

# The Control of *Lactobacillus* sp. by Extracellular Compound Produced by *Pseudomonas aeruginosa* in the Fermentation Process of Fuel Ethanol Industry in Brazil

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## ABSTRACT

This work evaluated the effect of secondary bacterial metabolites produced by *Pseudomonas* sp LV strain in control of *Lactobacillus* sp. population in the microcosm of the vat during ethanol fermentation. The fraction F4 produced by *Pseudomonas aeruginosa* was extracted with dichloromethane and fractionating by vacuum liquid chromatography obtained in a methanol phase. The evaluation of antibiotic activity of F4 fraction mixed or not with sulphuric acid and Kamoram<sup>®</sup>. The antibiotic activity of F4 fraction was determined as well as the fermentation efficiency. Also was determined yeast cell viability, budding formation, the viability of budding cells, and number of populations of *Saccharomyces cerevisiae* and *Lactobacillus* sp. The results showed that the F4 fraction had high selective antibiotic activity against *Lactobacillus* sp. but not for *S. cerevisiae*, and no inhibitory effect was observed in the fermentation process by yeast. Also F4 fraction decreased flocculation and foam formation. The F4 has an antibiotic activity against *Lactobacillus* sp. and should be used as an alternative to control bacteria contamination and foam and flocculation formation in the fuel ethanol fermentation process. The F4 fraction could reduce the use of antibiotics in the control of *Lactobacillus* sp. population during the fuel ethanol production.

**Keywords:** Fuel Ethanol; Fermentation; Contamination; Lactobacillus; Yeast; Pseudomonas

## 1. Introduction

The sugar cane (*Saccharum officinarum*) culture is largely used to produce sugar and fuel ethanol. Today, sugar cane is an important crop in Brazil occupying 8 million hectares producing more than 600 million tons per year, making the country the largest sugar cane producer worldwide [1].

Bacterial contamination is a continual problem in commercial fermentation cultures, particularly in fuel ethanol fermentation that is not performed under sterile and pure culture conditions [2]. A variety of Gram-positive and Gram-negative bacteria have been isolated from fuel ethanol fermentations including species of *Pedococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, and others [3,4]. The most common contaminant bacteria are species of *Lactobacillus*, which show fast growth rate

and tolerance to alcohol, low pH and effectively compete with the yeast [4].

During fuel ethanol production the contamination level can increase the gum formation which can obstruct pipes, sieves, centrifuge, heat sinks, increasing the flocculation of yeast, decreasing fermentation process activity, and losing yeast cells for pellet formation on the fermentator depth. Also the flocculation decreases the centrifugation efficiency reducing the yeast viability and/or increases a formation of compounds which decreases fermentation activity and ethanol production [5]. The formation of organic acid decreases ethanol production and unfeasible yeast, resulting in an operational problem in the industry facilities [6].

Chronic bacterial contamination causes a constant draining of available sugar that is converted to ethanol by yeast, and the bacteria compete for essential micronutrients required for optimal yeast growth and ethanol pro-

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duction. Bacterial contamination occurs unpredictably, and produces acetic and lactic acids inhibiting yeast growth and may result in “stuck” fermentations that require costly shutdowns of facilities for cleaning [7,8].

Despite efforts to prevent contamination with extensive cleaning and disinfecting procedures, saccharification tanks, continuous yeast propagation systems, and notoriously resistant biofilms can act as reservoirs of bacteria that continually reintroduce contaminants [4]. For this reason, antibiotics are frequently used to prevent and treat contamination [9,10]. The most common commercially available products used to control contamination in fuel ethanol facilities are based on the antibiotics virginiamycin and penicillin [2,3] and sodium monensin crystalline [11]. The emergence of drug resistant strains, however, may limit the effectiveness of these agents. Decreased susceptibility to virginiamycin has been observed in *Lactobacillus* isolated, and the emergence of isolates with multi-drug resistance to both virginiamycin and penicillin has been reported [12]. Therefore, new antibacterial agents and new drug management methods will need to be developed to effectively control bacterial infections and also are not harmful to the environment.

In this way, the challenge is developing a new product which has antibiotic activity to control bacteria contamination on the fermentator and is not harmful to the environment. Compounds produced by secondary metabolism of antagonist microorganisms during competition in the environment could show antimicrobial activity [13,14]. The use of secondary metabolites with antibiotic activity is against bacterial contamination during fuel ethanol production. The introduction of new biological products with antibiotic activity makes the industrial process more sustainable reducing the residues formation and eliminating the antibiotic in the vinasse that is sprayed on the soil as fertilize and the yeast cake that is used in the animal food industry, in the future both of which will be free antibiotics, decreasing environmental contamination [15,16].

The ethanol industry in Brazil is a very important renewable energy source and the bacterial contaminations that compete for C source with yeast is the most important problem for industrial process and represents great losses of ethanol production. To reduce the losses in the future the objective of this work was to evaluate the effect of fractions obtained from *Pseudomonas aeruginosa* (LV strain) culture after fractioned by liquid vacuum chromatography using dichloromethane on the control of *Lactobacillus* sp., the effect on *Saccharomyces* sp. and the influence in the physicochemical properties of wine during industrial production of fuel ethanol.

## 2. Material and Methods

### 2.1. Bacterial Strains

The strain of *Lactobacillus* sp. used in this study was

isolated from wine during fermentation at Cooperativa Agroindustrial Vale do Ivaí LTDA (COOPERVAL), and cultivated in MCC media, (sugar cane wine diluted with distilled water  $5.0^\circ \pm 0.1^\circ$  brix; yeast extract  $10 \text{ g}\cdot\text{L}^{-1}$ , peptone  $10 \text{ g}\cdot\text{L}^{-1}$ ) (Nobre 2005). The antagonistic strain was a *Pseudomonas aeruginosa* LV strain isolated from citrus canker lesions in orange [17].

The microbial community in the vat during wine fermentation process was determined and the *Lactobacillus* sp. was the most representative population around 90% of the bacterial community. Five strains of *Lactobacillus* sp. and one of *S. cerevisiae* were most representative bacteria were isolated, selected and criopreserved with glycerol solution 20% in liquid nitrogen.

### 2.2. Production, Purification and Fractioned of Secondary Metabolites with Antibiotic Activity

The production, extraction and obtation of fractions with antibiotics compounds was realized the according to [14] which method was patented [18]. After treat the supernatant with dichloromethane, the phase obtained was treated with six different solvents with different polarity resulting in six different fractions as following (v/v): hexane (100; F1), dichloromethane (100; F2), ethyl acetate (100; F3), methanol (100; F4), methanol: water (1:1; F5) and water (100; F6). The six fractions was used in the next tests.

### 2.3. Thin Layer Chromatography (TLC)

The TLC was used as qualitative analysis of fractions obtained during vacuum liquid chromatography fractionated with different liquid phases using solvents with different polarity. The TLC was carried out using chromatoplates of silica gel 60 F<sub>254</sub> (Merck®) fixed in aluminum support. The eluent system (mobile phase) used was a mix of chloroform/acetone/methanol. The spots obtained was revealed by the exposition of UV light in two different wavelength ( $\lambda = 254 \text{ nm}$  and  $366 \text{ nm}$ ).

### 2.4. TLC Bioautography

After done the TLC of DP, the antibiotic activity of spots present on chromatoplates was checked. The spots corresponding to all fractions obtained from DP. The chromatoplates was left in Petri dishes and covered with MCC agar plus cells suspensions of *Lactobacillus* sp. ( $10^8 \text{ CFU mL}^{-1}$ , D.O 0.14,  $\lambda = 590 \text{ nm}$ ) or *S. cerevisiae* ( $10^8 \text{ CFU mL}^{-1}$ , D.O 0.22,  $\lambda = 590 \text{ nm}$ ) by pour plate technique (Rahalison et al. 1991). After inoculation, the Petri dishes were incubated at  $32^\circ\text{C}$  per 24 h, and the result was evaluated by presence or absence of inhibition halo formed around the spot after revealed with de 2, 3, 5-triphenil-1H-tetrazolium chloride (TTC) 1%. The re-

sult was considered (–) with absence of halo and (+) with presence of halo.

## 2.5. Evaluation of Antibiotic Activity

### 2.5.1. Agar Diffusion Technique

The evaluation of antibiotic activity of all fractions obtained was realized using antibiogram paper disc. The experimental design was composed by 2 *Lactobacillus* sp. strains, 6 fractions obtained from DP of each fraction in duplicates ( $2 \times 6 \times 10 \times 2$ ,  $n = 48$ ). The inoculum of *Lactobacillus* sp. ( $10^8$  CFU mL<sup>-1</sup>; O.D. 0.14,  $\lambda = 590$  nm) and *S. cerevisiae* ( $10^8$  CFU mL<sup>-1</sup>; O.D. 0.22,  $\lambda = 590$  nm) was inoculated in MCC agar in Petri dishes with sterile swab. Aliquot of 5  $\mu$ L of each fraction containing 500  $\mu$ g, were added in paper filter discs, dried in sterile conditions and left on the inoculated plates and incubated at 32°C for 24 h. The results was considered as (–) with absence of inhibition halos and (+) with presence of inhibition halos.

### 2.5.2. Determination of Minimum Inhibitory Concentration (MIC)

After selected the F4 fraction that showed antibiotic activity only for *Lactobacillus* sp. but not for *S. cerevisiae* a MIC was determined. The experimental design was eight concentrations of F4 with four replicates ( $8 \times 4$ ,  $n = 32$ ) and respective controls.

The MIC was carried out in cell culture plates with 24 wells, non-inoculated MCC and cell suspensions of *Lactobacillus* sp ( $10^8$  CFU mL<sup>-1</sup>, D.O 0.14,  $\lambda = 590$  nm) were considered negative and positive controls, respectively. In each well, 1.8 mL of MCC inoculated with 100  $\mu$ L cell suspension of *Lactobacillus* sp. ( $10^8$  CFU mL<sup>-1</sup>, D.O 0.14,  $\lambda = 590$  nm) plus 100  $\mu$ L of F4 concentrations as following 48; 97; 195; 390; 781; 1562; 3125 and 6250  $\mu$ g·mL<sup>-1</sup>. Plates were incubated at 32°C for 48 h. Afterwards, 20  $\mu$ L of 1% 2%, 3%, 5-triphenyltetrazolium chloride (TTC) was added in the wells and incubated again at 32°C for 20 min. After that the wells that showed pink colour was considered resistant (+) and sensitive (–) when no colour changed was observed. The experiment was carried out three times.

## 2.6. The Influence of Fraction F4 on Fermentation Process

The experiment was carried out during the harvests of 2009/10 and 2010/11 at the facilities industry of Cooperativa Agroindustrial Vale do Ivaí Ltda (COOPERVAL). In the fermentation experiments 500 mL of wine composed by 60% of sugar cane juice and 40% of yeast was kept in an orbital shaker at 32°C for 24 h. The incubation conditions were the same used in the fuel ethanol industry process. The crystalline sodium monensin used was a

commercial product named Kamoran<sup>®</sup>. Before begin the fermentation process aliquots of 500 mL of wine was treated with H<sub>2</sub>SO<sub>4</sub> (98%) until the wine came to pH 2.5; 10 ppm antimicrobial Kamoran<sup>®</sup>; 1562  $\mu$ g·mL<sup>-1</sup> F4 fraction; H<sub>2</sub>SO<sub>4</sub> plus F4 fraction; 10 ppm Kamoran<sup>®</sup> plus 1562  $\mu$ g·mL<sup>-1</sup> F4 fraction. Non treated fermented wine was considered as control.

After incubation time was determined the wine pH, ethanol content (%), concentration of total residual reducing sugar (%). The experimental design was in block with five treatments with three replicates and the results were evaluated by analyses of variance (ANOVA) and Tukey test ( $p < 0.05$ ).

### 2.6.1. Evaluation of Foam Formation and Flocculation during Fermentation Process

The foam formation was evaluated by personal scale (1 to 5) for visual observation and the flocculation was analyzed by optical microscopy using a personal scale of 1 to 5, where 1 corresponding to low loam/flocculation and 5 high loam/flocculation.

### 2.6.2. Determination of Alcohol Content

The alcohol content of wine was determined with electronic hydrometer (Anton-Paar model dma 4500) after distillation of 25 mL of sample at Kjeldhal microdestilator adapted by alcohol. The alcohol content was calculated with densimeter after distillation of 25 mL of wine fermented (% v/v ethanol at 20°C).

### 2.6.3 Determination of Total Acidity

The total acidity was determined by titulation with NaOH 1N and bromothymol blue 1% as indicator [19]. The titulation was carried out with 10 mL of wine diluted five times with distilled water plus three drop of bromothymol blue 1%.

The total acidity was express by H<sub>2</sub>SO<sub>4</sub> g·L<sup>-1</sup>, that corresponding to the volume of NaOH 0.1N spent by mg of acetic acid L<sup>-1</sup>, based in the equation  $Ta = (V_{NaOH} \times N \times 60 \times k \times 1000)/20$ , Ta = total acidity (mg acetic acid L<sup>-1</sup>);  $V_{NaOH}$  = volume of NaOH 0.1 N solution spent (mL); N = NaOH solution normality; 60 = molecular weight of acetic acid; k = correction factor of NaOH 0.1 N; 20 = sample volume (mL).

### 2.6.4. Determination of Total Reducing Sugar (TRS)

The TRS (%) was determined by Lane-Eynon method [20], where 50 g of wine previously filtrated was transferred to the volumetric balloon and added 20 mL of inverted sugar 1% plus 4 mL of EDTA 4% and shaken by hand, after that was filtered and added 10 mL of Fehling liquor. The solution was heated until changed the color and added five drops of methylene blue 1% following by titulation.

## 2.7. Microbial Analysis

### 2.7.1. Cellular Viability of *Saccharomyces cerevisiae*

After finished the fermentative cycling, the cellular viability of *S. cerevisiae* (%) was determined in aliquots of 0.2 mL of wine plus erythrosine diluted in phosphate buffer in a Neübauer camera observed in microscope 400×. The cellular viability was express by the ratio between viable and unviable cells [21]. Also were evaluated budding rate, cell number and viability of *S. cerevisiae* (UFC. mL<sup>-1</sup>).

The budding tax was estimated by the ratio between viable cell and bud numbers. The viability of budding was determined by the ratio of number of viable bud and number of viable plus unviable bud. All evaluation was carried out with five replicates. The results were evaluated by analyses of variance (ANOVA) and Tukey test ( $p < 0.05$ ).

### 2.7.2. *Lactobacillus* sp. Population Evaluation

The number of colony forming units (CFU mL<sup>-1</sup>) was estimated by serial dilution 1:10 by *pour plate* method [22] in Petri dishes with MCC agar. Each sample was diluted of 10<sup>-1</sup> to 10<sup>-9</sup> and 50 µL was mixed with MCC agar in melt point and incubated at 32°C for 24 h. All dilutions of samples were in three replicates. The results were expressed by CFU mL<sup>-1</sup> and were evaluated by analyses of variance (ANOVA) and Tukey test ( $p < 0.05$ ).

## 3. Results

### 3.1. Evaluation of Antibiotic Activity

The DP and all six fractions obtained was evaluated the antibiotic activity (500 µg·mL<sup>-1</sup>) by agar diffusion using paper filter disc that showed different effect on *Lactobacillus* sp and *S. cerevisiae*. Six fractions obtained from DP, only F3 and F4 showing antibiotic activity against *Lactobacillus* sp. and no effect was observed by F1, F2, F5 and F6. However, F4 showed selective effect inhibiting only *Lactobacillus* sp. growth and did not show any effect against *S. cerevisiae* (Table 1).

In TLC the antibiotic activity observed in the spot corresponding to DP and F4 for *Lactobacillus* sp. and DP and F3 for *S. cerevisiae*. The MIC of F4 was 1562 µg·mL<sup>-1</sup> for *Lactobacillus* sp. and sample of all wells of plates that showed no growth was plated in MCC agar in Petri dishes and incubated at 32°C for 24 h and no colonies was observed in any concentration up to the MIC, indicating that F4 showed bactericidal effect. In all concentrations was observed *S. cerevisiae* growth.

### 3.2. The Influence of Fraction F4 on Fermentation Process

In the non-treated wine, the foam formation was very

high as well as in the wine treated with H<sub>2</sub>SO<sub>4</sub> and KM. The wine treated with F4 fraction did not formed foam even when combined with KM, on the other hand the F4 plus HS a foam formation was low (Table 2).

The wine treated with F4 and KM + F4 showed low flocculation during the final of fermentative process with level 1. All of those treatments showed high flocculation level among 3 and 5 except for F4 (Table 2). No differences were observed in the e pH of wine for all treatments except when was added H<sub>2</sub>SO<sub>4</sub> that the pH decreased significantly. The addition of H<sub>2</sub>SO<sub>4</sub> increased total acidity when compared with KM, F4 and control (Figure 1(a)).

The F4 fraction and KM + F4, showed significant differences on ethanol production when compared with control and wine treated with HS plus or not F4 (Figure 1(b)). The total reducing sugar no differences was observed except for KM + F4 that decreased the amount in the wine (Figure 1(c)).

### 3.3. Microbial Analysis

In all treatments including control was observed high cellular viability of *S. cerevisiae* that was more than 75% (Figure 2(a)).

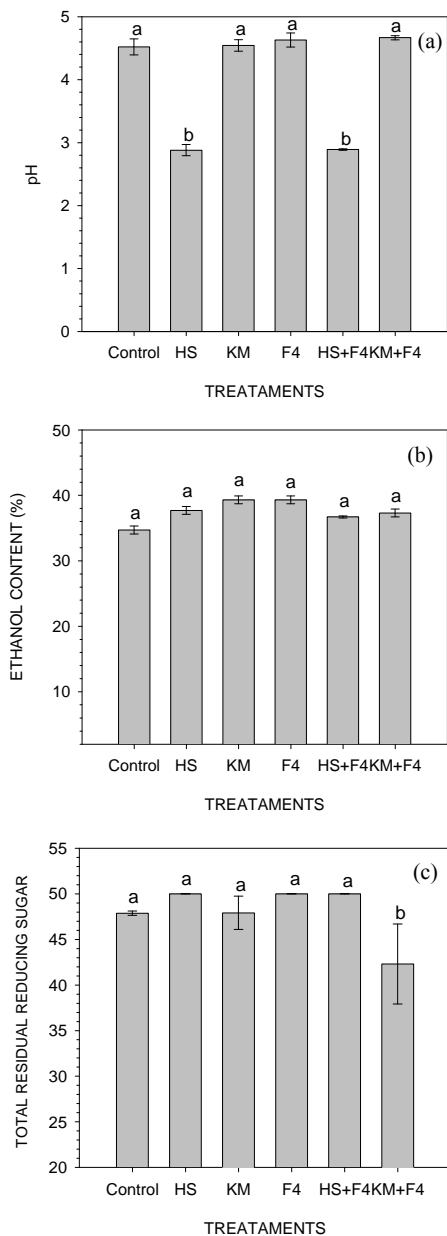
**Table 1. Evaluation of antibiotic activity of dichlorometane phase (DP) and fraction purified by vaccum liquid chromatography using six mobile phase with differents polarity (F1, 100% hexane; F2, dichlorometane 100%; F3 ethyl acetate 100%; F4, methanol 100%; F5 methanol/water (1:1, v/v) e F6, water 100%), against *Lactobacillus* sp. and *Saccharomyces cerevisiae*.**

	DP	F1	F2	F3	F4	F5	F6
<i>Lactobacillus</i> sp.	+	-	-	+	+	-	-
<i>S. cerevisiae</i>	+	-	-	+	-	-	-

(-) Absence of halo; (+) Presence of halo; C Control.

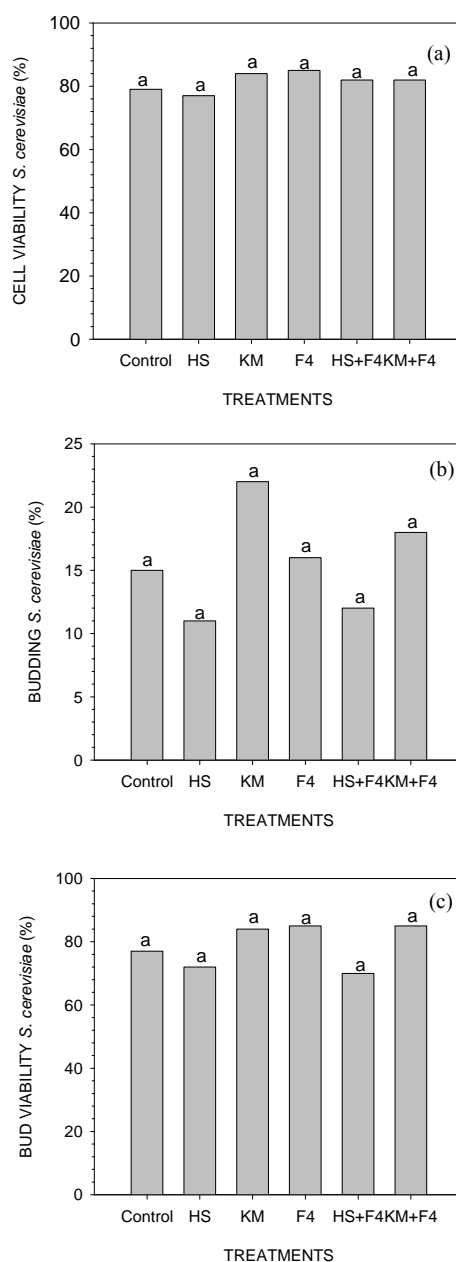
**Table 2. Foam and flocculation formation in the wine after fermentation process (32°C per 6 h) treated with sulphuric acid 98% (HS); Kamoran® 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. The numbers corresponding to: none (1); low (2); medium (3); high (4); very high (5).**

Treatments	Foam	Flocculation
Controle	5	5
HS	5	3
KM	5	5
F4	1	1
HS + F4	3	5
KM + F4	1	1



**Figure 1.** The pH, ethanol content and total residual reducing sugar formation in the wine after fermentation process (32°C per 6 h<sup>-1</sup>) treated with sulphuric acid 98% (HS); Kamoran® 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. A. Wine pH after fermentation process. B. Ethanol content. C. Total residual reducing sugar. After fermentation process (32°C per 6 h) treated with sulphuric acid 98% (HS); Kamoran® 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. Values are the means of 3 replicates ± SE. Means for each treatment with the same letter are not significantly different of Tukey test (p < 0.05).

No effect was observed on *S. cerevisiae* among treatments and control. The cell viability was high in all treatments included control (Figure 2(a)), the budding

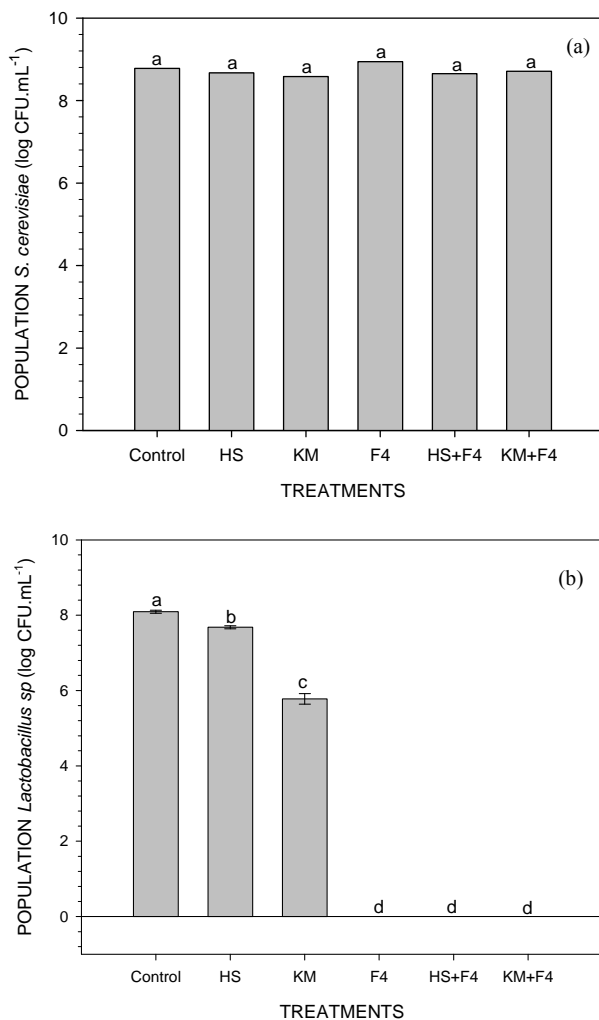


**Figure 2.** Cell viability, budding formation and bud viability of *S. cerevisiae* in the wine after fermentation process (32°C per 6 h<sup>-1</sup>) treated with sulphuric acid 98% (HS); Kamoran® 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. A. Cell viability of *S. cerevisiae*. B. Budding of *S. cerevisiae*. C. Bud viability of *S. cerevisiae*. After fermentation process (32°C per 6 h) treated with sulphuric acid 98% (HS); Kamoran® 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. Values are the means of 3 replicates ± SE. Means for each treatment with the same letter are not significantly different of Tukey test (p < 0.05).

formation of yeast was low around 20% and no significant differences were observed (Figure 2(b)). Cell vi-

ability of yeast was high and no differences were also observed (Figure 2(c)). The population of *S. cerevisiae* was high and no differences were observed among treatments (Figure 3(a)).

The *Lactobacillus* sp. population high decrease when the wine was treated with F4 fraction when compared with KM, H<sub>2</sub>SO<sub>4</sub> and control. The same effect was observed in the treatments that contained F4 (KM + F4 and HS + F4), showing that F4 fraction maintained antibiotic activity even was mix with KM or H<sub>2</sub>SO<sub>4</sub> (Figure 3(b)).



**Figure 3.** The populations of *S. cerevisiae* and *Lactobacillus* sp. in the wine after fermentation process (32°C per 6 h<sup>-1</sup>) treated with sulphuric acid 98% (HS); Kamoran<sup>®</sup> 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. A. Population of *S. cerevisiae*. B. Population of *Lactobacillus* sp. After fermentation process (32°C per 6 h) treated with sulphuric acid 98% (HS); Kamoran<sup>®</sup> 10 ppm (KM), F4 fraction 1562 µg·mL<sup>-1</sup> (F4); HS 98% + KM 10 ppm, KM 10 ppm + F4 1562 µg·mL<sup>-1</sup>. Values are the means of 3 replicates ± SE. Means for each treatment with the same letter are not significantly different of Tukey test ( $p < 0.05$ ).

## 4. Discussion

Many studies reported bacteria with antagonistic activity against others bacteria or fungi. The genera *Bacillus* and *Pseudomonas* are the most common microorganisms used as biocontrol agents [23-26]. Otherwise, few studies tested the effect of secondary metabolites produced by bacteria in the control of contaminants microorganisms during the fuel ethanol production [27].

The antibiotic activity against *Lactobacillus* sp. observed in CCD bioautography, antibiogram and MIC, indicated that F4 fraction showed a high antibiotic activity against *Lactobacillus* sp. but not for *S. cerevisiae* which did not decrease growth in all concentration tested. The fact of F4 showed a high effect against *Lactobacillus* sp. and no effect against *S. cerevisiae*, which indicates that the F4 fraction is a potential compound to use in the control of bacteria population during fuel ethanol production. In the attempt to control contaminants bacteria in the wine during fuel ethanol production [28] were tested many biocides such as methyl dithiocarbamate, thiocyanate, bromophenate, penicilin V acid, clindamicine, sulphite, nitrite, copper sulphate; and it is observed that many of the biocides also affect *S. cerevisiae* growth in the same doses that inhibit bacteria growth, and the fact was not observed with F4 fraction. Kamoran<sup>®</sup> (sodium monensine) is largely used as bactericidal in fuel ethanol production industry, but this product leaves residues in the vinasse that is used as fertilizer in the sugar cane culture and in the yeast cake that is used in animal nutrition.

The antibiotic activity of Kamoran was the same when compared with F4 fraction, and also F4 decreased flocculation and foam formation a fact not observed in the wine treated with Kamoran, and the yeast cake in this case could be used as animal food source.

The wine treated with F4 did not form foam even when combined with KM, this result suggested that KM did not influence F4 fraction against foam formation. On the other hand, the action of F4 decreased when combined with H<sub>2</sub>SO<sub>4</sub>, probably because the sulphuric acid highly decreased the pH. No difference was observed among KM, H<sub>2</sub>SO<sub>4</sub> and control that showed a high foam formation, showing that these compounds were efficient in bacteria control, but did not influence foam formation.

H<sub>2</sub>SO<sub>4</sub> showed medium flocculation, and this process occurred when the pH decreased [29]. Two factors should be involved, first the addition of sulphuric acid and a presence of *Lactobacillus* could decrease the pH until pH = 2.0 [30]. Also low pH influenced F4 fraction action, which showed high flocculation. On the other hand, F4 or F4 combined with KM the wine did not flocculate, and was very high with KM. It's clear that the action of F4 against flocculation was very high, and KM

did not influence F4 action. In the literature, we did not find information that related the action of compound produced by bacteria, which showed action against *Lactobacillus* sp. population, foam formation and flocculation, and it showed this effect first time.

The wine that showed more alcohol content was treated with Kamoram<sup>®</sup> and F4 fraction when compared with control. The reduction of bacteria population increases alcohol production, because it reduces losses of sacharose by CO<sub>2</sub> and/or lactic acid formation for bacteria population [9]. In this way in the wine treated with KM+F4 the *S. cerevisiae* took a sugar substrate most easily due to a low contamination level of *Lactobacillus* sp., but no difference of alcohol content was observed among treatment and control. The cellular viability of *S. cerevisiae* was high (up to 75%), and no differences were observed among treatments, this result indicates that F4 is completely safe for yeast.

The fraction F4 showed highest antibiotic activity against *Lactobacillus* sp. when compared with control and others treatments. The *Lactobacillus* sp. population was lowest in the wine treated with F4 fraction following by KM, H<sub>2</sub>SO<sub>4</sub>, and control. Also F4 highly decreased flocculation and loam formation, a fact not observed in the wine treated with Kamoran<sup>®</sup>. However, the amount of F4 used is high, because the fraction is semi purified. Probably when we obtain a pure molecules involved in this process, the amount used will decrease, which was observed in the other fraction (F3) that control Gram native bacteria [14].

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