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Chemical Inhibition of the Contaminant *Lactobacillus fermentum* from Distilleries Producing Fuel Bioethanol

Pedro de Oliva Neto[1*], Fabíola Aliaga de Lima[1], Ketrin Cristina da Silva[1], Douglas Fernandes da Silva[1], Ana Flavia Azevedo Carvalho[2] and Catarina dos Santos[1]

[1]Departamento de Ciências Biológicas; Laboratório de Biotecnologia Industrial; Universidade Estadual Paulista; Assis - SP - Brasil. [2]Faculdade de Engenharia de Alimentos; Universidade Estadual de Campinas; Campina - SP - Brasil

ABSTRACT

The purpose of this study was to determine the Minimum Inhibitory Concentration (MIC) of pure or mixed chemicals for Saccharomyces cerevisiae *and* Lactobacillus fermentum *in the samples isolated from distilleries with serious bacterial contamination problems. The biocides, which showed the best results were: 3,4,4' trichlorocarbanilide (TCC), tested at pH 4.0 (MIC = 3.12 mg/l), TCC with benzethonium chloride (CBe) at pH 6.0 (MIC = 3.12 mg/l) and TCC mixed with benzalkonium chloride (CBa) at pH 6.0 (MIC = 1.53 mg /l). If CBa was used in sugar cane milling in 1:1 ratio with TCC, a 8 times reduction of CBa was possible. This formulation also should be tested in fermentation steps since it was more difficult for the bacterium to develop resistance to biocide. There was no inhibition of* S. cerevisiae *and there were only antibiotics as an option to bacterial control of fuel ethanol fermentation by* S. cerevisiae.

Key words: Ethanol, lactic acid bacteria, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*

INTRODUCTION

Currently, bioethanol is considered an important recycled fuel and an alternative to fossil fuels. In Brazil, it is produced by fed-batch or continuous fermentation process from sugar cane using *Saccharomyces cerevisiae* in cell recycles. In this process, the microbial contaminants are also recycled. Bacterial contamination is an aggravating factor associated with several problems such as the consumption of sugar, alcohol and other medium components, reduction in ethanol yield (Oliva-Neto and Yokoya 1994, 1996); release of toxins and organic acids in the work, and decrease in the viability of yeast cells (Maiorella et al. 1983, Essia-Ngang et al. 1989, Dorta et al. 2007). It is well known that in the fermentation process, Gram-positive bacteria are the main agents of contamination, especially *Lactobacillus* spp. (Galo 1989; Oliva-Neto 1990). The antagonism between *L. fermentum* and *S. cerevisiae* is due to organic acids produced by the bacterial cells (Oliva-Neto and Yokoya 1994, 1996). Furthermore, *L. fermentum* is a contaminant agent responsible for yeast flocculation, which is associated with corrosion and obstruction of pumps and centrifuges and a decrease in antibiotic effectiveness and fermentation yield (Oliva-Neto and Yokoya 1991; Ludwig et al. 2001).

In the fermentation process, antibiotics are added to control bacterial contamination, but use of these compounds is limited. This method of control is limited to a few products. Currently, HJ Kamoran is widely used in Brazilian distilleries, but at a

*Author for correspondence: poliva@assis.unesp.br

high cost. Thus, the discovery of new products with antimicrobial activity against *L. fermentum* is necessary. Inhibitory action against the growth of *L. fermentum* has been determined for acid penicillin V (MIC 0.10-0.20 mg/L), clindamycin (MIC 0.05-0.40 mg/L), sulphite (MIC 10-40 mg/L), thiocianate (1.2-5.0 mg/L), formaldehyde (11.5-23 mg/L), cephamandole(0.26-1.45 mg/L), bromophenate (9-18 mg/L), methyldithiocarbamate (2.5 mg/L), copper sulphate (75-300 mg/L) and N-alkyl dimethyl-benzyl ammonium chloride or benzalkonium chloride (MIC 8.0 mg/L) (Oliva-Neto and Yokoya 2001). Polymixin B sulphate showed MIC of 64 mg/L against *L. fermentum*, which was more than 1024 mg/L for other species of *Lactobacillus* spp. (Flores et al. 2008). The MIC of gentamicin against *L. plantarum* was 128 mg/L, but this antibiotic did not perform well against other species of *Lactobacillus* spp. (Rojo-Bezares et al. 2006). According to Danielsen and Wind (2003), metronidazole was not effective in inhibiting *Lactobacillus* spp. (MIC> 200 mg/L). The surfactants sodium lauryl sulphate, benzethonium chloride and benzalkonium chloride were also evaluated for inhibiting the growth of *L. fermentum* and *S. cerevisiae* (Silva et al. 1997). The 3,4,4' trichlorocarbanilide demonstrated a selective inhibition against *L. fermentum* and *Leuconostoc mesenteroides* (Oliva-Neto and Yokoya 1998).

In this work, *in vitro* antimicrobial activity (MIC) against *L. fermentum* and *S. cerevisiae* was determined for several antimicrobial compounds aiming to find new alternatives for the control of bacterial contamination in fuel ethanol production.

MATERIALS AND METHODS

Microorganisms

The bacterial cultures used in this study were *L. fermentum* CCT 1396, isolated from an alcohol distillery by Oliva-Neto and Yokoya (1994), and *L. fermentum* CCT 0559, obtained from the Tropical Culture Collection (CCT), Campinas – Brazil. Both the strains were activated and maintained in Man Rogosa, Sharpe medium (Difco) at pH 4.5. The yeast strains used were *S. cerevisiae* CCT 4370, obtained from the Tropical Culture Collection, and *S. cerevisiae* FCLA M26, also isolated from an alcohol distillery (Oliva-Neto et al. 2004) and obtained from the Culture Collection of the Laboratory of Industrial Biotechnology, São Paulo State University (UNESP), Assis – Brazil. Yeast strains were activated and maintained in nutrient medium consisting of (%, w/v) 2 sucrose, 0.5 yeast extract, 0.1 $(NH_4)_2SO_4$, 0.114 $K_2HPO_4.3H_2O$, 0.0017 $MnSO_4.7H_2O$, 0.0028 $ZnSO_4.7H_2O$, 0.024 $MgSO_4.7H_2O$ and distilled water. All the cultures were incubated at 30°C for 24-48h.

Minimum inhibitory concentration (MIC)

The following chemicals were tested for MIC at 1-40 mg/l: nalidixic acid (Sanofi-Synthelabo Ltda.), pipemidic acid (Zambon Pharmaceutical Laboratories Ltda), phenazopyridine hydrochloride (Blanver Farmoquímica Ltda.), metronidazole (Aventis Pharma Ltda.), nitrofurantoin (Laboratory Teuto Brasileiro S/A), sulphasalazine (Apsen Farmacêutica S/A), sulphamethoxazole/ trimethoprim (Roche Chemicals and Pharmaceutical Products S.A), sulphadiazine silver (Pharma Nostra), gentamicin sulphate (Lab Duct Pharmaceutical Industry Ltda), sulfacetamide sodium (Henrifarma) and polymyxin B sulphate (American Farmasa Laboratory of pharmacotherapy SA). Pipemidic acid and nitrofurantoin were formulated with sodium dodecyl sulfate (SDS) (3:1 and 1:1, w/w) to evaluate the synergistic effect with this compound. The other tested chemicals were at 250– 4000 mg/L tartaric acid and sodium gluconate, and at 0.78-12.5 mg/L 3,4,4' trichlorocarbanilide (TCC), benzethonium chloride (Cbe), benzalkonium chloride (Cba), cetyl trimethyl ammonium chloride (CTA) and Hj Kamoran® (the antibiotic Monensin – Química Real - Ribeirão Preto - SP - Brazil). The products Cbe, Cba, tartaric and gluconate acids were dissolved in distilled water. TCC was dissolved in acetone. All the chemicals were autoclaved (121°C/15 min) or microfiltered (0.22 µm membrane Millipore - EUA).

The MIC of these chemicals was determined by adapted macrodilution broth method (Jones et al. 1985). Assays were performed in the tubes containing 6.0 mL medium. The inoculum was standardized according to MacFarland 0.5 standard in aseptic conditions. The cultures were incubated at 30°C for 24 h in an incubator and bacterial growth was aseptically measured by absorbance at 600 nm wavelength (A_{600}) by using a spectrophotometer (Spectronic 1100 Pharmacia - USA). The MIC was defined as the minimum

concentration able to inhibit at least 90% the microbial growth and performed in triplicate. The growth reduction was calculated as [1 − [(A_{600} 24 h with antimicrobial compound – A_{600} 0h with antimicrobial compound)/(A_{600} 24 h control – A_{600} 0h control)]] x 100 and expressed in %.

Tartaric acid showed a MIC of 2000 mg/L for *S. cerevisiae (*CCT 2652) and 4000 mg/L for *S. cerevisiae* M26 (Table 1). For sodium gluconate, a MIC more than 2000 mg/l was found for all the strains tested. These values of MIC, therefore, were not recommended for bacterial control, because the industrial dosage of biocides and antibiotics used in Brazilian distilleries have been 10-20 mg/L, and 3-4 mg/L, respectively. Furthermore, these products inhibited the yeasts as well as the bacteria.

From the pure cationic surfactants (Table 1) available, only cetyl trimethyl ammonium chloride - CTAC (Fig. 1) inhibited *L. fermentum* with a MIC between 3.12-6.25 mg/L. However, these values were close for *S. cerevisiae* (MIC 6.25-12.5 mg/L). Autoclaved or microfiltered Cba or Cbe showed higher values (MIC ≥ 6.25 mg/L) than CTAC for both *L. fermentum* and similar MIC for *S. cerevisiae*. These results showed that pure cationic surfactants (Cbe, Cba and CTAC) could not be not recommended for the control of bacterial contamination in the fermentation process of fuel ethanol by *S. cerevisiae*. However, Cba is currently used in cane milling steps of fuel ethanol production to bacterial control, mainly for the control of *Leuconostoc mensenteroides* and *Lactobacillus* sp at 10-20 mg/L. Ammonium quaternary (QA) compounds, dithiocarbamate and halogenated phenols have been used in Brazilian distilleries (Cereda et al. 1981). Sanitization with QA compounds has been performed and has saved as much as 60% of sugar loss from lactic acid bacteria contaminants in the sugar industry (Tilbury et al. 1977). N alkyl-di-methyl-benzyl ammonium chloride (Cba) showed a similar MIC (8.0 mg/L) for *S. cerevisiae*, *L. fermentum* and *L. mesenteroides* in fuel bioethanol distilleries, therefore, it would not be a practical additive for alcoholic fermentation process, since yeast cells could also be inhibited by this compound at the dosage necessary for bacterial growth inhibition (Oliva-Neto and Yokoya 1998).

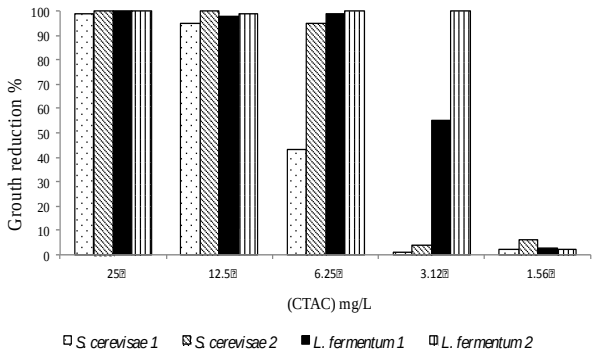

Figure 1 - Effect of cetyl trimethyl ammonium chloride (CTAC) on growth reduction (%) of *Saccharomyces cerevisiae* CCT 2652 (1) and FCLA M26 (2), and *Lactobacillus fermentum* CCT 0559 (1) and CCT 1396 (2).

Pure TCC presented an MIC of 6.25 mg/L for the tested bacterium at pH 6.0 and 3.12 mg/L at pH 4.0 (Table 1). The latter pH was similar to industrial conditions. This compound was effective against Gram positive bacteria. For example, an MIC against *Staphylococcus aureus* of 0.078 mg/L has been reported by Hamilton (1971) and TCC action against *L. mesenteroides* (MIC = 0.5 mg/L) and *L. fermentum* (0.5-2.0 mg/L) has been reported by Oliva-Neto and Yokoya (1988), which were similar to the levels reported in the present work. TCC did not inhibit *S. cerevisiae* growth in concentrations similar to *L. fermentum* (MIC= 12.5 mg/L). An MIC higher to 200 mg/L against *S. cerevisiae* has been reported by Oliva-Neto and Yokoya (1988).

The synergism between TCC and cationic surfactants CBe and CBa inhibited bacterial growth. A formulation with a mixture of these compounds was justified because TCC was not water soluble and pure ammonium quaternary compounds inhibited yeast growth. The combination of TCC and CBe (1:1 w/w) showed a decrease in the MIC of TCC against *L. fermentum* compared to pure TCC (Table 2). This microfiltered formulation was more effective (MIC 3.12 mg/l) than the autoclaved (MIC = 6.12 g/L). Nevertheless, the TCC/CBe formulation did not inhibit *S. cerevisiae* (MIC >12.5 mg/l) in the tested concentrations, which was an important condition for the application of this product on an industrial scale for fuel ethanol production.

Table 1 - Minimum Inhibitory Concentration (MIC) for several chemicals against *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, at 32°C for 24 h.

Chemicals	MIC (mg/L)		Cultures	
	S. cerevisiae 1 CCT 2652	***S.cerevisiae 2 FCLA M26***	***L. fermentum 1 CCT 0559***	***L. fermentum 2 CCT 1396***
Tartaric acid	2000	4000	≥ 4000	≥ 4000
Sodium gluconate	≥2000	≥2000	≥2000	≥2000
Acetone	>12.5	>12.5	>12.5	>12.5
CBe[1]	>12.5	>12.5	>12.5	>12.5
CBa[2]	>12.5	12.5	12.5	12.5
CBa[3]	12.5	6.25	>12.5	12.5
CTA[1]	12.5	6.25	6.25	3.12
TCC[1]	>12.5	>12.5	12.5	12.5
TCC[2]	>12.5	>12.5	6.25	6.25
TCC[3]	>12.5	>12.5	3.12	3.12

Symbols: TCC - 3,4,4' trichlorocarbanilide, CBe - benzethonium chloride, CBa - benzalkonium chloride, CTA – Cetyl trimethyl ammonium chloride. [1] - autoclaved product, culture of pH 6.0 for *L. fermentum*, [2] – microfiltered product, culture of pH 6.0 for *L.fermentum* [3] – microfiltered product, culture of pH 4.0 for *L. fermentum*.

Table 2 - Minimum Inhibitory Concentration (MIC) for different formulations of TCC and ammonium quaternary compounds and Hj Kamoran® against *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, at 32°C for 24 h.

Chemicals	MIC (mg/L)		Cultures	
	S. cerevisiae 1 CCT 2652	***S.cerevisiae 2 FCLA M26***	***L. fermentum 1 CCT 0559***	***L. fermentum 2 CCT 1396***
TCC+CBe[1]	>12.5	12.5	6.25	6.25
TCC+CBe[2]	>12.5	>12.5	3.12	3.12
TCC+CBe[3]	>12.5	>12.5	6.25	6.25
TCC+CBa 5:1[2]	>12.5	>12.5	6.25	6.25
TCC+CBa 2.5:1[2]	>12.5	12.5	3.12	3.12
TCC+CBa 1:1[2]	>12.5	12.5	1.56	1.56
TCC+ CBa 2.5:1[1]	>12.5	>12.5	12.5	12.5
HJ Kamoran[1]	>0.312	>0.312	0.156	0.078
HJ Kamoran[2]	>0.625	>0.625	0.312	0.156

Symbols: TCC - 3,4,4' trichlorocarbanilide, CBe - benzethonium chloride, CBa - benzalkonium chloride, CTA – Cetyl trimethyl ammonium chloride. Hj Kamoran – commercial product (antibiotic Monensin). [1] - autoclaved product, culture of pH 6.0 for *L. fermentum*, [2] – microfiltered product, culture of pH 6.0 for *L. fermentum*, [3] – microfiltered product, culture of pH 4.0 for *L. fermentum*.

The synergistic effect between TCC and CBa was also observed. The proportional increase of TCC in relation to CBe caused an increase in the MIC against *L. fermentum* (Table 2). The MIC observed with TCC/CBa at 5:1 (w/w) was 6.25 mg/L, but the best MIC was 1:1 (w/w) (1.56 mg/L) for *L.fermentum* and only 12.5 mg/L for *S. cerevisae* (Fig. 2). This result was probably observed because the surfactant led a better dilution of TCC in the broth, which was associated with the inhibitory action of these products. According these results, if CBa was used in sugar cane milling at 1:1 ratio with TCC, it resulted 8 times reduction of CBa since this value was the quotient of pure CBa and CBa:TCC (1:1) MIC. The MIC of autoclaved TCC/CBa (2.5:1, w/w) decreased four times in relation to this microfiltered formulation, probably because this formulation was not heat-resistant. However, TCC/CBa formulation showed a higher MIC (≥12.5 mg/L) against *S. cerevisiae* than *L. fermentum*. In this work, a ratio lower to 1:1 TCC:CBa was not available, but it was possible that the MIC against *L. fermentum* could be even lower. The improvement in antibacterial action of TCC when combined with surfactants follows the same pattern of TCC/Cbe. The surfactant probably improved the TCC solubility in bacterial cells, since the MICs of the chemicals CBa, Cbe, and TCC against *L. fermentum* were higher than those of the combined products.

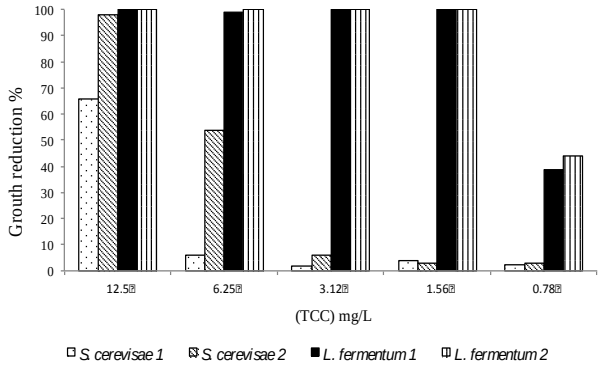

Figure 2 - Effect of 3,4,4' trichlorocarbanilide (TCC) and benzalkonium chloride (CBa) (1: 1 w/w) on growth reduction (%) of *Saccharomyces cerevisiae* CCT 2652 (1) and FCLA M26 (2) , and *Lactobacillus fermentum* CCT 0559 (1) and CCT 1396 (2).

The MIC of autoclaved Hj Kamoran® against *L. fermentum* was the lowest found in this study (0.078-0.156 mg/L) and higher to the microfiltered formulation (0.156-0.312 mg/L), probably due to its heat-resistance and loss in the microfiltration process (Fig. 3). Hj Kamoran® (antibiotic monensin) is currently used in bioethanol distilleries in Brazil and is the most important product for the control of bacterial infections in industrial alcoholic fermentation. The usual dosage in distilleries was formerly 1.0-3.0 mg/L (Oliveira et al. 1996) but has now been increased to 3.0-4.0 mg/l. This was probably due to an increase in bacterial resistance to the antibiotic.

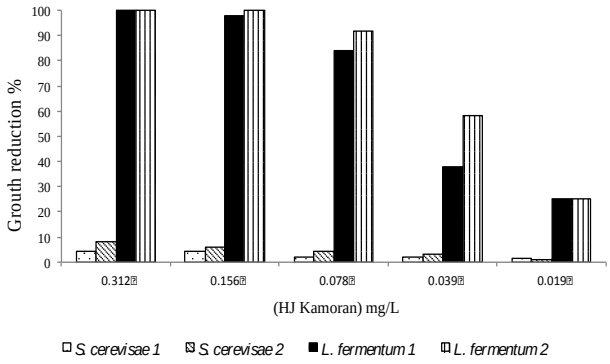

Figure 3 - Effect of HJ Kamoran® (monensin) on growth reduction (%) of *Saccharomyces cerevisiae* CCT 2652 (1) and FCLA M26 (2), and *Lactobacillus fermentum* CCT 0559 (1) and CCT 1396 (2).

Autoclaved Hj Kamoran® presented an average MIC against *L. fermentum* (0.117 mg/L) 13.3 times lower than formulated TCC/CBa (1:1) MIC. Nevertheless, despite the efficiency of Hj Kamoran®, the formulation TCC/Cba could be improved. It would be worth considering that these types of biocides were usually cheaper than antibiotics. Furthermore, the biocide mechanism of bacterial growth inhibition is generally less specific than that of antibiotics, and this factor may make it more difficult for bacteria to become the resistant to TCC/CBa.

Table 3 shows the MICs obtained for chemotherapeutic agents. Nalidixic acid, pipemidic acid, phenazopyridine, sulphadiazine silver, sulphasalazine, gentamicin sulfate, sulfamethoxazol/ trimethoprim, sulfacetamide sodium and polymyxin B sulphate showed MICs higher than 40 mg/L against *L. fermentum*. Since the usual concentrations of antibiotics used in fuel ethanol fermentation were lower than 4.0 mg/L, these compounds could not be recommended to control lactic acid bacterial contaminants of alcoholic fermentation. As for gentamicin, some authors have reported a resistance of 57.7% in *Lactobacillus* spp. from the fermented products (Olukoya et al. 1993). Moreover, it has been shown that the MIC of gentamicin for *L. plantarum* was almost 128 mg/L and indeed this antibiotic did not perform well against other species of the *Lactobacillus* genus (Rojo-Bezares et al. 2006). The MIC of polymyxin B sulphate for *L. fermentum* has been reported as 64 mg/l, which was higher than 1024 mg/L for other species of this genus (Florez et al. 2008). An MIC >1024 mg/L for sulphamethoxazole against *Lactobacillus* spp. has also been reported. These results confirmed previous reports about the intrinsic resistance presented by the genus *Lactobacillus.* Sulfas are competitive inhibitors of enzymes that convert the substrate para-aminobenzoic acid (PABA), an essential nutrient used by many bacteria, for use in the synthesis of the coenzyme folic acid (Tortora et al. 1998). Lactic acid bacteria, however, do not produce folic acid, and so sulfonamides such as sulfamethoxazol and sulfacetamide are inactive against *Lactobacillus* spp. (Katila et al. 2001).

Resistance to gentamicin is associated with membrane impermeability of the *Lactobacillus* spp. (Elkins and Mullis 2004). With respect to metronidazole, this antibiotic was not effective in inhibiting either bacterial or yeast growth,

presenting an MIC > 200 mg/l for *Lactobacillus* spp. Furthermore, it has been reported that 128 mg/l metronidazole may actually stimulate *Lactobacillus* growth (Choi et al. 2003). Nitrofurantoin was the only chemoterapeutic agent that provided an inhibitory effect against *L. fermentum*, (MIC= 15 mg/L), but it took higher concentrations than the antibiotics normally used in fuel ethanol fermentation. The effectiveness of nitrofurantoin against *Lactobacillus* spp. has been previously reported (MIC 8.0 mg/l). More specifically, for six strains of *L. acidophilus*, its MIC was reported as 2.0 mg/L (Danielsen and Wind 2003).

Table 3 - Minimum Inhibitory Concentration (MIC) of several chemotherapeutic agents against *Lactobacillus fermentum* and *Saccharomyces cerevisiae.*

Antimicrobial Agents	**Minimum Inhibitory Concentration (mg/L) Cultures**			
	***L. fermentum* CCT 1396**	***L. fermentum* CCT 0559**	***S. cerevisiae* CCT 4370**	***S. cerevisiae* FCLA M26**
Gentamicin sulphate	>40	>40	>40	>40
Metronidazole	>40	>40	>40	>40
Nalidixic acid	>40	>40	>40	>40
Nitrofurantoin	15	15	>40	>40
Nitrofurantoin+SDS(1:1)	15	15	>40	>40
Nitrofurantoin+SDS(3:1)	15	15	>40	>40
Phenazopyridine hydrochloride	>40	>40	>40	>40
Pipemidic acid	>40	>40	>40	>40
Polymyxin B sulphate	>40	>40	>40	>40
Silver sulphadiazine	>40	>40	>40	>40
Sulfacetamide sodium	>40	>40	>40	>40
Sulphamethoxazole/trimethoprim	>40	>40	>40	>40
Sulphasalazine	>40	>40	>40	>40

CONCLUSION

There was a synergistic effect between 3,4,4' trichlorocarbanilide (TCC) and benzethonium chloride (CBe) or benzalkonium chloride (CBa), improving the inhibitory action against the growth of *L. fermentum*. These formulations also increased the selective action only on bacteria, not inhibiting the growth of *S. cerevisiae*. The best MIC (1.56 mg/L) of the combined chemicals against *L. fermentum* was obtained by the formulation TCC: CBa 1:1 (w/w). CBa could be tested in sugar cane milling in 1:1 ratio with TCC since a reduction of 8 times in the use of this product was possible. This formulation also should be tested in fermentation steps since it was more difficult for the bacteria to develop resistance to biocide. There was no inhibition of *S. cerevisiae*. Currently, there are only antibiotics as an option to bacterial control of fuel ethanol fermentation by *S. cerevisiae.* The present results demonstrated the importance of further studies about the TCC mixed with surfactants for the improvement of fuel bioethanol technology.

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