COMBINED USE OF ETHANOL AND SODIUM CHLORIDE IN THE CONTROL OF BACTERIAL CONTAMINATION IN ETHANOLIC FERMENTATION

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ABSTRACT

Ethanol production in Brazil faces challenges due to the presence of microbial contaminants, especially lactic acid bacteria. Traditional control approaches, such as sulfuric acid and antibiotics, have limitations, including high costs, potential risks, and antibiotic impregnation in yeast mass intended for sale. This study proposes the combined use of ethanol and sodium chloride (NaCl) as an antimicrobial strategy to control the growth of *Limosilactobacillus fermentum*, verifying the efficacy in non-proliferative conditions, and during batch fermentation with cell recycle in co-culture with *Saccharomyces cerevisiae*. The results showed a bacterial decrease of almost 2 logarithmic cycles under non-proliferative conditions with 100 g/L NaCl + 4% ethanol (v/v). In fermentation tests, a significant reduction in the bacterial number was observed after the 2nd fermentation cycle, with no effect on *S. cerevisiae* number an on the fermentation parameters. A less costly and more environmentally friendly solution such as NaCl and ethanol rather than sulfuric acid could be a good option to reduce bacterial contamination to manageable number without the need for additional antimicrobial.

Keywords: Ethanol. Microbial contamination. *Limosilactobacillus fermentum*. Antimicrobial. Batch fermentation.

1 INTRODUCTION

Ethanol production in Brazil faces significant challenges related to microbial contamination, from the initial stages in the field to fermentation steps. This issue is attributed to non-aseptic conditions during harvesting, substrate treatment, and bioprocess operation.[1]. Prevalent contaminations in these steps are often caused by lactic acid bacteria such as *Lactobacillus*, with *Limosilactobacillus fermentum* highlighted as one of the main culprits impairing fermentation.[2].

It is crucial to control contaminating microorganisms in the fermentation tank to ensure the efficiency of the fermentation process. For this purpose, the predominant practice in bioethanol industries in Brazil involves the use of antimicrobial treatments such as sulfuric acid or antibiotics to control the bacterial contaminants. These methods, besides representing a high cost to the industry, can lead to additional problems, such as the risk to employees in the case of sulfuric acid. Furthermore, antibiotics can result in losses to the commercialization of dried yeast at the end of the harvest due to the impregnation of cells with these substances.[3].

Sodium chloride (NaCl) is utilized for food preservation since the Neolithic era. This substance is able to reduce the growth of a number of bacteria, including lactic acid bacteria.[4]. A reduction up to 68% in growth of lactic acid bacteria at 5% NaCl was demonstrated.[5]. In association with ethanol, Albers et al.[6] demonstrated the efficacy of using these substances as antimicrobials in wood hydrolysate medium, dropping the bacterial viability without decreasing yeast viability when 50 g/L of NaCl combined with 20 g/L of ethanol was used.

This antimicrobial treatment using NaCl together with ethanol has not yet been tested in the bioethanol industry. The aim of this study was first to determine the NaCl concentration in combination with 4% ethanol (v/v) that would result in the greatest reduction of *L. fermentum* in non-proliferative conditions. Further, the efficacy of the combined treatment and the effects on the fermentative parameters were evaluated in batch fermentation with cell recycling conducted by an industrial strain of *Saccharomyces cerevisiae* under bacterial contamination.

2 MATERIAL & METHODS

Strains of *L. fermentum* (CCT0559/ATCC9338) and *S. cerevisiae* (PE-2) were used in the experiments. Slants were maintained onto MRS and YPD media, respectively for bacterium and yeast, at 4°C, and frequently transferred to fresh medium for assays.

First experiment: the bacterium was cultured in MRS broth at 35°C overnight, and the optical density (at 600 nm) was adjusted to approximately 0.8 (approximately 10^9 CFU/mL) and used as inoculum. A volume of 5 mL was added to 125-mL Erlenmeyer flasks containing 45 mL of NaCl solution at various concentrations (25 g/L, 50 g/L, 75 g/L, 100 g/L) with the addition of 4% ethanol (v/v). The flasks were maintained for 2 h at 160 rpm and 30°C. Samples were taken, diluted serially, and plated onto MRS medium to determine the bacterial number (CFU/mL). The results were expressed as logarithmic reduction in relation to the number of bacteria in the control treatment (only water).

Second experiment: for the fermentation test, the methodology of Raghavendran et al.[7] was used in batch system with sugarcane juice, and cell treatments consisting of 100 g/L NaCl + 4% ethanol (v/v) or only water, for 2 h. One cycle of cell

treatment and two fermentation cycles were carried out. The bacterium was inoculated in the *pé-de-cuba* step at 5 x 10^8 CFU/mL. Initial yeast concentration was around 1 x 10^8 cells/mL. In the cell mass, the number of *L. fermentum* was determined as described above. Yeast cell number was determined in Neubauer chamber after methylene blue staining. In the supernatant after fermentative cycles, pH (determined in pH-meter), alcohol content (distillation of samples and density determination), and total reducing sugar concentration (by 3,5-dinitrosalicylic acid method after sample hydrolysis[8]) were determined. Fermentative efficiency (%) was calculated based on the theoretical Gay-Lussac equation (0.51 g ethanol/g total reducing sugar consumption). Statistical analysis consisted in Analysis of Variance and Tukey´s test ($p<0.05$).

3 RESULTS & DISCUSSION

The reduction in the number of *L. fermentum* in non-proliferative conditions reached almost 2 log cycles with the concentration of 100 g/L NaCl + 4% ethanol v/v (Figure 1). A reduction of 3 log cycles in the viable bacteria was obtained by Albers et al.[6] with the combined use of 50 g/L NaCl + 20 g/L ethanol (approximately 2.5% v/v ethanol) in hydrolysate medium, i.e., in proliferative conditions. These authors also used a mixture of *L. fermentum, Lactobacillus buchneri, Acetobacter syzygii* and *Acetobacter tropicalis*. A similar effect was obtained with 25 g/L NaCl + 40 g/L ethanol (approximately 5.0% v/v ethanol).

Comparing to the treatment with sulfuric acid in the industrial conditions (pH 2.5 solution with an approximate concentration of 4-5% ethanol v/v present in the *wine* that comes along with the yeast mass after centrifugation), there was a substantial difference in the logarithmic reduction by using the combined use of NaCl and ethanol once a complete loss of bacterial viability is obtained in acid condition (Figure 1).

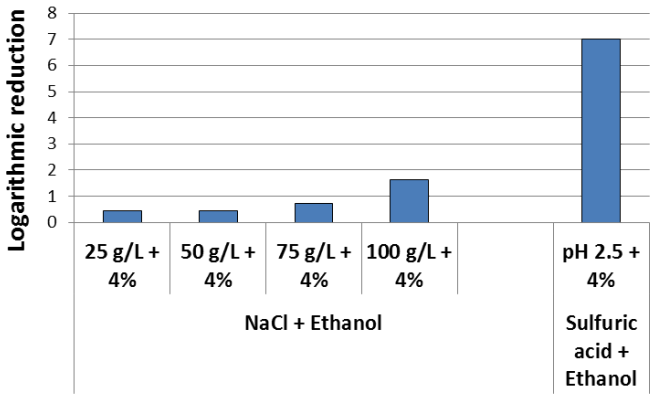

Figure 1 Logarithmic reduction of *L. fermentum* number (CFU/mL) in non-proliferative conditions consisted of sodium chloride (NaCl) in different concentrations and 4% ethanol (v/v) compared to the acid treatment with sulfuric acid (pH 2.5) and 4% ethanol (v/v). Conditions: initial number of cells of 10^7 CFU/mL; 35ºC; 150 rpm; 2 h.

A batch fermentation with cell recycle was carried out to evaluate the effect of the treatment 100 g/L NaCl + 4% ethanol on the *S. cerevisiae* and *L. fermentum* in co-culture, and on the fermentative parameters. When comparing the yeast number after fermentation in cycles 1 and 2 and between the two cell treatments (water as control), there was no significant difference among them. It means that the combined treatment of NaCl and ethanol did not influence the yeast viability (Figure 2a). Regarding *L. fermentum*, a significant decrease in the number was verified after the 2nd fermentative cycle (1 log cycle). Comparing the bacterial number before and after the combined treatment of NaCl and ethanol, a drop of 0.8 log cycles was observed (Figure 2b), which means that the treatment had a long term effect on the bacterium once the fermentation takes place in the same tube as the treatment step was developed. The salt – NaCl – remained in the tube along the fermentation process, with continuous ethanol production, what had effect on the bacterial viability.

Ethanol production was not affected significantly by the cell treatment. Final pH and fermentative efficiency did not differ substantially between the control (water) and the combined treatment of NaCl and ethanol (Figure 3). Albers et al.[6] observed an increase in the ethanol production using the combined treatment but the authors credited this result to the fact that the treatment reduced the bacterial viability with a positive impact on yeast performance in lignocellulosic hydrolysate medium.

This work demonstrated by the first time the combined treatment of NaCl and ethanol as an alternative to acid treatment in the alcoholic fermentation to control bacterial contamination. Further studies should evaluate the long lasting effect of the treatment on the bacterial viability and on fermentative efficiency by testing more fermentation cycles. Even with the more remarkable effect of acid solution on *L. fermentum*, it must be considered that reducing the number of bacteria to values below 10^8 CFU/mL (above which results in fermentative loss[3]) allows the industry to continue the fermentation process without the need to use antibacterial, and thus, a less costly and more environmentally friendly solution such as NaCl and ethanol could be a good option.

4 CONCLUSION

The combined use of 100 g/L NaCl and 4% ethanol (v/v) reduced the number of *L. fermentum* by almost 2 log cycles in non-proliferative conditions. During batch fermentation with cell recycle in sugarcane juice, this cell treatment did not affect *S. cerevisiae* number nor the fermentative parameters but it reduced significantly the bacterial number. A less costly and more environmentally friendly solution such as NaCl and ethanol rather than sulfuric acid could be a good option to reduce bacterial contamination to manageable number without the need for additional antimicrobial.

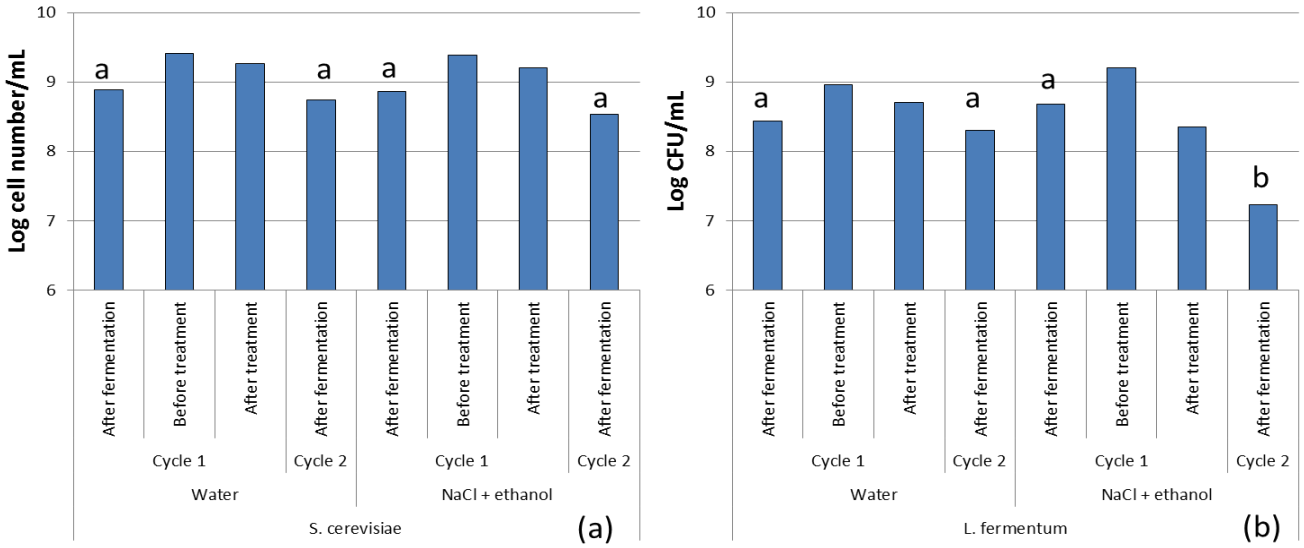

Figure 2 Log of *S. cerevisiae* (a) and *L. fermentum* (b) numbers during batch fermentation in sugarcane juice with cell recycle. The cell treatments between the fermentative cycles were water (control) or 100 g/L sodium chloride (NaCl) and 4% ethanol (v/v). Different letters above the bars indicate significant difference by Tukey´s test ($p<0.05$) for each microorganism. Initial number of microorganisms: 10^8 cells/mL or CFU/mL (for yeast and bacterium, respectively).

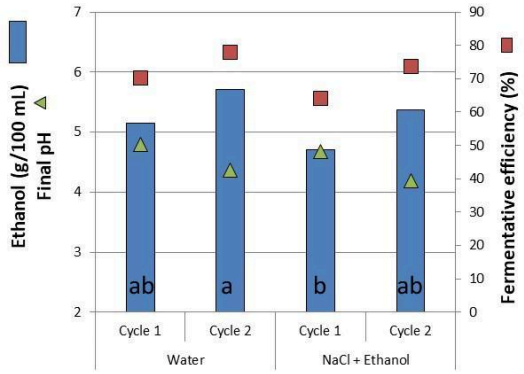

Figure 3 Ethanol production, final pH and fermentative efficiency of the batch fermentation in sugarcane juice carried out by *S. cerevisiae* contamined with *L. fermentum.* The cell treatments between the fermentative cycles were water (control) or 100 g/L sodium chloride (NaCl) and 4% ethanol (v/v). Different letters inside the bars indicate significant difference by Tukey´s test ($p<0.05$) among the ethanol concentration.

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