

EFFECTS OF BIOCIDES ON LACTIC ACID AND ACETIC ACID BACTERIA, POLLUTANTS IN THE PRODUCTION OF FUEL ALCOHOL.

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ABSTRACT

Within the group of biofuels of plant origin, fuel alcohol from sugar cane stands out. It is used as a source of sugars, producing byproducts such as honey A, which is extracted from 75% of the recoverable sugar, honey B when it completes 86 % crystallization and molasses. During the production of ethanol by fermentation with *Saccharomyces cerevisiae*, bacterial contamination on the raw material must be kept controlled, because the bacteria reduce the availability of carbon sources for the yeast to carry out its fermentation process, therefore, The most common contaminants are Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (BAA), due to this the biocide effect on the contaminating bacteria BAL and BAA was evaluated as a control measure in the fermentative stage of fuel alcohol production. The ideal concentration of the biocide PROQUAT BC 50 and the exposure time of PROCIDE BIOQ were found, the effect of which was to reduce the contaminant load produced by BAL and BAA in the fuel alcohol production process, at the selected points (fermenter and vinasse), likewise, the interaction between yeast-bacteria and yeast-yeast, as well as the effect on the performance of *S. cerevisiae* with the previous selection of bacteria and the classification of yeast-bacteria interactions, developing interaction studies in two different fermentation media, in a first stage of study, the interactions were analyzed in MRS and WL medium; means used to evaluate the behavior of interactions.

Keywords: Sugars, biocides, fermentation, interaction and byproducts.

Introduction

Biofuels of vegetable origin are widely used nowadays, within this group the alcohol fuel stands out, this is a fuel that has been implemented several years ago in developed countries such as the United States, from corn. Russia, central and northern Europe, of the beet. In countries located in the tropics the raw material has been cassava. In Latin America, Brazil started using it more than 25 years ago, the Dominican Republic and Ecuador are starting to implement it. (Peña M, 2020)

Colombia is no stranger to this movement and begins to write its history with Law 693 of 2001 in which it was stipulated that, as of September 2005, cities with more than 500,000 inhabitants must have their gasoline mixed with 10% ethyl alcohol. On April 14, 2003, the joint resolution between Minminas and Minambiente N° 447 determined the quality requirements of alcohol. Subsequently, by means of Resolution 180687 of June 17, 2003, the technical and safety requirements were established in relation to the production, collection, distribution and mixing points of fuel alcohols. Finally, by Resolution 180836 of July 25, 2003, the Ministry of Mines and Energy defined the price per gallon for alcohol fuel at the refinery gate, which is equivalent to approximately US\$ 1.20 per gallon, for the year 2022

through Resolution 40095, of March 11 of the same year, the income to the producer of alcohol fuel was set at COP 13,048.21 an equivalent to US\$ 3.22 as indicated by the Colombian Association of Sugar Cane Producers and Suppliers (PROCAÑA), “Ingenios como Manuelita, Incauca, Mayagüez and Providencia allocate a percentage of their crude sugar production to the elaboration of this biofuel that allows the incursion into the production of renewable energies.

Fuel alcohol is a flammable compound that has no color and has an odor characteristic of alcohols. It is a product from sugar cane used as a source of sugars that act as a carbon source (Suarez, A., 2020). As stated by the Ministry of Agriculture in 2020 with Decree 527, this type of renewable biofuel is the result of the fermentation of the by-products of the sugar production process, such as Honey A, Honey B and molasses reaching a production of 412 million liters per year, this process occurs by the action of the yeast *Saccharomyces Cerevisiae*, which takes these sugars and converts them into alcohol, which is subjected to a distillation, evaporation and dehydration process, with the aim of to purify it, thus remaining, the fuel alcohol or anhydride with a purity of 99.6%.

During the process of ethanol production by fermentative route with *S. cerevisiae*, bacterial contamination on the raw material must be kept controlled, because the bacteria create a constant decrease of the carbon sources available for the yeast to carry out its fermentative process (Camacho, M., 2021). Among the most common contaminants are Lactic Acid Bacteria (LAC), they are a group of Gram-positive microorganisms, not forming spores, cocci or bacilli, catalase-negative, oxidase, with high tolerance to low pH and Acetic Acid Bacteria (BAA) as contaminants in fermentation, these are Gram-negative bacteria, ellipsoidal or cylindrical in shape that can be isolated, in pairs or forming chains, they are mobile by flagellation, have positive catalase, negative oxidase activity. The BAL are characterized by the production of lactic acid as the only product, which is why they are called homofermentative, on the other hand, there are the heterofermentative ones, which produce acetate, ethanol and carbon dioxide. Among the best known genera are *Lactobacillus* sp, *Sporolactobacillus* sp, *Zymomonas* sp, *Micrococcus* sp, *Acetobacter* sp and *Gluconobacter* sp, the most important genera being (Pilco et al., 2023).

On the other hand, Acetic Acid Bacteria (AAC) are known to oxidize sugars and alcohols, obtaining as a final product an accumulation of organic acids, a capacity that is used in the food industry for the production of vinegars and fruit wines, however, in the bioprocess of ethanol production they negatively affect the yield. Through enrichment and isolation techniques in plates, it is possible to identify at the species level those bacteria (genera *Acetobacter* sp, *Gluconobacter* sp and *Gluconacetobacter* sp) that could be used for the development of a starter culture suitable for the biooxidation of alcoholic musts (Gómez, J., 2020). In addition, bacterial contamination causes a significant loss of time, as well as an increase in the cost of the final product. The presence of these pollutants generates considerable economic losses for industries, because they compete with yeasts for fermentable sugars and other nutrients present in the fermentation raw materials, resulting in a reduction in ethanol production (Díaz, p., 2021; Reisman, 2019).

In view of the above, technological progress and research in fermentations have been of great importance, since significant contributions were made to the development and evolution of the alcohol industry (Portocarrero, et al., 2018). Such technological advances have focused on obtaining a better control of the fermentation process mitigating the contamination by BAL and BAA, controlling the fermentation process by reducing yeast stress conditions guaranteeing a high yield in terms of the transformation of a substrate such as molasses,

which is a dense and blackish liquid constituted by the residue that remains in the vats after the extraction of most of the cane sugars, into liquid fuel (Gómez, A., and Pérez, A., 2018).

For the control of contamination different methods are used that include antibiotic treatments and for the purposes of this work two biocides were used, which are substances of general or specific action against harmful organisms such as: bacteria, fungi and other microorganisms. The first biocide used is PROQUAT BC 50 based on fifth generation quaternary ammonium and the second is PROCIDE BIOQ which, according to Patiño, D et al., in the year 2018, is a disinfectant that combines the potential of polymeric guanidines with the disinfecting power of fifth generation quaternary ammoniums, of low toxicity, but, broad spectrum at low concentrations.

Therefore, the objective of this research was to evaluate the biocidal effect on BAL and BAA, pollutants, in the production of fuel alcohol as a control measure for contaminating bacteria in the fermentation stage of fuel alcohol production.

Materials and Methods

La metodología The methodology was carried out in accordance with the established parameters and the norms that are consigned, both in the area of the Microbiology Laboratory of Valle del Cauca, and in the same processes that are carried out; giving an accurate compliance to what is allowed.

Sampling

The samples were taken at the alcohol production plant of a mill in the Valle del Cauca, for this purpose the area where the samples are taken with 97% alcohol was disinfected, from the tanks of raw materials of final Honey, Vinasse, and from the fermenter for their respective analysis.

Isolation of contaminating bacteria from the fermentation process

An aliquot of 100 mL of sample was taken at the points where there was evidence of a decrease in yeast yield in terms of fermentation and biomass production, such points were the fermenter and the Vinasse tank. (Estrada, D et al., 2016; Prado, L., 2018)

In the first stage, the colonies of the contaminating bacteria were characterized in selective media, for the BAL the MRS Agar developed by Man, Rogosa and Sharpe was used in which it is an isolation medium for the growth and enumeration of Lactobacilli and other lactic acid bacteria in samples from different sources, for BAA the WL differential Agar was used being this the indicated medium for the counting of bacteria in fermentation processes (Pilco, C., et al., 2023). For this they were left at rest, inhibitors such as cyclohexamide (MRS and WL) and aniline blue (MRS) were added, thus guaranteeing the isolation only of bacteria with the inhibition of molds and yeasts. (Gil M, 2019).

Serial dilutions were made for the vinasse and fermenter sample of 10⁻² and 10⁻³ by seeding each dilution in duplicate on WL Agar, in the same way serial dilutions of 10⁻² and 10⁻⁴ were made and seeded in the MRS medium in duplicate in sterile environment, incubated at 36°C and reading was performed between 24 to 48 hours on the bacterial isolates. (Camacho, M., 2021).

As inclusion criteria, the macroscopic and microscopic characteristics of reaction in the culture medium of the colonies were taken into account, in the selective solid media, which showed typical characteristics of lactic acid and acetic acid colonies, the selection of the

desirable species in the research was indispensable to take into account the physicochemical characteristics that occur in the fermentation process when obtaining the alcohol.

Yeast isolation and stress test

In the fermentation process on a laboratory scale, it was based on the study of Aminahue, C Et al 2017, where from the samples of Vinasse and Fermenter N° 4, 10-1, 10-2 and 10-3 dilutions were made and the plate seeding technique was used. The dilutions were seeded on Chloramphenicol Glucose Agar (YGC), Subsequently, the colonies that were morphologically different from the yeast isolates were chosen and seeded at different temperatures of 25°C , 35°C, and 40°C for 72 hours, under microaerophilic conditions, in order to guarantee the desired conditions for their respective growth, this was done in order to compare at which temperature they developed better, in this way to perform the count of yeast-like colonies

Once the incubation time had passed, the first thing that was done was the observation of the reverse and the obverse of the plates, also to see if there was presence or absence of growth, the size, the color and the appearance of the yeasts, for which fresh mounts were made with methylene blue and observed under a microscope. (Daza & Meyer, 2019).

Evaluation of the MIC (Minimum Inhibitory Concentration) by the microdilution technique in tubes for lactic acid and Acetic bacteria

The methodology described by Guerrero (2018) was used with modifications relevant to this study and to the needs of the microbiology laboratories of the fuel alcohol plant where the present research work was developed.

To evaluate the MIC, subcultures of young strains of BAL and BAA, isolated from the fermentation process, were made in MRS and WL selective media respectively; then concentrations between 0.78 ppm and 200 ppm were prepared (See Table 1) of the biocides, PROCUAT BC 50 and PROCIDE BIOQ, then the inoculum was prepared, which, was standardized in the HermleZ206A spectrophotometer using a wavelength of 560 nm, until reaching absorbance of 0.8 ABS equivalent to 1x10⁸ CFU/mL, consecutively 0.1 mL of the microorganism inoculum was taken and added to each tube. Tube number 10 was used as a control. Subsequently, plating was performed in MRS and WL medium of each of the tubes, After 72 hours of interaction in incubation at 37°C, between the different concentrations of the biocide and the inoculum, the turbidity of each tube was observed, and, finally, the variation of contamination was compared and evaluated, using the following formula:

$$\text{Efficiency} = \frac{\frac{\text{CFU}}{\text{ml}} \text{With application of biocide}}{\frac{\text{CFU}}{\text{ml}} \text{Without application of biocide}} \times 100$$

After obtaining the data when calculating the efficiency, the concentrations were compared using analysis of variance (ANOVA) and Tukey's test.

Table 1

CMI (Minimum Inhibitory Concentration) Distillery Laboratory, Ingenio Valle del Cauca.

Tube No.	1	2	3	4	5	6	7	8	9	10
Concentration	200 ppm	100 ppm	50 ppm	25 ppm	12,5 ppm	6,25 Ppm	3,12 ppm	1,56 Ppm	0,78 ppm	Control

Note: Obtained by the authors from different tests, 2022.

PPM: Parts per million. Lines with a negative sign (-) indicates the absence of turbidity or absence of Counting.

Effect of PROCIDE BIOQ biocide on vinasse

To determine the effect of the biocide PROCIDE BIOQ, a sample of vinasse was taken, when the lactic acidity at 0 hours reached a value of 5040 ppm and the fresh bacillus count at 0 hours was 1.01×10^8 CFU/mL by the plate counting technique. It was compared with a blank containing no biocide, and with two doses of PROCIDE BIOQ at 300 ppm and 600 ppm, directly to 2 L of wort. All treatments were shaken at 70 rpm at $30 \pm 2^\circ\text{C}$, after 24 hours, a bacillus count, lactic acid determination and cell density determination were performed.

Results and Discussion

Selection of bacteria

Las cepas The bacterial strains with phenotypic characteristics selected were blue colonies, circular shapes, symmetrical and asymmetric edges, creamy and dry consistency, convex elevation, Gram-positive colorations, microscopic morphology of coconuts and bacilli forming chains (Gómez, J, 2020).

Green colonies, convex elevation, creamy consistency, slow colony growth, Gram negative coloration, coconuts and bacilli were observed on WL agar, in addition to an agar clearance that indicated a pH change in the medium, slow colony growth, as demonstrated in the studies of Salazar, L in 2017 in his article, for the characterization of acid-acetic bacteria in cocoa fermentations, where they are important for the production of acetic acid, since this oxidizes ethanol and acids (citrus, malic and lactic), and finally this acetic acid manages to become carbon dioxide and water, to continue with the cocoa production process.

For his part, in the research of Villalba, M., in 2022, he states that the number of lactic acid bacteria during alcoholic fermentation is usually very low, at most 102 per mL, since most are inhibited by ethanol and by the SO₂ added to the wort to control the bacterial population, especially the acetic ones. When the alcoholic one ends and the yeasts die, some lactic bacteria can thrive and achieve a certain growth, sometimes up to 107 per mL. These lactic acid bacteria produce some transformations in wine, the most interesting of which is the so-called malolactic fermentation (MLF).

These bacteria have also been found in juice, honey and cane sugar that participate in ethanol production, where they compete with yeast for nutrients and generate compounds that inhibit the growth of this such as acetic acid and acetaldehyde, also causing the increase of volatile acidity in the wort. Also, in the morphological characterization of lactic and acetic bacteria a significant increase was evident, on the other hand, the amount of contamination indicated a growth of 3.00×10^5 CFU/ml for the BAL and 2.40×10^4 CFU/ml for the BAA in the samples of the fermentates and the tanks. Also, proving to be one of the results of great importance because lactic acid and acetic bacteria become contaminants of the fermentation system due to their high capacity to inhibit the processes of massification of yeast which intervenes in the continuous production of ethanol.

Therefore, once the morphological characterization and the count of the yeasts and bacteria were carried out, it could be observed that after the treatment of the biocide PROQUAT BC 50, there was a decrease in the contaminant bacterial loads such as 6.30×10^4 CFU/mL for the BAL and 5.40×10^3 CFU/mL for the BAA, which represents a decrease of 91.4%. This led to an increase in yeast biomass by 96%. It should be noted that so far it is the first time that tests have been carried out in the alcohol plant with the use of the Biocides previously exposed, to determine such interactions, which occur between bacteria-yeast in the process of generating fuel alcohol.

Consequently, the interaction of *Saccharomyces cerevisiae* and contaminating yeasts affects the fermentation process to a lesser extent compared to the interaction of *Saccharomyces cerevisiae* and contaminating bacteria (Pilco et al; 2023), so the impact of the biocide used on other species could reveal behaviors of greater interest, due to the contribution of volatile compounds that are mainly produced by non-*Saccharomyces* species. (Riascos and López, 2016).

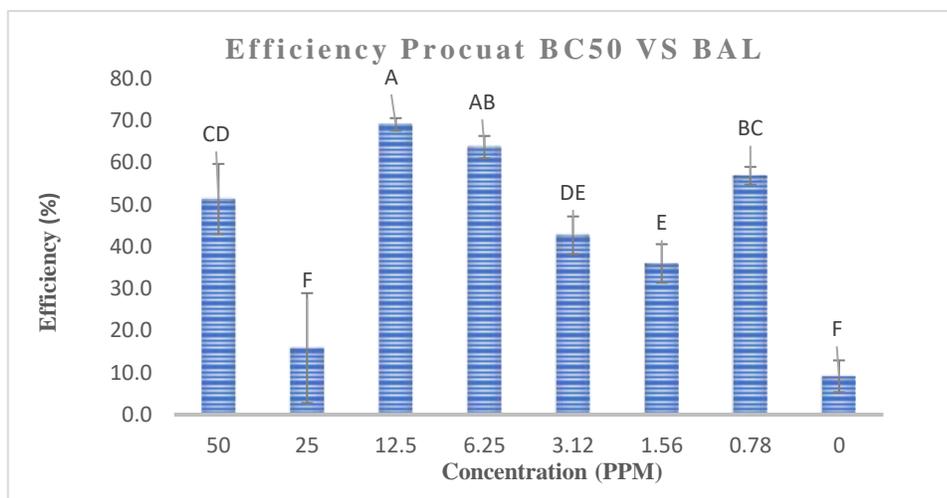
Biocides tests and analysis: The samples collected for the tests and evaluation of the biocides, PROQUAT BC 50 and PROCIDE BIOQ, were chosen from the fermentates N°4 and the vinasse tank, the main reason why this was chosen is because lactic and acetic acid allows to conceptualize higher levels of contamination, for which a value of 400 CFU/ml for the BAL and 389 CFU/ml for the BAL in the fermentation processes could be found.

Another reason for choosing these samples is because, if the vinasse is not free of contamination, in the fermentation chain, it does not work and the production does not reach the adequate levels that are required daily, affecting the performance of the yeast, which by their nature are resistant to antibiotics (Zielińska, M., Bernat, K and Mikucka, W., 2020).

The decrease in the yield of the process can occur when the yeast faces acidic conditions during fermentation, with contamination levels between 105-106/ml in the tanks.

As for the biocide PROQUAT BC 50 tested in the BAL samples and the PROCIDE BIOQ in the BAA obtained in the fermenter N°4 and vinasse, a MIC of 13.1 ppm was obtained for lactic acid bacteria in the tube test and 25 ppm for the plate evaluation, from there it was observed a decrease in microbial growth. This biocide was chosen for the BALS, because the concentration of the biocide affected their growth (See Figure 1). On the other hand, the effectiveness of their product was considered to verify which was the best contraction of the same. All this in order to give decrease to the contamination of lactic acid bacteria.

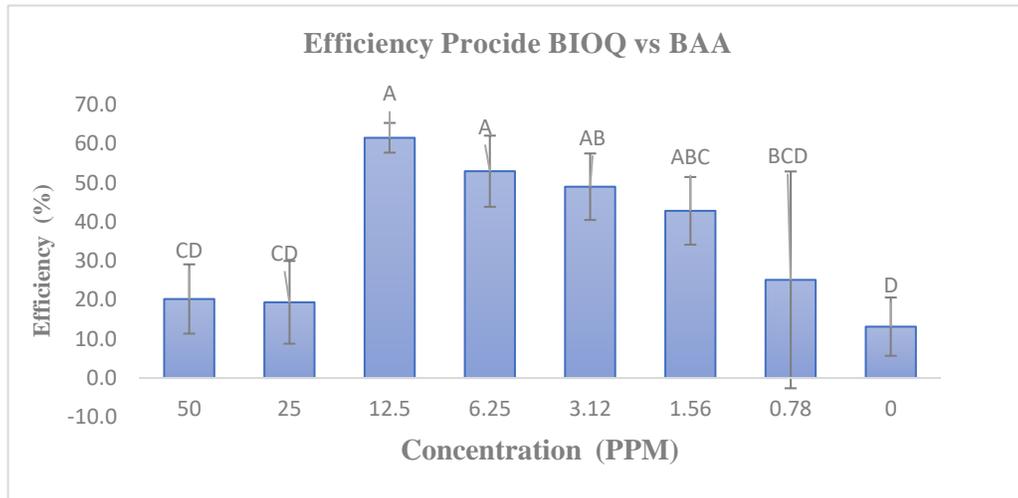
Figure 1. MIC of the tested Biocide (PROQUAT BC 50) for contaminating lactic acid bacteria in the: Fermenter N°4 and vinasse, was evaluated by turbidity (Microbiology Laboratory Distillery/ Ingenio Valle del Cauca).



On the other hand, for acetic bacteria in tubes the MIC was 12.5 ppm, this biocide was evaluated only in the test in tubes, the reason is given because this biocide affects its growth by the type of biocide and by the active component found in it, for this type of bacteria, since yeast is more decreased in its concentration is by lactic acid bacteria than by acetic acid bacteria, see figure 2. With this biocide, BAAS were studied, where it affects their growth by the type of biocide used and more by the active component found in it, for this type of

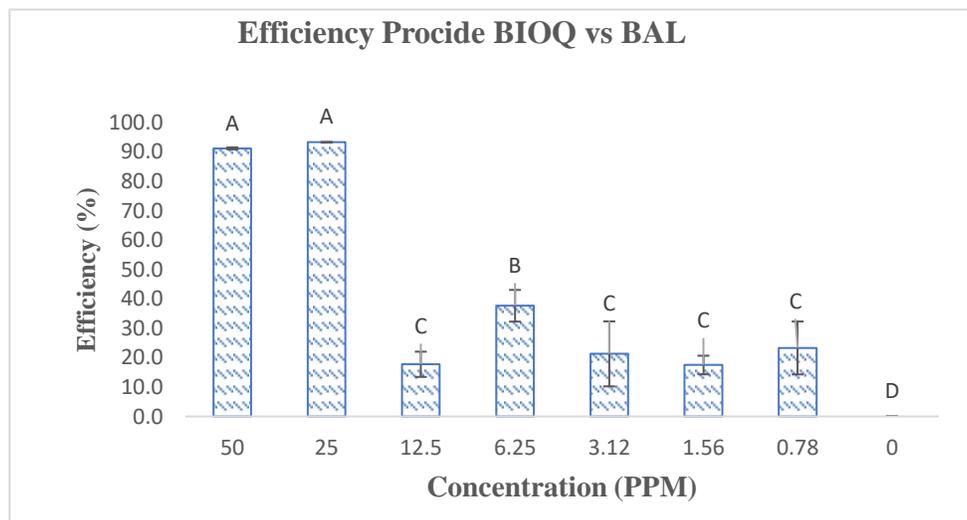
bacteria. also in the box count, this biocide was used again due to its great effectiveness, because it reduces the burden of contamination without affecting the yeast population so much.

Figure 2. (PRICIDE BIOQ) for contaminating acetic acid bacteria in the N°4 and vinasse fermenter. It was evaluated for turbidity.



Likewise, for the PROCIDE BIOQ biocide tested in the fermenter and the vinasse tank, it was found that there was an inhibitory effect of contaminating microorganisms from 0.78 ppm, a visible decrease in turbidity was observed. The effect of the biocide on the two groups of contaminating microorganisms had a proportional relationship with the amount of it used, this effect could be appreciated when it was used from a concentration of 6.25 ppm to reduce the contaminating load which could be evidenced by the decrease in turbidity.

Figure 3. MIC of the tested Biocidal Product (Procide BIOQ). for bacteria lactic acid contaminants in the: fermenter N°4 and vinasse, counting in boxes.



Similarly, in the research of Castillo and Peláez (2018), PROCIDE BC 800 30 H, BACTOL and PROQUAT DDAC 52 were used where they obtained a biocide activity of 50% at the laboratory and field level on the population of lactic acid bacteria, *Leuconostoc* spp and yeasts in the juice of *Saccharum officinarum* “sugarcane”; they have used this type of biocide for the control of the contaminating microbial population. In comparison with this research, a reduction of approximately 91% was achieved, that is, a high biocidal activity, however,

the BALS evaluated by the same authors are different from those evaluated in this test, which, it is recommended to carry out an identification and characterization of the population in order to choose an appropriate biocide that can achieve the reduction of the contaminant population residing in the bioreactors.

On the other hand, Freitze, N et al (2019) in their study refers to the use of natural biocides in the ethanol production process from various sources, to control the proliferation of harmful microorganisms to the fermentation process of cane, starch and other sugars.

On the other hand, reference is taken from authors (Capote et al., 2020; Castaño; Díaz et al., 2015; Delnois and De la Cruz., 2019; Montealegre 2019); determine the appropriate times and analyses for antibiotic and biocide treatments, emphasizing their ability to generate more appropriate conditions for other contaminants that affect the processes and interaction of yeast in the process of obtaining alcohol. In our case the studied biocides had an excellent receptivity to the samples taken, it is important to understand that this research does not seek to affirm or establish that these biocides work always and in all samples.

This is how in the study carried out in 2018 by Orozco et Al, the effect of antibiotics in different concentrations was evaluated on the control or inhibition of the growth of lactic acid bacteria (LAC) over time (0, 4, 6 and 16 hours), where they used the same strain of *S. cerevisiae*, thus demonstrating that biocides are efficient in the control of contaminating bacteria exerting the greatest effect when used in high concentrations (15 ppm) and in the antimicrobial the lowest concentration (15 ppm). It was also determined that, in the times that biocides best exert or effect the action are between 6 and 24 hours, this research validates the results obtained in ours because in it the efficiency of biocides was demonstrated.

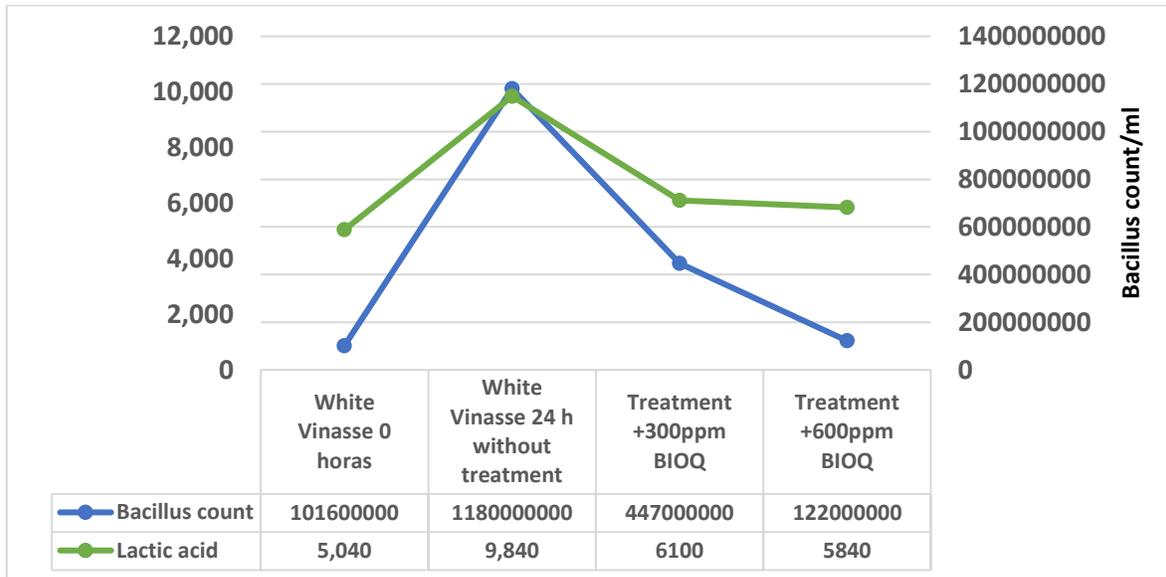
But, these treatments are not enough to control or avoid the contamination that is generated, not only by bacteria and other yeasts that are evident in the processes, but also by improper handling and by the liquid effluent, as is the case of vinasse, this is how authors such as Camacho, et al. currently., 2018; Pabòn, O, 2021 and Pilcon et al., 2023 mention that, in recent times it has generated great impact at the levels of interactions, which has led the sugar agribusiness to rethink and look for new ways to deal with these problems that produce secondary waste and effluents, which must be treated or used appropriately to avoid environmental pollution.

Therefore and as mentioned, the action of the biocide is mediated by factors, which allow cell injury, irreversible cell damage, achieving the control of the microorganism and thus generate the elimination of the bacteria or the control of the microbial load in a successful way, these factors correspond to those related to the composition of the biocide, the structure and ultra cellular structure. Bactericides destroy microorganisms with their power and surfactant faculties, eliminating fungi, yeasts and bacteria that cause the inversion of sucrose into glucose, levulose and other sugars that do not crystallize. Bactericides mainly control *Leuconostoc mesenteroides*. Bacteria develop with a high proportion between cane cutting and its process (Benincore, J and Padierna, L., 2023).

Evaluation of the Vinaza by time.

The fresh count from the vinasse samples at zero hours was 1.01×10^8 bacilli/mL, increasing an exponent in the bacilli count (1.18×10^9 bacilli/mL) at 24 hours. The same behavior is observed with the production of lactic acid, which increases from 5040 ppm to 9840 ppm after 24 hours in the sample of vinasse that had no treatment.

Figure 4. Behavior of bacilli and lactic acid production at 0 hours and after 24 hours.



From the above, with the application of 300 ppm of PROCIDE BIOQ, a decrease in the bacillus count of 1.6142×10^2 bacilli/ml was achieved, with respect to the results obtained from the vinasse, which had no treatment after 24 hours. On the other hand, the treatment with 600 ppm of BIOQ managed to decrease 1.06×10^9 bacilli/mL with respect to the vinasse without treatment at 24 hours, and additionally, an increase of 2.6310×10^2 that were obtained in the sample without treatment after 24 hours is evidenced, this represents approximately 91%.

In the same way, the treatment with the PROCIDE BIOQ biocide was evident when observing at the microscopic level, a high count of live bacilli at zero hours and a decrease of the bacilli by microscopic fields due to the effect caused by the treatment when the exposure time to the biocide concentrations was prolonged over a period of 24 hours. Viable bacilli are observed bright and without coloration, this is because they have enzymes that reduce methylene blue to its colorless derivative unlike non-viable cells, which take on the blue coloration characteristic of this dye, as described in the Resolution OIV/206 of 2010. In general, it was found that the biocide PROCIDE BIOQ significantly affects, not only by the concentration, but also by the exposure time, both the concentration of acetic acid bacteria, and of *S. cerevisiae* ($p < 0.05$).

Conclusions and Recommendations

In this study it was possible to find the ideal concentration and the exposure time of the biocides PROQUAT BC 50 and PROCIDE BIOQ, whose purpose was to reduce the contaminant load that is produced by lactic and acetic acid bacteria in the production process of fuel alcohol, demonstrating significant differences in the concentrations 12.5 and 6.25 ppm in both PROQUAT BC 50 and PROCIDE BIOQ. It should be noted that in the selected points (fermenter and vinasse), the interaction between yeast-bacteria and yeast-yeast was observed, as well as the effect on the yield of *Saccharomyces cerevisiae*, with the previous selection of bacteria and the classification of yeast-bacteria interactions, with this, it was possible to develop interaction studies in two different fermentation media, in a first stage of study, the interactions were analyzed in MRS and WL medium; means used to evaluate the behavior of the interactions. The effect exerted by the biocides presented differences in terms of concentration and contact time, finding an inversely proportional relationship, a higher concentration of the biocide showed a decrease in contaminating bacteria, which affect the fermentation process to obtain the alcohol.

Therefore, it is necessary to carry out scaling studies in order to evaluate the impact of the interaction on the distillate in the process of making cane juice and in its system. Also, evaluate in detail the behavior of each interaction in terms of cell development, production of volatile compounds and their ethanol production.

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