60.0 $\mu g \cdot L^{-1}$) but not enough to reduce the current over the carbon steel. This result is not common but factors as temperature, pH and type of medium used can influence the efficiency of natural product on corrosion mainly due to the interaction of the components of the natural product[33,34].

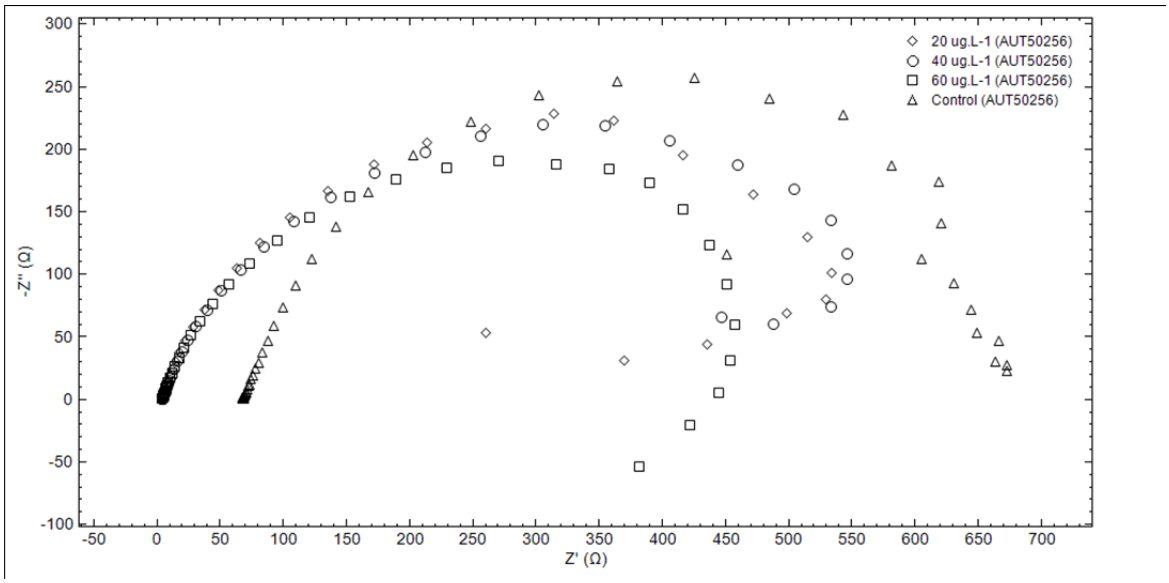

Figure 3. EIS diagram for AISI 1020 carbon steel in salting medium (NaCl 0.5 M) in the presence of different concentrations of the *L. gracilis* Schauer essential oil.

On the other hand, LPP analysis as shown by Tafel curves for essential oil of *L. gracilis* Schauerat different concentrations, compared to the control, showed at 60.0 $\mu g \cdot L^{-1}$ the essential oil was able to reduce the current (I_{corr} = 2.59) increasing the potential and thus reducing the mass loss of the AISI 1020 carbon steel (Data not shown). As shown in **Figure 4**, the anodic and cathodic polarization curves showed reduction of the current intensity in both branches, with the cathodic showing higher inhibition due to the use of 60.0 $\mu g \cdot L^{-1}$ of essential oil.

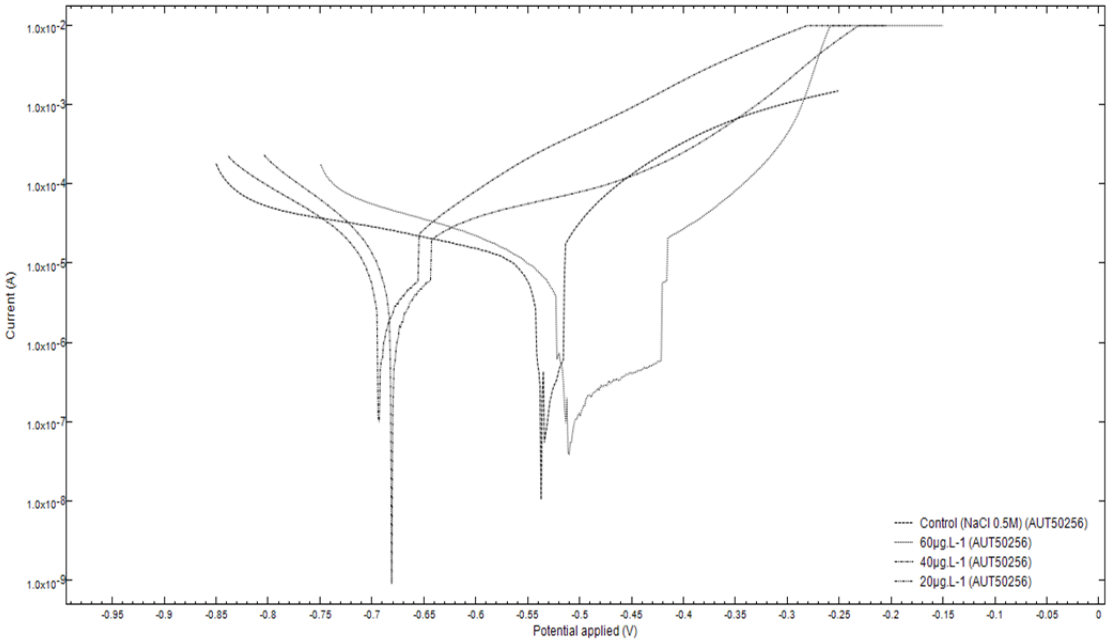

Figure 4. Polarization curves for AISI 1020 carbon steel in the salting medium (NaCl 0.5M) in the presence of different concentrations of *L. gracilis* Schauer essential oil using ascontrol NaCl (0.5M).

Gravimetric assays by mass loos performed out of the electrochemical cell ans using a higher range of contact time for the essential oil of the *L. gracilis* Schauer showed that this oil was able to reduce the corrosion rate compared to the control (NaCl 0.5M). In this case, the corrosion rate was kept near zero during all the time of contact (**Figure 5**). Therefore, the gravimetry assays confirm the results of LPP analysis, showing that essential oil of the *L. gracilis* Schauer can act as a corrosion inhibitor even in a medium with corrosive characteristics[35] acting even at lower concentration.

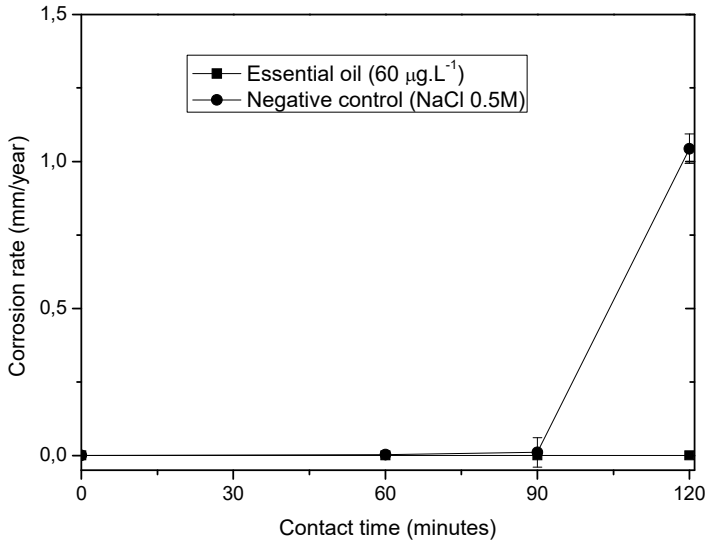

Figure 5. Corrosion rate of essential oil of L. gracilis Schauer (60 μg·L-1) in different contact time (in minutes).

6. Conclusion

In this study it was investigated the anti-microbial potential of the essential oil of the *L. gracilis* Schauer on microbial biofilms. This oil was able to inhibit the *C. bifermentans*, showing a MIC of 20 $\mu g \cdot L^{-1}$. Anti-fungal activity was also observed in biofilms in which 120 minutes was found to completely reduce the CUF to zero. The biocide activity of this essential oil is mainly due to presence of carvacrol acting in synergism with others molecules such as *p*-cymene e γ-terpinene. An oil concentration of the 60.0 $\mu g \cdot L^{-1}$ was able to reduce the mass loss of 1020 AISI carbon stell as shown by LPP and gravimetry analysis. The use of essential oil of *L. gracilis* Schauer can be alternative to the MIC control since is environmentally-friend, renewable and shows lower toxicity.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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