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IDENTIFICATION AND EVALUATION OF CONTAMINATING BACTERIA IN THE FERMENTATIVE PROCESS OF A FUEL ALCOHOL PLANT IN THE VALLE DEL CAUCA, COLOMBIA

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Abstract: The addition of various nutrients such as vinasse, honey and water in the fermentative process for the production of fuel alcohol can favor the growth of organic acid-producing microorganisms that generate stress for the yeast: *Saccharomyces cerevisiae* and therefore the reduction of performance in terms of alcohol production. For this reason, this work aimed to identify and evaluate contaminating bacteria in the fermentation process of a fuel alcohol production plant located in the department of Valle del Cuaca, managing to identify 5 bacterial species molecularly through PCR: *Lactobacillus hilgardii* and *Acetobacter tropicalis* with a frequency of 33.3%, followed by *Lactobacillus plantarum, Acetobacter pasteurianus* and *Leuconostoc mesenteroides;* with a frequency each of 11.1 % of the total isolated strains, finding that of these the one that generates the most losses in production is *L. hilgardii* producing 1060 p.p.m ±335 of lactic acidity and 1338 p.p.m ±1059.2 of volatile acidity consuming the 2% ±*0.*6 of the sugars present in the medium. Therefore, we continued to evaluate the effect of three antibiotics about *L. hilgardi* using the spectrophotometry technique, showing that the antibiotic with active principle b Hop acid was the one that managed to obtain better control over the growth of this bacterium, followed by the antibiotic with Clindamycin and finally the antibiotic with Monensin sodium. Therefore it can be concluded that *L. hilgardii* is the cause of decreased productivity *Saccharomyces cerevisiae* in the fermentative process due to its high production of organic acids that are detrimental to the enzymatic activity of the yeast, but by using antimicrobial substances, especially of vegetable origin, such as hop acid, it is possible to reduce and control the presence of this bacterium and thus improve the efficiency of the company.

Keywords: fermentation, contamination, organic acids, antibiotic.

INTRODUCTION

Currently the rate of environmental pollution on the planet is very high and one of the main causes of this problem is the gases emitted by cars, to solve this some countries have chosen to use an alternative which consists of the use of oxygenates. in gasoline, within these the most used is fuel alcohol since it helps to minimize energy consumption and contributes to improving the environment. (Higuera, et al., 2007).

Fuel alcohol is a product generated from fermentation by microorganisms especially *Saccharomyces cerevisiae* using organic matter. This product became important again in the middle of the 20th century after the increase in the price of oil, after this, countries around the world have begun to develop its production, among which the United States is in first place followed by Brazil. Colombia is in tenth place with a participation of 0.4% of production worldwide (CEPAL, 2011).

However, within the fermentation process, in addition to the alcohol-producing yeast, contaminating microorganisms such as *Leuconostoc, Bacillus* y *Lactobacillus,* (De Oliva, et al., 2013), that by not having control over them, they generate low yield of the process because they assimilate glucose from the medium, decreasing the physical and nutritional space, altering the physicochemical characteristics of the must and reducing the enzymatic activity of the yeast, (Ferreira et al., 2010). For this reason, it is vitally important to use antimicrobials that manage to stabilize the fermentation and thus be able to have a single microorganism (yeast) present in the medium. For this reason, the objective of this work was to identify and evaluate contaminating bacteria in the fermentation process of a fuel alcohol production plant in Valle del Cauca.

METHODOLOGY

LOCATION AND SAMPLING

The study was carried out in a distillery that produces fuel alcohol in Valle del Cauca, Colombia. Obtaining samples of the must from the fermenter number 3 (R313), the cream from the settling tank (S331), the honey and the stillage used in the fermentation process in the plant which has a production capacity of 250,000 liters per day.

ISOLATION AND PURIFICATION OF BACTERIAL STRAINS IN THE FERMENTATION PROCESS

Serial dilutions (10-1 - 10-6) were made of each sample collected in Man, Rugosa and Sharpe MRS agar (Merck) and incubated at 30°C for 48 hours in microaerophilia (Ramos, et al., 2009). Then the isolated and morphologically different colonies were taken and successive passages were carried out by depletion in MRS agar (Merck), until a pure culture was obtained.

The purified strains were preserved in MRS medium in a flute-shaped inclined tube, forming a workbench and keeping them at a temperature of 4°C. (Buzon, 2007).

IDENTIFICATION OF BACTERIAL STRAINS

Molecular identification was carried out with the help of an external laboratory because the necessary equipment for this type of procedure was not available. The laboratory used the PCR technique with amplification of the gene encoding the 16S rDNA using the PA (5' AGAGTTTGATCCTGGCTCAG 3') and PC5B (5'TACCTTGTTACGACTT 3') primers, followed by sequencing. They used the databases: National Center for Biotechnology (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cg) and Ribosomal Database Project (RDP, https://rdp.cme.msu.edu/) for the taxonomic determination of microorganisms.

DETERMINATION OF THE CONSUMPTION OF SUGARS AND THE PRODUCTION OF ORGANIC ACIDS OF THE IDENTIFIED BACTERIA

A solution of honey B (50-55% sucrose) was prepared at a concentration of 11.5% (m/v) to which 60 mL of the inoculum was added with a concentration of 3*106 CFU/mL representing 10% of the work volume, this was done by washing the bacterial colonies with sterile distilled water and seeding in MRS agar (Merck) to determine the initial and final concentration of each of the bacteria. To this dilution, 300 p.p.m. of commercial Urea was added and the pH was adjusted to 4.5. Incubating at 30°C in anaerobiosis, performing the following procedures:

RESIDUAL TOTAL SUGARS (ICUMSA GS METHOD 7/8/4-23)

Analytical grade fructose, glucose and sucrose standards were used.

The samples of each bacterium and blanks were dissolved in deionized water prior to their analysis by high performance liquid chromatography.

The chromatography conditions were as follows:

- Chromatograph: High resolution liquid chromatograph with WATERS I refractive index detector.
- Flow: 0.5 to 0.6 mL/min.
- Mobile phase: EDTA (Calcium Salt) at 5 p.p.m.
- Column: Column filled with calcium-based exchange resin, with a particle size of 10 – 15 μm, length 250-300m (Sugar Pack I).
- Oven temperature: 80 to 85°C.
- Injection volume: 20 μL.

LACTIC ACIDITY

A total of 5 mL of each sample were taken at the beginning and at the end of the test and centrifuged at 5000 rpm for 10 minutes to separate the biomass from the supernatant.

Lactic acid production was measured by reflectometry (Reflectoquant Merck - RQflex Plus 10, Germany). The supernatant was filtered (0.45 μm) before measurement (Serna, et al., 2013).

VOLATILE ACIDITY

It was done through a distillation and later a titration with NaOH 0.02N, in a Metrohm 719S automatic titrator (Maturana, 2004). The analyzes were performed on each sample at the beginning (0hrs) and at the end (48hrs) of the simulation.

EVALUATION OF THE EFFICIENCY OF ANTIBIOTICS AGAINST THE STRAINS IDENTIFIED WITH THE HIGHEST PRODUCTION OF ORGANIC ACIDS AND CONSUMPTION OF SUGARS

This stage was done using the spectrophotometry method (Lopes, et al., 2011).

Three types of antibiotics were evaluated, Monensin sodium (10, 20 and 30 p.pm.), β hop acids (30, 70 and 100 p.p.m.) and Clindamycin (3, 5, 7 p.p.m.). These doses were taken taking into account the consumption of these products in the fermentation process of the distillery.

In screw cap tubes, 15 mL of sterile GLT (Glucose, Tryptone and Yeast Extract) broth were added, plus the doses of antibiotics to be evaluated, to which 1 mL of the inoculum adjusted on Mac Farland scale 1 was added.

Through a spectrophotometer with a wavelength of 520 nm, the readings were made at 0, 4, 6 and 8 hours, during each reading the tubes were incubated at 30ºC. In addition, blanks of each antibiotic were left for the equipment calibration process before each reading.

EXPERIMENTAL DESIGN

A completely randomized design with three replicates was used to evaluate the effect of antibiotics against the strains identified with the highest production of organic acids and consumption of sugars.

Each treatment consists of the bacteria to be evaluated inoculated in the GLT culture medium plus the dose of antibiotic which will be left exposed for 8 hours.

STATISTIC ANALYSIS

Given the lack of normal distribution of the data due to the fact that the concentrations of the evaluated antibiotics are different, a comparison of medians was made through the non-parametric Kruskal-Wallis test to determine significant differences between treatments (type, concentration and time of exposure).) with a confidence of 95% using the Minitab 17 computer application.

RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION OF BACTERIAL STRAINS IN THE FERMENTATION PROCESS

From the sowings carried out in MRS agar, 9 bacterial strains were isolated and purified, to which a code was given taking into consideration, the place where it was isolated (Table 1).

Sampling number	Sampling place	Strain Code
1	STILL EXIT H401	VZCP01
2	STILL EXIT H401	VZCP02
3	FERMENTER R313	FRCP03
4	FERMENTER R313	FRCP04
5	SETTLER S331	SDCP05
6	HONEY B TANK	MBCP06
7	HONEY B TANK	MBCP07
8	HONEY B TANK	MBCP08
9	STILL EXIT H401	VZCP09

Table 1: *Registration and Coding of the strains isolated and purified from the fermentation process of the Alcohol Fuel Distillery plant, 2017.*

The strains underwent a morphological characterization with Gram stain, which showed that seven of these strains are Gram-positive bacilli and two Gram-positive cocci.

On the other hand, for molecular identification, the National Center for Biotechnology (NCBI) and Ribosomal databases were used.

Database Project (RDP) for the taxonomic determination of the microorganism, obtaining as results the presence of 5 different bacterial species as *Acetobacter tropicalis* y *Lactobacillus hilgardii* with a frequency of 33.3%, followed by: *Lactobacillus plantarum*, *Acetobacter pasteurianus* and *Leuconostoc mesenteroides*; with a frequency each of 11.1 %.

The most frequent microorganisms in the process as contaminants are: *Lactobacillus hilgardii* and *Acetobacter tropicalis*. These were found from the beginning of fermentation mainly in the raw materials and final product of the process, such as honey and vinasse respectively, being able to grow and proliferate until they were established in the fermentation system and making their control more difficult, as demonstrated in a study. carried out by Dias et al., (2015), when evaluating the effect that lysozyme has on various strains of Lactobacillus in a wine sample, finding that various strains of *Lactobacillus hilgardii*, they are resistant to high concentrations of this enzyme, such as 2000 mg/L, due to the presence of a double layer of protein S on the cell surface, which hinders bacterial lysis and therefore its elimination in the fermentation process.

On the other hand, acetic acid bacteria such as *Acetobacter tropicalis* can grow in fermenters up to a 85 x 10^4 UFC/mL. This genus can overoxidize ethanol to acetic acid and ultimately to CO_2 and H_2O through the Krebs cycle, affecting the viability of the yeast by 30%, lowering its performance in the reproduction and fermentation stages (Hurtado et al., 2011).

Comparing the results generated in this stage of the investigation with previous studies, it can be said that the high frequency of *Lactobacillus hilgardii* y *Acetobacter tropicalis* in the fermentative process they generate drops in the efficiency of the plant.

EVALUATION OF THE CONSUMPTION OF SUGARS AND THE PRODUCTION OF ORGANIC ACIDS OF THE IDENTIFIED BACTERIA

An analysis of variance (ANOVA) was performed on the data obtained in the trials with a reliability percentage of 95%, resulting in significant differences (p>0.05%) for Total Residual Sugars, Lactic Acids, and Volatiles. Given the existence of these differences, an analysis of multiple comparisons was carried out using Tukey's HSD test in order to further delve into the existing differences.

Regarding the ATR, a significant difference was found (*p*>0.05%) between: *Lactobacillus hilgardii* y *Acetobacter tropicalis* with respect to the Control at 48 hours of the test. On the other hand, in lactic and volatile acids the microorganism: *L. hilgardii* presents significant differences (p>0.05%) with respect to the Control at 48 hours. The other bacteria had a significant difference (p >0.05%).

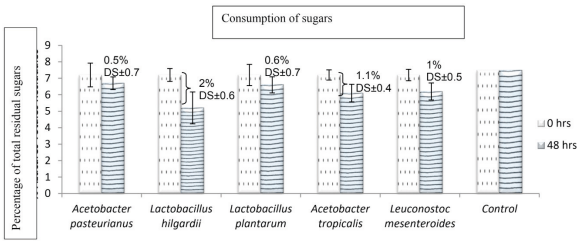

Figure 1. Sugar consumption of the identified bacteria present in the fermentation process, 2017.

After making the final analyzes and comparing them with the initial state of the medium, it was found that all the bacteria were able to consume the fermentable sugars present in the medium, mainly Lactobacillus hilgardii with 2% ±0.6 of consumption equivalent to 17.2 g, taking into account the amount of honey used at the beginning of the trial, which was 62 g; followed by *Acetobacter tropicalis* with 1.1% ±0.4 (9.5 g); Leuconostoc mesenteroides with 1% ±0.5 (8.6 g), *Lactobacillus plantarum* with 0.6% ±0.7 (5.2 g) and finally Acetobacter pasteurianus with 0.5% ±0.7 (4.3 g), which indicates that among the bacteria evaluated: *L. hilgardii* represents more competition for *Saccharomyces cerevisiae,* since it does not leave enough sugars to meet the alcohol production goal that the company has, decreasing its percentage of efficiency (Figure 1).

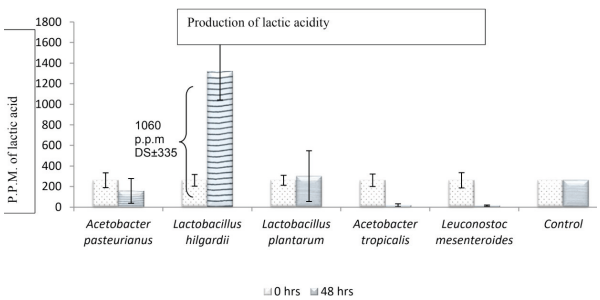

Figure 2. Production of lactic acidity of the identified bacteria present in the fermentation process, 2017

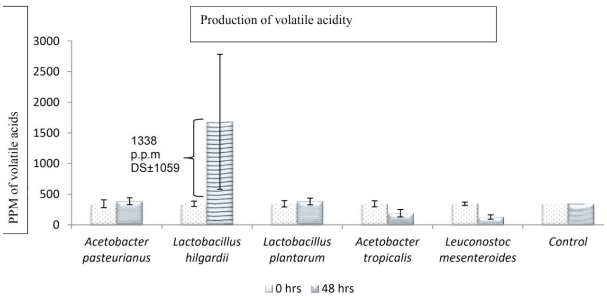

Figure 3. Production of volatile acidity of the identified bacteria present in the fermentation process of the Alcohol Fuel Distillery plant, 2017.

Taking into account the production of organic acids, it was found that *Lactobacillus hilgardii* represents a danger for the fermentative process since the concentration of acids increased in greater proportion with a delta of 1060 p.p.m ±335 for lactic acidity and 1338 p.p.m ±1059 for volatile acidity. followed by *Lactobacillus plantarum* with a delta of 40 p.p.m ±174.5 for lactic acidity and 38 p.p.m ±42 for volatile acidity (Figure 2 and 3), generating a decrease in the efficiency of the process of approximately 4% with respect to that used in the plant, which is 91 %, according to the performance balances of the plant during the dates that the bacteria were isolated.

This is reflected in a study carried out by Sossa et al., (2009) where they indicate that the presence of bacteria in the process, especially *Lactobacillus* sp., create an efficiency drop of 7.81%, using a theoretical efficiency of 88%. The foregoing corroborates the results obtained in this investigation, confirming that these contaminating bacteria consume the sugars present in the medium to produce mainly organic acids that affect the yeast, decreasing its performance.

On the other hand, we see that *Acetobacter pasteurianus,* it only produced volatile acidity and not lactic acidity, this is because this bacterium is characterized by producing mainly acetic acid in the presence of 6%

ethanol at 37ºC, it can also grow in a culture medium containing 1.5% acetic acid or 4% ethanol at 39°C, according to studies carried out by Kanchanarach et al., (2010). Finally, it was observed that *Acetobacter tropicalis y Leuconostoc mesenteroides,* they did not produce organic acids, but used the acid present in the medium, as food for the production of other substances or to survive in that medium due to stress conditions. In the case of *Leuconostoc mesenteroides,* in addition to acids, it can produce large amounts of dextran, in a study conducted by Rodríguez & Hanssen, (2007) The above was verified, since this bacterium was able to produce 3.4 g/L of dextran in a medium containing 20 g/L of sucrose at 30ºC.

EFFECT OF THE CONCENTRATION OF ANTIBIOTICS ABOUT THE BIOMASS OF *LACTOBACILLUS HILGARDII*

After obtaining the results of each antibiotic, the statistical analysis continued with the non-parametric Kruskal-Wallis test, making comparisons of medians.

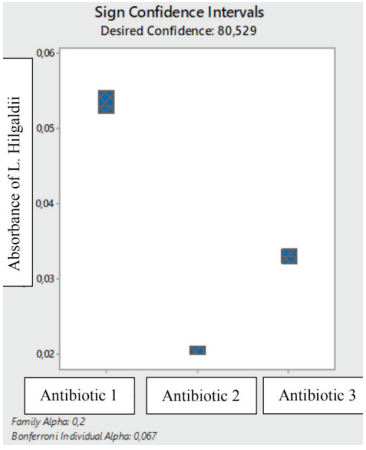

Figure 4. Comparison of the effect of the three antibiotics on the biomass of Lactobacillus hilgardii. Source: Minitab 17 software.

The Kruskal-Wallis test and the multiple comparison of medians showed that there were significant differences in absorbance between the three antibiotics. ($p<0,05$).

The best of all was the antibiotic 2 (**β**- hop acids) since the absorbances thrown have a much smaller range of difference compared to the absorbances of the other two antibiotics. In second place is antibiotic 3 (Clindamycin), followed by antibiotic 1 (Monensin sodium) (Figure 4).

The results found in this study agree with those carried out by Freitas & Romano, (2014) where they evaluated three natural products such as β-acid derived from hops, citrus extracts and pure oil of *Mentha piperita* to control the presence of bacteria in alcoholic fermentation, through sensitivity techniques, antimicrobial tests and fermentation on a laboratory scale, resulting in that β-acid was the most efficient for process control because it completely decreased the presence of bacteria in the alcoholic fermentation. fermentation, did not affect the viability and enzymatic activity of *Saccharomyces cerevisiae* contributing to the increase in efficiency compared to the other two antibiotics that failed to control the bacterial concentration and also the product with *Mentha piperita* presented an antifungal activity because it affected the viability of the yeast.

Corroborating the above, in a study carried out with the purpose of increasing the yield in the production of fuel ethanol with flocculant yeasts with organic by-products of agro-industrial processes, Santos et al, (2014) simulated fermentations for the control of contaminating bacteria with Kamoran antibiotics. (sodium monensin) at 3 and 6 p.p.m and Beta Bio (hop extract) at 35 p.p.m, which significantly controlled the contaminants present in the fermentation system with a total yield of 80 and 90% respectively.

CONCLUSION

After carrying out the morphological and molecular identification, it was found that there are a great variety of contaminating microorganisms in the fermentation process, with the following being more frequent: *Lactobacillus hilgardii* y *Acetobacter tropicalis* which are harmful since they help to reduce the performance of the plant representing economic losses.

Considering the results obtained, it can be said that *Lactobacillus hilgardii* represents a danger in the fermentation process because with the amount of organic acids it produced and the sugars it consumed, it affects the viability and activity of the yeast, generating economic losses since alcohol production is reduced and the investment of money is increased in treatments to reduce contamination.

The use of antibiotics of plant origin such as β-acid from hops represents a good alternative to improve the yield of alcohol production because it reduces and controls the presence of contaminating bacteria in the fermentation system, as is the case with *Lactobacillus hilgardii.*

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