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CONTROL OF CONTAMINATION IN DILUTION WATER USED IN MOLASSES MUST PREPARATION

CONTROLE DA CONTAMINAÇÃO NA ÁGUA DE DILUIÇÃO USADA NA PREPARAÇÃO DE MOSTO DE MELAÇO

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ABSTRACT

The amount of sugar present in molasses requires a reduction in order to be used for ethanolic fermentation by yeasts. However, dilution water added could be contaminated by microorganisms (bacteria and wild yeasts). This study aimed to reduce water contamination using two filtering systems: composite membranes of TiO_2/SiO_2 and filter with activated carbon. The water samples from both filtering systems were compared to conventionally treated water, in sugar mill using chlorine (positive control) and untreated water (negative control). It was observed a reduction in the quantities of microorganisms in all kind of treated water compared to the negative control. Then, all samples were added to molasses and underwent fermentation in a laboratory scale. As result, low contamination was observed in all treatments. Some substance from molasses reduced contamination even in the untreated water sample. It is known that SO_2, used in juice purification in a process called sulphitation at sugar production remains in molasses after crystals production. Bearing in mind, another study was accomplished using three different types of molasses added to sterile (by high temperature) or non-sterile water. There were applied two molasses from the production of raw sugar (VHP – very high polarization) that is used reduced quantity of SO_2. The third type of molasses was from white sugar process that uses high amounts of SO_2 at purification. There was a reduction in contamination from samples of raw sugar molasses using sterile water compared to non-sterile water. When we use molasses from white sugar, sterile and non-sterile water samples did not differ in bacterial contamination in the must. As a consequence, there is not necessary treat dilution water, when we using molasses from sugar production with sulphitation. Residual sulfur could reduce contamination in must preparation.

Keywords: Microorganisms. Composite membrane. Carbon filter. Sugar VHP. White sugar. Biofuel.

RESUMO

A quantidade de açúcar presente no melaço requer uma redução para ser usada na fermentação etanólica pelas leveduras. No entanto, a água de diluição adicionada pode estar

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contaminada por microrganismos (bactérias e leveduras selvagens). Este estudo teve como objetivo reduzir a contaminação da água por meio de dois sistemas de filtragem: membranas compósitas de TiO2 / SiO2 e filtro com carvão ativado. As amostras de água de ambos os sistemas de filtragem foram comparadas com a água tratada convencionalmente, em usina de açúcar usando cloro (controle positivo) e água não tratada (controle negativo). Observou-se redução nas quantidades de microrganismos em todos os tipos de água tratada em relação ao controle negativo. Em seguida, todas as amostras foram adicionadas ao melaço e submetidas à fermentação em escala laboratorial. Como resultado, baixa contaminação foi observada em todos os tratamentos. Alguma substância do melaço pode ter reduzido a contaminação mesmo na amostra de água não tratada. Sabe-se que o SO2, utilizado na purificação de caldo em um processo denominado sulfitação na produção de açúcar, permanece no melaço após a produção de cristais. Tendo isso em mente, outro estudo foi realizado utilizando três tipos diferentes de melaço adicionado a água estéril (por alta temperatura) ou não estéril. Foram aplicados dois melaços a partir da produção de açúcar bruto (VHP - polarização muito alta) que é utilizada em quantidade reduzida de SO2. O terceiro tipo de melaço foi do processo de açúcar branco que usa grandes quantidades de SO2 na purificação. Houve uma redução na contaminação de amostras de melaço de açúcar bruto usando água estéril em comparação com a água não estéril. Quando usamos melaço de açúcar branco, amostras de água estéreis e não estéreis não diferiram na contaminação bacteriana do mosto. Como conseqüência, não é necessário tratar a água de diluição, quando usamos o melaço da produção de açúcar com sulfitação. O enxofre residual pode reduzir a contaminação na preparação do mosto.

Palavras-chave: Microrganismos. Membrana compósita. Filtro de carvão. Açúcar VHP. Açúcar branco. Biocombustível.

1 INTRODUCTION

Ethanol is gaining popularity because it reduces environmental impacts when compared with fossil fuels (SANGWAN *et al*., 2014). This biofuel is mainly obtained by fermentation process, which involves sugar monomer oxidation, resulting in ethanol, carbon acid and other by-products (DELLA-BIANCA, 2013). *Saccharomyces cerevisiae* are the main microorganisms involved in sugar conversion into ethanol (NIELSEN *et al*., 2013).

In order to enable fermentation, must is prepared, and then adjusted for sugar level, nutrients content, and pH to optimize the process. Molasses, residue of sugar production, is the most common feedstock used for the must preparation in Brazil (BASSO *et al*. 2011).

The mixture of molasses and water is made to provide the ideal amount of sugars for the fermentation process (REIN, 2007). High levels of sugar in must are responsible for losses, causing slow and incomplete fermentation. This happens because yeasts metabolism is inhibited by the high amount of ethanol (DASHKO *et al*. 2014). However, dilution water used to reduce sugar quantity could be contaminated by microorganisms. The main contamination sources in fermentation comes from poor quality of sugarcane, water used in must preparation and from unclean tubing and equipments of industry (OLIVEIRA *et al*., 2013).

Microorganisms such as bacteria and wild yeasts are the contaminants (MUTHAIYAN *et al*., 2011) and can cause several problems such as sugar loss, yeast flocculation, gum production, yeast growth inhibition or even reduction in fermentation yield (RAVANELI *et al*., 2011). The use of antibiotic drugs has been the most common control method against these contaminants, but because of their high cost, there has been a constant search for other viable alternatives (MADALENO *et al*., 2016).

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The physical filtration methods may be used mainly to control microorganisms in water used in molasses dilution. The quality of water is extremely important, so many sugar mills use water from nearby rivers, and its treatment is normally made with chlorine addition. However, current studies have shown that chlorine is not efficient to control some microorganisms (MADALENO *et al*., 2016). Also, chlorine could be toxic to yeasts, causing fermentation inhibition, significant process losses and corrosion of equipments.

Therefore, the objective of this study was to evaluate the efficiency of decontaminating dilution water using two filter systems, TiO_2/SiO_2 composite membranes, and activated carbon filter. The different molasses used as must feedstock and their effects as far as contamination is concerned was also evaluated.

2 MATERIAL AND METHODS

Experimental design

Three experiments were conducted at Fatec Nilo De Stéfani – Jaboticabal – SP to verify: a) the ability of two different filters to control the microorganism amount; b) observe the impact of filtered water samples in the fermentation process and c) evaluate the use of sterile and non-sterile water on the dilution of three types of molasses.

First experiment

Four different treatments of water, obtained from a local river in a sugar mill next to Jaboticabal-SP, were submitted to a microorganisms count. The first water sample did not receive any treatment and it was considered the negative control. Samples 2, 3 and 4 underwent the addition of chlorine (positive control, performed in a different tank by the sugar mill), filtration with activated carbon filter and TiO2/SiO2 composite membrane filter, respectively. The last two treatments and microorganism count were made at Fatec. Calculations of the amount of microorganisms were performed using the PetrifilmTM system (SANT'ANA *et al*. 2002). In order to avoid over counting, besides using the water as it was after treatments, we also measured using three different dilutions with saline solution 1% as shown in table 1 (results and discussion).

Second experiment

A must, which is the dilution of molasses with water and others adjusting, was made using the four different water samples from experiment 1 reduced molasses from 82,5o to 14 °Brix (soluble solids) and pH was adjusted to 4.5. The molasses originally used was from a sugar mill which produces very polarization sugar (VHP) (season 2013/2014). The must were then analyzed for acidity (g H2SO4 L-1) and Total Reducing Sugars (TRS) conducted according to Lane and Eynon (1934).

The pressed baker yeast (Saccharomyces cerevisiae) was prepared to be used in the fermentation process being firstly diluted in a glucose solution. For each 15g of yeast, 50ml of 1% glucose solution were used and the mixture was kept for an hour. After this pre-fermentation process, the mixture of yeast and glucose solution was centrifuged (Hettich Rotina 420®) with 3000rpm in order to separate the solid yeast part from the glucose solution. The supernatant was discarded and the 15g of yeasts left in each tube were removed

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with 100 ml of must and set in a 1L Erlenmeyer and waiting for 15 minutes, for yeast adaptation.

An amount of 100ml of must was subsequently added to this mixture every 15 minutes until the final volume of 500ml (AMORIM *et al*. 1996). The four Erlenmeyer containers with mustes made with the 4 water samples from experiment 1 and the yeasts were kept under stir at 32°C (CT-712, Cienter). The process was monitored by calculating the soluble solids (oBrix) after 6 hours and every hour thereafter. Values lower than 4 were considered to characterize the end of fermentation. The analysis of yeast cell viability in the beginning (after 15 minutes of the last must addition) and at the end of fermentation was performed according to Lee et al.'s (1981) analysis. Contamination was also measured at the beginning and at the end of the fermentation process and it followed the same protocol of experiment 1.

After fermentation, the mixture is named wine. The wines from the 4 samples were submitted to analysis of acidity, total residual reducing sugars (TRRS) and ethanol content (CTC 2011). The produced and ethanol yield was calculated according to Fernandes (2006) method.

Third experiment

Three different molasses provided by sugar mills from Jaboticabal-SP region, Brazil were analyzed: a) Molasses 1 (season 2013/2014, the same used in second experiment); b) molasses 2 (obtained from the same sugar mill of experiment two, but another season 2014/2015) and c) molasses 3 (obtained from other sugar mill, that applies sulfur treatment in purification on sugar production, season 2014/2015). Molasses were diluted with sterile (by high temperature in autoclave) or non-sterile water to obtain the must at 14 °Brix and pH adjusted to 4.5. The effects of the three different molasses contamination were evaluated in the must preparation. The microorganisms were counted also using the PetrifilmTM system (SANT'ANA *et al*. 2002).

Statistical analysis

The assays were conducted with five repetitions. The results were analyzed by ANOVA and means were compared by Tukey test at 5% (BARBOSA; MALDONADO JÚNIOR, 2015).

3 RESULTS AND DISCUSSION

Water dilution treatment

In the first study, the quality of the water submitted to all treatments was evaluated: 1- non-treated, 2- chloride, 3- activated carbon and 4- composite. Table 1 shows the quantification of total aerobic bacteria in the water using PetrifilmTM system which has been found the most efficient for the water contaminants studies (BELOTI *et al*. 2003; SCRAFT; WATTERWORTH, 2005; SAKAI *et al*. 2013) and in foods (LAKIMINI; MADHUJITH 2012.

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Table 1 - Quantification of total aerobic microorganisms in water by Petrifilm™ system (CFU/mL)

Sample Dilution*	No-treated	Chloride	Activated carbon filter	Composite filter
No-dilution	900	67	420	7
10^{-1}	371	3	15	0
10^{-2}	39	0	0	0
10^{-3}	2	0	0	0

***dilutions with saline solution 1%.**
Source: authors (2019)

The results showed that the filter systems were able to reduce 114% of the contaminants when used the active carbon filter and 128.57% with the composite membrane one when compared to no-treated water (Table 1). In relation to the positive control treatment (chloride), the composite filter is found 9.57 times more efficient in the contaminants removing and could be used as an alternative to must dilution water.

Use of treated water in fermentation

The water samples obtained from different treatments were used in the molasses dilution for must preparation. Table 2 shows the results obtained in the must quality analysis. The amount of total reducing sugars (TRS) was higher in the must prepared with water treated with chlorine. This result is because the chloride treated water was collected in a tank in a sugar mill which receives residual water from other processes and from the river, prior to the addition of chloride. Residual water from sugar production could contain sugar from industrial losses, as condensed water from the juice evaporation process (REIN, 2007) increasing the TRS in this sample.

There is no difference in total acidity observed in the treatments. The acidity concentration found are common to the ethanol process using molasses for must preparing (SANTOS *et al*. 2012).

Table 2 - ANOVA results to TRS and must total acidity. The same letters do not differ by Tukey test (P≤0.05)

Variation Sources	TRS (%)	Acidity (gH_2SO_4 L^{-1})
Treatments (F)	17.7242 **	1.0580^{ns}
No-treated	11.51 B	2.69 A
Chloride	12.56 A	2.57 A
Activated carbon filter	11.54 B	2.64 A
Composite filter	11.60 B	2.76 A
CV	2.28	6.44

ns: no- significant; * significant (P≤0.05); ** significant (P≤0.01).
Source: authors (2019)

The analysis of wine, after the fermentation process, showed there is no significant difference for final soluble solids (°Brix), total residual reducing sugars (TRRS), acidity, alcohol by volume and ethanol yield (Table 3).

As for the ethanol yield, a higher value is observed when molasses was blended with composite membrane filter, in relation to chlorine treatment (Table 3), despite its higher TRS values (Table 2). Probably, part of the glucose in chlorine was used to produce secondary

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compounds by yeasts, as glycerol and others that are produced in stressful medium by these fungi (AMORIM *et al*. 1996). In this treatment, a reduction on yeast cell viability in the beginning of fermentation is also observed (Table 4).

Ethanol yield of all treatments is very low compared to sugar mill brazilian aim that is 92%. Its happing because molasses is a liquid with many impurities, which causes inhibition in fermentation by yeasts (REIN, 2007; BASSO *et al*. 2011). In this study we didn't do correction in nutrients, as nitrogen and phosphorus concentration that could improve ethanol yield.

The initial yeast cell viability was higher in the dilution water treated with carbon filter compared to chloride and control treatments (Table 4). In the end of fermentation, the higher values of the composite membrane filter treatment indicate that the fermentation would be more favorable to yeasts metabolism, which was further confirmed by the higher ethanol yield (Table 3). It is probably because of the fact that this system can remove some substances as toxic molecules and heavy metals, which are toxic to yeasts (DOLEZALOVA; RUMLOVA 2014).

Table 3 - ANOVA results for the Technological analysis of wine. The same letters do not differ by Tukey test (P≤0.05)

Variation Sources	°Brix	TRRS (%)	Acidity (gH_2SO_4/L)	Ethanol (% v v^{-1})	Ethanol Yield (%)
Treatments (F)	1.5556 ^{ns}	0.4298 ^{ns}	0.3311 ^{ns}	2.0306 ^{ns}	3.5195 *
No- treated	3.7200 A	0.6430 A	3.9102 A	6.03 A	64.64 AB
Chloride	3.5600 A	0.7050 A	3.8259 A	6.03 A	59.32 B
Activated carbon	3.5200 A	0.6650 A	3.8338 A	5.86 A	62.71 AB
Composite membrane	3.6000 A	0.6670 A	3.8044 A	6.83 A	72.62 A
CV	4.3	13.1	4.66	11.04	10.38

ns: no- significant; * significant (P≤0.05); ** significant (P≤0.01).
Source: authors (2019)

Table 4 - ANOVA results for the Microbiological analysis. The same letters do not differ by Tukey test (P≤0.05)

Variation Sources	Initial viability (%)	Final viability (%)	Initial contamination (CFU/mL)	Final contamination (CFU/mL)
Tratments (F)	5.2761*	3.8810*	2.1387 ^{ns}	1.7265 ^{ns}
No- treated	83.3980 B	83.5480 B	273.8000 A	399.6000 A
Chloride	82.2940 B	88.4280 AB	175.4000 A	250.0000 A
Activated carbon	92.7380 A	91.6540 AB	224.4000 A	338.8000 A
Composite membrane	85.1140 AB	91.8760 A	212.0000 A	343.2000 A
CV	5.34	4.96	28.08	31.6

ns: no- significant; * significant (P≤0.05); ** significant (P≤0.01)
Source: authors (2019)

For all treatments, except the activated carbon (where yeast viability was high since the beginning), the yeast viability was found higher at the end of fermentation as compared to the beginning. This parameter is important because, in the brazilian ethanol production process, yeasts are reused after a treatment with water and sulfuric acid (BASSO *et al*. 2011).

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The yeast cell viability in the end of the process is directly related to the ethanol yield of the next fermentation cycle, and its values should be higher than 85% (RAVANELI *et al*. 2011; DELLA-BIANCA *et al.* 2013).

Despite the differences of water samples added to molasses in the must preparation, no reduction was found in the contaminants concentration during the fermentation. The contaminants concentration found in molasses was low in the four samples when compared to the values commonly observed in sugar mills, higher than 106 CFU mL-1 of must (RAVANELI *et al*. 2011).

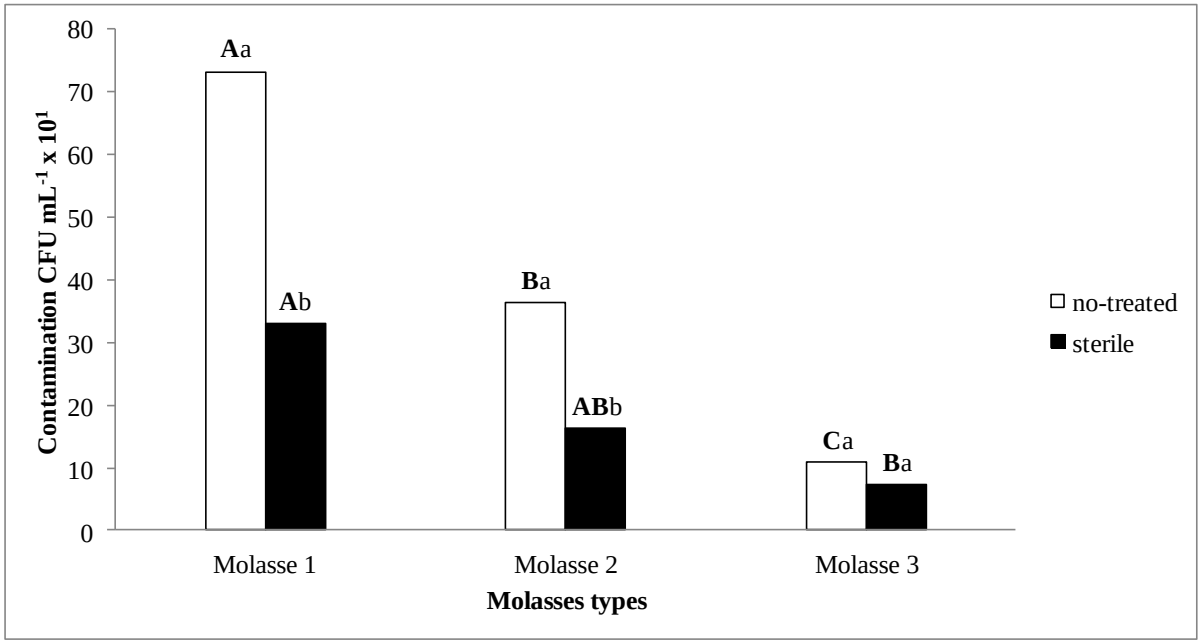
We also observed that the concentration of microorganism of the negative control reduced from 900 CFU/ml (Table 1) to 273.8 CFU/ml (Table 4) when the molasses was added to it. Probably one or some compounds of the molasses were reducing the microbial population.

Different types of molasses and dilution water

Whenever there is a difficulty in obtaining the specified sugar color, the industry usually uses sulfur for VHP sugar purification, a treatment up to 500mg L1 doses (REIN, 2007). Some of the sulfur used in this process remains in the molasses and could help to reduce the microbial population.

The influence of molasses in the must contamination process has been observed in the third study. In order to evaluate the influence of molasses in the contamination, three different molasses were diluted in sterile and non-sterile water (Figure 1). Molasses 1 and 2 are from the same sugar mill, although molasses 1 had been stored for one year at Fatec. These molasses are from VHP sugar production that could use sulphitation in purification. Molasses 3, however, was provided by another sugar mill which uses sulfur treatment for white sugar production, with sulfation; therefore, some of this sulfur was expected to remain in the molasses and in the must, consequently.

Figure 1 - Contamination observed in the must produced with three different molasses diluted in sterile and no-treated water. The same letters do not differ by Tukey test (P≤0.05). Capital letters compare molasses types with non-treated and sterile water. Lower case compares water treatments on molasses 1, 2 or 3



Source: authors (2019)

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It is possible to verify that must prepared with molasses 1 (the same of the second experiment, stored at Fatec for one year approximately) showed higher contamination than the others, even being the by-product of the same mill as molasses 2, but approximately a year older. Despite of the high concentration of soluble solids found in molasses constitution, contamination could increase slowly and that was what probably happened with molasses 1 compared with 2. Molasses 2 showed an intermediate contamination in relation to 1 and 3.

Figure-1 clearly shows that the use of sterile water has reduced significant the contamination only in samples 1 and 2. The reason for low microorganisms' contamination in molasses 3 is due to the fact that the molasses have been taken directly from the white sugar production process which uses SO_2 to purify the juice (REIN, 2007). Probably, sulfur reduced contamination in no treated water, which was expected to show higher microorganism concentration when compared with sterile water.

The amount of microorganisms found in the molasses 3 (around 100 CFU/ml) was similar to what has been observed in the fermentation process of the second study. The initial contamination with molasses diluted in non-treated water was 273.8 CFU/mL (Table 2) while after the fermentation process, the contamination was 399.6 CFU/mL (Table 4; higher mean observed in the molasses diluted with non-treated water), but this value is considered low as compared to the industrial process contamination, which has around 1,000,000 CFU/mL (RAVANELI *et al*., 2011).

Therefore, it is clear that the use of molasses with sulfur can reduce the microorganisms' population in the must which lessens the significance of the water treatment for the molasses dilution. Yet being harmful to yeasts (Table 4), the use of chloride treatment in order to reduce water contamination is a regular practice among Brazilian sugar mills. However, this practice has been demonstrated useless herein whenever molasses with sulfur is used. Industries that use sulfur in juice purification for sugar production could avoid chloride treatment in the water used for molasses dilution.

However, molasses with residual sulfur reduce microorganims contamination, in the second study, probably molasses has residual sulfur (not measured) and it could caused inhibition in yeasts and reduced ethanol yield (Table 3). Finally, the idea of using untreated water in the molasses dilution should be tested in an industrial-scale to confirm these findings.

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