UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências - Rio Claro/SP

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

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PROSPECTING NEW COMPOUNDS WITH ANTIMICROBIAL ACTION TO CONTROL OF CONTAMINANTS IN FUEL ETHANOL FERMENTATION

Rio Claro – SP - Brasil

Novembro – 2021

PROSPECTING NEW COMPOUNDS WITH ANTIMICROBIAL ACTION TO CONTROL OF CONTAMINANTS IN FUEL ETHANOL FERMENTATION

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Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do Título de Doutora em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia).

Orientador: Prof. Dr. Pedro de Oliva Neto

Rio Claro - SP

2021

R198p

Ranke, Fabiane Fernanda de Barros
Prospecting new compounds with antimicrobial action to control of contaminants in fuel ethanol fermentation / Fabiane Fernanda de Barros Ranke. -- Rio Claro, 2021
159 p. : tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, Rio Claro
Orientador: Pedro de Oliva Neto

1. Biotecnologia industrial. 2. Microbiologia industrial. 3. Antibacterianos. 4. Fermentação. 5. Drogas Resistência em micro-organismos. I. Título.

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TÍTULO DA TESE: PROSPECTING NEW COMPOUNDS WITH ANTIMICROBIAL ACTION TO CONTROL OF CONTAMINANTS IN FUEL ETHANOL FERMENTATION

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Aprovada como parte das exigências para obtenção do Título de Doutora em CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA), área: Estrutura, Função e produção de Biomoléculas pela Comissão Examinadora:

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Rio Claro, 12 de novembro de 2021

Will

There is no chance, no destiny, no fate,
Can circumvent or hinder or control
The firm resolve of a determined soul.

Ella Wheeler Wilcox

AGRADECIMENTOS / ACKNOWLEDGMENT

Agradeço inteiramente a Deus, por nunca soltar minhas mãos e por ser absolutamente tudo em minha vida. Trabalhar com pesquisa científica por si só já é desafiador, mas com um adicional de "Pandemia Covid-19", foi golpe duro. Segui firme porque apesar de todos os apesares, Ele sempre está.

Agradeço ao Prof. Dr. Pedro de Oliva Neto por confiar em meu trabalho e por me aceitar em seu grupo de pesquisa há 10 anos. Minha construção profissional foi muito enriquecida por ter sido responsável pela evolução do Instituto de Pesquisa em Bioenergia (IPBEN) – Unidade de Bioprocessos da Universidade Estadual Paulista (UNESP/Assis), ao qual eu orgulhosamente faço parte. Obrigada pela paciência e orientação, e por ser meu exemplo de dedicação no campo de atuação do presente estudo.

Ao Instituto de Biociências (IB-UNESP/Rio Claro), Programa de Pós Graduação em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia) pela oportunidade da realização deste doutorado, e a todos os docentes e profissionais envolvidos no programa, especialmente ao Felipe Marchi, pelo auxílio na sessão técnica de pós-graduação. Agradeço também a Faculdade de Ciências e Letras de Assis – UNESP, por disponibilizar as instalações e recursos para realização dos experimentos.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001.

Agradeço aos membros da banca examinadora por suas valiosas contribuições.

Agradeço as empresas Sabará Químicos e Ingredientes (São Paulo/SP/Brasil) e a Usina Cocal – Energia Responsável (Paraguaçu Paulista/SP/Brasil) por fornecer gentilmente amostras de Dióxido de Cloro e Melaço de Cana. Agradeço também ao Prof. Dr. Regildo Márcio Gonçalves da Silva, coordenador do Laboratório de Fitoterápicos e Produtos Naturais (UNESP/Assis), que de forma solícita contribuiu com alguns extratos vegetais mucilaginosos. Todas estas amostras fornecidas foram fundamentais para a parte de experimentação.

Agradeço a disposição do Prof. Dr. Eduardo Festoso Vicente do Departamento de Engenharia de Biossistemas (UNESP/Tupã), que sintetizou um dos compostos que foi estudado, bem como sua parceria com o Prof. Dr. Giuliano C. Clososki e Franco J. Caires (USP/Ribeirão Preto), que prontamente fizeram testes com corantes halogenados que também foram objeto de estudo. Agradeço também a Profa. Dra. Juliane C. Forti do

Grupo de Pesquisa em Química Ambiental (UNESP/Tupã), que contribuiu para a realização de algumas análises com degradação de corantes.

Meu imenso reconhecimento aos técnicos de laboratório Gilberto Millani, Allan, aos eletricistas e profissionais da manutenção responsáveis por todo funcionamento das nossas pesquisas, além dos porteiros do nosso Campus que sempre me recebem de forma muito gentil.

Agradeço carinhosamente as minhas amigas de laboratório Thaís Shinya, Franciane Figueiredo, Joyce Souza e Tania Campioni, que se tornaram parte da minha história e que levo para a vida. Obrigada por todo apoio, sugestões e pela parceria diária.

Aos meus coorientados em Iniciação Científica: Thainá Oliveira, Gabriel Guimarães, Amanda Ribeiro e Ana Laura Silvério, que fizeram parte ativa desta pesquisa e sem os quais, as fermentações extensas deste trabalho não teriam sido possíveis. Obrigada pela parceria e pelas madrugadas no laboratório.

As minhas amigas Larissa Lara, Natália Píccolo e Amanda Santilho, que me deram o suporte de suas amizades sólidas, e de fé em Deus, para que o caminho fosse mais leve e feliz.

À melhor família que poderia ter, agradeço. A minha mãe Solange Barros, que foi a maior incentivadora de todos os meus sonhos. Aos meus avós preferidos Agenor e Evanilda e a minha madrinha Eleusa, por serem também meus pais, muito presentes em tudo e sempre – as minhas conquistas são de e para vocês. Agradeço especialmente a Nicellen, que é minha prima-irmã e está diariamente por perto me apoiando e incentivando. Amo vocês.

Por fim, agradeço ao meu marido Renan Ranke, por ser minha família, meu porto seguro, por entender a minha ausência constante e por sonhar os meus sonhos comigo. Sem você e a cia da Mel, nada disso seria igual. Te amo!

"Eu tenho pensado nas pessoas que me somam. Naquelas que perdem seu tempo e energia comigo, insistindo de várias formas, para que o meu riso seja constante e as preocupações pequenas. Naquelas pessoas que me pegam pela mão e me ajudam a atravessar abismos. Eu tenho pensado nas pessoas que fazem abrigo no coração, pra eu morar. Naquelas que tecem milhares de sorrisos no meu rosto. Naquelas que constroem inúmeras certezas em cima do meu medo. Naquelas que falam bonito, depois de uma tempestade emocional desabar sobre o meu quintal. Naquelas que plantam pés de esperança, no vaso de entrada, pra encantar meu olhar. Por aquelas pessoas que não desistem de mim, eu agradeço!".

GENERAL ABSTRACT

Lactic acid bacteria are common contaminants in the bioethanol industry. Some treatments have been used to reduce bacterial contamination in fermentation, but their uses are limited due to high cost, bacterial resistance, toxicity to yeast, regulations and environmental aspects. This research aimed to study new products (chemically synthesized and natural) with antimicrobial properties, free or immobilized on natural supports, in order to replace or improve the efficiency of the molecules currently used in the industry for the control of *Limosilactobacillus fermentum*. The in vitro antimicrobial activity tests determined the Minimal Inhibitory Concentration (MIC) of 0.8 to 0.9 mg L^{-1} of Sodium Monensin (SM) and 150 mg L^{-1} to 230 mg L^{-1} of Chlorine Dioxide (CD), some of the main molecules currently used. The dyes Gentian Violet (GV) and Methylene Blue (MB) were also studied as an alternative and showed antibacterial action at 20 mg L^{-1} and 60 mg L^{-1}, respectively. Furthermore, GV and SM can act synergistically with MICs of 10 and 0.5 mg L^{-1}. Tests with MB and CD showed synergy with MIC of 40 mg L^{-1} for MB and 125 mg L^{-1} for CD. Immobilization of GV in Alginate/Avocado seed beads (Al/Av) showed 100% inhibition at 100 mg L^{-1} of GV Al/Av beads after 48 h of incubation at 37 °C in MRS medium. This technique was not effective in adsorbing MB, which easily detached from the gel during the incubation process. The GV Al/Av beads showed efficacy in 42 recycle, proving their stability and efficacy. Some free-cells culture of the yeasts *Sporobolomyces japonicus*, *Sporidiobolus pararoseus*, *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* were tested, which showed no inhibition of *L. fermentum* under the conditions testesd. In screening of the plant extracts, *Eucalyptus urograndis* inhibited *L. fermentum* and *Agave americana* extract inhibited *S. cerevisiae*, both at concentrations of 10,000 mg L^{-1}. *Eucalyptus* extract was no toxic to *S. cerevisiae*, however there was a partial inhibition of *L. fermentum* in mixed culture with *S. cerevisiae* in Fed-batch fermentation. Rosemary essential oil showed antibacterial action with MIC of 103 g L^{-1} in Fed-batch process in mixed culture after 5 recycling, with high ethanolic yield, suggesting that this oil should be further studied for industrial applications.

Keywords: *Lactobacillus fermentum*; Inhibition; Bacterial Contamination; Lactic Acid Bacteria; Alcoholic Fermentation.

RESUMO

As bactérias ácido láticas são contaminantes comuns na indústria do bioetanol. Alguns tratamentos têm sido utilizados para reduzir a contaminação bacteriana na fermentação, porém seus usos são limitados devido ao alto custo, resistência bacteriana, toxicidade para a levedura, regulamentações e aspectos ambientais. Essa pesquisa teve como objetivo estudar novos produtos (de síntese química e natural) com propriedade antimicrobiana, livres ou imobilizados em suportes naturais, a fim de substituir ou melhorar a eficiência das moléculas atualmente utilizadas na indústria para o controle de *Limosilactobacillus fermentum*. Os testes de atividade antimicrobiana in vitro determinaram a Concentração Inibitória Mínima (CIM) de 0,8 a 0,9 mg L^{-1} de Monensina Sódica (MS) e 150 mg L^{-1} a 230 mg L^{-1} de Dióxido de Cloro (DC), algumas das principais moléculas utilizadas atualmente. Os corantes Violeta Genciana (VG) e Azul de Metileno (AM) também foram estudados como alternativa e demonstraram ação antibacteriana em 20 mg L^{-1} e 60 mg L^{-1}, respectivamente. Além disso, VG e MS podem atuar sinergicamente com CIM de 10 e 0,5 mg L^{-1}. Testes com AM e DC mostraram sinergia com CIM de 40 mg L^{-1} para AM e 125 mg L^{-1} para DC. A imobilização de VG em grânulos de Alginato/Semente de Abacate (Al/Av) apresentou 100% de inibição a 100 mg L^{-1} de corante, após 48 h de incubação a 37 °C em meio MRS. Esta técnica não foi eficaz na adsorção do AM, que se desprendeu facilmente do gel durante o processo de incubação. Os grânulos de VG Al/Av mostraram eficácia em 42 reciclos, comprovando sua estabilidade e eficácia. Foram testados os sobrenadantes delevurados do cultivo das leveduras *Sporobolomyces japonicus, Sporidiobolus pararoseus, Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* M-26, que não mostraram inibição de *L. fermentum nas condições testadas*. Na triagem dos extratos vegetais, *Eucalyptus urograndis* inibiu *L. fermentum* e o extrato de *Agave americana* inibiu *S. cerevisiae*, ambos nas concentrações de 10.000 mg L^{-1}. O extrato de eucalipto não foi tóxico para *S. cerevisiae*, no entanto houve inibição parcial de *L. fermentum* em cultura mista com *S. cerevisiae* em fermentação Fed-batch. O óleo essencial de alecrim mostrou ação antibacteriana com CIM de 103 mg L^{-1} em processo de Fed-batch em cultura mista após 5 reciclagens celulares, com alto rendimento etanólico e sem efeito contra *S. cerevisiae*, sugerindo que esse óleo deve ser mais estudado para aplicações industriais.

Palavras-chave: *Lactobacillus fermentum*; Inibição; Contaminação Bacteriana; Bactérias Ácido Láticas; Fermentação Alcoólica.

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SUMMARY

1 GENERAL INTRODUCTION

Climate change, toxic substances from the use of fossil fuels and high energy demand had intensified the search for greener alternatives of fuel sources (KHAN et al., 2021). Thus, the importance of ethanol as a renewable biofuel, economically viable and less polluting source is given (KARP et al., 2021). In addition, ethanol can be used in the manufacture of paints, varnishes, solvents, among other products (CONAB, 2021; SANTOS, 2016).

In response to these needs, ethanol production is expanding steadily around the world, driven by significant investments. Studies show that for large-scale ethanol production, biological synthesis is more viable and adopted in almost every industry in the sector, especially where biomass is available (LOPES et al., 2016; VENTURA, 2015). Plant sources rich in carbohydrates continue to outperform all other alternatives in terms of productivity and efficiency at the desired scales (KIM et al., 2018).

As a tropical country, Brazil can be considered one of the largest producers of residual biomass energy and precursors in the policy of alternative forms of renewable fuels for its energy matrix. A large industry emerged from this initiative and today holds the most economically viable process for the production of bioethanol (MENDONÇA; LEAL JUNIOR, 2010). Sugarcane is one of the main raw materials for obtaining ethanol and the southeastern region concentrates the largest share of production (58.11% of the total produced in the country) (CONAB, 2017). In the 2018/2019 harvest, production reached a record 33.14 billion liters and an important factor that supports high production is the practically instantaneous flow of sales that increases cash flow more quickly (CONAB, 2020).

Agricultural processes generate residues rich in sugars, being an alternative for the production of bioethanol by yeast (SHARMA et al., 2007). Sugars are complex and difficult to access in this waste. Ethanol from these materials does not compete with the food industry and, for this reason, is known as second generation ethanol (NASSAR; MOREIRA, 2013). Ethanol production on an industrial scale is conducted by yeasts, more commonly *Saccharomyces cerevisiae*. The fermentation process is the most critical stage, where yeasts convert sugars into ethanol and presents a unique fermentation niche in which the substrate is unsterile and the conditions are not aseptic. The rich medium and favorable conditions encourage contaminating bacteria to grow and produce metabolites

that significantly decrease the fermentation yield (AMORIM et al., 2011; LOPES et al., 2016).

Among the factors that interfere with ethanolic fermentation, bacterial contamination stands out, as the quality of biofuel and alcohol yield can significantly decrease and the demand for chemicals and antimicrobials can increase, generating additional costs (AMORIM; BASSO; LOPES, 2009; OLIVA-NETO et al., 2012). It is estimated that 10^8 bacteria/mL decrease ethanol production by about 10,000-30,000 L a day in a distillery able to produce one million liters a day (AMORIM et al., 2011). The fermentative process may be monitored to indicate the potential source of contamination and the factors further aggravating it, in that way we can devise a strategy to control the bacterial growth (BASSO et al., 2008; COSTA; CERRI; CECCATO-ANTONINI, 2018) but sometimes it may not be totally effective, and may also affect the starter yeast culture and compromise the fermentation performance.

Lactic acid bacteria (LAB) compete for sugars with yeasts and for each glucose molecule they consume, two of lactic acid can be produced and two molecules of ethanol will no longer be produced. The lactic acid excreted in the environment compromises the reproduction, nutrition and synthesis of ethanol and, at a high level can cause the death of yeast (DORTA et al., 2005; NARENDRANATH et al., 1997). LAB can also cause cell flocculation leading to yeast loss via centrifuge, and increased spending on defoamers. For all these reasons, microbiological control is extremely important, ensuring the industrial productivity of this biofuel (BASÍLIO et al., 2008; OLIVA-NETO et al., 2013).

The management of microbial contamination in an industrial environment is highly complex, due to the factors that cause the selection and dissemination of contaminants (EGUCHI, 2007; OLIVA-NETO et al., 2012). In addition, a serious health risk to the workers is the corrosive nature of sulfuric acid that is often used, besides making the final disposal of the effluent a costly affair. Antibiotics have also been used in distilleries to control bacterial growth but the emergence of drug-resistant strains has limited their use. Besides that, the antibiotics are retained in the yeast cell mass that is later utilized for livestock and poultry feeds, and it could induce resistance in the animal and poultry pathogens (MUTHAIYAN; LIMAYEM; RICKE, 2011).

The availability of antimicrobials for ethanol production is very limited (OLIVA-NETO et al., 2012), either due to the low supply or the toxicity to yeast and its possible use as animal feed, which further limits the type of product that can be used industrially.

In this context, the improvement and discovery of new alternatives of chemical and natural products for bacterial control in fuel ethanol industries are strategic for Brazil. In the present study a selection of new alternative chemicals for controlling the growth of *Limosilactobacillus fermentum* without causing injury to *Saccharomyces cerevisiae* was evaluated. The synergism between conventional molecules, currently used in the control of industrial bacteria, and the new alternatives were also evaluated as immobilized dyes, natural ones as plant extracts and essential oils. Besides these, yeast extracts and immobilization processes were tested to avoid the waste of compounds with bactericidal action, as well the possibility of their reuse in new cyclic batches.

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3 OBJECTIVES

3.1 General objective

Evaluate new chemical alternatives for controlling the growth of *Limosilactobacillus fermentum* in ethanol fermentation by *Saccharomyces cerevisiae*.

3.2 Specific objectives

- Evaluate of the Minimum Inhibitory Concentration (MIC) of the compound's sodium monensin, chlorine dioxide, gentian violet and methylene blue in isolated tests for bacterial control.
- Evaluate the synergy of the chemicals by MIC.
- Test the ability of the dyes immobilized on Alginate/Avocado seed powder pellets to inhibition of bacteria.
- Evaluate the antibacterial activity of yeast cell filtrates.
- Screening of plants extracts to evaluate the antibacterial potential and their antimicrobial properties in Fed-batch fermentation.
- Evaluate the antimicrobial ability of rosemary and clove essential oils MIC and in Fed-batch fermentation.

4 CHAPTER 1 – BACTERIAL CONTAMINATION IN FUEL ETHANOL FERMENTATION: CHARACTERIZATION AND STRATEGIES FOR ITS CONTROLLING

ABSTRACT

Bacterial contamination in fuel ethanol bio-production is a significant problem for industries. In particular, *Limosilactobacillus fermentum* is the main bacterium of concern. In the process there are some features that inevitably lead to the production of organic acids, biofilms and yeast flocculation, decreasing the fermentation yield and producing metabolites other than ethanol. Some treatments are used at each fermentation cycle to reduce bacterial contamination, such as acids, antibiotics or biocides like chlorine dioxide. However, all compound alternatives have limited effectiveness, as they could generate residues in yeast biomass or damage for yeast metabolism. Furthermore, the environmental contamination of these compounds is another concern, in addition to the high costs for their application at industry. The development of an alternative of chemicals to prevent bacterial multiplication with low cost, high efficiency and environmentally correct aspects could bring benefits to the industry. This review analyzes the potential strategies with some compounds with antibacterial action that can be used to control the growth of lactic acid bacteria, in addition to those already known. We emphasize the issue of bacterial contamination in bioethanol industries and the efficiency of new compounds other than traditional ones.

Keywords: *Limosilactobacillus fermentum*; antimicrobial; biofuel; antibiotics.

5 INTRODUCTION

In the search for renewable sources to replace petroleum-based fuel, ethanol from plant sources rich in carbohydrates continues to outperform alternatives in terms of production and efficiency at the desired scales (KIM et al., 2018). The United States of America and Brazil are the largest producers of bioethanol from corn and sugarcane, respectively, and both are responsible for 83% of world production of ethanol (AFDC, 2021; BERTRAND et al., 2016; MORAIS et al., 2017). Brazilian knowledge in ethanol production from sugar cane began to be developed in the colonial period, when farmers produced Brazilian sugarcane spirit, called "cachaça" (GONÇALVES et al., 2021). In addition, the sugar-energy sector in Brazil represents 2% of the Gross Domestic Product (GDP), according to data from the 2018/2019 harvest. This sector is also one of the most employed in Brazil, generating approximately 4.5 million jobs, in addition to bringing together more than 72,000 farmers and 373 industries and distilleries, in operation or project.

The alcoholic fermentation, most commonly conducted with the yeast *Saccharomyces cerevisiae*, is applied to ethanol production processes on an industrial scale. Fermentation is done naturally in response to the medium in which the yeast is inserted, and the fermentation product increases or decreases according to the changes in that medium (LIN et al., 2012).

The industrial and agricultural environments have ubiquitous microorganisms as bacteria. Bacteria are widely present in fermented foods, beverages and fuels, both as fermentative agents and contaminants. The efficacy of these microorganisms is determined by the composition of the fermentation medium, culture conditions, interactions with other microorganisms, and concentration of the final product (AMORIM et al., 2011; LOPES et al., 2016).

The Gram-positive group represents 65% of the total number of bacteria that predominate in the contamination of alcoholic fermentation, with the genus *Lactobacillus* and *Bacillus* being one of the most prevalent (BONATELLI et al., 2017; OLIVA-NETO; YOKOYA, 1994). The *Limosilactobacillus fermentum* stands out, causing a drop in yeast viability and ethanol yield in fermentations, among other characteristics (OLIVA-NETO et al., 2012; ZHENG et al., 2020). The presence of active bacteria such as *L. fermentum* and *Bacillus subtilis* are not sufficient factors to decrease the percentage of live yeast cells. It is necessary the action of a set of metabolites associated to the acidity of the

medium for the yeast *Saccharomyces cerevisiae* to become unviable (NOBRE; HORII; ALCARDE, 2007).

Antibiotics have been used in distilleries to control bacterial growth but some negatives like the emergence of drug-resistant strains, the toxicity of the compounds and environmental pollution have limited their use. In addition, antibiotics are retained in the cell mass of the yeast that is subsequently used for cattle and poultry feeds. These compounds could induce resistance in the animal and poultry pathogens (MUTHAIYAN; LIMAYEM; RICKE, 2011). Alternative plant-derived antimicrobial compounds have been tested to improve both the fermentation process and its economic value. In addition, new chemical compounds have been studied on their antibacterial potential, representing a new path to greater efficiency combined with environmental sustainability and low costs. This review aims to examine the issue of bacterial contamination in fuel ethanol production, focusing on both conventional and unconventional strategies to control bacterial growth.

5.1 The fermentation process for fuel ethanol production

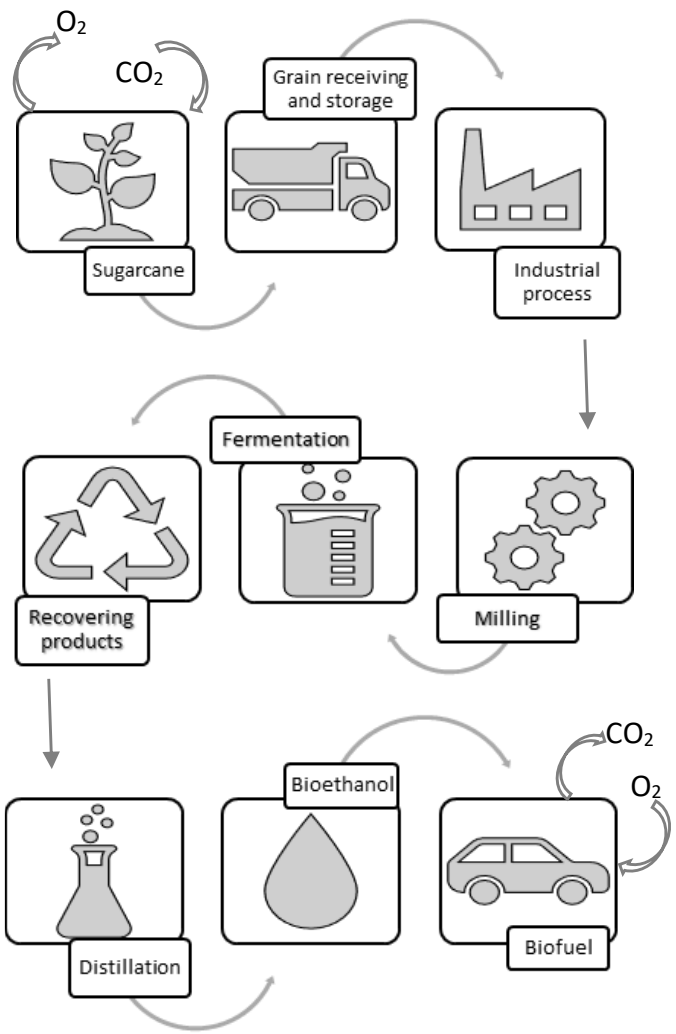
Traditionally, ethanol production is linked to the sugar industries in Brazil (LOPES et al., 2016; PEREIRA; SILVEIRA, 2016). At mills, sugarcane is pressed (some industries use diffusion), resulting in sugarcane juice and a solid fibrous residue, sugarcane bagasse. After clarification, the juice is concentrated by evaporation until the sucrose crystallizes. The sucrose crystals are collected by centrifugation, generating a viscous saturated sucrose phase, called cane molasses, with 45 to 60% sucrose and 5 to 20% glucose and fructose. Initially, ethanol production was established as a way to process molasses resulting from the sugar industry, but due to the growing importance of ethanol in the 1980s, many mills started as autonomous ethanol producers (LOPES et al., 2016).

The main industrial route used for ethanol production worldwide is the microbiological process, also referred to as alcoholic or ethanol fermentation (HAHN-HÄGERDAL et al., 1991; MADALENO et al., 2016). In this anaerobic process, sugars are converted into ethanol by yeast cells and they can come from different raw materials and crop residues (CAETANO; MADALENO, 2011; WANG et al., 2016). The “wine” (fermented must) obtained in the fermentation process is subjected to distillation, where hydrated ethanol (used in flex vehicles) and anhydrous ethanol (used in mixture with

gasoline) are obtained (CHUM et al., 2014). The liquid flow called vinasse is produced in the proportion of 10 to 15 L per liter of ethanol. Vinasse can be delivered to sugarcane fields for use as irrigation water and fertilizer, containing potassium, calcium, magnesium, other micronutrients and organic matter (LOPES et al., 2016).

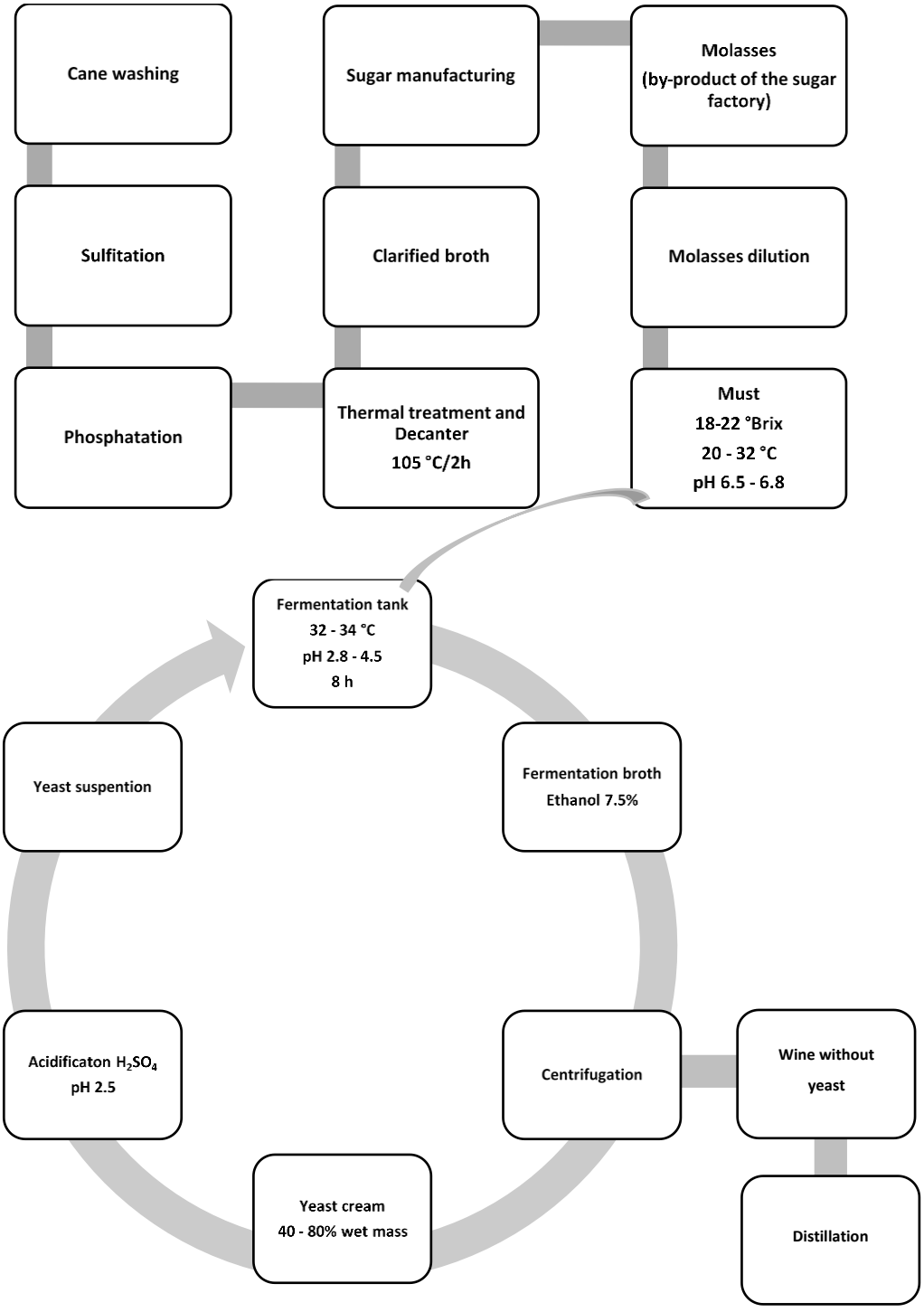
Technically, ethanol can be produced from a wide variety of renewable raw materials, which can be classified into three main groups: (1) those containing considerable amounts of readily fermentable sugars (sugar cane and sugar beet); (2) starches (corn, potatoes, rice and wheat); and (3) cellulosic (straw, grasses, ears of corn, wood and sugarcane bagasse) (MARTINS; OLIVETTE, 2015; WANG et al., 2016). Sugarcane, corn and sorghum are C4 plants with high efficiency to convert atmospheric CO_2 and water into sugars and polymers such as starch, cellulose and hemicellulose through photosynthesis. This process uses sunlight energy to fix carbon and release oxygen (O_2) into the atmosphere (LOPES et al., 2016). So, the CO_2 resulting from the production of ethanol is recycled through photosynthesis. After harvesting and crushing, sugars are fermented by yeasts and converted into ethanol. It is separated by the distillation and used as biofuel by cars (LOPES et al., 2016) (Figure 1). In Brazil, the main raw material predominantly sugar cane, while the United States of America (USA) produces ethanol from corn (AMORIM; LOPES, 2009). The general flowchart of bioethanol production in Brazil is presented in the Figure 2.

Figure 1. General industrial bioethanol production steps.



Source: The author.

Figure 2. General flowchart of distillery attached to a sugar factory.



Source: The author.

5.2 Yeast fermentation processes

In traditional and industrial fermentation processes a microbiota with specific characteristics is found. In particular, stress-tolerant yeast strains are found during ethanolic fermentation, which are exposed to high osmotic pressure caused by high sugar concentration, nutrient starvation, lack of oxygen, accumulation of high ethanol concentrations (TEIXEIRA; MIRA; SÁ-CORREIA, 2011; THANONKEO et al., 2007). An adaptive evolution of multi-tolerant strains is carried out in yeast cell recycling, leading to a higher tolerance to fermentative stress (BASSO et al., 2011; COSTA et al., 2008; GOMES et al., 2021).

Yeasts of the genus *Saccharomyces* are considered the most important for obtaining ethanol through fermentation in the manufacture of beverages and fuel (MAICAS, 2020). The biochemical process is carried out by the species *Saccharomyces cerevisiae* that has the ability to hydrolyze sucrose into glucose and fructose, hexoses easily used by it, in addition to mannose and galactose (CHEN, 2011). The yeast converts these sugars into ethanol, energy, biomass, CO_2 and other by-products (DASHKO et al., 2014). The yeast cell cytoplasm has an enzymatic apparatus that can readily ferment glucose, fructose, mannose, galactose, sucrose, maltose and maltotriose into ethanol and CO_2, in a sequential enzyme-catalyzed reactions, each by a specific enzyme (WALKER; STEWART, 2016).

During the production of bioethanol, several factors affect the fermentation yield, that is, the efficiency of the conversion of glucose into ethanol. These factors can be physical (temperature, osmotic pressure), chemical (oxygenation, pH, mineral and organic nutrients, inhibitors) and microbiological (species, strain and concentration of yeast, bacterial contamination) (ALMEIDA et al., 2016). The composition of the raw material is usually molasses, which is not altered to meet the needs of the starter yeast and is used according to the balance between sugar and alcohol production (REIS et al., 2018).

The bacterial contamination causes a reduction in ethanolic yield and inhibition of yeast growth, as it generates undesirable metabolites that can be toxic, causing a decrease in yeast cell viability (NOBRE; HORII; ALCARDE, 2007). As well as resulting in different end products than ethanol, such as lactic and acetic acids, harm the yield on the fermentative performance of yeast, resulting in reduced ethanol yield, yeast cell flocculation, foaming and low viability of yeasts (EGGLESTON et al., 2007;

NARENDRANATH et al., 1997; OLIVA NETO; YOKOYA, 1997a). This process is non-aseptic and conducive to the establishment of contaminating microbial communities that interact with each other (BREXÓ; SANT'ANA, 2017). Additionally, factors such as high cell density (8 to 17% v v^{-1}) and temperatures between 33 to 35 °C contribute to the productivity of yeast and high ethanolic yield in a short-term fermentation, from 6 to 10 h. This factor compensates the few days of the harvest and industrial operation, about approximately 200 per year (WHEALS et al., 1999).

Many different types of ethanol fermentation processes have been proposed such as continuous fermentation (using flocculant yeast strains) (VIEGAS; ANDRIETTA; ANDRIETTA, 2002), but this process has not been very successful due to bacterial contamination. Other processes include single batch fermentation, continuous fermentation with cell recycling and fed batch. Despite higher investments for installation, fed-batch fermentation processes shown better results when compared to continuous fermentation processes. Using a fed-batch strategy allows regulation of microorganism's physiology and can maintain inhibitory products at lower concentrations, as well as direct metabolic flux for the production of desired products. The highest fermentation yields were achieved by fed-batch processes, according to a years-long study of 62 distilleries with fed-batch and continuous fermentation processes (GODOY et al., 2008).

About 83% of the ethanol-producing fermentation units operate in the fed-batch process in Brazil (ANDRIETTA; ANDRIETTA; STUPIELLO, 2011; DE SOUZA DIAS et al., 2015) which has lower bacterial contamination, higher yields and lower consumption of chemicals, such as sulfuric acid and antibiotics, when compared to continuous fermentation (GODOY et al., 2008; LOPES et al., 2016). In all kinds of processes, yeast cell recycling is a common practice which avoids intense yeast propagation and less deviation of sugar for biomass production (OLIVA-NETO; YOKOYA, 1994). The recycling of cells in the Melle-Boinot model made to increase the productivity and ethanol yield with the gradual feeding of substrate, reducing the fermentation time (BASSO et al., 2011; OLIVA-NETO et al., 2012). In addition, enrichment of the medium with sugars and nutrients for cell propagation has been eliminated. In this process, however, microbial contaminants are also recycled. Associated with other problems such as sugar consumption and decreased ethanol production, release of organic acids, toxins and decreased viability of yeast cells, bacterial contamination is an aggravating factor in the bioprocess (DORTA et al., 2006).

Generally, fermentation process begins by broth composed of sugarcane molasses diluted with water or sugarcane juice and usually has a sugar concentration of 18 to 22% (w v^{-1}), mixed to a yeast suspension (AMORIM; BASSO; LOPES, 2009). This yeast suspension (with about 30% yeast cell, on a wet basis) represents 25 to 30% of the total fermentation volume, and this process is carried out in tanks of 300 to 3,000 m^3. Large fermenters normally exceeding 500 litres and are fed fresh broth for 4 to 6 hours (BASSO et al., 2011), and fermentation is completed within 6 to 12 h, as mentioned earlier (AMORIM et al., 2011). The ethanol fermentation process reach final concentrations of 7 to 11% (v v^{-1}) with fermentation yields as high as 92% of total theoretical yield (AMORIM et al., 2011). Stirring the tank is also desirable at low power, just to avoid compacting the cells at the bottom of the tank and to maintain a larger contact surface with the substrate. After the end of fermentation process, the yeast cells are collected by centrifugation and acidified with a sulfuric acid solution (1.8–2.5 pH) about two hours. After that, up to 90-95% of the acid-treated yeast mass is re-used for a new fermentation cycle with the fresh substrate (from 10 to 14% - w v^{-1}) (BASSO et al., 2011).

The yeast biomass increases from 5 to 10%, in relation to the initial biomass, during a fermentation cycle. This biomass increase is sufficient to replace the yeast cells lost during the centrifugation step. The high yeast biomass inside the fermenter is responsible for a very short fermentation time of 6 to 10 hours, when compared to 40 to 50 hours in the corn fermentation process (BASSO et al., 2011). Normally, the temperature is maintained at around 32 to 35 °C, but due to the short fermentation time, cooling is not always efficient enough to remove heat, and the temperature can reach 40 °C, especially in the humid season (AMORIM; LEÃO, 2005). In this condition there is a strong stimulation of bacterial growth and additional problems in the process. Additionally, the recover and recycle of the yeast contributes to recover part of the bacterial contamination since they are together in the wort.

5.3 Mixed culture of yeast and contaminating bacteria in a fuel ethanol bioprocess

The production of ethanol that occurs through fermentation has been constantly improved. In distilleries, contamination can occur through the quality of the raw material, from the stalks of the contaminated plant in the field, as in sugar cane, in the processing of corn kernels, which despite being less subject to deterioration, can be affected by the starch saccharification stage and by the long fermentation time, variations in pH,

temperature, nutrient concentration, water used in maceration and the industry itself through tubes and parts exposed to microorganisms (OLIVEIRA et al., 2013). Once present in the wort, bacteria find an environment conducive to proliferating quickly, competing with yeasts to obtain sugars, which should be converted into ethanol (VENTURA, 2015).

The hydrogen potential of the medium is of great relevance for the production of ethanol, both for the control of bacterial contamination, yeast growth, fermentation rate and product formation (MOHD AZHAR et al., 2017). Bacteria have a slower growth rate than yeasts, as they do not have much resistance to acidic pH. In fermentation for ethanol production, the optimum pH range of *S. cerevisiae* is 4.0 (LIN et al., 2012), while for LAB the ideal pH is in the range of 5.0 – 7.0 (MELO et al., 2017).

Non-sterile fermentation offers several benefits compared to sterile operation which can significantly reduce fermentation costs including reduced maintenance requirements, simplified operation, simple bioreactor design and elimination of sterility (CHEN; WAN, 2017; PAN et al., 2019). The use of two or more strains of different microorganisms has been increasing over time in fermentation processes, especially those developed from non-sterile sources and conditions. The different microorganisms present in mixed cultures can suffer interactions and the most interfering in fermentation is competition (TANGYU et al., 2019). The influence of the type of carbon source available as a substrate is one of the operating conditions of the fermentative processes involving mixed cultures that stand out the most. This is because this condition favors the growth of microorganisms with greater specific affinity for the substrate, or source of nutrients. Parameters such as pH and temperature are also relevant in this type of fermentation, since they can change the maximum specific growth rate of the microorganisms involved and the enzyme-substrate conversion factor (OLIVA-NETO et al., 2012).

Bacterial contamination in fermentation can cause damage to the process, such as: sugar consumption, inhibition and drop in the viability of yeast cells due to toxins and organic acids excreted in the medium, flocculation of the yeast and, consequently the reduction in the yield of ethanolic productivity (MUTHAIYAN; LIMAYEM; RICKE, 2011; NOBRE; HORII; ALCARDE, 2007). In addition, there may be the formation of gums and biofilms that increase viscosity and can cause operational problems (SKINNER-NEME et al., 2007), reducing ethanol production (MADALENO et al., 2016).

Several studies prove the influence of acetic and lactic acids in inhibiting growth

and decreasing cell viability of *S. cerevisiae*, when mixed with contaminating bacteria. At levels above 10^6 or 10^7 cells/mL of must, bacterial contamination can cause a significant drop in ethanol yield (NOBRE; HORII; ALCARDE, 2007).

The most commonly found bacteria in contaminations is *Limosilactobacillus fermentum*, also acting as an agent responsible for yeast flocculation, which is associated with corrosion and obstruction of pumps and centrifuges and reduced fermentation efficiency and efficiency (OLIVA NETO et al., 2014). *L. fermentum* receives this name for its association with yeasts in fermentative processes of vegetables, is an obligately heterofermentative species (CALASSO; GOBBETTI, 2011). The metabolic products of this bacterial growth, mainly organic acids, are harmful to the growth of yeasts and affect the yield and productivity of alcohol. Besides that, stressed yeast cells tend to release nutrients, particularly amino acids and peptides, which can stimulate bacterial growth (OLIVA NETO; YOKOYA, 1997a). Consequently, many problems are manifested in industrial operations, such as increased demand for chemicals to control fermentation, failure in the centrifugal cell separator and malfunction of the heat exchanger (YOKOYA; OLIVA NETO, 1991).

5.4 Bacterial diversity and its effects on alcoholic fermentation

The contaminant microbial communities interact with the yeast cells and can cause decrease of ethanol production and inhibition of yeast growth and additional undesirable metabolites are produced which may be toxic, causing a decrease in yeast cell viability (NOBRE; HORII; ALCARDE, 2007). As well as resulting in different end products than ethanol, such as lactic and acetic acids, harm the yield on the fermentative performance of yeast, resulting in reduced ethanol yield, yeast cell flocculation, foaming and low viability of yeasts (EGGLESTON et al., 2007; NARENDRANATH et al., 1997; OLIVA NETO; YOKOYA, 1997b). Factors such as high cell density (8 to 17% v v^{-1}) and temperatures between 33 to 35 °C contribute to yeast inhibition and high ethanolic yield and a short-term fermentation, from 6 to 10 hours, compensates for only approximately 200 days of harvest (WHEALS et al., 1999).

In ethanol production there are several contaminants and among the most harmful are the lactic acid bacteria (LAB) (LI; HEIST; MOE, 2016; SKINNER; LEATHERS, 2004). The specificities of LAB were studied from the early years of the 20th century. This group consists mainly of Gram-positive, non-sporulating,

carbohydrate-fermenting bacteria, acid-producing, anaerobic, acid-tolerant, catalase-negative bacteria, mostly without mobility and nitrate-reducing (BINTSIS, 2018; INGRAM, 1975). LAB is microaerophilic or facultative anaerobic and they may have cocci or rod-shaped cells, have complex nutritional requirements and produce lactic acid as the major end product of carbohydrate metabolism (AXELSSON, 2004). They form a diverse group with few common characteristics, except for their metabolism (BINTSIS, 2018; RADLER, 1975). LAB have an efficient metabolism of carbohydrates and the time of population increase is much faster than that of yeast (NARENDRANATH; THOMAS; INGLEDEW, 2001).

The genus *Lactobacillus* comprises rod-shaped cells measuring 0.5 - 1.2 x 1.0 - 10 µm, occasionally coccoid, which can occur in short, but mostly long chains. It has an optimum temperature for growth between 30 and 40 °C and pH about 5.5 and 6.0 (HOLT et al., 1994). Due to its specificities, LAB are practically the only non-catalase that can grow aerobically (LEHNINGER; NELSON; COX, 2000). Although they do not synthesize ATP by respiratory means, LAB is not influenced by the presence or absence of oxygen. During bioethanol fermentation, a dynamically structured bacterial community is therefore expected. LAB strains are classified as homofermentative when they convert glucose almost quantitatively to lactic by the Embden-Meyerhof pathway or heterofermentative when they have an equimolar mixture of lactic acid, ethanol and CO_2 by the pentose phosphate oxidative pathway. The homofermentative, such as *L. plantarum*, *L. paracasei*, *L. delbrueckii* and *L. vini*, break down hexoses through glycolysis into lactic acid and ATP. Heterofermentative, including *L. fermentum*, *L. buchneri*, and *L. brevis* convert hexoses into a mixture of lactic acid, ethanol or acetate, carbon dioxide and ATP (FELIS; DELLAGLIO, 2007). Heterofermentative species have more detrimental effects on the fermentation yield than the homofermentative species have more detrimental effects on fermentation yield than homofermentative species, although both LAB groups are common during fuel ethanol fermentations (BASSO et al., 2008; COSTA et al., 2008).

The main difference between homofermentative and heterofermentative LAB is the absence or presence of the enzyme aldolase (NARENDRANATH; THOMAS; INGLEDEW, 2001). Homofermentative species possess enzymes that convert hexoses such as glucose, fructose and mannose; disaccharides such as lactose, maltose and sucrose and polysaccharides such as starch and dextrin into intermediates for the glycolytic pathway (WOODS, 1961). In industrial processes of ethanol production, bacterial strains

belonging to the genus *Limosilactobacillus* was isolated and it was identified identical proportions between homo and heterofermentative, as well as observed the predominance of bacteria forming D-lactate and L-lactate (COSTA et al., 2008).

LAB are very relevant in the production of several cheeses, breads, drinks, milk, plants, silage, wine, beer and in the human and animal intestines (BINTSIS, 2018). They are able to live in the presence of oxygen or not, however they obtain their energy by substrate-level phosphorylation (WHITE, 2000). Even though LAB are traditionally fastidious from a nutritional point of view (FITZPATRICK; O'KEEFFE, 2001), they find in cane and milled juice, sufficient amounts of amino acids, vitamins and minerals for their development and cause significant losses of sucrose (SOLOMON, 2009). Sometimes some strains secrete exopolysaccharides (EPSs) into their extracellular environment for cell protection (EVIVIE et al., 2017).

There is a bacterial diversity in typical fermentation conditions where the majority of yeast cells are in suspension, and in co-aggregated fermentation with visible sedimentation/flocculation of the yeast cells. Even though the distribution of bacterial species was quite similar in both conditions, *L. fermentum* was the most common species in co-aggregated fermentation. Under laboratory conditions, yeast cells displayed sedimentation and co-aggregation when the number of *L. fermentum* was $>1\times10^5$ CFU/mL (CARVALHO-NETTO et al., 2015). From samples of wort, wine from ethanolic fermentation, centrifuged yeast and yeast treated with sulfuric acid from the industrial process, there was isolated several contaminating bacteria (VENTURA, 2015). Among these, *Lactobacillus sp* (45.04%) stands out; *Leuconostoc mesenteroides* (14.41%); *Bacillus sp* (9.46%), among others such as *Acetobacter* and *Clostridium*, proving the predominance of LABs in the ethanolic fermentation process. Brazilian industry of ethanol production have a predominance of Gram-positive bacteria (98.52%) and one of the predominant genera was *Lactobacillus* (59.75%), with the species *L. fermentum* in evidence (15.04%) (ANDRIETTA; ANDRIETTA; STUPIELLO, 2011). All of these produce appreciable amounts of organic acids (formic, butyric, acetic, lactic) (ANDRIETTA; STECKELBERG; ANDRIETTA, 2006).

A predominance of contaminants from the *Limosilactobacillus* group was also found after isolation of these microorganisms from samples of "yeast milk" in industries at State of São Paulo. The predominant bacterium was *L. fermentum* (62%), followed by *L. murinus* (9%), *L. vaccinostercus* (9%), *L. plantarum* (2%) and *Leuconostoc sp* (2%). In addition, the authors simulated the fed-batch process with cell recycling using *L.*

fermentum mixed with the yeasts and after 18 consecutive cycles, the yeast was seriously compromised and the *L. fermentum* grew very high, about 10^9 cells/mL, demonstrating their high competitiveness rate (OLIVA-NETO; YOKOYA, 1994).

A study conducted on four distilleries found $6.0\times10^5 – 8.9\times10^8$ CFU/mL of LAB in their fermentation tanks (LUCENA et al., 2010). The most common species of LAB were *L. fermentum* and *L. vini*, which especially emerge at the end of the harvest period. Most studies have identified bacterial species in fermentation tanks on the basis of culture-dependent methods. In contrast, there is a diverse bacterial community in corn-based bioethanol fermentation in five facilities utilizing 16S gene amplicon sequencing. Although the predominance of *Lactobacillus spp*. was verified in two facilities, the other three were dominated by *Proteobacteria* mostly belonging to the genera *Pseudomonas* and *Escherichia–Shigella*, that were positively correlated with each other but negatively correlated with LAB (LI; HEIST; MOE, 2016).

On the other hand, more recently a co-inoculation of *L. fermentum* did not potentiate the effects of a contaminating wild strain of *S. cerevisiae* (REIS et al., 2018). However, co-inoculation of *Dekkera bruxellensis* and *L. fermentum* led to a significant decrease in the CFU of an industrial strain of *S. cerevisiae*, with effects on the fermentation parameters (REIS et al., 2018). The latter study indicated that compounds other than organic acids were released into the medium as a part of the metabolism of the contaminants, because the organic acid production was low but the pH of the fermentation medium, irrespective of molasses or juice, was greatly reduced.

5.5 Bacterial resistance

According to the most used criterion multi-resistant bacteria are microorganisms resistant to most antimicrobials to which other are commonly sensitive (ROBERTS et al., 2009). The indiscriminate and irresponsible use of antibiotics has favored the selective pressure and predominance of increasingly resistant bacterial species (DEL FIOL et al., 2010). In fact, the discovery of antibiotics was an essential part in combating bacterial infections that have always plagued humanity. However, the worldwide concern with the development and spread of bacterial resistance to antibiotics is currently growing (FAIR; TOR, 2014).

Many international bacterial resistance programs were launched in the late 1990s, such as the Sentry Antimicrobial Surveillance Program (1997); Europeau

Antimicrobial Resistance Surveillance System (EARSS) (1998), among others. In Brazil, the National Health Surveillance Agency (ANVISA) created the National Network for Monitoring Antimicrobial Resistance in Health Services, in partnership with the Pan American Health Organization and the General Coordination of Health Laboratories Ministry of Health. All of these programs aiming to raise awareness of the rational use of antibiotics, in addition to the development of research on new methods for detecting the transfer mechanism of bacterial resistance (TENOVER, 2006).

The epidemiology of antibiotic resistance varies by region and country (NGWOKE; ODIMEGWU; ESIMONE, 2011). While some countries are declining, others are experiencing increased bacterial resistance. However, the increase or decline is always linked to the indiscriminate use of antibiotics (TACCONELLI et al., 2009). In 1945 when he received the Nobel Prize, Fleming warned in his speech that the misuse of antibiotics would result in resistance. A few decades later, the problem had already become widespread and became a relevant issue in medicine (SPELLBERG; GILBERT, 2014; VENTOLA, 2015).

The first case of antimicrobial resistance was reported in 1947, when isolation of *Staphylococcus pyogenes* (currently *Staphylococcus aureus*) resistant to penicillin in 38 of 100 patients with staphylococcal infections in England (BARBER, 1947; POUPARD, 2015). In 1957, the 6-aminopenicillanic acid lactam ring was purified by opening research for semi-synthetic penicillin. The synthesis of methicillin and ampicillin in 1960 and 1961 marked the introduction of semi-synthetic penicillin and, subsequently, there were reports of a decrease in the incidence of resistance as a result of the introduction of new antibiotics (ROLINSON; GEDDES, 2007). However, after these dates, strains of *S. aureus* resistant to methicillin and other semi-synthetic penicillin were isolated (SENGUPTA; CHATTOPADHYAY; GROSSART, 2013). Before 1946 the sensitivity of *S. aureus* to these antibiotics was estimated in 85% and in 1984, the sensitivity was reduced between 20% and 30%. In 2003, resistant methicillin-resistant *S. aureus* occurred, which was isolated from a nasal swab from healthy individuals, about 91% in Korea (JEONG et al., 2007) and 82.1% in Germany (FLUEGGE et al., 2006). The high incidence in healthy individuals was related to the overuse of antibiotics (MICHAEL; DOMINEY-HOWES; LABBATE, 2014; VISWANATHAN, 2014).

5.6 Control of bacterial growth in bioethanol industry

5.6.1 Conventional strategies

Antibiotics and antiseptics which act differently on one or more groups of microorganisms can be used to control the contamination problem in ethanolic fermentations, when they exceed acceptable limits (MUTHAIYAN; LIMAYEM; RICKE, 2011). However, they can leave residues in the distillates. Researches point out that among the commercial chemicals most used for bacterial control in fermentation are monesin antibiotics (Kamoran®), with about 80% efficacy against *Limosilactobacillus*, without interfering with ethanol-producing yeasts, and virginiamycin (harmless to yeast) (LEITE et al., 2013; OLIVA-NETO et al., 2013; OLIVEIRA et al., 1996), in addition to the chlorine dioxide biocide that affects *Saccharomyces cerevisiae* in higher dosages at 50 mg L^{-1}, partially inhibiting *L. fermentum* (MENEGHIN et al., 2008).

Penicillin has been studied in the past and interfered with active transport by inhibiting the biosynthesis of essential membrane constituents (EGUCHI, 2007), however, over time, continuous use on an industrial scale has led to a biological adaptation of contaminants, so this method is no longer it is more employed (OLIVA-NETO et al., 2012; SPELLBERG; GILBERT, 2014). The pentachlorophenol was used for years in proportions of 0.01 to 0.05 g L^{-1} of must, with excellent results, but its use was prohibited due to toxic and cumulative effects (LIMA et al., 2001). Hexachlorophene, which at doses of 4 mg L^{-1} of wort, contributed to good fermentations also subsequently had toxic effects attributed to this compound, which was banned in Brazil (GONÇALVES; GRAZIANO; KAWAGOE, 2012; LIMA et al., 2001). The antibiotics chloramphenicol and tetracycline were also used, although penicillin is widely more economically advantageous, but all of them had its uses limited due to bacterial resistance (KARIKARI et al., 2017).

In addition, the acid treatment of the yeast cells at the end of each fermentation cycle is crucial to reduce bacterial contamination and the harmful effects on yeasts, the reduction of pH 2.5 using sulfuric acid is used (BASSO et al., 2014). This method is used not only to reduce contaminants, but also to prevent yeast flocculation (OLIVA NETO; YOKOYA, 1997a). In vitro experiments showed a 3-log reduction in *L. fermentum* numbers with sulfuric acid treatment (COSTA; CERRI; CECCATO-ANTONINI, 2018); however, in the industrial set-up the numbers are not known. Acid washing of cells to decrease bacterial contamination is not exclusive to fuel ethanol fermentation. The

breweries have been using this technique for many years to eliminate bacteria from the pitching yeast. In spite of the negative effects of acid on yeast viability and physiology, especially in unfavorable conditions (long-term storage of inoculum, high contamination level, and slow fermentations), acid treatment in appropriate conditions (stirring, washing temperature, no storage) does not affect its physiology (SIMPSON; HAMMOND, 1989).

However, all the methods adopted above, including monensin, can leave residues attached to yeasts and generate, among other factors, the impossibility of using them as an animal supplementation (OLMSTEAD, 2012), preventing economic gains with this by-product. Due to the residual detection of antibiotics higher than expected, as well as the increase in microbial resistance and toxicity, several compounds are with restricted or prohibited use (CAETANO; MADALENO, 2011).

Actually, among the antimicrobial chemical compounds that have been tested on the industrial scale, chlorine dioxide has been most successful one. This molecule is a strong oxidant with antimicrobial properties against bacteria, virus, spores, and algae, and generally used in water systems (CECCATO-ANTONINI, 2018; LAPOLLI et al., 2005; OLMSTEAD, 2012). Chlorine dioxide can control the population of contaminants used in a concentration about 400 mg L^{-1} in fermentation. Researches pointed out that the effect of chlorine dioxide was the inhibition of protein synthesis and the extent of inhibition is proportional to the initial dosage of chlorine dioxide (ROLLER; OLIVIERI; KAWATA, 1980). This is due to the fact that there is an impediment to bacterial population growth in fermentations. However, chlorine dioxide can affect yeast in the process causing inhibition of cell viability (MENEGHIN et al., 2008), and its use should be limited.

Brazilian distilleries have replaced antibiotics and 40% of acid washing by chlorine dioxide at 30 mg L^{-1} (CECCATO-ANTONINI, 2018; FURTADO, 2013). In 2012 the patent of chlorine dioxide was approved in Brazil and commercialized for use in fermentation as DuPont™ Fermasure® (HARIS et al., 2018). It leaves no residue in yeast and other by-products and avoids the problem of antibiotic resistance and risks of sulfuric acid wash (MENEGHIN et al., 2008).

In this context, the use of antimicrobial is uncertain, which highlights the importance of studies of antimicrobials of natural origin as an effective and economical alternative (VARGAS et al., 2004).

5.6.2 Natural antimicrobials

Plant metabolism is divided into primary or macromolecules and secondary or micro molecules. The first includes proteins, lipids and carbohydrates, which are widely distributed in living beings, and the second involves alkaloids, terpenoids and flavonoids, which have a restricted occurrence, although they are essential for the organisms that produce them (HASSANPOUR et al., 2011). Secondary metabolites have shown to possess various biological effects, as well as antibiotic, antifungal, antiviral and able to protect from pathogens (HUSSEIN; EL-ANSSARY, 2019). For humans, these metabolites have important therapeutic properties, such as soothing, antiviral, contraceptive, antibacterial, antifungal, anti-inflammatory and insecticide that are widely used in the pharmaceutical, chemical, food and cosmetic industries (NAMITA; MUKESH, 2012).

Plant-derived products with antibacterial properties have a very long history and have been utilized in the last fifth century B.C. for treatment and prevention of diseases. Many studies in recent times have investigated the antimicrobial activities of several plant species and their active chemical constituents (MAHADY et al., 2008). An increasing number of infectious agents are becoming resistant to commercial antimicrobial compounds (HANCOCK; NIJNIK; PHILPOTT, 2012). To counteract the lack of new antibacterials and the rise of antibiotic resistance, plants could represent a potential solution (CHASSAGNE et al., 2021), for this reason, the need to develop new drugs requires varied strategies, such as the bioprospecting of secondary metabolites produced by medicinal plants (DIONISI; LOZADA; OLIVERA, 2012).

The search for compatible natural alternatives in an attempt to control invading microorganisms in the industrial process has been constant (OLIVEIRA et al., 2013). Numerous antimicrobial compounds offer a novel and safer option against bacterial contamination in the bioethanol industry. They are found in herbs, spices, fruits, vegetables, seeds, and leaves, and they can exert either direct or indirect effects (GYAWALI; IBRAHIM, 2014; MADALENO et al., 2016). Antibacterial plant-based can be used in ethanolic fermentation maintaining yeast viability, decrease the level of acids and increase the production process yield (MUTTON et al., 2014).

Biocides are specific formulations or natural products used to disinfect the must in tanks and equipment used in ethanol fermentation. More and more countries are demanding in relation to the regulatory security of their industrial processes and these

products meet the food requirements of some countries, although in others they do not reach the minimum necessary standards for use in the food industry (VENTURA, 2015). Unlike synthetic antibiotics, plant antibiotics have a chemical structure that can regulate the intermediate metabolism of pathogens, activating or blocking reactions and enzymatic synthesis or even changing the membrane structure (MICHELIN et al., 2005). The researches demonstrated the antimicrobial activity of plant extracts against *Salmonella spp*. and found that among the aqueous alcoholic/hydroalcoholic extracts of 86 plants under study, 58% had some inhibition or inactivation over *Salmonella sp* (WIEST et al., 2009).

Some biocides such as hops extract, propolis, pomelo and others that have been studied, show relative efficiency in the control of contamination in fermentations. Of these, the hop extract was the most efficient so far (CAETANO; MADALENO, 2011). Hops (*Humulus lupulus*) belong to the *Cannabinaceae* family and to the order *Urticales* (ALONSO-ESTEBAN et al., 2019). Its female flowers have lupulin glands in the petals which consist of an essential bitter resin rich in α-acids (humulones) and β-acids (lupulones) that are able to interfere with the transport of metabolites in the cell membrane and modify the intracellular pH, causing the death of bacteria through insufficiency nutritional. Gram-positive bacteria were highly inhibited by β-acids in concentrations over 8 μg L^{-1}, that does not alter the growth of *Saccharomyces cerevisiae* (ASTRAY et al., 2020). Commercial preparations of these acids (IsoStab®, LactoStab®, and Betabio 45®) are used in the bioethanol industry to combat bacterial infections at concentrations ranging from 10 to 50 mg L^{-1} in both fermentation tanks and during acid cell treatment with no effect on yeast viability (LEITE et al., 2013).

Propolis is a resinous substance collected by honeybees from buds and tree leaves, mixed with pollen and enzymes (CIFTCI-YILMAZ et al., 2017). It is an example of an animal-derived natural product with good potential in controlling bacterial contamination. Since the low numbers of microorganisms seen within beehives are associated with propolis, it has been evaluated for its antimicrobial properties (ANJUM et al., 2019; KALOGEROPOULOS et al., 2009; RUFATTO et al., 2017; SFORCIN; BANKOVA; KUROPATNICKI, 2017), it also surfaced that ethanolic extracts of propolis were more effective against Gram-positive than Gram-negative bacteria, and showed activity against different yeasts and molds (FRANCHIN et al., 2016; HARFOUCH; MOHAMMAD; SULIMAN, 2016). There are many applications of propolis in medicine, food industry, agriculture, cosmetology, as antifungal, anti-inflammatory, antiviral,

antioxidant (OMAR et al., 2017). Although studies on its application in ethanol fermentation processes have been restricted to small-scale experiments, they have yielded positive results without disturbing fermentation yields and controlling bacterial contamination (CECCATO-ANTONINI, 2018).

Chitosan is derived from the chitin of crustacean shells and fungal mycelia by deacetylation and is a very versatile compound with antimicrobial activity (BATISTA; SOUZA; PAIVA, 2018). It is able to inhibit the growth of LAB at a concentration of 0.1 g L^{-1} during brewing without affecting yeast viability (GIL et al., 2004). This compound is more effective against Gram-positive than Gram-negative bacteria (GOY; MORAIS; ASSIS, 2016). A special characteristic of chitosan is that its action increases greatly at low pH, which is often present during fermentation, due to the solubility of the charged amino acid groups (RABEA et al., 2003). The action of chitosan on the microbial contaminants of wine and beer fermentation has been reported (BAĞDER ELMACI et al., 2015; FERREIRA et al., 2013; GIL et al., 2004; GÓMEZ-RIVAS et al., 2004; PAN; REZAEI; SOOR, 2011; VALERA et al., 2017), and its commercial preparations such as Enartis Stab Micro M® are available for vinification. Doses of 0.1 and 1 g L^{-1} are required to inhibit bacteria such as *Pediococcus* and *L. plantarum*, respectively (GIL et al., 2004). In spite of the potential antimicrobial activity of chitosan, it has not been explored in the bioethanol industry.

Essential oils (EOs) have different biological properties, including fungicidal and bactericidal actions (AL-SHUNEIGAT et al., 2020). Oregano (*Origanum vulgare*) is an example of herb globally used as food flavoring. Her essential oil has a recognized action against bacteria. Oregano essential oil mainly contains carvacrol (more than 60% of the oil composition), a phenolic compound known for its broad-spectrum anti-infective properties, which makes it a much more effective bacterial agent (MAN et al., 2019). During ethanol fermentation, if used in the ideal dosage, it can favor bacterial control and preserve yeast.

Lignin is a sustainable biopolymer that can be found as a waste product from lignocellulosic biorefineries. A new study showed that technical lignin and their smaller phenolic subunits exhibit broad-spectrum antimicrobial properties. The corn stover lignin was oxidatively depolymerized using an environmentally benign organic oxidant, peracetic acid, into a bio-oil that has antimicrobial properties against LAB (including antibiotic-resistant strains) up to 90% at 4 mg L^{-1} with no inhibition against an industrial yeast strain (KALINOSKI et al., 2021).

Currently, the amount of waste produced by the food industry, in addition to being a huge loss of valuable materials, also poses serious management problems for both the economy and the environment (BUELVAS SALGADO; PATIÑO GÓMEZ; CANO-SALAZAR, 2012). The husks and seeds of avocado fruit are the largest by-product of this large industrial production (FERNÁNDEZ-CASTAÑEDA et al., 2018). The peel: seed: pulp ratio is 8.5: 11.5: 72%, respectively (DANE, 2015). The avocado is grown in both in subtropical and tropical regions of 59 countries. More than 60% of avocado plantations are in America (RAMÍREZ-GIL; GILCHRIST RAMELLI; MORALES OSORIO, 2017). In 2016, the FAO estimated an overall production of 5.57 million tons (FERNÁNDEZ-CASTAÑEDA et al., 2018).

The husks and seeds have antioxidant activity and are rich in bioactive substances such as polyphenols and chlorophylls (RAIGOZA MONTOYA et al., 2016). The avocado seed (AS) contains natural products such as phytosterols and triterpenes, fatty acids with olefinic, acetylenic, furanoic acid, oligomeric proanthocyanidin flavonole dimers, 8-hydroxyabscysic acid β-d-glucoside and epi-dihydro-d-glucoside acid (CEBALLOS; MONTOYA, 2013). It is possible to identify the presence of 3-O-caffeoylquinic acid, 3-O-p-coumaroylquinic acid, and procyanidintrimers by characterization of phenolic compounds (KOSIŃSKA et al., 2012). Seed and exocarp have higher total antioxidant levels than mesocarp (DAIUTO et al., 2014), predominantly in ascorbic acid AS and total phenolic compounds (TESFAY; BERTLING; BOWER, 2010). AS has a phytochemical composition of saponins 19.21, tannins 0.24, flavonoids 1.9, alkaloid 0.72, and phenols 6.14: all of which are measured in mg/100 g AS. These act as antioxidants as a result of the interaction between them (KOSIŃSKA et al., 2012). In addition, Hass avocado seed extract presented the potential antimicrobial activity against the bacteria *Salmonella enteritidis*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, and the yeast *Zygosaccharomyces bailii*. It could potentially be used as a food additive (RAYMOND CHIA; DYKES, 2010), or to avoid the growth of *L. fermentum* in fuel ethanol fermentation depending on some evaluations.

5.6.3 Nonconventional strategies to control bacterial growth in bioethanol industry

Novel antimicrobial products are constantly being tested against bacterial contamination in bioethanol fermentation to limit antibiotic usage and emergence of resistant strains. In addition, acid cell washing is occasionally inefficient and the corrosive acids are a serious safety risk. More recently, a series of chemical agents with bacteriostatic and bactericidal actions have been tested in small-scale experiments to evaluate their potential in bioethanol industry (Table 1).

Table 1. Antibacterial action of chemical agents in the bioethanol industry.

Product	Bacteria	Fermentation medium	Main results	Reference
Sodium monensin (HJ Kamoran®)	*L. fermentum*	Man-Rogosa-Sharpe	Monensin (Kamoran®) at 0.8 to 0.9 mg L^{-1} were able to completely inhibit *L. fermentum* growth	(RANKE et al., 2019; RIBEIRO et al., 2019)
Industrial chlorine dioxide	*L. fermentum*	Man-Rogosa-Sharpe	The growth inhibition of *L. fermentum* occurred about 200 mg L^{-1}. There was no decrease on the yeast viability	(RANKE et al., 2019)
Ethanol and low pH	*L. fermentum*	Sugarcane juice	Yeast cell washing with acid solution (pH 2.0) containing 5% ethanol resulted in bacterial inhibition after the first fermentative cycle. There was no effect on the ethanol yield	(COSTA; CERRI; CECCATO-ANTONINI, 2018)
3,4,4′-Trichlorocarba-nilide (TCC) + benzethonium chloride or benzalkonium chloride	*L. fermentum*	Man-Rogosa-Sharpe and nutrient medium	The addition of TCC in association with other compounds to sugarcane milling showed an intense bacterial reduction. The yeast *S. cerevisiae* was not affected	(OLIVA NETO et al., 2014)
Performic acid	*L. fermentum, L. paracasei, L. planetarium, L. meseteroides*	Sucrose medium	After bacterial exposure for 10 minutes to the performic acid (DesinFix TM 135) there was a decrease of four log cycles in the number of bacteria. No negative effects on yeast growth and fermentation	(BARTH et al., 2014)

Ethanol and sodium chloride	*L. fermentum, L. buchneri, L. plantarum, Acetobacter tropicalis, Acetobacter syzygii*	Wood hydrolysate	The synergistic action between NaCl (25 g L^{-1}) and ethanol (40 g L^{-1}) resulted in decreased bacterial viability and maintained yeast viability. There was no effect on the fermentation parameters	(ALBERS et al., 2011)
Chlorine dioxide	*L. fermentum, L. plantarum, L. mesenteroides* and *Bacillus subtilis*	Nutrient Broth and Man-Rogosa-Sharpe	The effects of chlorine dioxide (75–125 mg L^{-1}, respectively for *L. fermentum* and *L. plantarum*) were comparable to those of monensin (Kamoran®). *B. subtilis* (10 mg L^{-1}) and *L. mesenteroides* (50 mg L^{-1}) were more susceptible to the chemical compound	(MENEGHIN et al., 2008)
3,4,4′-Trichlorocarba-nilide (TCC)	*L. fermentum*	Molasses	TCC (1.8 g L^{-1}) entrapped in calcium alginate inhibited *L. fermentum* growth. A much lower concentration (0.075 g L^{-1}) combined with Sodium Dodecyl Sulfate (1.67 mg L^{-1}) controlled bacterial growth for 19 fermentation cycles. Improvement in the ethanol efficiency was observed	(OLIVA-NETO; YOKOYA, 1998)
Sulfite and hydrogen peroxide	*L. fermentum and L. casei*	Glucose-based semi synthetic medium	*L. casei* was more susceptible to sulfite (100 a 300 mg L^{-1}). *L. fermentum* growth was controlled by hydrogen peroxide at the concentrations of 1–10 mM	(CHANG; KIM; SHIN, 1997)

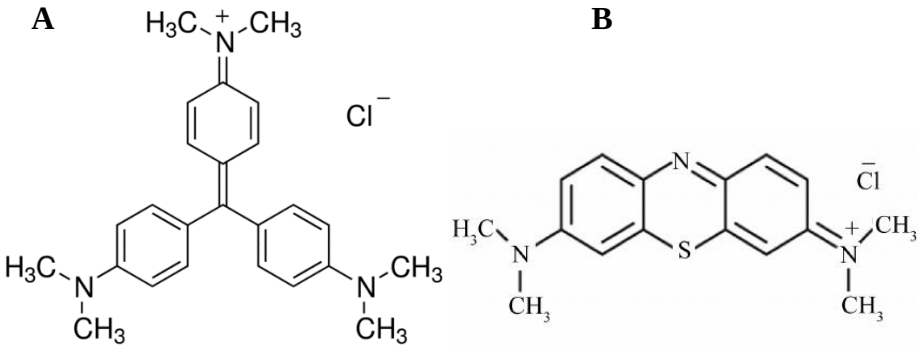
Source: The author.

Chemical compounds such as gentian violet and methylene blue dyes have shown positive results in the control of microorganisms (TAMAKI et al., 2017). Research shows that gentian violet - also called crystal violet - has antibacterial action against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Bacillus subtilis*. The effect of the dye, measured with the Minimum Inhibitory Concentration (MIC) or with growth retardation, increases as the pH increases from 6 to 8. Of the four species mentioned, *E. coli* is more resistant to the dye; the resistances of other microorganisms are similar (ADAMS, 2011). This inhibitory potential is likely to apply positively to other species of bacteria, *L. fermentum* being one of these.

Gentian violet (Figure 3A) is a purple dye derived from aniline with antimicrobial properties. This dye dissociates into positive and negative ions, which penetrate Gram-positive and Gram-negative bacterial cells. Positive ions interact with negatively charged components of the bacterial cell wall, including lipopolysaccharide, peptidoglycan and DNA. This agent is also a mutagenic and mitotic poison. Gentian violet causes a photodynamic action mediated by a free radical mechanism and, in addition, this agent dissipates the action potential in prokaryotic or eukaryotic membranes, inducing permeability, leading to respiratory inhibition and subsequent cell death (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2021a). GV can causes damage to DNA or mitochondrial dysfunction and the death of microorganisms. Another hypothesis assumes that are formation of complexes with the cell wall of bacteria or disorders of glutamic acid metabolism with high bactericidal or bacteriostatic activity. It was also noted that GV is able to combine with proteins (GARUFI et al., 2014; PERRY et al., 2006).

Methylene blue (Figure 3B) is a compound capable of reducing oxidation. The intravenous form of methylene blue is approved by the Food and Drug Administration (FDA) for the treatment of pediatric and adult patients with acquired methemoglobinemia. Historically, it has been widely used in Africa to treat malaria (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2021b).

Figure 3. Chemical structure of gentian violet (A) and methylene blue (B).



Source: (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2021a-b).

Finally, industrial strains of *S. cerevisiae* should be more investigated for antibacterial control, especially against LAB, since an inhibitory action against *L. fermentum* was proved (OLIVA NETO; FERREIRA; YOKOYA, 2004). In addition, the fermentation supernatant can act against lactic acid bacteria through toxins produced by *S. cerevisiae* or other yeast species are also a good perspective against contaminating bacteria (MENEGHIN; REIS; CECCATO-ANTONINI, 2010). A recent data indicated the possibility of producing ethanol under high acetate accumulation and inhibiting *Lactobacillus sp*. growth in molasses medium or acetate-containing condition. The co-fermentation was conducted with *L. fermentum* IFRPD and the acetate-tolerant yeast *S. cerevisiae* KU 002-3 at 0.8% (v v^{-1}) acetate (130 mM of the undissociated form of acetate) (SAITHONG; VANICHSRIRATANA; MORAKUL, 2021). This approach would be of great interest and should be incorporated as a criterion for yeast strain selection in fermentation.

Developing alternatives for bacterial control of alcoholic fermentation for the production of fuel ethanol is a strategic and very difficult task, given the few and limited products already available. Among the main alternatives to antibiotics are dyes, essential oils and other plant and microbial extracts, which need to be tested and evaluated not only against the growth of contaminating bacteria, but also, whether they affect the yeast metabolism and leave toxic residues that may compromise the yeast trade for animal purposes, or impact the environment, as well as being economically competitive.

6 CONCLUSIONS

The production of fuel ethanol by fermentation process is sensitive to microbial contamination, mainly bacteria, since this process is not aseptic and a recycled cell yeast allows the contaminant to be recycled. The most important group of microorganisms in this process is the genus *Lactobacillus* and *Limosilactobacillus fermentum* is one of the most important species which causes problems of decrease of ethanol efficiency, lower productivity and increased chemical use. All these problems increase the cost of fuel ethanol. In addition, there are few chemicals for bacterial control such as monensin, virginiamycin, chlorine dioxide or resins rich in α-acids (humulones) and β-acids (lupulones). These few options are currently used in Brazilian distilleries but their use is limited due to different reasons. Therefore, new alternatives need to be evaluated. Some antimicrobial compounds extracted from natural sources like oils and/or extracts, chemical dyes, or microbial extracts may be a new candidate for use in this area depending on further research in this field.

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8 CHAPTER 2 – NEW CHEMICAL ALTERNATIVES IN THE CONTROL OF BACTERIAL CONTAMINANTS OF FUEL ETHANOL FERMENTATION

ABSTRACT

Fuel ethanol distilleries have some chemical alternatives to avoid lactic acid bacteria contamination in a non-sterile bioprocess. The antibiotics Sodium Monensin (SM) produced by *Streptomyces cinnamonensis* and Chlorine Dioxide (CD) acts on Gram-positive bacteria such as *Limosilactobacillus fermentum*, the major contaminant in Brazilian distilleries. However, its uses are limited due to some problems such bacterial resistance and/or toxicity to yeast. In this sense, new alternatives are needed to replace or improve the current molecules, such as the use of Gentian Violet (GV) and Methylene Blue (MB) dyes. In this work the Minimal Inhibitory Concentration (MIC) for some antimicrobials in pure and mixed culture conditions was used for *L. fermentum*. The in vitro antimicrobial activity tests showed significant results for each compound, 0.8 to 0.9 mg L^{-1} of SM were able to completely inhibit *L. fermentum*. Currently the concentration used in distilleries represents 3-4 mg L^{-1}. CD had the inhibitory concentration about 230 mg L^{-1} for trade A (CDA) and the trade B (CDB) about 150 mg L^{-1}. GV was efficient at 20 mg L^{-1} and MB inhibited the bacteria at 60 mg L^{-1}. GV and SM could act synergistically with MICs of 10 and 0.5 mg L^{-1}, respectively (almost 50% reduction at both concentrations individually). Tests with MB and CDA showed synergy with MICs of 40 mg L^{-1} for MB and 125 mg L^{-1} for CDA (45% reduction in CDA concentration). The same concentrations of all the compounds were tested for yeast, which was not affected. The results point out a higher sensitivity of *L. fermentum* to antibiotic or biocide in synergistic process using dyes. Considering the lower toxicity of dyes to *S. cerevisiae* than CDA, or the lower use of SM in combination with dyes, these results can be promised as an alternative for bacterial control in fuel ethanol fermentation.

Keywords**:** Antimicrobials; Dyes; Chlorine Dioxide; Monensin; Bioethanol.

9 INTRODUCTION

The *Limosilactobaillus fermentum*, *Lactiplantibacillus plantarum* (ZHENG et al., 2020) and *Bacillus* (*B. subtilis*, *B. megaterium*, *B. coagulans*) represent the majority of contaminating bacteria in Brazilian fuel ethanol distilleries (DELLIAS et al., 2018; OLIVA-NETO et al., 2013). *L. fermentum* uses carbohydrates contained in the medium to produce lactic acid, which, in addition to reducing alcohol yields, inhibits yeast fermentation (BREXÓ; SANT'ANA, 2017; DA SILVA-NETO et al., 2020; REIS et al., 2018). This contamination can result in the loss of 10,000 to 30,000 liters of bioethanol per day in a production of one million liters (AMORIM et al., 2011; OLIVA-NETO et al., 2013). In addition, *L. fermentum* causes serious yeast flocculation problems, resulting in decreased viability of *S. cerevisiae* during fermentation and additional chemical expenses leading to increase of ethanol cost (CECCATO-ANTONINI, 2018; LOPES et al., 2016; OLIVA NETO; YOKOYA, 1997).

The treatments to control bacterial contamination from fuel ethanol fermentation broth include measures against *L. fermentum* and other contaminating lactic bacteria, to be effective. The explored methods to combat contamination include pre-treatment of the raw material, changes in the process conditions, such as solids and pH content, cleaning of fermentation equipment, antibiotics, bactericides and washing of the yeast used in sugarcane fermentations (KIM et al., 2018). Currently, acid treatment of yeast, cleaning equipment and antibiotics are the most used measures in the industry. However, acid washing stresses fermenting yeast and can lead to a decrease in the efficiency of ethanol production (BASSO et al., 2011; CECCATO-ANTONINI, 2018). Increased development of antibiotic resistance in contaminating strains has also been observed (MURPHREE; HEIST; MOE, 2014).

Monensin is the one of the most widespread chemical antibiotics in distilleries in fermentations contaminated with *L. fermentum*, however its commercial use is currently limited by restrictions on health or resistance to strains (SOUSA et al., 2014). Particularly, there is a maximum permitted limit of residues of this antibiotic in commercial yeasts that is exported. The monensin concentration used in the broth during fuel ethanol fermentation is 3 mg L^{-1} (OLIVA NETO et al., 2014). Researches studied the toxicology of sodium monensin in various species of laboratory animals and there was considerable variation of the species in the acute values of LD50 orally, with consistent signs of acute toxicity such as: anorexia, skeletal muscle weakness, ataxia, diarrhea,

decreased weight gain and late death (TODD; NOVILLA; HOWARD, 1984). In this same study, dogs underwent research and after treatment with monensin, for one month, developed anorexia, weakness, ataxia, difficult breathing, loss of body weight, increased values of serum muscle enzymes, severe skeletal muscle degeneration and necrosis with less severe cardiac injuries and deaths. If considered that the antibiotics are retained in the yeast cell mass that is later utilized for livestock and poultry feeds, and it could induce resistance in the animal and poultry pathogens (MUTHAIYAN; LIMAYEM; RICKE, 2011) than, it can not be commercialized.

Another alternative currently used in Brazilian distilleries to control the bacterial population in fuel ethanol fermentation is chlorine dioxide. Chlorine dioxide acts by inhibiting protein synthesis and microbial growth and the extent of inhibition is proportional to the initial dosage (ROLLER; OLIVIERI; KAWATA, 1980). However, chlorine dioxide can affect yeast in the process causing inhibition of cell viability (MENEGHIN et al., 2008), so this product has its use limited by the dosage.

The spread of antibiotics resistance in bacteria cause enormous economic and social burdens to health care systems worldwide (KASHEF; HAMBLIN, 2017). There is a challenging task to develop effective strategies for the efficient use of existing antibiotic resources avoiding the development of bacterial resistance with environmental conscious (LIU et al., 2020). This fact limited the use of antibiotics for industrial purposes opening opportunities for other molecules. New chemical alternatives for bacterial inhibition in the fuel ethanol industry are an example of this demand, so some dyes may have antimicrobial potential.

Gentian Violet (GV), also known as gentian or crystal violet is a purple dye derived from aniline with antimicrobial properties. This dye dissociates into positive ions, which penetrate Gram-positive and Gram-negative bacterial cells, interacting with negatively charged components of the bacterial cell wall, including lipopolysaccharide, peptidoglycan and DNA (DRAGAN; MICHALAK, 2019). GV can causes DNA damage or mitochondrial dysfunction and the death of microorganisms. Another hypothesis assumes that are formation of complexes with the cell wall of bacteria or disorders of glutamic acid metabolism with high bactericidal or bacteriostatic activity. It has also been observed that GV is able to combine with proteins (GARUFI et al., 2014; PERRY et al., 2006). This inhibitory potential is likely applied positively to other Gram-positive bacteria such as *L. fermentum*. Methylene Blue (MB) also known as methylthionine chloride ($C_{16}H_{18}N_3SCl$) is a basic dye that has a pH of 11.4 in solution. MB is an

antimicrobial with ability to puncture and penetrate bacterial endospores (OKTARI et al., 2017). The intravenous form of MB is approved by the Food and Drug Administration (FDA) for the treatment of pediatric and adult patients with acquired methemoglobinemia (LUDLOW; WILKERSON; NAPPE, 2020) and may be an alternative against lactic acid bacteria.

There is an increasing demand for less harmful antibacterial for the realization of fermentative processes, in an attempt to control invading microorganisms and increase yield, with the use of new alternatives being a promising option, because, in addition to avoiding or reducing the use of antibiotics based on monensin, distilleries could use yeast dry biomass for animal feed (LOPES et al., 2016). In this sense, antimicrobials inert to yeast metabolism should be sought for this application, since the antibacterial inhibitory action on contaminants should not affect yeast metabolism (MUTTON et al., 2014), as well as actions to eliminate the use of these antibacterial or using them in lower concentrations may contribute to the sugar and alcohol sector in Brazil.

This work aimed to evaluate the antimicrobial potential of gentian violet and methylene blue dyes applied against *Limosilactobacillus fermentum* and its effect on the yeast *Saccharomyces cerevisiae*, responsible for ethanol production. These antimicrobials were tested in synergistic action with sodium monensin and chlorine dioxide in order to broaden the spectrum of action and to decrease the efficient concentrations of each one in addition to reduce the damage effect on yeast.

10 MATERIALS AND METHODS

10.1 Microorganisms and maintenance of strains

The yeast strain *Saccharomyces cerevisiae* M-26 was previously isolated from bioethanol distillery (OLIVA NETO; FERREIRA; YOKOYA, 2004). Yeasts were stored at -80 °C in Eppendorf on YM medium containing 2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.087% dipotassium phosphate, 0.024% magnesium sulfate heptahydrate, 0.0017% manganese sulfate heptahydrate, 0.0028% zinc sulfate heptahydrate to 100 mL of distilled water (with 10% of glycerol) at pH 5. The culture medium for the propagation of yeast and inoculum production was prepared by (w v^{-1}): 2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.1140% dipotassium phosphate $3H_2O$, 0.024% magnesium sulfate $7H_2O$, 0.0012% manganese sulfate H_2O, 0.0028% zinc sulfate $7H_2O$ (pH 5) (DORTA et al., 2006). The lactic acid bacteria

Limosilactobacillus fermentum CCT 0559 was obtained by André Tosello Culture Collection (Campinas, Brazil) and cultivated at pH 6 on MRS medium (DE MAN; ROGOSA; SHARPE, 1960).

10.2 Conventional and non-conventional antibacterial compounds

For the first screening tests the chemical dyes tested were phenolphthalein, safranin, acid fuchsin (Quimibras), red congo (Neon), bromocresol green, coomassie blue (Dinâmica), methyl red (Synth), orcinol, aniline blue, evans blue (Vetec), purple bromocresol, eosin, erythrosine, malachite green and shiny violet remagol (Sigma-Aldrich). After that, gentian violet (GV) (Synth) and methylene blue (MB) (Biotec) was evaluated just like sodium monensin – Kamoran (Química Real) at 80% concentration and chlorine dioxide – ClO_2 (trade A) (Aratrop) at 32% concentration. Samples of industrial monensin and ClO_2 (trade A) was kindly supplied by Usina Cocal - Energia Responsável (Paraguaçu Paulista/SP/Brazil). The other chlorine dioxide (trade B) tested was provided by Sabará Químicos e Ingredientes (São Paulo/SP/Brazil) at 25% concentration.

10.3 Minimum Inhibitory Concentration Test (MIC)

The MIC of these chemicals was determined by adapted macrodilution broth method (JONES et al., 1985). Assays were performed in the tubes containing 8 mL of MRS medium (DE MAN; ROGOSA; SHARPE, 1960) for bacteria and Formulated medium (DORTA et al., 2006) for yeast with concentrations according to promising studies already produced at the laboratory. The inoculum was standardized according to McFarland 0.5 standard in aseptic conditions. The MIC was defined as the minimum chemical compounds concentration able to inhibit at least 90% the microbial growth and performed in triplicate.

Microbial growth was measured by turbidity of the medium by reading the absorbance at 600 nm wavelength using a spectrophotometer (Pharmacia - Ultrapec), in addition to checking under a microscope.

10.4 Preparation of solutions and cultures

The processes included preparation of solutions diluted in MRS medium of dyes (100 mg L^{-1}) each one, the industrial reagent of sodium monensin (SM) (4 mg L^{-1}) and chlorine dioxide trade A (CDA) (400 mg L^{-1}) and the chlorine dioxide solution trade B (CDB) (300 mg L^{-1}). After preparation, the solutions were stirred for at least 30 minutes after tests. The following concentrations were tested for each compound: GV by 60 to 10 mg L^{-1}, MB by 100 to 10 mg L^{-1}, SM by 4 to 0.1 mg L^{-1}, CDA by 270 to 180 mg L^{-1} and CDB by 300 to 30 mg L^{-1}. The phenolphthalein, safranin, acid fuchsin, red congo, bromocresol green, coomassie blue, methyl red, orcinol, aniline blue, evans blue, purple bromocresol and shiny violet remagol were all tested by 100 mg L^{-1}.

The assays were sterilized at 121 °C for 15 minutes. The inoculum was aseptically added and the cultures were incubated at 30 °C for yeast and 37 °C for bacteria for 48 hours.

10.5 Antimicrobial compounds analyzed in synergy

After obtaining the isolated MIC of the dyes, theses concentrations were tested in combination between the dyes and with de MIC of sodium monensin and chlorine dioxide (CDA) to analyze the occurrence of antagonism or synergism by the MIC of different mixtures (Tables 2a, 2b and 2c). Isolated MIC tests were conducted using two different chlorine dioxide reagents (CDA and CDB), the industrial and the commercial one with a high purity grade. For tests in synergy with other compounds, the reagent obtained from industry (CDA) was chosen.

Table 2a. Variable concentrations to test synergy or antagonism between gentian violet, methylene blue, sodium monensin and chlorine dioxide trade A (CDA).

Treatment	Gentian Violet (mg L^{-1})	Methylene Blue (mg L^{-1})	Sodium Monensin (mg L^{-1})	Chlorine Dioxide (CDA) (mg L^{-1})
1	3	-	0.4	-
2	5	-	0.5	-
3	8	-	0.6	-
4	10	-	0.7	-
5	13	-	0.8	-
6	15	-	0.9	-
7	18	-	0.9	-
8	20	-	0.9	-
9	10	-	-	230
10	15	-	-	230
11	30	-	-	230
12	25	-	-	230
13	30	-	-	230
14	35	-	-	230
15	40	-	-	230
16	45	-	-	230
17	50	-	-	230
18	55	-	-	230
19	60	-	-	230
20	20	-	0.35	100
21	12.5	-	0.1	100
22	5	-	0.35	100
23	12.5	-	0.35	100
24	12.5	-	0.35	75
25	12.5	-	0.6	100
26	5	-	0.1	125
27	20	-	0.1	125
28	20	-	0.6	125
29	20	-	0.1	75
30	20	-	0.6	75
31	5	-	0.6	75
32	12.5	-	0.35	125
33	5	-	0.1	75
34	5	-	0.6	125

Source: The author.

Table 2b. Variable concentrations to test synergy or antagonism between gentian violet, methylene blue, sodium monensin and chlorine dioxide trade A (CDA).

Treatment	Gentian Violet (mg L^{-1})	Methylene Blue (mg L^{-1})	Sodium Monensin (mg L^{-1})	Chlorine Dioxide (CDA) (mg L^{-1})
35	5	60	-	-
36	40	5	-	-
37	23	43	-	-
38	32	8	-	-
39	31	34	-	-
40	20	30	-	-
41	40	60	-	-
42	5	5	-	-
43	-	35	0.024	-
44	-	5.27	0.5	-
45	-	35	0.976	-
46	-	10	0.1	-
47	-	60	0.9	-
48	-	10	0.9	-
49	-	60	0.1	-
50	-	35	0.5	-
51	-	64.73	0.5	-
52	-	70	-	125
53	-	60	-	125
54	-	50	-	125
55	-	40	-	125
56	-	30	-	125
57	-	20	-	125
58	-	10	-	125
59	-	5	-	125
60	-	70	-	230
61	-	60	-	230
62	-	50	-	230
63	-	40	-	230
64	-	30	-	230
65	-	20	-	230
66	-	10	-	230
67	-	5	-	230

Source: The author.

Table 2c. Variable concentrations to test synergy or antagonism between gentian violet, methylene blue, sodium monensin and chlorine dioxide trade A (CDA).

Treatment	Gentian Violet (mg L^{-1})	Methylene Blue (mg L^{-1})	Sodium Monensin (mg L^{-1})	Chlorine Dioxide (CDA) (mg L^{-1})
68	-	22.5	0.3	177.5
69	-	22.5	0.5	230
70	-	22.5	0.5	125
71	-	5	0.5	177.5
72	-	40	0.1	177.5
73	-	5	0.3	125
74	-	40	0.5	177.5
75	-	5	0.3	230
76	-	22.5	0.3	177.5
77	-	22.5	0.3	177.5
78	-	40	0.3	125
79	-	22.5	0.1	125
80	-	22.5	0.1	230
81	-	5	0.1	177.5
82	-	40	0.3	230

Source: The author.

10.6 Analytical procedures

10.6.1 Growth and viability of microorganisms

After 48 hours of incubation at the optimum temperature, microbial growth was verified by turbidity of the medium with the naked eye and reading in a spectrophotometer (600 nm). In addition, verification under a microscope was performed to determine cell growth and viability by counting live cells/mL of extract by adding 1 mL of each sample to 1 mL of methylene blue dye solution (LEE; ROBINSON; WANG, 1981). After homogenization, live cells were counted (uncolored) in a Neubauer Chamber, and cell viability was determined by the ratio of living/total cells per mL of extract (DORTA et al., 2006). The determination of the quantity was found according to the equations:

$$Number\ of\ cells/mL\ =\ \sum of\ the\ quadrants\ x\ 5\ x\ 10^4\ x\ DF \qquad (1)$$

$\sum$ of squares = sum of the number of cells counted in the five quadrants;

10^4 = volume adjustment on the camera;

DF = sample dilution factor.

$$Cell\ viability\ (\%) = \frac{total\ number\ of\ living\ cells\ x\ 100}{total\ number\ of\ cells\ (live+dead)} \qquad (2)$$

10.6.2 Growth inhibition of microorganisms

Antibacterial efficiency was calculated using the following equations:

$$Inhibition\ abs\ (\%) = \frac{(Abs\ treatment\ 48\ h - Abs\ treatment\ 0h)\ x\ 100}{(Abs\ control\ 48\ h - Abs\ control\ 0\ h)} \qquad (3)$$

Abs of the treatment 48 h - Abs of the treatment 0 h = absorbance of the treatment means the growth in 48 h culture;
Abs of the control 48 h – Abs of the control 0h = absorbance of the control group means the growth in 48 hours;
Inhibition abs (%) = percentage of growth inhibition of the microorganism.

$$Bacterial\ reduction\ (\%) = \frac{(Abs\ control - Abs\ treatment)\ x\ 100}{Abs\ control} \qquad (4)$$

11 RESULTS

11.1 Initial screening of dyes for growth inhibition of *L. fermentum* and *S. cerevisiae*

The results of the selection of different dyes in inhibition of *L. fermentum* and *S. cerevisiae* are shown in Table 3. The dyes bromocresol green and malachite green showed microbial inhibition of *S. cerevisiae* at concentration of 100 mg L^{-1} and no inhibition of the other dyes evaluated. Gentian violet and Methylene blue dyes inhibited *L. fermentum* without affecting yeast viability at 100 mg L^{-1}.

Table 3. Initial prospecting of 12 chemical dyes at 100 mg L^{-1} concentration to evaluate the growth control in the cultures of *L. fermentum* at 37 °C and *S. cerevisiae* at 30 °C for 48 hours.

Dyes	***L. fermentum***	***S. cerevisiae***
Phenolphthalein	+	+
Red congo	+	+
Bromocresol green	+	-
Methyl red	+	+
Safranin	+	+
Orcinol	+	+
Purple bromocresol	+	+
Acid fuchsin	+	+
Evans blue	+	+
Aniline blue	+	+
Coomassie blue	+	+
Eosin	+	+
Erythrosine	+	+
Malachite green	+	-
Shiny violet remagol	+	+
Gentian violet	-	+
Methylene blue	-	+

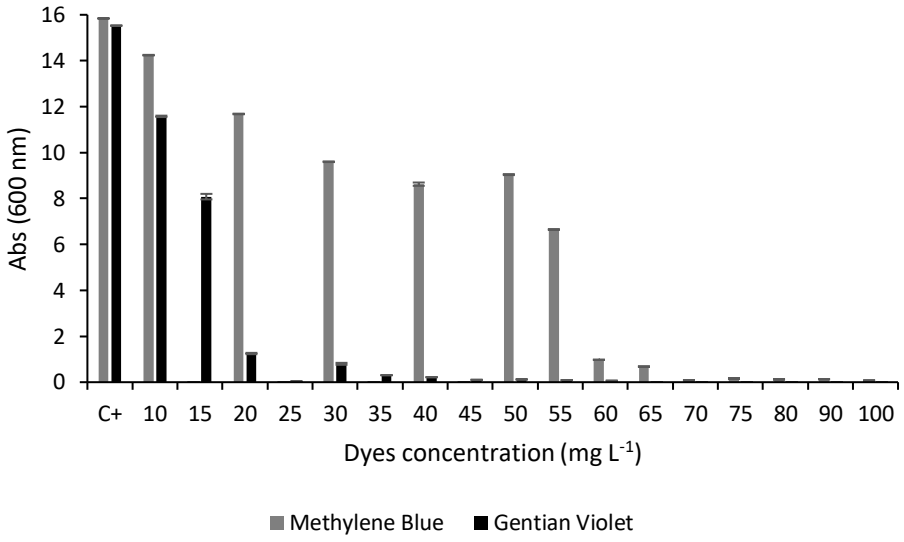
Where (+) indicates microbial growth; (-) indicates microbial inhibition.

Source: The author.

11.2 Analysis of isolated compounds

Tests with gentian violet evaluated from 10 to 60 mg L^{-1} resulted in 20 mg L^{-1} of MIC against *L. fermentum.* There was growth only with 10 mg L^{-1}. This result did not affect *S. cerevisiae* as showed by the turbidity of the medium and microscope analysis. Methylene blue showed 60 mg L^{-1} of MIC. The test was carried out from 10 to 100 mg L^{-1}. The growth was also verified in a spectrophotometer with absorbance at 600 nm, and the results can be seen in the graph (Figure 4).

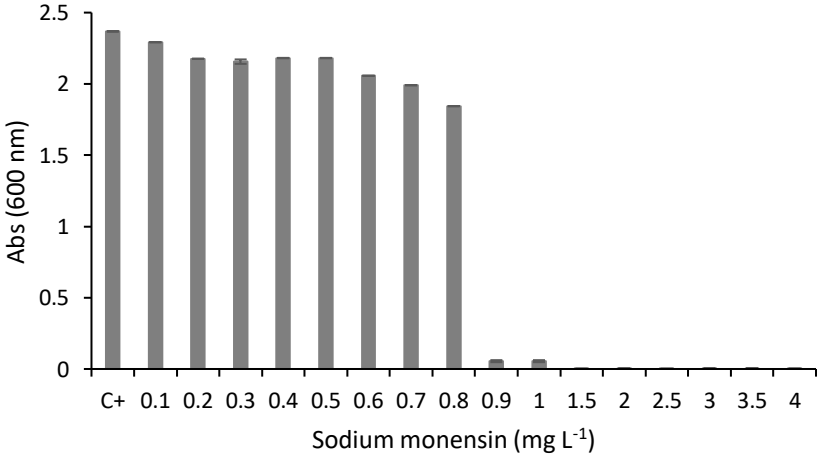
Figure 4. Growth-related absorbance of *L. fermentum* in different concentrations of gentian violet or methylene blue in MRS medium at 37 °C.



Source: The author.

Industrial sodium monensin and chlorine dioxide (CDA) showed MIC of 0.9 and 230 mg L^{-1}, respectively and the chlorine dioxide (CDB) presented MIC of 150 mg L^{-1}, for bacterial inhibition. In isolation, the research results showed that SM, CDA and CDB have a lower solute concentration than that applied in the ethanolic fermentation process by distilleries, since the common industrial dosage is 3 mg L^{-1} from MS and 400 mg L^{-1} from CD (Figures 5, 6 and 7).

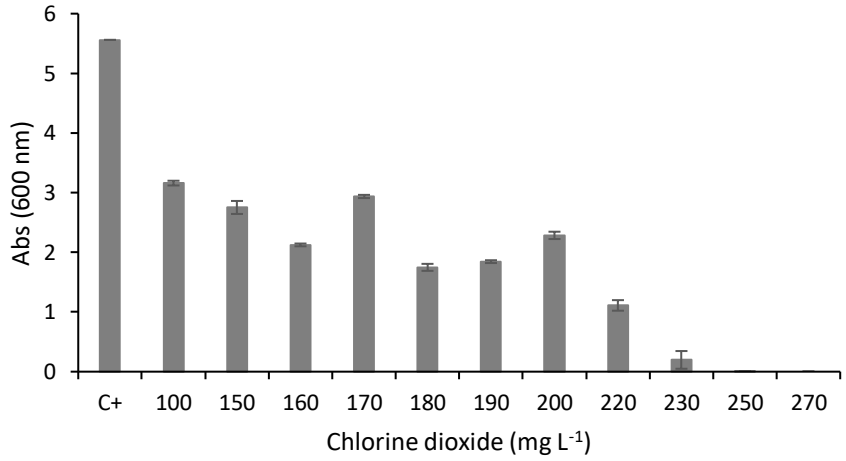
Figure 5. Growth-related absorbance of *L. fermentum* in different concentrations of sodium monensin in MRS medium at 37 °C.



Obs. Positive control (C+). Source: The author.

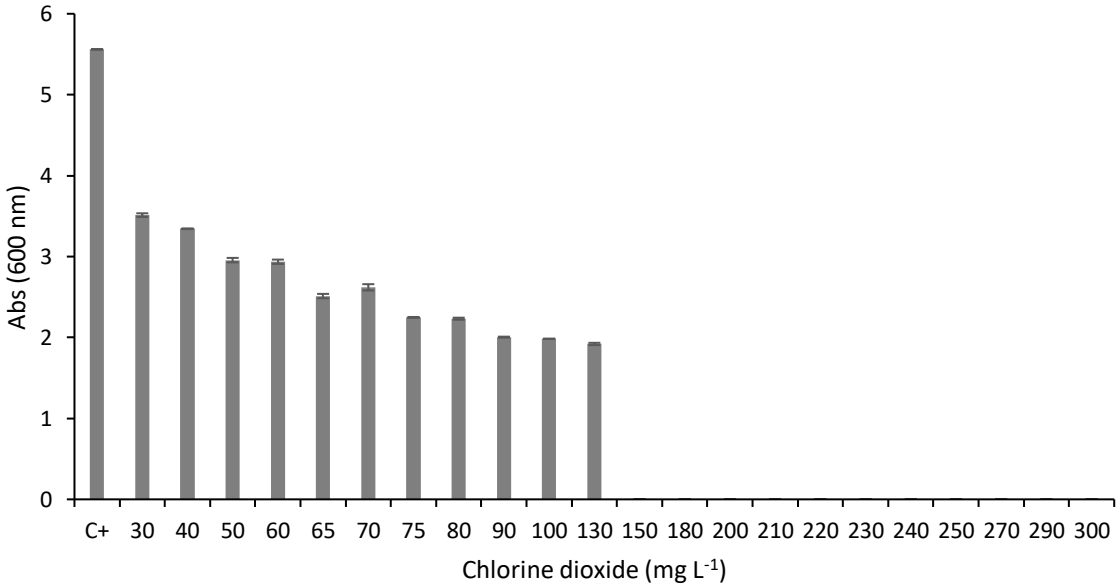
There was a remarkable reduction in bacterial growth from 0.9 to 4 mg L^{-1} of sodium monensin, which demonstrated the effectiveness of this compound with 0.9 mg L^{-1}.

Figure 6. Growth-related absorbance of *L. fermentum* in different concentrations of industrial chlorine dioxide trade A (CDA) in MRS medium at 37 °C.



Obs. Positive control (C+). Source: The author.

Figure 7. Growth-related absorbance of *L. fermentum* in different concentrations of industrial chlorine dioxide trade B (CDB) in MRS medium at 37 °C.



Obs. Positive control (C+). Source: The author.

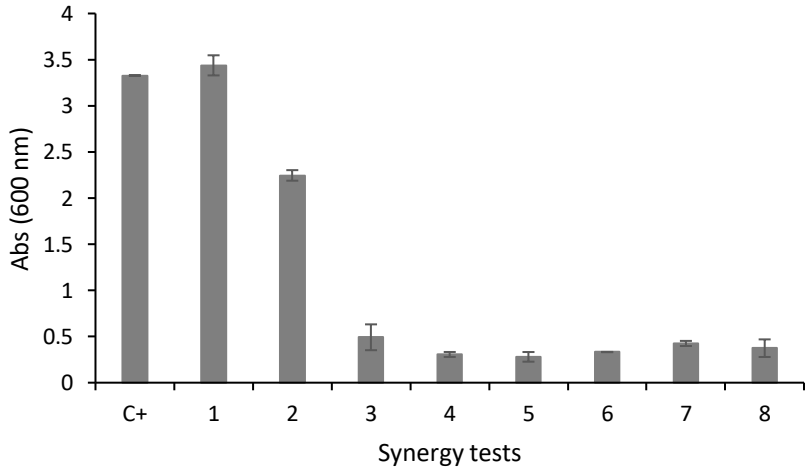
Industrial chlorine dioxide (CDA) had no antibacterial action at concentrations below 230 mg L^{-1}. However, tests performed with commercial chlorine dioxide (CDB) had a lower MIC around 150 mg L^{-1}, both calculated on a pure active compound basis.

All these chemical compounds evaluated did not affect the growth and viability of *S. cerevisiae* in any of the tested concentrations.

11.3 Analysis of the synergy of the mixture of antimicrobial compounds in *L. fermentum* culture in MRS medium

The results showed synergy between gentian violet and monensin sodium and indicated inhibition of *L. fermentum* growth in the minimum concentrations of 10 and 0.5 mg L^{-1} of the compounds, respectively. A decrease of almost 50% in the concentration of both testes separately was verified in comparison with the mixture (Table 2a). The synergy between these two compounds did not affect the growth of *S. cerevisiae* (Figure 8), indicating the possibility to use in fuel ethanol fermentation.

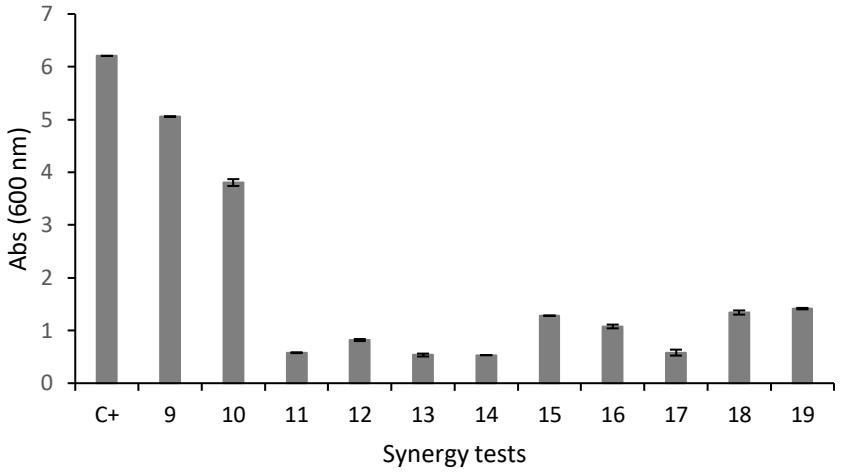
Figure 8. Growth-related absorbance of *L. fermentum* at different concentrations between gentian violet and sodium monensin in MRS medium at 37 °C. Concentrations on Table 2a.



Obs. Positive control (C+). Source: The author.

The synergy between gentian violet and chlorine dioxide (CDA) showed MIC against *L. fermentum* in the range of 30 to 35 mg L^{-1} gentian violet with 230 mg L^{-1} of industrial chlorine dioxide (CDA) (Figure 9).

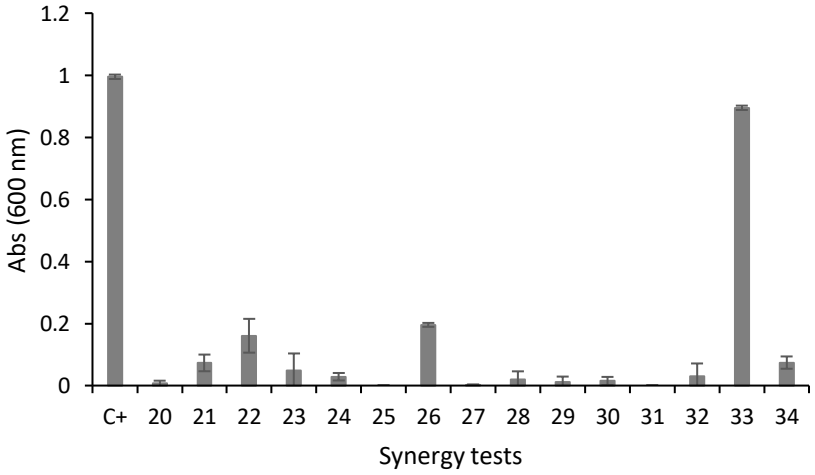
Figure 9. Growth-related absorbance of *L. fermentum* at different concentrations between gentian violet and industrial chlorine dioxide trade A (CDA) in MRS medium at 37 °C. Concentrations on Table 2a.



Obs. Positive control (C+). Source: The author.

A more detailed test was carried out with the synergy of gentian violet, monensin sodium and industrial chlorine dioxide (CDA). The results show that at 5, 0.6 and 75 mg L^{-1} of the three antimicrobials, respectively, there was bacterial inhibition (Figure 10).

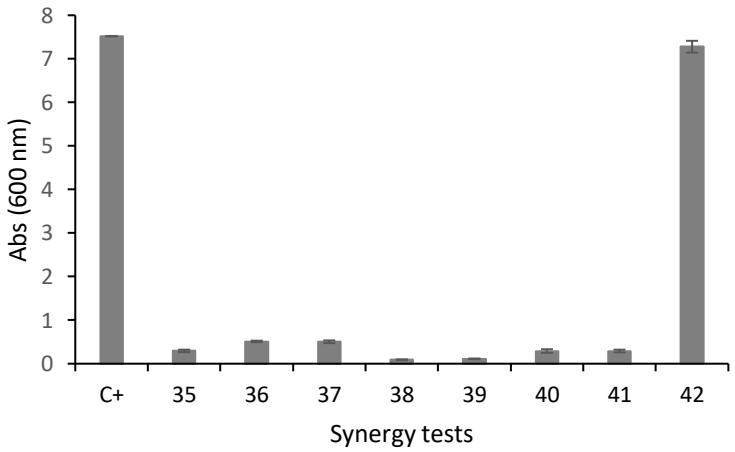
Figure 10. Growth-related absorbance of *L. fermentum* at different concentrations between gentian violet, sodium monensin and industrial chlorine dioxide trade A (CDA) in MRS medium at 37 °C. Concentrations on Table 2a.



Obs. Positive control (C+). Source: The author.

There was a *L. fermentum* growth in the mixture with 5, 0.1 and 75 mg L^{-1} of gentian violet, sodium monensin and industrial chlorine dioxide (CDA), respectively. This fact proves the need to use at least 0.6 mg L^{-1} sodium monensin to obtain antibacterial efficiency. In another experiment was possible to reduce the concentration of methylene blue acting with gentian violet (Figure 11).

Figure 11. Growth-related absorbance of *L. fermentum* at different concentrations between 5 to 40 mg L^{-1} gentian violet and 5 to 60 mg L^{-1} methylene blue in MRS medium at 37 °C. Concentrations on Table 2b.

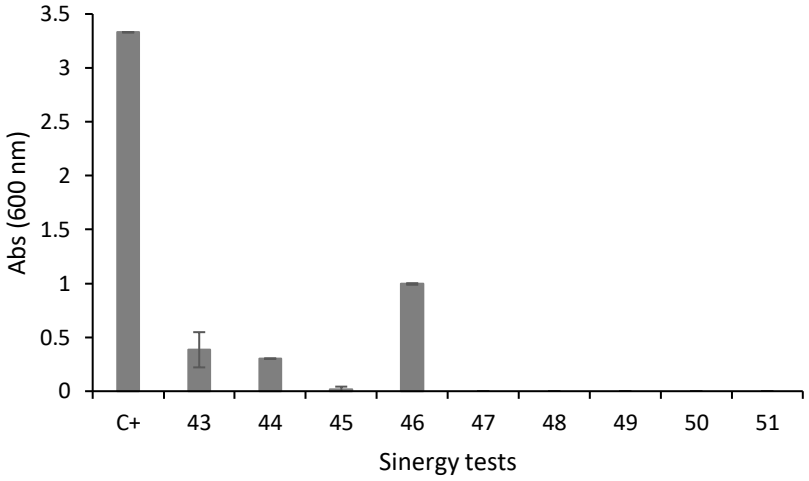


Obs. Positive control (C+). Source: The author.

In test tube 35, which contained 5 mg L^{-1} gentian violet and 60 mg L^{-1} methylene blue, bacterial growth was inhibited. In test 40, which contained 20 and 30 mg L^{-1} for gentian violet and methylene blue, respectively, there was a decrease of *L. fermentum* growth by half, demonstrating synergy between the compounds. The bacterial growth was observed only at the lowest tested concentrations of 5 mg L^{-1} for both dyes.

The Figure 12 showed synergy between methylene blue and monensin sodium and proved by inhibition of *L. fermentum* in the concentrations of 10 to 60 mg L^{-1} and 0.1 to 0.9 mg L^{-1} of the compounds, respectively. This test had MIC with a decrease of almost 55% in the concentration of both tested separately.

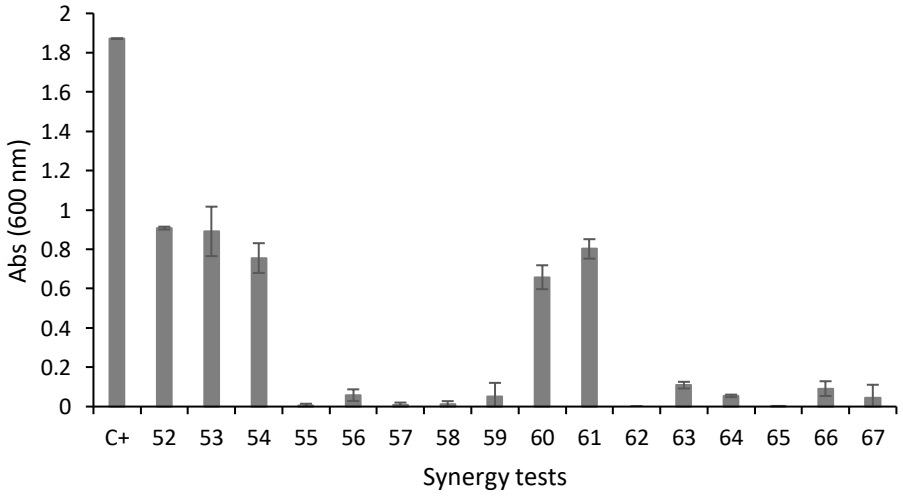
Figure 12. Growth-related absorbance of *L. fermentum* at different concentrations between 10 to 60 mg L^{-1} methylene blue and 0.1 to 0.9 mg L^{-1} sodium monensin in MRS medium at 37 °C. Concentrations in Table 2b.



Obs. Positive control (C+). Source: The author.

Methylene blue and industrial chlorine dioxide (CDA) acted in synergy against *L. fermentum* with MIC about 20 mg L^{-1} of methylene blue and 230 mg L^{-1} of industrial chlorine dioxide (CDA). When the dioxide concentration was halved, the MIC result was 40 mg L^{-1} methylene blue and 125 mg L^{-1} chlorine dioxide (CDA) (Figure 13).

Figure 13. Growth-related absorbance of *L. fermentum* at different concentrations between 5 to 70 mg L-1 methylene blue and 125 to 230 mg L-1 industrial chlorine dioxide trade A (CDA) in MRS medium at 37 °C. Concentrations in Table 2b.

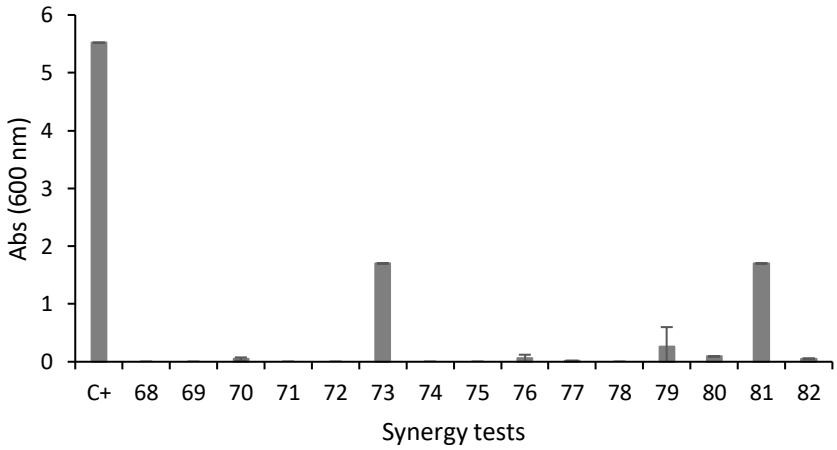


Obs. Positive control (C+). Source: The author.

The experiment showed how efficient the combination of these two compounds was, since the industrial chlorine dioxide (CDA) MIC in synergy with methylene blue was much lower than the dosages when they were evaluated isolated.

Finally, the test was carried out with the synergy of methylene blue, monensin sodium and industrial chlorine dioxide (CDA). The results show that at 5, 0.5 and 177.5 mg L^{-1} in 71 or 75 test number at 5, 0.3 and 230 mg L^{-1} of the three antimicrobials, respectively, there was the best bacterial inhibition (Figure 14).

Figure 14. Growth-related absorbance of *L. fermentum* at different concentrations between 5 to 40 mg L^{-1} methylene blue, 0.1 to 0.5 mg L^{-1} sodium monensin and 125 to 230 mg L^{-1} industrial chlorine dioxide trade A (CDA) in MRS medium at 37 °C. Concentrations in Table 2c.



Obs. Positive control (C+). Source: The author.

The MIC for the yeast *S. cerevisiae* has not been determined since it is higher than the maximum tested concentrations.

12 DISCUSSION

This study showed for the first time the tests for the use of dyes to control bacterial growth in fermentative processes. The results obtained with bromocresol green and malachite green had an inhibitory action for the yeast *S. cerevisiae*, as expected since the WLN medium is composed of them and is therefore good as a differentiation medium for observing mixed yeast cultures. On the other hand, chemical compounds such as gentian violet and methylene blue dyes have shown positive results in the control of

microorganisms (TAMAKI et al., 2017) which motivated the choice for using this products in the present study.

In fact, the results of the present study showed *Limosilactobacillus fermentum* inhibition with MIC of 20 mg L^{-1}. Another studies achieved greater antibacterial potential with MIC of 20 mg L^{-1} gentian violet (TAMAKI et al., 2017), the same concentration obtained by studies carried out recently (GUIMARÃES et al., 2018; RANKE et al., 2019; RIBEIRO et al., 2019). This corroborates the literature that highlights gentian violet antibacterial action against other Gram positive as *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis* and *Bacillus subtilis.* The effect of the dye, measured with the Minimum Inhibitory Concentration (MIC) or with growth retardation, increases as the pH increases from 6 to 8. Of the four species mentioned, *E. coli* is more resistant to the dye; the resistances of other microorganisms are similar (ADAMS, 2011). In addition, GV was recently considered a safe drug with a limited number of side effects (BHATE; WILLIAMS, 2013).

Methylene blue is also known to be an effective antibacterial agent. In the present study we evaluated the MIC of 60 mg L^{-1}. It is used as a bacterial inactivator for skin treatment, dental therapy and other needs of bacterial disinfection (LI et al., 2016). Besides that, MB have been used to perform photodynamic therapy (DAI; HUANG; HAMBLIN, 2009) and had its chemical properties tested in several studies that proved its antimicrobial efficacy (KOMINE; TSUJIMOTO, 2013).

The mechanisms of action of GV/MB are not entirely understood, but some of mechanisms have been proposed (EDWARDS, 2016). GV and MB have an alteration in redox potential and it has been suggested that theses dyes operate by “short circuiting” electron transport pathways. Bacteria need a certain level of balance between reductive and oxidative actions to be able to survive. The dyes could inhibit of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (PERRY et al., 2006). Both GV and MB dyes showing differential activity toward Gram-negative versus Gram-positive bacteria and they are basic with a positive charge (EDWARDS, 2016). Gram-positive bacteria form adducts with GV, due to its ability to penetrate the bacterial cell wall and covalently bond to proteins. GV is far less effective against Gram-negative bacteria and *Mycobacterium*, due to its inability to penetrate the lipids surrounding the cell wall. This is the basis of the Gram stain, which is still in clinical use for over a century (MALEY; ARBISER, 2013).

The usual dosage of sodium monensin in distilleries was formerly 1.0 to 3.0 mg L^{-1} (OLIVEIRA et al., 1996) but was increased to 3.0 to 4.0 mg L^{-1} (OLIVA NETO et al., 2014). This was probably due to an increase in bacterial resistance to the antibiotic. The study conducted in this work showed the MIC of 0.9 mg L^{-1} of SM under the tested conditions. This concentration was also found in previous studies (RANKE et al., 2019; RIBEIRO et al., 2019).

Acting in synergy with other compounds, the decrease in the application of these biocides was even greater about 35 and 0.5 mg L^{-1} of MB and SM, respectively, almost 55% in some cases, demonstrating an excellent potential for reducing the dosage of conventional chemicals in the production of fuel ethanol. In this way, distilleries could use dry yeast biomass for animal feed (LOPES; GABRIEL; BORGES, 2011) because there would not be a high residual degree of the conventional chemicals.

This work showed a MIC against *L. fermentum* of 230 mg L^{-1} for industrial chlorine dioxide (CDA) and 150 mg L^{-1} for commercial chlorine dioxide (CDB), individually tested. These results corroborates the same range of results found by the same research group around 200 mg L^{-1} (RANKE et al., 2019). In synergistic action, the concentration of chlorine dioxide reached levels of up to 75 mg L^{-1}, ensuring antibacterial efficacy and reducing the concentration of the compound. Although chlorine dioxide has many advantages, the growth of yeast can also be affected by chlorine dioxide in concentrations over 50 mg L^{-1} (MENEGHIN et al., 2008). Therefore, the use of chlorine dioxide should be used in association with other antimicrobials to avoid the inhibition of yeast metabolism. In the present work some successful mixtures were presented.

The chlorine dioxide is a strong oxidant with antimicrobial properties against bacterial and has the ability to oxidize other substances through a transfer mechanism of only an electron, when the dioxide is reduced to chlorite without hypochlorite or gaseous chlorine production (LAPOLLI et al., 2005; OLMSTEAD, 2012). Therefore, this chemical shows reduced formation of organochloride products and besides it does not react with ammonia preventing chloramine formation, which is potentially toxic. When chlorine dioxide is used for drinking water systems chlorine and chlorate can be formed, however, in low concentrations with no risk to human health (MENEGHIN; REIS; CECCATO-ANTONINI, 2010).

Research has shown that the concentration of chlorine dioxide to inhibit *Lactiplantibacillus plantarum* in the tested concentrations of 100 to 200 mg L^{-1} was sufficient to prevent its growth (MENEGHIN et al., 2008). In addition, in another

experiment carried out by the same author for *L. fermentum*, total growth inhibition was achieved with 100 mg L^{-1} of chlorine dioxide. However, studies have shown that the genus *Limosilactobacillus* is more adapted to the conditions of the fermentation process (NARENDRANATH, 2003) and, therefore, more resistance to antimicrobial agents. Then, the bacterial resistance was responsible to the increase of dosage of chemicals in a fermentation process for ethanol production over time.

13 CONCLUSIONS

The antibacterial potential of gentian violet (GV) and methylene blue (MB) has been proven to control the growth of *Limosilactobacillus fermentum* individually or in synergistic processes. This is the most important contaminant in Brazilian fuel ethanol distilleries and there are few viable chemicals for bacterial inhibition on an industrial scale that do not affect yeast metabolism. As a new alternative, these same molecules can act in synergy with sodium monensin and/or chlorine dioxide, currently used in the industry, but with some limitations. The synergy verified in GV and/or MB with monensin and/or chlorine dioxide can drastically decrease the individual chemical dosage needed in industrial scale with benefits for its use, since the latter chemical inhibits the metabolism of *S. cerevisiae*, especially when the yeast viability is low or when there are some physiological stressed factors involved.

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15 CHAPTER 3 – IMMOBILIZATION OF ANTIBACTERIAL DYES TO CONTROL THE GROWTH OF *Limosilactobacillus fermentum* IN FUEL ETHANOL FERMENTATION

ABSTRACT

Fuel bioethanol is produced in Brazil from sugar cane molasses by *Saccharomyces cerevisiae* fermentation. The industrial process is characterized by non-aseptic operation, high concentration of cells and yeast recycle. *Limosilactobacillus fermentum* is the main contaminants of distilleries. This bacterium can cause industrial contamination resulting in decreased alcoholic efficiency, increased fermentation time, yeast inhibition and increased chemicals consuption. There are few alternatives to control this bacterium and this is carried out with limitations. Therefore, the search for new alternatives is needed. Dyes can be an alternative to solve this problem if they have the ability to inhibit only bacterial growth. The Minimal Inhibitory Concentration (MIC) was evaluated for some dye immobilization protocols and to assess their ability to control the growth of *L. fermentum* ATCC 9338. The immobilization of Gentian Violet (GV) and Methylene Blue (MB) in alginate spheres containing avocado seed powder was individually studied. The in vitro antimicrobial activity tests showed significant results on immobilization of GV with Alginate/Avocado (Al/Av) spheres in three steps of chemical modification. *L. fermentum* was inhibited by 100 mg L^{-1} of GV Al/Av beads after 48 h of incubation at 37 °C in MRS medium. This technique was not effective in adsorbing MB, which easily detached from the gel during the fermentation process. The results of GV Al/Av beads showed high efficacy in 42 recycle. The protocol developed in this study was capable to inhibit bacteria at pH 6, and also to use the technique in other cycles.

Keywords: Bacterial inhibition; Gentian Violet; Avocado; Immobilization; Bioethanol.

16 INTRODUCTION

The improvement and discovery of new alternatives of chemicals for the bacterial control in fuel ethanol industries are strategic for the producers of bioethanol. The lactic acid bacteria *Limosilactobacillus fermentum* and some of the genus *Bacillus* (*B. subtilis*, *B. megaterium*, *B. coagulans*) represent about 62% of contaminating microorganisms in Brazilian fuel ethanol-producing distilleries (DELLIAS et al., 2018; OLIVA-NETO et al., 2013). *L. fermentum* uses carbohydrates contained in the medium to produce lactic acid, which, in addition to reducing alcohol yields, inhibits yeast fermentation (BREXÓ; SANT'ANA, 2017; DA SILVA-NETO et al., 2020; REIS et al., 2018). The bacterial contamination can result in the loss of 10,000 to 30,000 liters of bioethanol per day in a production of one million liters (AMORIM et al., 2011; OLIVA-NETO et al., 2013). In addition to acid production, *L. fermentum* causes serious yeast flocculation problems, resulting in decreased viability of *Saccharomyces cerevisiae* during fermentation and additional chemical expenses leading to increase of ethanol cost (CECCATO-ANTONINI, 2018; LOPES et al., 2016; OLIVA NETO; YOKOYA, 1997).

The treatments of bacterial control in fuel ethanol fermentation to be effective include the growth control of *L. fermentum* and other lactic acid bacteria. The explored methods to combat contamination include pre-treatment of the raw material, changes in the process conditions, such as solids and pH content, cleaning of fermentation equipment, antibiotics, bactericides and washing of the yeast used in sugarcane fermentations (KIM et al., 2018). Currently, acid treatment of yeast, cleaning equipment and antibiotics are the most used measures in the industry. However, acid washing stresses fermenting yeast and can lead to a decrease in the efficiency of ethanol production (BASSO et al., 2011; CECCATO-ANTONINI, 2018). Increased development of antibiotic resistance in contaminating strains has also been observed (MURPHREE; HEIST; MOE, 2014).

The control of bacterial contamination in fuel ethanol fermentation is an effective method to stabilize the bioprocess. However, it is responsible an increase in the cost of the final product (EGUCHI, 2007; OLIVA-NETO et al., 2012; SEO et al., 2020). There is an increasing demand for less harmful antibacterial for the fuel bioethanol production to avoid bacterial infection and decrease of ethanol production. The use of new alternatives is a promising option, because, in addition to avoiding or reducing the

use of antibiotics based on monensin, distilleries could use yeast dry biomass for animal feed (LOPES et al., 2016).

Some antimicrobial compounds extracted from natural sources like oils and/or vegetal extracts, chemical dyes like gentian violet and methylene blue or microbial extracts could be a new alternative to antibiotics for use in this area. In fact, new alternatives could be useful and efficient to avoid bacterial resistance to drugs used for human and animals (LIU et al., 2020). Immobilization technology for these molecules may also offers advantages, as the use of immobilized particles can be reused or easily separated from the process. Reuse of the biocatalyst can help bridge the gap between the costs of chemical and enzymatic processes (ANDLER; GODDARD, 2018).

In this sense, antimicrobials inert to yeast metabolism should be sought for this application, since the antibacterial inhibitory action on contaminants should not affect yeast metabolism (MUTTON et al., 2014). Therefore, new chemical alternatives for the control of bacteria in ethanol fermentation will contribute to the sugar and fuel bioethanol industry.

16.1 New alternatives for the bacterial control

In the era of the growing resistance of bacteria and fungi, the re-discovery of drugs can be an effective alternative in the treatment of many infections or use in industrial bioprocess. Dyes are broadly used in the textile, food, pharmaceutical, cosmetic, printing and leather industries. Chemical compounds such as gentian violet and methylene blue dyes have shown positive results in the control of microorganisms (TAMAKI et al., 2017) and they could be an alternative to *L. fermentum* control in ethanol fermentation.

Gentian violet (GV) and methylene blue (MB) are used to incorporate innovative technologies such as antibacterial agents in wound care (WOO; ALAM; MARIN, 2014). GV/MB foam dressings they have demonstrated antibacterial activity against a broad spectrum of yeast and bacteria commonly found in wounds, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (EDWARDS, 2016). Positive ions dissociated by the GV dye penetrate Gram-positive and Gram-negative bacterial cells and interact with negatively charged components such as lipopolysaccharide, peptidoglycan, and DNA (DRAGAN; MICHALAK, 2019). Recent studies showed that 20 mg L^{-1} of GV was able to completely inhibited *L. fermentum*

growth in Man, Rogosa e Sharpe medium (GUIMARÃES et al., 2018; RIBEIRO et al., 2019). This inhibitory potential is likely to apply positively to other Gram-positive bacteria.

Methylene Blue (MB) also known as methylthionine chloride ($C_{16}H_{18}N_3SCl$), an aromatic hydrocarbon compound, and at room temperature appears as a solid, odorless, dark, and green colored powder. It is an alkaline solution, which has a pH of 11.4, and is soluble in water and alcohol. MB is frequently used because its production is economic and easy. This dye has many uses in different fields, such as biology and chemistry, including the process of dyeing leather, cloth and cotton cloth (SALEM et al., 2018). Some tests showed that a solution of MB with a concentration of 0.5%, 0.7%, and 1% can pierce and penetrate the bacterial endospores so endospores appear blue (OKTARI et al., 2017). Methylene blue is a compound capable of reducing oxidation. The intravenous form of methylene blue is approved by the Food and Drug Administration (FDA) for the treatment of pediatric and adult patients with acquired methemoglobinemia (LUDLOW; WILKERSON; NAPPE, 2020) and could be an alternative against lactic acid bacteria. The studies showed MB potential inhibition against *L. fermentum* at 60 mg L^{-1} (GUIMARÃES et al., 2018).

The avocado (*Persea americana)* belongs to the family Lauraceae and is native to Central America. Mexico is the largest producer of avocados worldwide (VILLAMIL et al., 2018) but it is currently cultivated worldwide (GARCÍA-VARGAS; CONTRERAS; CASTRO, 2020). At present, Brazil occupies the sixth position in avocado production with 3.2% of world (PIO; MAGALHÃES, 2020). In the industry, the pulp is used, while the skin and the seeds are discarded. Condensed tannins, phenolic acids, and flavonoids are the most representative groups in avocado seed. Among the polyphenols, (+)-catechin, (−)-epicatechin, and 3-leucoanthocyanidin are found (FIGUEROA et al., 2018). The proximate composition of the seeds from the Hass and Fuerte varieties are similar: 52.7 to 54.1% moisture, 1.2% ash, 2.4 to 2.5% protein, 42.5% nitrogen-free extract, 27.5% starch and 0.5% fat (lower fat content than the pulp) (DABAS et al., 2013; SAAVEDRA et al., 2017). The majority of the lipids (77-80%) in the seeds are neutral lipids, whereas glycolipids and phospholipids represent the 7.4 and 10.9%, respectively (DABAS et al., 2013). Also, the seed has a high content of dietary fiber (34.8%) (PAHUA-RAMOS et al., 2012).

Exploring the possible dietary and therapeutic potentials of especially underutilized agro-food by-products will in addition reduce the possible environmental waste burden (EGBUONU, 2017).

Avocado seed is underutilized and represents a large portion of the fruit, thus its use can be an alternative to reduce the production cost of some products like edible oil (DUARTE et al., 2016). These residues of the fruit are rich in polyphenols with many functional properties such as antioxidant, antimicrobial, analgesic (RODRÍGUEZ-CARPENA; MORCUENDE; ESTÉVEZ, 2011; SETYAWAN; SUKARDI; PURIWANGI, 2021), anti-carcinogenic, anti-inflammatory (AHMED et al., 2018), antidiabetic, antihypertensive, cholesterol reducer, dermatological effects, insecticidal and even as a colourant (DABAS et al., 2013). These features are attributed to several bioactive compounds groups (phytochemicals) as polyphenols, carotenoids, tocopherols, among others (JIMENEZ et al., 2021). Organic extracts from seed avocado inhibited the growth in vitro of strains of *Candida* spp., *Cryptococcus neoformans* and *Malassezia pachydermatis* (LEITE et al., 2009), *S. aureus*, *S. pyogenes*, *C. ulcerans*, *C. albicans*, *E. coli*, and *S. typhi* (IDRIS; NDUKWE; GIMBA, 2009). In vitro assays have shown the antimicrobial activity of the avocado suggesting that the avocado could be a potential antimicrobial agent for pathologies produced by bacteria, fungus, and others (JIMENEZ et al., 2021).

16.2 Adsorption process in antimicrobial immobilization and reuse

Adsorption processes are attractive approaches for water treatment, particularly because of the simplicity of the design, low-cost, availability, and ease of operation (NAUSHAD et al., 2019; PANG et al., 2019). Adsorption, despite its problems associated with adsorbent disposal and post contamination using adsorbents, is an effective and simple method that is relatively free from the concerns of generating unwanted by-products and a good percentage of dye removal (BURAKOVA et al., 2018; MOOSAVI et al., 2020). In turn, is indispensable a complete understanding of adsorption mechanisms for an industrial application.

Conventionally, activated carbon has been excessively used to remove dyes from wastewater. Despite the popularity of activated carbon in adsorption, it can cause pollution, it has poor regeneration capacities, and its use and re-use are costly (AICHOUR; ZAGHOUANE-BOUDIAF, 2020; FAZAL et al., 2020). Hence, it is

desirable to use alternative adsorbents that can couple an higher efficiency and many studies have shown that the bio-adsorbents, such as those deriving from coconut husk, palm bark and sugarcane bagasse, chitosan, avocado seeds and others (CHE BT MAN; AKINBILE; XUN JUN, 2015). These materials possess high carbon content, but low inorganic content. They can have a large application since they are abundant, eco-friendly, nontoxic, with a low cost and usually show a high adsorption potential (PAVAN et al., 2014; PROLA et al., 2013; SILVEIRA et al., 2014) and can be utilized in their native forms as an alternative for the removal of several dyes from aqueous solutions (BAZZO et al., 2016; PAVAN et al., 2014; SILVEIRA et al., 2014).

The kernel of avocado (*Persea americana*), which is discarded in the garbage after consumption, is about 10–13% of the fruit (ELIZALDE-GONZÁLEZ et al., 2007). The current production of avocado in Brazil generates tons of kernel (a waste). Therefore, it is necessary to utilize this waste to avoid environmental problems that may be associated with the decomposition of the waste. Due to usage of dyes in different industrial applications, various adsorbents have been used to remove GV from aqueous solutions (ALJEBOREE; ALKAIM; AL-DUJAILI, 2015). Degradation of avocado kernel could result in accumulation of unwanted chemicals and microbial growth. Therefore, the use of avocado kernel for wastewater decontamination (removal of dyes) is economical and eco-friendly (BAZZO et al., 2016).

Immobilization is an important tool capable of rendering the application of these antimicrobial compounds, as it allows reuse and may improving the operational stability, which, in turn, enables the development of continuous operation systems (REHMAN et al., 2013). Furthermore, the immobilization eases the dye separation from the reaction environment and eliminates the risk of environmental damages. Treatment with dyes for bacterial control could be an adequate alternative, since this method does not affect yeast metabolism. However, industrial scale application will only be economically viable if this process will be cost-effective. For this reason, the reuse of the dye is an important strategy for this process, which can be achieved with immobilization on solid supports.

16.3 Types of immobilization process

Some compounds can be immobilized by various methods that can be classified as physical, with a weak interaction between the molecule and support, and chemicals whose covalent bonds are formed with the compound. Some examples of physical

methods are: containment of a molecule within a membrane reactor, adsorption (physical, ionic) on a water-insoluble matrix, inclusion (or gel entrapment), microencapsulation with a solid membrane (KRAJEWSKA, 2004).

Various immobilization supports such as chitosan, calcium alginate, microporous exchange resins have been used for immobilization of compounds (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011) and the use of low-cost supports, such as gelatin, starches and agar, is an important alternative to industrial use of immobilized molecules. Of all these compounds, the most widely used for encapsulation is calcium alginate, an heteropolysaccharide, non-toxic, with very simple preparation and low cost (BLANDINO; MACÍAS; CANTERO, 2001; SIMÓ et al., 2017). Thus, these supports have a high immobilization potential for the dyes as well. Nevertheless, these supports present limitations due to their low physical stability, which can be minimized with the use of crosslinking and linking agents, such as glutaraldehyde and concanavalin A (ConA) (HUSAIN; SALEEMUDDIN, 1986; SALEEMUDDIN; HUSAIN, 1991).

A major challenge for the use of immobilizes dyes in bacterial control during fuel ethanol fermentation is to retain their stability and performance under the conditions typical of ethanol mills, reuse of dye as well as avoiding the disposal of this material in effluents. Considering the end application during the fermentation process a design of stabilized dyes systems is therefore imperative for the success of this technology. In this context, the aim of this research was to evaluate the parameters involved in the process of the entrapment of gentian violet and methylene blue in alginate spheres with avocado seeds powder and the evaluation of antimicrobial effect of this Alginate/Avocado (Al/Av) spheres against *Limosilactobacillus fermentum* and *Saccharomyces cerevisiae*.

17 MATERIALS AND METHODS

17.1 Microorganisms and maintenance of strains

The yeast strain *Saccharomyces cerevisiae* M-26 was previously isolated from bioethanol distillery (OLIVA NETO; FERREIRA; YOKOYA, 2004). Yeasts were stored at -80 °C in Eppendorf on YM medium containing 2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.087% dipotassium phosphate, 0.024% magnesium sulfate heptahydrate, 0.0017% manganese sulfate heptahydrate, 0.0028% zinc sulfate heptahydrate to 100 mL of distilled water (with 10% of glycerol) at pH 5. The culture medium for the propagation of yeast and inoculum production was prepared by (w v^{-1}):

2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.1140% dipotassium phosphate $3H_2O$, 0.024% magnesium sulfate $7H_2O$, 0.0012% manganese sulfate H_2O, 0.0028% zinc sulfate $7H_2O$ (pH 5) (DORTA et al., 2006). The lactic acid bacteria *Limosilactobacillus fermentum* CCT 0559 was obtained by André Tosello Culture Collection (Campinas, Brazil) and cultivated at pH 6 on MRS medium (DE MAN; ROGOSA; SHARPE, 1960).

17.2 Preparation of Solutions and Tests

The processes included preparation of gentian violet and methylene blue solutions (100 mg L^{-1}) each one diluted in distillated water. After preparation, the solutions were stirred for at least 30 minutes before tests.

The assays containing bacteria were performed in triplicate and maintained at 37 °C and the yeasts at 30 °C. After 48 hours the growth was verified by the turbidity of the medium with the naked eye and reading in a spectrophotometer (600 nm), in addition to the verification under a microscope.

17.3 Isolated MIC analysis of non-immobilized antimicrobials

The Minimal Inhibitory Concentration (MIC) of gentian violet and methylene blue was determined by adapted macrodilution broth method (JONES et al., 1985). GV were tested by 60 to 10 mg L^{-1} and MB by 100 to 10 mg L^{-1} in MRS medium. Performed concentrations are shown on Table 4.

Table 4. Concentrations tested by MIC in isolated assays for non-immobilized gentian violet and methylene blue.

Test	Gentian Violet (mg L^{-1})	Methylene Blue (mg L^{-1})
1	-	100
2	-	90
3	-	80
4	-	75
5	-	70
6	-	65
7	60	60
8	55	55
9	50	50
10	45	-
11	40	40
12	35	-
13	30	30
14	25	-
15	20	20
16	15	-
17	10	10

Source: The author.

17.4 Alginate/Avocado beads development

17.4.1 Avocado seed powder extract

The avocados (*Persea americana*) used in this study was obtained from local supplier. The fruits were selected according to the similarity of ripening stage and peel color, fresh and green, in order to obtain a homogeneous batch.

In order to obtain avocado seed extract, the seeds were initially separated from the pulp, washed, fragmented in a small piece, dried in a drying oven at 50 °C for 24 hours. The dried seeds were crushed in a 900 W power household blender for 10 min and ground in a porcelain bowl and sieved through 35 mesh to obtain a powder that was stored in a clean dry container at room temperature and protected from light until use. This methodology was inspired by the literature for dye adsorption and was developed with several adaptations and modifications (BAZZO et al., 2016).

17.4.2 Steps of immobilization development

This methodology is according to literature (GONDIM et al., 2014) with adaptations. A suspension containing sodium alginate (2.5% w v^{-1}) was prepared in 100 mL of distilled water at 60 °C. Afterwards two different concentrations (1 and 4%) of avocado seed powder were tested. Firstly, 1 or 4 g of avocado seed powder was mixed in a sodium alginate solution and stirred at least for 30 minutes. The resulting mixture was slowly dripped into a 0.3 M calcium chloride solution with glutaraldehyde (2% v v^{-1}) under moderate agitation, using a disposable needle (25x7 mm) inserted into a 5 mL syringe or a burette. The formation of Alginate/Avocado (Al/Av) spheres through electrostatic interaction was instantaneous. After 2 hours of contact with the immobilization support at 25 °C, the resulting biocatalyst was separated through a sieve and rinsed with a distillated distilled water.

17.4.3 Chemical modification of alginate/avocado beads

The chemical modification of Al/Av wall beads was carried out in a glass beaker (100x40 mm) in four phases, tested individually and subsequently (Table 5).

Table 5. Four steps for chemical modification of Al/Av beads.

Phases	Chemicals	Reaction time	Temperature
F1	0.3 M calcium chloride solution with 2% glutaraldehyde (v v^{-1})	2 hours	25 °C
F2	1% polyethyleneimine solution (v v^{-1})	2 hours	25 °C
F3	10% dimethylformamide solution (v v^{-1})	30 minutes	60 °C
F4	10% epichlorohydrin solution (v v^{-1})	Overnight	25 °C

Source: The author.

After each four phases, the solid beads were washed again with distilled water until neutrality.

17.4.4 Immobilization of dyes on alginate-avocado beads

Gentian violet were immobilized into Al/Av beads according to the procedure described by (GONDIM et al., 2014) with modifications. Firstly, 1.0 g of Al/Av beads

was added to 100 mL dye solution (100 mg L^{-1}) and stirred at 60 °C for 1 hour. After that, the dyed beads were maintained in the solution at 25 °C overnight. Then, it was washed with distilled water and stored in a 0.3 M calcium chloride solution with 2% glutaraldehyde.

17.5 Adsorption protocols of gentian violet and methylene blue on Al/Av beads

The chemical conditions used at the protocols tested for better dye adsorption are correlated and shown in Tables 6 and 7.

Table 6 Chemical conditions used in the adsorption protocols of Gentian Violet and Methylene Blue on Al/Av beads.

Operation numbers	**Chemical conditions**
OP1	Al/Av beads development (1% of avocado seed powder)
OP2	Al/Av beads development (4% of avocado seed powder)
OP3	Chemical modification of Al/Av in 4 phases
OP4	Adsorption of GV solution (0.1 mg L^{-1}) using 20% (w v^{-1}) of NaCl at 60°C for 1 hour
OP5	Adsorption of GV solution (0.1 mg L^{-1}) at 60 °C for 1 hour
OP6	Adsorption of GV solution (0.1 mg L^{-1}) at 60 °C for 1 hour and overnight
OP7	Only Phase F1: 0.3 M $CaCl_2$ solution with 2% (v v^{-1}) glutaraldehyde for 2 hours at 25 °C
OP8	Test tubes with 1 g of GV Al/Av beads
OP9	Test tubes with 5 g of GV Al/Av beads

Source: The author.

Table 7. Protocols for adsorption of Gentian Violet on Al/Av beads.

Steps	**Protocol 1**	**Protocol 1.1**	**Protocol 2**	**Protocol 2.2**	**Protocol 3**
1st	OP1	OP1	OP1	OP1	OP2
2nd	OP3	OP3	OP4	OP4	OP6
3rd	OP4	OP5	OP3	OP3	OP7
4th	OP7	OP8	OP8	OP8	OP9

Source: The author.

17.6 Antibacterial activity tests

In order to equal amounts of yeast and bacteria to be distributed in test tubes, the inoculum was standardized according to McFarland 0.5 standard in aseptic conditions. Assays were performed in test tubes containing 5 mL MRS medium with dyes beads of gentian violet and methylene blue. The MIC was defined as the minimum chemical compounds concentration able to inhibit at least 90% the microbial growth and performed in triplicate.

For each dye and for each concentration of Al/Av beads (1 and 4% of avocado seed powder), individual tests were performed to assess the antibacterial activity of each four phases of chemical modification of the gel. First tests: 5 mL of MRS medium were added to each test tube and 1 g (approximately 15 and 25 beads for 1 and 4% of avocado seed powder, respectively) of Al/Av beads previously treated in four chemical modification stages and fixed with 100 mg L^{-1} of methylene blue and gentian violet dyes. The experiments were incubated at 37 °C for 48 hours, performed in triplicate. Second tests: the same methodology was applied but using 5 g (almost 75 beads) of 4% of avocado seed powder into alginate beads. Microbial growth was verified by turbidity of the medium in the naked eye and reading on a spectrophotometer (600 nm). All tests were performed in triplicate.

17.6.1 Bacterial inhibition test associating Al/Av beads with gentian violet and pH variation in a mix culture with *L. fermentum* and *S. cerevisiae*

Firstly, assays were carried out with pre-inoculum of *L. fermentum* and *S. cerevisiae* in test tubes containing 5 mL of liquid MRS medium and incubated at 37 and 30 °C, respectively, for 48 hours. After growth, a mix biomass (bacteria+yeast) was prepared by dilution in 500 mL of sterile distilled water, where 0.665 g of bacteria was equivalent to 10 g of yeast (on a wet basis). The pH of bacteria and yeast suspension was corrected to pH 5.0, 5.5 and 6.0 with HCl and NaOH 1 M. For the antimicrobial tests, in each test tube were placed 5 mL of mixed suspension of each pH together with 5 g of Al/Av beads (4% avocado seed powder) stained with gentian violet. Assays were incubated at 25 °C for 0, 3 and 6 hours and 1.5 mL samples were collected for subsequent plating for bacterial colony count, cell/mL microscopic count and for yeast viability analysis. All the tests were performed in triplicate.

After the incubation of cells suspensions treated with the Al/Av beads with GV, a serial dilution was made using 0.1% peptone diluted in water. Three dilutions of broth were selected (10^{-1}, 10^{-3} and 10^{-7}) as samples for the spread-plate technique. The plates with 30 mL of previously melted MRS medium with 0.01% of ketoconazole were placed at room temperature until total solidification and then seeded in triplicate with 0.5 mL of each concentration of diluted broth for each pH. The plates were incubated inverted to 37 °C for 48 hours. The positive control (C+) (without the addition of beads) and the negative control (C-) (with undyed beads) were also performed.

17.7 Recycling of gentian violet Alginate/Avocado beads

After each growth cycle, the beads were removed from the test tubes, washed with distilled water and placed back in a 0.3 M $CaCl_2$ solution with 2% glutaraldehyde for 2 hours. After that, the beads were able to be used again in the next cycles.

17.8 Bactericidal action test with Al/Av beads with gentian violet

The tests were carried out in two stages with a pre-inoculum of *L. fermentum* in 5 mL of liquid MRS medium. In the first stage of the experiment, after microbial growth, 50 and 75 beads (5 g) of beads per test tube with pH 6.0 were added. The tubes were incubated at 37 °C for 1.5, 3 and 6 hours and 0.5 mL samples were removed and inoculated in a Petri dish containing solid MRS medium, in order to quantify if there was bacterial inhibition. In the second stage, the pH variation of 2.5, 4.5 and 6.0 was tested, using the same method with approximately 75 beads (5 g) per test tube. The tests were carried out in triplicate.

17.9 Analytical procedures

17.9.1 Growth and viability of microorganisms

After 48 hours of incubation at the optimum temperature the microbial growth was verified by the turbidity of the medium with the naked eye and reading in a spectrophotometer (600 nm). In addition, the verification under a microscope was performed to determination of cell growth and viability by counting live cells/mL of extract by adding 1 mL of each sample to 1 mL of methylene blue dye solution (LEE;

ROBINSON; WANG, 1981). After homogenization, living cells were counted (not stained) in a Neubauer Chamber, and cell viability was determined by the ratio of living cells/total cells per mL of extract (DORTA et al., 2006). The determination of the quantity was found according to the equations:

$$Number\ of\ cells/mL\ =\ \sum of\ the\ quadrants\ x\ 5\ x\ 10^4\ x\ DF \qquad (5)$$

$\sum$ of squares = sum of the number of cells counted in the five quadrants;
10^4 = volume adjustment on the camera;
DF = sample dilution factor.

$$Cell\ viability\ (\%)\ =\ \frac{total\ number\ of\ living\ cells\ x\ 100}{total\ number\ of\ cells\ (live+dead)} \qquad (6)$$

17.9.2 Growth inhibition of microorganisms

Antibacterial efficiency was calculated using the following equations:

$$Inhibition\ abs\ (\%)\ =\ \frac{(Abs\ treatment\ 48\ h - Abs\ treatment\ 0h)\ x\ 100}{(Abs\ control\ 48\ h - Abs\ control\ 0\ h)} \qquad (7)$$

Abs of the treatment 48 h - Abs of the treatment 0 h = absorbance of the treatment means the growth in 48 h culture;
Abs of the control 48 h – Abs of the control 0h = absorbance of the control group means the growth in 48 hours;
Inhibition abs (%) = percentage of growth inhibition of the microorganism.

$$Bacterial\ reduction\ (\%)\ =\ \frac{(Abs\ control - Abs\ treatment)\ x\ 100}{Abs\ control} \qquad (8)$$

17.9.3 Spread-plate technique and bacterial Colony Forming Units (CFU)

The spread-plate technique was used for bacterial estimations. The results were expressed in Colonies Forming Units/mL (CFU/mL) and %.

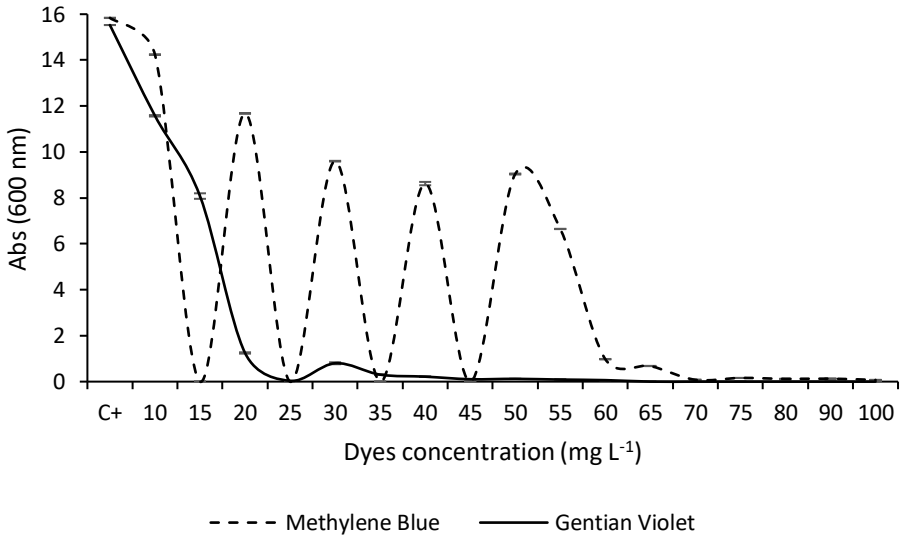
$$Bacterial\ reduction\ (\%) = \frac{CFU\ Test\ x\ 100}{CFU\ Control} \quad (9)$$

18 RESULTS

18.1 Analysis of isolated non-immobilized compounds

Tests with gentian violet performed in MRS medium from 60 to 10 mg L^{-1} resulted in 20 mg L^{-1} of MIC against *L. fermentum.* There was growth only with 10 mg L^{-1}. This result did not affect *S. cerevisiae* viability. The MIC of methylene blue showed 60 mg L^{-1}. The test was carried out from 100 to 10 mg L^{-1}. The growth was also verified in a spectrophotometer with absorbance at 600 nm (Figure 15). There was notable bacterial growth from 15 and 55 mg L^{-1} for GV and MB, respectively.

Figure 15. Growth-related absorbance of *L. fermentum* in different concentrations of gentian violet or methylene blue in MRS medium at 37 °C. (Table 4)



Obs. Positive control (C+). Source: The author.

18.2 Adsorption of gentian violet in alginate/avocado beads

In order to choose the best sequence of steps for chemical modification of the beads, tests were performed with the phases F1, F2, F3 and F4 for gentian violet and methylene blue at 100 mg L^{-1} concentration (Table 8).

Table 8. Dye's adsorption in four chemical phases using 100 mg L^{-1} of Gentian Violet and Methylene blue in Al/Av beads.

Dye	Phase of bead treatment	% of dye adsorption (1% of avocado seed powder)	% of dye adsorption (4% of avocado seed powder)
Gentian violet	F1	20	100
	F2	15	100
	F3	80	100
	F4	10	100
Methylene blue	F1	10	-
	F2	10	-
	F3	10	-
	F4	10	-

Obs. F1: $CaCl_2$ 0.3 M with 2% (v v^{-1}) glutaraldehyde; F2: 1% (v v^{-1}) polyethyleneimine; F3: 10% (v v^{-1}) dimethylformamide and F4: 10% (v v^{-1}) epichlorohydrin. Spheres of alginate 2.5% with 1% and 4% of avocado seed powder. Source: The author.

The Al/Av structure proved to be more porous allowing for better internal mass-transfer diffusion of the MB dye to the culture medium. The technique used in each of the phases was not efficient to trap the dye inside the beads, and for this reason MB was not used in the later stages of this study. The adsorption of GV was more efficient in phase F3, considering 1% of avocado seed powder and efficient in all of them for 4% of avocado seed powder. The results show that all the dispersed dye was adsorbed inside the Al/Av beads. This factor is a differential because there is no loss in the concentration of dye used, ensuring the reuse of the beads in next cycles.

Afterwards, the protocol sequence was performed (Tables 6 and 7). Protocols 1 and 2 were not efficient, as the use of NaCl did not show an increase in the retention capacity of the dye in the Al/Av spheres with 1% of avocado seed powder. Therefore, it was proven that the tests without NaCl were more efficient (Protocols 1.1 and 2.1), even though the dye has come off the spheres during the process.

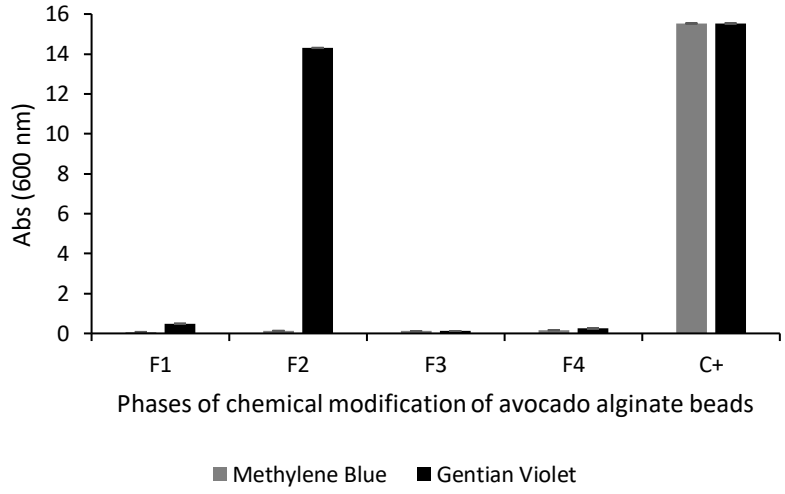
Thus, Protocol 3 (Table 7) was performed using 4% avocado seed powder in alginate spheres (2.5%) without the use of salt. In addition to increasing the dye retention capacity inside the beads, the structure was strengthened and made more resistant to new reuse cycles. It is noteworthy that without the addition of heat in the dye adsorption phase, the process is not as effective and the exposure time retains all of the dye dispersed in the

liquid, about 95% in first cycle. In addition, the order of the processes was fundamental, as the process of chemical modification of the bead in F1 (solution of $CaCl_2$ 0.3 M with 2% (v v^{-1}) of glutaraldehyde – Table 5) necessarily needs to occur after the adsorption of the dye on the beads so that there is no detachment of dye in the growth medium.

18.3 Antibacterial action of immobilized dyes

There was no growth in the tests containing methylene blue. All phases released a lot of dye in the medium, proving that the dye trapping technique in Al/Av beads was not efficient. In tubes with gentian violet, on the other hand, a bacterial inhibition was verified in phases 1, 3 and 4. Phase F2 had a high growth showing that treatment with polyethyleneimine 1% (v v^{-1}) was not effective (Figure 16).

Figure 16. The effect of immobilized methylene blue and gentian violet in Alginate/Avocado beads in the growth of *L. fermentum* at 37 °C. Phases F1: $CaCl_2$ 0.3 M solution with 2% (v v^{-1}) glutaraldehyde; F2: 1% (v v^{-1}) polyethyleneimine; F3: 10% (v v^{-1}) dimethylformamide and F4: 10% (v v^{-1}) epichlorohydrin.



Obs. Positive control (C+). Source: The author.

Regarding the dye retention capacity in Al/Av beads, it was possible to notice that phase 1, 3 and 4 was effective, since it had an action against *L. fermentum* and little loss of GV concentration in the culture medium. Even so, it was tested several times to use F1 directly to F3, not to make F2, but in addition to bacterial inhibition, there was also yeast inhibition and the process was not satisfactory. Thus, after several tests and

experimental validation, it was determined that phase F1 was enough to be used in protocol 3 not requiring the use of other subsequently steps.

Smaller amounts of beads were tested in the culture medium, that is, lower dye concentration, but apparently the MIC for bacterial inhibition using 4% avocado seed powder in the beads needs to be in a 1:1 ratio of dye and medium culture.

18.3.1 Recycle of Al/Av beads with gentian violet

Forty-two cycles of the Al/Av beads with 4% of avocado seed powder were performed in the bacterial growth inhibition. In the first cycle, Protocol 3 (Table 7) was carried out and, on average, 95% of the dye was adsorbed on the beads. At each recycle, the spheres were washed in water and kept in phase F1 for 2 hours at room temperature. Then, they were kept in a laminar flow hood with UV light for 20 minutes. After this period, they were included in the assays with *L. fermentum* suspension and incubated at 37 °C for 48 hours. Moreover, after storage for 4 months, the antibacterial action of GV-immobilized was still active. Therefore, this last process demonstrated that the beads can be stored for long periods without loss of activity and reused in several cycles.

18.4 Bactericidal action test with Al/Av beads with gentian violet

The first stage of the experiment had an inhibitory action against *L. fermentum* at 1.5, 3 and 6 hours, in MRS medium at pH 6.0 with the addition of 75 beads (5 g) of beads with 4% avocado seed powder and gentian violet. The assays with only 50 Al/Av beads were not able to inhibit the growth bacteria. In the second stage there was 100% bacterial inhibition at pH 6.0, in all exposure times, corroborating the results previously obtained.

18.5 Bacterial inhibition test associating Al/Av beads with gentian violet and pH variation in a mix culture with *L. fermentum* and *S. cerevisiae*

The inhibition of bacterial growth was very strong about 99.9% at pH 5.0 to 6.0 after 0 hours and about 100% after 3 and 6 hours of exposure of the mixed culture to the treatment with the Al/Av beads with VG dye. The Al/Av beads without dye addition also inhibited 100% of the bacteria after 3 and 6 hours of exposure (Table 9). This result shows

the efficiency of this technology in acid conditions, similar to industrial alcoholic fermentation.

Table 9. Growth reduction of *L. fermentum* using spread-plate technique after testing with a mix of cultures (*L. fermentum* and *S. cerevisiae*) and treatment with Al/Av beads with or without GV with pH variation (5.0, 5.5 and 6.0) and at 0, 3 and 6 hours of exposure.

Time (hours)	pH	Test	Dilution	CFU/mL	% Growth reduction
0	5	C+	1.0E+07	1.10E+08	99.94
		GVB	1.0E+03	6.40E+04	
		AV	1.0E+01	<1.10E+01	100
	5.5	C+	1.0E+07	2.60E+08	99.96
		GVB	1.0E+03	1.03E+06	
		AV	1.0E+01	<1.10E+01	100
	6	C+	1.0E+07	9.00E+07	99.99
		GVB	1.0E+01	1.36E+02	
		AV	1.0E+01	<1.10E+01	100
3	5	C+	1.0E+07	1.10E+08	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100
	5.5	C+	1.0E+07	2.60E+08	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100
	6	C+	1.0E+07	9.00E+07	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100
6	5	C+	1.0E+07	1.10E+08	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100
	5.5	C+	1.0E+07	2.60E+08	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100
	6	C+	1.0E+07	9.00E+07	100
		GVB	1.0E+07	<1.10E+01	
		AV	1.0E+01	<1.10E+01	100

Obs. (C+) Positive control; (GVB) Al/Av beads with GV; (AV) Al/Av beads with 4% of avocado seed powder. Source: The author.

19 DISCUSSION

The use of antimicrobials for ethanol production is very important as they need to be effective only in controlling bacterial growth without any toxicity to *S. cerevisiae*. The tests obtained with the GV dye achieved the highest antibacterial potential against *L. fermentum* with MIC of 20 mg L^{-1}, the same concentration obtained in other researches (GUIMARÃES et al., 2018; RANKE et al., 2019; RIBEIRO et al., 2019). Another study addressed the effective action of 0.01% (w v^{-1}) gentian violet in treating joint infection by methicillin-resistant *Staphylococcus aureus* - a Gram-positive bacterium, as well as *L. fermentum*. This study also obtained antibacterial action with MIC of 20 mg L^{-1} of gentian violet (TAMAKI et al., 2017). These results demonstrate a linear pattern of GV action, since in this study the same concentration was also effective against *L. fermentum* bacteria.

In preliminary tests with MB in this work, the MIC of 60 mg L^{-1} for this dye was found, the same presented by the same group of researchers previously (GUIMARÃES et al., 2018). These results corroborate those found in the present work. The results showed a notable antibacterial growth with 60 mg L^{-1} for non-immobilized methylene blue.

Particularly, in the case of dyes with antimicrobial abilities the problem is their use in free or soluble conditions since effluents containing 5-10% of dyestuffs are usually discharged into natural water bodies and may cause severe environmental pollution because of their carcinogenicity and toxicity (DIORIO; FRÉCHOU; LEVIN, 2021). However, a challenging task is to develop effective strategies for the efficient and cost-effective immobilization process, such as increased retention time in bioreactors, high cellular metabolic activity, high cell load and protection of the cells (SIRIPATTANAKUL-RATPUKDI, 2012).

Dye immobilization was successfully developed in the present study. The concentration of 2.5% alginate was used for immobilizing the dyes on the beads and concentrations below this are not effective in stabilizing the sphere structure, which breaks down throughout the fermentative cycle. Some research highlight that a Na-alginate concentration of 3 to 4% has also considered suitable for protease immobilization (GEETHANJALI; SUBASH, 2013). The Ca-alginate bound to a purified manganese peroxidase (MnP) was catalytically more vigorous, thermo-stable, reusable and worked over wider ranges of pH and temperature as compared to its free counterpart (BILAL;

ASGHER, 2015). However, not only must the dye be physically recoverable from the fermentation media, but the dye must also retain activity over multiple cycles. Choice of carrier material and immobilization method are therefore imperative to rational design of stable, reusable immobilized systems (ANDLER; GODDARD, 2018). To validate the antibacterial action, 42 cycles of bead reuse with 4% avocado seed powder and GV were performed and the results confirmed 100% inhibition of *L. fermentum* growth.

Although the Al/Av structure proved to be more porous allowing a mass-transfer diffusion of MB, this work showed that the MIC of MB is lower than 100 mg L^{-1}. Under free conditions in the culture medium, MB can kill the bacteria with MIC of 60 mg L^{-1}. Immobilized dye within Al/Av beads was studied to have a higher productivity, however some limitations, such as gel degradation, low physical strength and mainly a low dye adsorption, were often observed when using Al/Av-based beads with 1 or 4% of avocado seed powder.

The use of NaCl 20% (w v^{-1}) in Protocols 1 and 2 (Table 7) was not effective to increase the adsorption of dyes on Al/Av beads. The literature highlights that based on experimental and theoretical findings, the presence of salts did not improve the adsorption capacity of MB in the adsorbent of comminuted raw avocado seeds. Based on the application of the physical monolayer model, the presence of salts prevented the bonds between MB dye molecules and destabilized the MB dye orientation on the surface of the adsorbent. Furthermore, the interpretation of the adsorption energy indicated that this dye adsorption was a physical process, and confirmed that the presence of salts has no effect on the interaction energy (DHAOUADI et al., 2020). The same occurred for GV, and for this reason, Protocol 3 (Table 7) without the addition of salts was adopted for the subsequent studies.

The phenolic compounds and flavonoids are generally encountered in high levels in the pulp than the peel and seed of avocado. These properties can serve as protection in the epidermis of the avocado, but they can be found in all parts of the fruit. There is evidence that at high concentrations they can act as antimicrobials and antioxidants (AMADO et al., 2019; DAIUTO et al., 2014). Moreover, the seeds of Hass and Fuerte avocado varieties shows an higher antioxidant power (RODRÍGUEZ-CARPENA; MORCUENDE; ESTÉVEZ, 2011) and this fruit has bioactive compounds at the avocado seed as procyanidins (WANG; BOSTIC; GU, 2010). Thus, we can associate that avocado alginate beads may have a greater antibacterial power when associated with an immobilized agent. Studies prove that the phenolic compounds from the avocado seed

extract showed a marked effect in inhibiting the growth of *L. fermentum* for the ethanol and methanol extracts (ROSA et al., 2019).

Several tests were carried out using synergy between the best inhibitory tools found. Thus, the dyed beads were added to the culture media at three different pHs. The best bacterial inhibition results were found at pH 6.0 within 3 and 6h of dye/pH exposure (Table 9). Growth of *L. fermentum* was observed at 0 h in acidified medium at pH 5.0 and 5.5. At pH 6.0 there was no bacterial growth and this was a big difference for the other pHs, which after 3 and 6 hours of exposure were also able to inhibit *L. fermentum*. Compared to the control medium with pH 6.0 without dye treatment, a decrease of the growth was about 99.99%. Some investigation revealed that the cell concentration of ethanol-producing yeast with adjusted pH of 8.5 every 12 h was lower than that with adjusted pH of 5.5 (MA et al., 2021) and this indicates that in addition to bacterial control, there is great yeast viability at this range of pH. Besides that, the negative control without dye treatment also showed a bacterial inhibition. This last result showed the antibacterial contribution of other chemicals in the bead, probably in avocado seed powder for the *L. fermentum* inhibition.

Therefore, the best results were obtained by GV, both free and encapsulated at pH 6.0 and on the Al/Av beads without dye addition. This demonstrates the high adsorbent capacity and strong dye trapping mechanisms on the Al/Av beads by Protocol 3 (Table 7) and emphasizes the applicability of this protocol.

20 CONCLUSIONS

The immobilization of gentian violet in Al/Av beads was efficient and proved an antibacterial action against *L. fermentum* without affecting the viability of *S. cerevisiae* at in vitro cultures. The immobilization technique was not effective for methylene blue, since the dye was not well adsorbed and the entire content of the dye was lost from the bead to the liquid culture medium.

This study showed that the best results of bacterial inhibition were obtained by GV, both free and encapsulated preferably at pH 6.0. There was a high potential for reusing this gel beads, reducing the cost of the process and applying the GV dye, preventing chemical contamination with dye residues and damage to the environment.

The test using beads with 2.5 alginate and 4% avocado seed powder without dye adsorption also inhibited the bacteria in the last stage of the study, demonstrating that it

is possible to carry out industrial tests in the future to further assess the potential of this technique.

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22 CHAPTER 4 – BIOPROSPECTION OF ANTIBACTERIAL COMPOUNDS AGAINST LACTIC ACID BACTERIA FROM THE FUEL ETHANOL INDUSTRY

ABSTRACT

Lactic acid bacteria are among the major contaminants in the ethanol industry, an important energy pathway. *Limosilactobacillus fermentum* represents the main contaminating microorganisms in Brazilian fuel ethanol-producing distilleries. Natural extracts oils are being studied to control the contaminating microorganisms in fermentation. A screening of fungal supernatants, plant extracts and essential oils were performed against *L. fermentum* in order to find new alternatives for bacterial control in industrial process of fuel ethanol. The antibacterial activity of the supernatants of *Sporobolomyces japonicus, Sporidiobolus pararoseus, Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* cultures against *L. fermentum* was not found in the concentrations and conditions tested in the present work. In preliminary tests with plant extracts, an antimicrobial activity of extracts of *Eucalyptus urograndis* was found against *L. fermentum* and *Agave americana* against *S. cerevisiae*, both at concentrations of 10,000 mg L^{-1}. Although the *Eucalyptus* extract is not toxic to *S. cerevisiae*, it was not so effective in a Fed-batch fermentation process. The analyzes of the fermentation in first screening, which was more efficient, indicated the need for further studies under different conditions on the use of *E. urograndis* extract, possibly at lower concentrations in the Fed-batch process. As for essential oils, the Rosemary essential oil met the criteria for application in cyclic Fed-batch fermentation tests at a concentration of 103 mg L^{-1} suggesting that this oil should be further studied for industrial applications.

Keywords: Natural antimicrobial; Plant extracts; Essential Oils; Antibiotics; Ethanol.

23 INTRODUCTION

The need to make standardized plant extracts for large-scale use in the ethanol production industry is now evidenced. Natural extracts are being used with great success in the control of contaminating microorganisms in fermentation, but the results are shown in isolation (LOPES et al., 2016). The antimicrobial activity of a plant extract can be conferred by the presence of tannins, terpenes, flavonoids, phenolic compounds and phenols (SATISH; FARHA; UROOJ, 2018). With the application of a natural product, there is a great chance of getting help in decision making for a type of control that is in accordance with current waste reduction requirements. Furthermore, the use of acids and antibiotics in the microbiological control of ethanol production has a very high cost, while the scenario for plant extracts may be not (CAETANO; MADALENO, 2011).

Among the natural products that can have an antibacterial action are those obtained from microbial cultures. Several yeast are responsible for the production of natural antibiotics from the fermentation process, where these metabolites can be effective in controlling unwanted microorganisms, and several compounds have already been produced for this purpose (CLEMENTINO et al., 2015). The fungi *Sporobolomyces japonicus, Rhodotorula mucilaginosa* and *Sporidiobolus pararoseus* are responsible for producing orange pigments when submitted to fermentation, showing the presence of carotenoids, a compound that has antioxidant properties, in addition to the possible existence of antimicrobial properties (ARUNKUMAR; GORUSUPUDI; BERNSTEIN, 2020; YOO; ALNAEELI; PARK, 2016). A study showed the existence of antibacterial factors produced by the yeast *Saccharomyces cerevisiae* M26, with a bacterial reduction around 50% (OLIVA NETO; FERREIRA; YOKOYA, 2004).

Several plant extracts have antimicrobial potential. *Eucalyptus* leaves are approved for use as food additives and several species of the plant can be found in all regions of Brazil (SILVEIRA et al., 2012). The essential oils found in eucalyptus leaves have terpenoids, known for their antimicrobial properties (GOLDBECK et al., 2014). *Eucalyptus urograndis* has a predominance of mono and sesquiterpenes, including 1,8-cineole compounds, with bactericidal, insecticide, herbicide and pharmacological properties, and thymol, which is vermicide, bactericide, fungicide, anthelmintic (ARAÚJO et al., 2010). Jambolão leaves (*Syzygium cumini* L.), for example, are rich in tannins and saponins. The antimicrobial action of tannins is explained by the inhibition of enzymes and the modification of microbial metabolism by this agent. Another plant

extract already used is hops, a flower of the Canabidaceae family. In its essential oil, β-acids have a bactericidal action, acting in the transport of metabolites in the cell membrane and changing the intracellular pH. The pronounced bacteriostatic action on Gram-positive bacteria seems to be related to the interference of the prenil group, present in the side chains of β-acids, on the plasma membrane of cells, strongly inhibiting their growth (CAETANO; MADALENO, 2011).

Studies have shown that compounds from ethanol extracts from the seeds and husks of the *Annona classiflora* plant (Annonaceae family) belonging to this family have an excellent antioxidant activity (BIBA et al., 2014). A test to evaluate the antibacterial activity of the *A. classiflora* plant was carried out using the alkaloid fraction of its wood, showing a biological activity against pathogenic bacteria such as *Staphylococcus aureus*, in addition to other tests carried out, which indicated this plant as a potential source of compounds for antimicrobial development (GONÇALVEZ; MOSQUEIRA; PIMENTA, 2010). Other studies carried out showed that the plant *Agave americana* (Agavaceae family) also showed antibacterial results against the *S. aureus* (KHAN et al., 2010), in addition to the bacteria *Escherichia coli* (KANDHASAMY; VASUDEVAN, 2015).

Belonging to the Bignoniaceae family, the plants *Pyrostegia venusta* and *Spathodea campanulata* were subjected to studies to assess their antimicrobial potential (OFORI-KWAKYE; KWAPONG; ADU, 2009). Tests using crude methanolic extract and ethanolic extract of *S. campanulata* were evaluated against isolated pathogenic bacteria showing high inhibitory potency (AKHARAIYI; BOBOYE; ADETUYI, 2012), emphasizing *Staphylococcus aureus*, in which the hexane extract of *P. venusta* showed moderate inhibition (SILVA et al., 2011). There are other reports in the literature on the antimicrobial activity evaluated using aqueous, methanolic, ethanolic and petroleum ether extracts of the plant *S. campanulata* (OFORI-KWAKYE; KWAPONG; ADU, 2009), but also flower extract and methanol extract of the *P. venusta* plant (BOUZADA et al., 2009; TORRES et al., 2013).

Tridax procumbens L. (Asteraceae family) was submitted to a study evaluating the ethyl acetate extract of the flowers and extract of its aerial parts, which showed antimicrobial activity against Gram-positive bacteria, but none of the plant extracts tested showed to be active against the yeasts used in the study (TADDEI; ROSAS-ROMERO, 2000). Responsible for being a source of saponins, the arboreal species *Sapindus saponaria* L (Sapindaceae family) can be used for pharmacological uses (ALBIERO; BACCHI; MOURÃO, 2001). The hydromethanolic extract of aerial parts of *S. saponaria*

was evaluated on *Bacillus cereus, Micrococcus flavus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae* and *Salmonella typhimurium*, showing a significant bacterial and fungi antimicrobial activity in all species tested. These results enable the discovery of new antimicrobial agents of plant origin (RASHED et al., 2013).

Essential oils are characterized by being secondary metabolites of various parts of plants, known for their biological properties such as the control of food oxidation and the control of resistant bacteria (CONTRUCCI et al., 2019; MIRANDA et al., 2016). The species *Rosmarinus officinalis* L. belonging to the Lamiacea family was used in antiquity due to its medicinal effects, and its oil (Rosemary essential oil) show an effective antimicrobial activity, mainly on bacteria, and its efficacy on *Salmonella sp* and on some Gram-positive bacteria such as *Staphylococcus aureus* (HENTZ; SANTIN, 2007; PORTE; GODOY, 2005).

The Clove (*Eugenia caryophyllus*) is known for its various applications in health, and its essential oil is described in the literature with antimicrobial, antioxidant and anesthetic properties. Studies have shown antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (SCHERER et al., 2009; SILVA et al., 2011).

In the present work, a screening of new alternative and biological compounds to replace traditional chemicals used in the bacterial control of fuel ethanol production was perfumed, particularly with high efficiency for the control of *Limosilactobacillus fermentum*, the most important contaminant in this bioprocess in Brazil.

24 MATERIALS AND METHODS

24.1 Microorganisms and maintenance of strains

The yeast strain *Saccharomyces cerevisiae* M-26 was previously isolated from bioethanol distillery (OLIVA NETO; FERREIRA; YOKOYA, 2004). Yeasts were stored at -80 °C in Eppendorf on YM medium containing 2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.087% dipotassium phosphate, 0.024% magnesium sulfate heptahydrate, 0.0017% manganese sulfate heptahydrate, 0.0028% zinc sulfate heptahydrate to 100 mL of distilled water (with 10% of glycerol) at pH 5. The culture medium for the propagation of yeast and inoculum production was prepared by (w v^{-1}): 2% sucrose, 0.5% yeast extract, 0.10% ammonium sulfate, 0.1140% dipotassium

phosphate $3H_2O$, 0.024% magnesium sulfate $7H_2O$, 0.0012% manganese sulfate H_2O, 0.0028% zinc sulfate $7H_2O$ (pH 5) (DORTA et al., 2006). The lactic acid bacteria *Limosilactobacillus fermentum* CCT 0559 was obtained by André Tosello Culture Collection (Campinas, Brazil) and cultivated at pH 6 on MRS medium (DE MAN; ROGOSA; SHARPE, 1960).

The culture media were sterilized in an autoclave at 121 °C, at 1 atm, for 20 minutes, inoculated and then incubated in an incubator (FANEM LTDA, model 002CB – São Paulo – SP) at 37 and 30 °C for *L fermentum* and *S. cerevesiae*, respectively, for 48 hours.

24.2 Screening of yeast

24.2.1 Fungi cultivation

The strains from the yeast collection of the Bioenergy Research Institute (IPBEN/Assis) identified as *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Rhodotorula mucilaginosa* LABI1 and *Saccharomyces cerevisiae* M-26 were submitted to the same culture cultivation conditions described in item 24.1, but maintained under constant agitation of 150 rpm for 48 hours.

24.2.2 Screening of supernatants with antimicrobial activity

After the incubation time, the yeasts were subjected to centrifugation (Thermo Scientific – Heraeus Megafuge 16R) at 5,000 rpm for 30 minutes at 25 °C. The wort supernatant from each yeast was extracted with a micropipette and used for dilution in MRS medium. The tests were performed in triplicate, and the test tubes were autoclaved at 121 °C, 1 atm, for 20 minutes. After sterilization, the tubes received 0.1 mL of the standardized inoculum of *L. fermentum* culture, and were subsequently incubated at 37 °C for 48 hours in a growth oven.

24.3 Plant extract

Plant extracts extracted and processed from different forms of plants were tested: *Annona crassiflora, Pyrostergia venusta, Spathodea campanulata, Sapindus saponaria, Tridax procumbens, Agave americana, Ananas comosus, Aloe arborescens, Bidens*

pilosa, Plectranthus barbatus, Solanum Americanum, Solanum paniculatum, Solanum mauritianum, Eucalyptus urograndis, Eucalyptus grandis, Corymbia citriodora, Vernonia polysphaera, Senna hirsuta, Acanthospermum australe, Melissa officinalis, Zea mays, Stryphnodendron, Saccarum offinarum and *Hymenaea courbaril.*

Plant extracts of *A. crassiflora*, *P. venusta*, *S. campanulata*, *S. saponaria* and *T. procumbens* were kindly provided by Prof. Dr. Regildo Márcio Gonçalves da Silva, coordinator of the Laboratory of Herbal Medicines and Natural Products at UNESP, Assis, São Paulo, Brazil. Paste extracts and powdered extracts were resuspended in water until the concentration of interest and homogenized until total dilution. Furthermore, the preparation of the plant extract of *A. americana* was made by cutting it into pieces of approximately 1 cm, with subsequent addition of water and grinding in a blender, ending with filtration to obtain the mucilage.

In natura leaves of of *A. comosus, A. arborescens, B. pilosa, P. barbatus, S. americanum, S. paniculatum, S. mauritianum, E. urograndis, E. grandis, C. citriodora, V. polysphaera, S. hirsuta, A. australe, M. officinalis, Zea mays, Stryphnodendron, S. offinarum, H. courbaril and* another sample of *A. americana* were dried in an oven for 24 hours at 50 °C and crushed in a blender and processed into powder form. The leaves of these last plants tested were collected at the Estância Esperança, Borá/SP.

24.3.1 Initial screening of plant extracts with antimicrobial activity

To verify the antimicrobial activity of different plant extracts, the initial dosage of 1,000 mg L^{-1} (w v^{-1}) was used for *A. crassiflora*, *P. venusta*, *S. campanulata*, *S. Saponaria*, *T. procumbens* and *A. americana* mucilage. The powdered extract samples from the plants *A. comosus, A. arborescens, B. pilosa, P. barbatus, S. americanum, S. paniculatum, S. mauritianum, E. urograndis, E. grandis, C. citriodora, V. polysphaera, S. hirsuta, A. australe, M. officinalis, Zea mays, Stryphnodendron, S. offinarum, H. courbaril and* a sample of *A. americana* powder were added to the culture medium at a concentration of 10,000 mg L^{-1} (w v^{-1}).

The tests were divided into two parts: samples sterilized by microfiltration (Millipore) and samples autoclaved at 121 °C, 1 atm, for 20 minutes, to verify whether the antimicrobial potential of plant extracts was degraded at high temperatures. After these procedures, the test tubes received an inoculum containing 0.1 mL of the

standardized *L. fermentum* culture, and subsequently incubated at 37 °C for 48 hours (RANKE et al., 2019).

24.3.2 Liquid-liquid extraction of plants

For the liquid-liquid extraction (LLE) of plant extract powder that showed antimicrobial action in the initial tests, four types of solvents were used: butanol, dichloromethane, hexane and heptane, with different polarities to increase the selectivity range. The test was carried out only with bacteria, since the aim of this study was the antibacterial action of the extracts. Solvents were tested in ascending order of polarity. First, the LLE was performed by mixing the plant extract powder with hexane (2:1 v v^{-1}), butanol and heptane both (1:1 v v^{-1}) and dichloromethane (1:4 v v^{-1}). The LLE were done both at 25 °C and 80 °C. For hot extraction, 5 flasks were separated. In the first flask, 1g of the plant extract powder was mixed with 50 mL of MRS medium, for the other four tests 1g of the plant extract was added in each one and 25 mL of the MRS medium and 25 mL of the respective solvent. Afterwards, the flasks were kept in a water bath at 80 °C for 20 minutes with constant agitation and manual homogenization. The extraction process at 25 °C was similar. After infusion, the mixture was vacuum filtered and the organic and aqueous fractions were obtained by separatory funnel. Afterwards, the flasks were conditioned in an oven at 80 °C for 5 days to obtain the dry extract, so that the dry mass was resuspended with MRS medium until 10,000 mg L^{-1} (w v^{-1}) concentration.

24.4 Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory concentration (MIC) was determined by adapted macrodilution broth method (JONES et al., 1985). The inoculum was standardized according to McFarland 0.5 standard in aseptic conditions. The MIC was defined as the minimum chemical compounds concentration able to inhibit at least 90% the microbial growth and performed in triplicate.

Microbial growth was measured by turbidity of the medium by reading the absorbance at 600 nm wavelength using a spectrophotometer (Pharmacia - Ultrapec), in addition to checking under a microscope.

24.5 Essential oils

24.5.1 Obtaining essential oils

Rosemary (*Rosmarinus officinalis*) belongs to the Lamiaceae family and the commomly knonw as Clove (*Eugenia caryophyllus*) belongs to Myrtaceae family. The essential oils (EO) of Rosemary and Clove Leaf were purchased from WNF Essential Oils (São Paulo), and they are pure and 100% natural. The lot of the products are 03370/19 and 03783/19, respectively.

24.5.2 Determination of dry mass and preparation of essential oils

In order to find the concentration (mg L^{-1}) of the essential oils, the dry mass was determined and 0.5 mL of them were dried at 120 °C for 4 hours until constant weight, with subsequent weighing and determination of concentration.

Due to the polarity of the essential oils and the difficulty of solubility in water, Tween 80 was added, as a process of emulsification of the oils was carried out, enabling the use in MRS medium and formulated medium, and the solution had a milky appearance. For the Rosemary essential oil, 25% of Tween 80 was added in relation to the volume of oil, however for the oil of Clove Leaf 50% was used due to its difficulty in emulsifying it in water.

24.5.3 MIC of essential oils

The essential oils of Clove Leaf and Rosemary were submitted to determination of the Minimum Inhibitory Concentration (MIC) by macrodilution in test tubes with mixed culture of *L. fermentum* and *S. cerevisiae*. Were added to each test tube 4 mL of culture medium with quadruple the concentration of solids, specific for each microorganism (according to item 24.1), and then 4 mL of the infusion of the compound with antimicrobial potential was then added to the first test tube. After homogenizing the first tube, 4 mL were pipetted into the second tube, which contained 4 mL of the same specific medium, but with twice the concentration of nutrients. The procedure was repeated successively for three more different tubes, in triplicate. In the end, the 4 mL removed from the last test tube were discarded, and for the control tube, without the

infusion of compounds, 4 mL of distilled water were added. For the preparation of control, the test tubes were sterilized, inoculated and incubated at the properly temperature.

24.6 Fed-batch fermentation with cell recycle

The alcoholic fermentation tests were based on the methodology described by literature, with some modifications (DORTA et al., 2006; OLIVA-NETO; YOKOYA, 1994). The wort was formulated with sugarcane molasses (supplied by Usina Cocal - Paraguaçu Paulista/SP), then diluted with distilled water to obtain the number of soluble solids of 18 °Brix checked in an analog refractometer (Biosystems, RSA- BR32T), and then the salts belonging to the formulated medium for yeast were added (item 24.1). Furthermore, the pH was corrected to 5.5 with HCl.

Five successive batches were carried out with cell recycling, through the Fed-batch process, with fermentation tests in 500 mL Erlenmeyer flasks. The tests were performed in triplicate, with the control tests. For the first fermentation cycle, the inoculum was prepared for each flask with 1.17 g of wet mass of *L. fermentum* and 17.5 g of wet mass of *S. cereviasie*, and later diluted with sterile distilled water. Fermentation was fed with must in order to avoid yeast inhibition by the substrate, which occurs at concentrations above 150 g L^{-1} (JONES et al., 1985). After feeding, the Erlenmeyer flasks were incubated in an orbital incubator (TE-421, Tecnal, Piracicaba, Brazil) with gentle agitation of 80 rpm at 30 °C. After the end of fermentation cycle, the fermented broth was centrifuged in a centrifuge for 25 minutes, 4 ºC and 3500 *x*g (Cryofuge 6000i, Heraeus, Germany). The supernatant was used for the other analysis and the wet mass, in each cycle, was collected and used as an inoculum in subsequent cycles.

24.6.1 Inoculum preparation

The strains of the yeast *S. cerevisiae* M-26 and the bacteria *L. fermentum* CCT 0559 were reactivated three times in tubes using an inoculation loop before their propagation. Pre-inoculum tubes were prepared by adding 0.1 mL of *L. fermentum* to 6 mL of liquid MRS medium under aseptic conditions and 0.1 mL of *S. cerevisiae* was inoculated into each test tube containing 6 mL of liquid formulated medium for yeast. Subsequently, the tubes with the bacteria and yeast were incubated in an oven at their optimal temperatures of 37 °C and 30 °C, respectively, both for 48 hours until cell growth.

The inoculum for the 1st cycle was propagated with 9 tubes of *L. fermentum* and 9 tubes of *S. cerevisiae* with a volume of 6 mL per test tube. Each one of *L. fermentum* was poured into a 1 L Erlenmeyer flask containing 500 mL of MRS medium and each tube of *S. cerevisiae* was poured into a 1 L Erlenmeyer flask containing 500 mL of formulated medium. The total volume obtained was 4.5 L for both microorganisms. The flasks were incubated for 24 to 48 hours with gentle shaking at 80 rpm at 37 °C (bacteria) and 30 °C (yeast). At the end of the incubation, the media were centrifuged for 25 minutes at 4 °C and 3500 *x*g. The supernatant was discarded and the wet mass was used as a mixed inoculum in the 1st cycle of alcoholic fermentation.

24.6.2 Alcoholic fed-batch fermentation using plant extract

The *Eucalyptus urograndis* was chosen to alcoholic fermentation in Fed-batch process and used at a concentration of 7,000 mg L^{-1}. Each Erlenmeyer flask initially contained 24 mL of inoculum (*L. fermentum* and *S. cerevisiae* mix) with 24 mL of previously autoclaved wort at 121 °C, 1 atm, for 20 minutes. The positive control was done in a similar way, except for the addition of eucalyptus extract to the wort. The tests were fed every hour with 24 mL of wort (with and without extract) during the first 8 hours which in the end resulted in 240 mL.

24.6.3 Fed-batch alcoholic fermentation using rosemary essential oil

Rosemary essential oil was added to the wort at a concentration of 103 mg L^{-1} considering the inoculum volume, and they were autoclaved at 121 °C, 1 atm, for 20 minutes. Each fermentation cycle lasted 12 hours, starting with 17.5 mL of wort and 35 mL of inoculum. The first 8 hours had 1 feeding of 17.5 mL of wort per hour, which in the end resulted in 140 mL.

24.7 Analytical procedures

24.7.1 Growth and viability of microorganisms

After 48 hours of incubation at the optimum temperature the microbial growth was verified by the turbidity of the medium with the naked eye and reading in a spectrophotometer (600 nm). In addition, the verification under a microscope was

performed to determination of cell growth and viability by counting live cells/mL of extract by adding 1 mL of each sample to 1 mL of methylene blue dye solution (LEE; ROBINSON; WANG, 1981). After homogenization, living cells were counted (not stained) in a Neubauer Chamber, and cell viability was determined by the ratio of living cells/total cells per mL of extract (DORTA et al., 2006). The determination of the quantity was found according to the equations:

$$Number\ of\ cells/mL\ =\ \sum of\ the\ quadrants\ x\ 5\ x\ 10^4\ x\ DF \qquad (9)$$

$\sum$ of squares = sum of the number of cells counted in the five quadrants;
10^4 = volume adjustment on the camera;
DF = sample dilution factor.

$$Cell\ viability\ (\%)\ =\ \frac{total\ number\ of\ living\ cells\ x\ 100}{total\ number\ of\ cells\ (live+dead)} \qquad (10)$$

24.7.2 Growth inhibition of microorganisms

Antibacterial efficiency was calculated using the following equations:

$$Inhibition\ abs\ (\%)\ =\ \frac{(Abs\ treatment\ 48\ h - Abs\ treatment\ 0h)\ x\ 100}{(Abs\ control\ 48\ h - Abs\ control\ 0\ h)} \qquad (11)$$

Abs of the treatment 48 h - Abs of the treatment 0 h = absorbance of the treatment means the growth in 48 h culture;
Abs of the control 48 h – Abs of the control 0h = absorbance of the control group means the growth in 48 hours;
Inhibition abs (%) = percentage of growth inhibition of the microorganism.

$$Bacterial\ reduction\ (\%)\ =\ \frac{(Abs\ control - Abs\ treatment)\ x\ 100}{Abs\ control} \qquad (12)$$

24.7.3 Total acidity quantification

The quantification of the total acidity produced was carried out at 0 and 12 hours by titration with 0.1 N NaOH standardized in a 25 mL graduated burette until reaching pH 7.0 measured with a pH meter (Bel, PHS3BW, Piracicaba, Brazil). The equation described was used to obtain the acidity results expressed in g L^{-1} (OLIVA-NETO; YOKOYA, 1994):

$$Total\ acidity = \frac{NaOH\ spent\ (mL)\ x\ 9.008\ x\ NaOH\ correction\ factor}{Sample\ volume\ (mL)} \qquad (13)$$

9.008 = neutralization in mass of conversion expressed in lactic acid

24.7.4 Determination of reducing sugars

The quantification of total reducing sugars (TRS) of the must and of total residual reducing sugars (TRRS) of the fermented broth was carried out by previous acid hydrolysis with subsequent application of the DNS method (MILLER, 1959). The acid hydrolysis of the samples was carried out using 2N HCl in a water bath at 100 °C for 5 minutes, followed by an ice bath and the addition of 2N NaOH, in the same proportions.

24.7.5 Quantification of ethanol produced

The determination of ethanol was made from samples of the centrifuged broth taken at the beginning of each ethanol fermentation cycle and samples of the centrifuged broth at the end of each fermentation cycle, to determine the initial and final mass of ethanol, respectively.

First, a simple distillation was carried out to obtain the hydroalcoholic fraction of each sample (LATYKI, 2017). The samples were kept in a heating mantle system until reaching temperatures of 80 to 100 °C until complete evaporation of the ethanol in a 100 mL flask. To determine the ethanol content, a portable digital hydrometer (DMA 35-Anton-Paar) was used.

The ethanol efficiency ($Y_{ethanol}$) was calculated based on the theoretical conversion of glucose in ethanol using the factor 0.511 ($\frac{g\ ethanol}{g\ glucose}$) (DORTA et al., 2006). The analyses of hydrolysis efficiency were calculated as the glucose concentration (g L^{-1}) obtained referred to the initial concentration of substrate (g L^{-1}) and was expressed in percentage (%). The ethanol productivity ($P_{ethanol}$) was calculated as the mass of produced ethanol by the fermentation time and the volume expressed in g L^{-1} h^{-1}. The equations are described below:

$$Y_{\ ethanol}\ (\%) = \frac{(P - P_0)}{(S_0 - S)\ x\ (0.511)} x\ 100 \qquad (14)$$

Where P and P_0 is the final and initial mass of ethanol inside a defined volume, respectively, in a fermentation process; S and S_0 is the final and initial mass of substrate inside the same defined volume for ethanol mass, respectively, in a fermentation process; and 0.511 ($\frac{g\ ethanol}{g\ glucose}$) is the theoretical ethanol yield value corresponding to glucose.

$$P_{\ ethanol} = \frac{(P - P_0)}{t_f\ x\ V} \qquad (15)$$

Where t_f is fermentation time (hours) and V is the working volume (L).

24.7.6 Statistical analyses

For the analysis and statistical tests, the Test T Sudent was used in the Bioestat 5.0 program.

25 RESULTS

25.1 Evaluation of free-cells yeast broth and plant extracts in the growth inhibition of *Limosilactobacillus fermentum and Saccharomyces cerevisiae.*

The tests using free-cells yeast broth of S. japonicus, *S. pararoseus, R. mucilaginosa and S. cerevisiae* against *Limosilactobacillus fermentum* growth showed no antibacterial action since there was the presence of high turbidity.

The plant extracts presented a certain difficulty in relation to sterilization, since part of the extract was retained. In addition, extracts from plants were submitted to sterilization by ultraviolet light for 20 minutes. Initial concentrations of 1,000 (mg L^{-1}) were tested, however several concentrations were tested from this and the maximum are described in Table 10.

Table 10. Antimicrobial activity against *Limosilactobacillus fermentum* and *Saccharomyces cerevisiae* from plant extracts at different maximum concentrations tested.

Plant extract	**Concentration (mg L^{-1})**	**Antibacterial action**	**Antifungal action**
A. americana	5,000	-	-
A. crassiflora	5,020	-	-
P. venusta	30,000	-	-
S. campanulata	13,500	-	-
S. saponaria	5,000	-	-
T. procumbens	10.500	-	-
A. comusus	10,000	-	-
A. arborescens	10,000	-	-
B. pilosa	10,000	-	-
P. barbatus	10,000	-	-
S. americanum	10,000	-	-
S. paniculatum	10,000	-	-
S. mauritianum	10,000	-	-
E. urograndis	10,000	+	-
E. grandis	10,000	-	-
C. citriodora	10,000	-	-
V. polysphaera	10,000	-	-
S. hirsuta	10,000	-	-
A. australe	10,000	-	-
M. officinalis	10,000	-	-
Zea mays	10,000	-	-
Stryphnodendron	10,000	-	-
S. offinarum	10,000	-	-
H. courbaril	10,000	-	-
A. americana	10,000	-	+

* (-) Visible turbidity, no antimicrobial action (+) Antimicrobial action. Source: The author.

The plant extract of *A. americana* (infusion) showed activity against yeast at 10,000 mg L^{-1} concentration. The results show that only *E. urograndis* plant extract at the same concentration was active against *L. fermentum*. For this reason, it was the only one submitted to solvent extraction in order to concentrate the active principle and to evaluate some chemical properties of the molecule, for example, its polarity.

25.1.1 Extraction of antimicrobial compounds from *Eucalyptus urograndis* infusion by liquid-liquid separation using organic solvent

After organic solvent extraction of *Eucalyptus urograndis* using butanol, dichloromethane, hexane and heptane, the organic and aqueous fractions of the solvents were tested at a concentration of 10,000 mg L^{-1} and only the organic butanolic fraction showed bacterial inhibition in first screening. Thus, this fraction was chosen to be applied in subsequent Fed-batch ethanol fermentation tests. Then, a mixed fermentation (*L. fermentum* and *S. cerevisiae*) of the wort (diluted molasses) was carried out to analyze the efficiency of the natural compound as an antimicrobial. The Table 11 shows the acidity results after alcoholic fermentation. The acidity of the mixed culture with the addition of *E. urograndis* extract proved to be lower than the control in 25 °C and 80 °C extraction, since the acidities decrease 35% and 23%, respectively, in comparison with the control. However, the bacterial growth had an increase in relation to the control as show at Table 12.

Table 11. Acidity results of first screening test in mixed culture fermentation of the *Eucalyptus urograndis* extract at 10,000 mg L^{-1}. Positive Control (C+), Negative Control (C-), Test Average (TA), Test acidity variation (ΔA), Control acidity variation (ΔC), % Acidity Reduction (%R) expressed in g L^{-1}.

Solvent extraction	**Extraction at 25 °C**		**Extraction at 80 °C**	
Time	**0 hour**	**24 hours**	**0 hour**	**24 hours**
C+	1.65	5.00	1.56	4.95
C-	1.10	1.33	1.05	0.87
TA	2.15	4.32	1.86	4.13
ΔA	2.17		2.61	
ΔC	3.35		3.39	
%R	35.16		23.00	

Source: The author.

Table 12. Growth of *L. fermentum* and *S. cerevisiae* and cell viability of the yeast in mixed culture fermentation of the *Eucalyptus urograndis* extract at 10,000 mg L^{-1}.

Assay	Bacterial growth	Yeast growth	Yeast viability (%)
C+	7.05E+08	1.95E+07	100
25 °C extraction	1.10E+10	1.69E+07	99.69
C+	6.15E+08	2.78E+07	96.55
80 °C extraction	1.19E+10	1.48E+07	99.21

Obs. Positive control (C+). Source: The author.

Due to the difference in behavior between acidity and bacterial growth in the mixed fermentation with *E. urograndis* extract, the experiments were repeated. Tables 13 and 14 show the results of a new test of mixed culture fermentation with 7,000 mg L^{-1} of *E. urograndis* extract. The presence *E. urograndis* extract at 25 °C and 80 °C showed again a decrease in acidity, respectively 6.1% and 22.2%, probably lower than the first test because in the second was used 30% less extract than the first.

Regarding cell growth, the yeast count in the second experiment was still below the controls, but with a smaller difference than in the first test, which indicates that perhaps in a lower concentration the extract is more beneficial for *S. cerevisiae*. By not having changed the high viability, it is possible to infer that within the two experiments there was a low toxicity for yeast or even absence. However, the main objective of the addition of the plant extract on wort was the bacterial control and, on the contrary, there was a strong increase in the bacteria number in comparison to the control.

Table 13. Acidity results of second tests in mixed culture fermentation of the *Eucalyptus urograndis* extract at 7,000 mg L^{-1}. Positive Control (C+), Negative Control (C-), Test Average (TA), Test acidity variation (ΔA), Control acidity variation (ΔC), % Acidity Reduction (%R) expressed in g L^{-1}.

Solvent extraction	**Extraction at 25 °C**		**Extraction at 80 °C**	
Time	**0 hour**	**24 hours**	**0 hour**	**24 hours**
C+	1.65	3.90	0.98	4.08
C-	0.92	1.05	1.42	1.01
TA	1.83	3.94	1.79	4.25
ΔA	2.11		2.46	
ΔC	2.25		3.16	
%R	6.12		22.2	

Source: The author.

Table 14. Growth of *L. fermentum* and *S. cerevisiae* and cell viability of the yeast in mixed culture fermentation of the *Eucalyptus urograndis* extract at 7,000 mg L^{-1}.

Assay	**Bacterial growth**	**Yeast growth**	**Yeast viability (%)**
C+	2.04E+09	2.09E+07	100
25 °C extraction	1.15E+10	1.66E+07	99.61
C+	2.80E+09	2.16E+07	100
80 °C extraction	1.76E+10	1.48E+07	99.51

Obs. Positive control (C+). Source: The author.

25.1.2 Fed-batch alcoholic fermentation with *Eucalyptus urograndis* extract

The plant extract of *Eucalyptus urograndis* was also evaluated as antimicrobial agent in Fed-batch mixed culture alcoholic fermentation. The dosage of 7,000 mg L^{-1} was performed in alcoholic fermentation tests.

25.1.2.1 Growth and cell viability

The Table 15 indicates the viability of yeast cells during the 5 fermentation cycles. The yeast viability remained high during all cycles, even though feedings were carried out every hour for 8 hours with the natural antimicrobial. This result corroborates the tests previously performed with the *Eucalyptus* extract, showing again that this plant

extract does not have fungicidal power. The yeast cells number showed a slight variation with some cycles, superior in the control (1st, 4th and 5th) and inferior to the treatment (2nd and 3rd). Probably, these differences are related to the difficult of the samples due to the distribution of the yeasts in the wort.

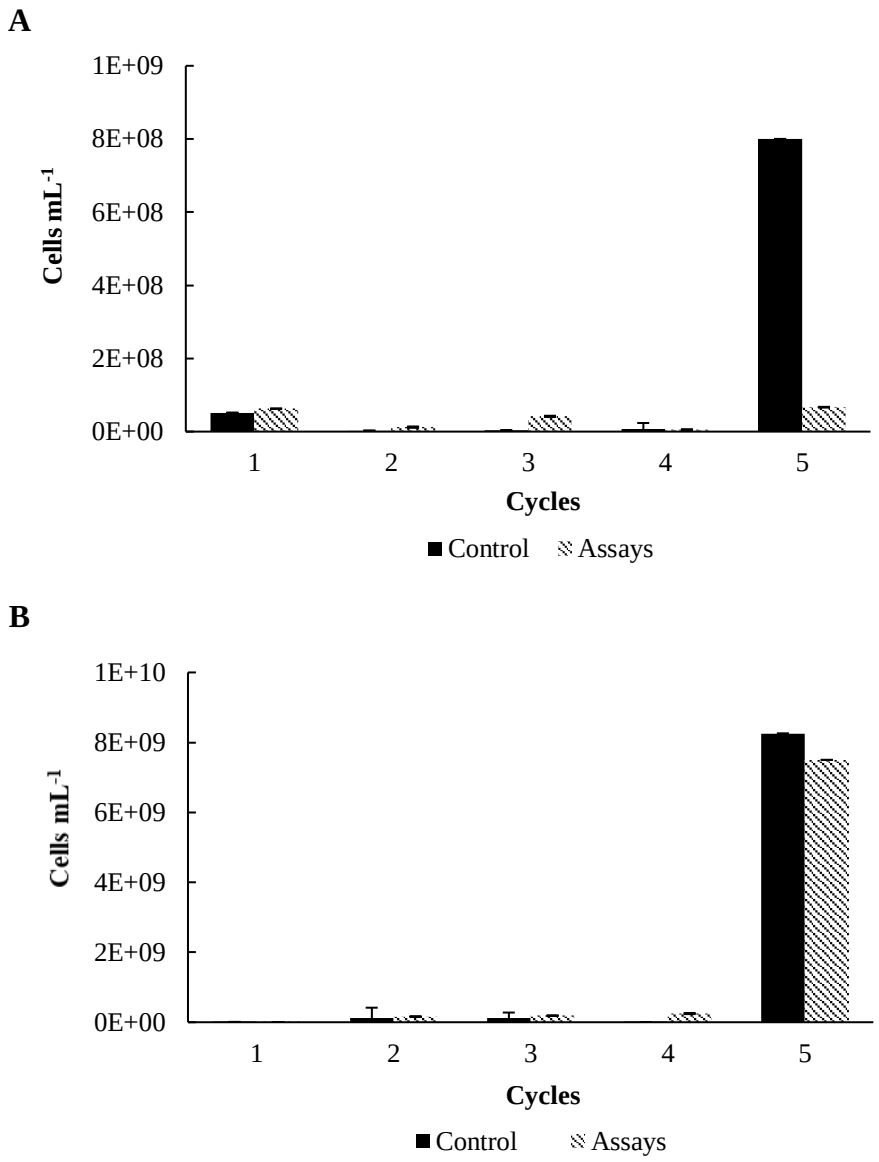
The bacteria growth (Figure 17) had a decrease in both, control and treatment, during the first 4 cycles, but a great increase was verified in the 5th cycle, when the fermentation with the *E. urograndis* extract partially could control the bacterial growth, since a great difference was verified in the control (10^8 cell mL^{-1}) and the treatment with *E. urograndis* extract (10^7 cell mL^{-1}).

Table 15. Growth and cell viability of *S. cerevisiae* in mixed culture fermentation with *Eucalyptus urograndis* extract.

Clycle	**Assay**	**Yeast growth**		**Yeast viability (%)**
1	Control	8.70E+06		100
	E. urograndis	4.32E+06	±1.50E+05	100
2	Control	1.19E+08		100
	E. urograndis	1.62E+08	±1.60E+05	100
3	Control	1.27E+08		100
	E. urograndis	1.89E+08	±1.60E+05	100
4	Control	3.95E+08		100
	E. urograndis	2.49E+08	±1.60E+07	100
5	Control	8.25E+09		99
	E. urograndis	7.50E+09	±1.60E+05	98

Source: The author.

Figure 17. Comparison of growth of *L. fermentum* (A) and *S. cerevisiae* (B) in each recycle of mixed culture alcoholic fermentation with *E. urograndis* extract as bacterial control.



Source: The author.

25.1.2.2 Total acidity

Another way to analyze the presence of lactic bacteria in the alcoholic fermentation was through the acidity evaluation (Table 16). There was an increase of the acidity during the culture and with the recycles. In addition, the acidity of the treatment with *E. urograndis* extract only was slightly inferior to the control in the 5th cycle when the number of bacteria was also lower in the treatment in comparison with the control.

According to these results, the acidity seems to decrease in relation to the control after some recycles suggesting a partial control of bacterial population. However, this test revealed the need of more recycles to evaluate the effectiveness of the *E. urograndis* extract as an inhibitory agent against bacteria.

Table 16. Total acidity during mixed culture (*L. fermentum* and *S. cerevisiae*) fermentation with *E. urograndis* extract as antimicrobial for 12 hours at 30 °C.

Clycle	**Assay**	**0 h (g L $^{-1}$)**	**8 h (g L^{-1})**	**12 h (g L^{-1})**	**Acidity reduction (%)**
1	Control	35.213	106.374	102.706	-168.12
	E. urograndis	38.148	226.930	219.105	
2	Control	50.619	159.561	267.035	-21.921
	E. urograndis	57.955	255.297	321.811	
3	Control	90.234	287.209	267.035	-63.624
	E. urograndis	66.759	304.938	356.046	
4	Control	81.431	319.121	287.576	-0.474
	E. urograndis	118.111	368.762	325.234	
5	Control	138.653	404.220	318.987	0.680
	E. urograndis	156.993	375.854	335.505	

Source: The author.

25.2 The effect of essential oils on the growth of *L. fermentum* and *S. cerevisiae*

25.2.1 Determination of essential oils concentration

The dry mass of the essential oils of Rosemary and Clove Leaf are described in Table 17.

Table 17. Dry mass of 0.5 mL of Rosemary and Clove Leaf essential oils.

Essential oils	**Dry mass (g)**	**Concentration of oils on dry basis (% w v^{-1})**
Rosemary	0.0116	2.32
Clove Leaf	0.0022	0.44

Source: The author.

1.1.1 MIC of antibacterial essential oils

From the analysis of the MIC tests with some essential oils (Table 18) Rosemary essential oil had a MIC of 103 mg L^{-1} against the bacteria (100% inhibition), but at this concentration there was a partial yeast inhibition on growth (40%). Clove Leaf essential oil showed inhibition against the yeast since the MIC (2.74 mg L^{-1}) was inferior to *L. fermentum* (> 4.11 mg L^{-1}). Then, Clove Leaf cannot be used in alcoholic fermentation because the yeast inhibition in dosages to inhibit bacterial growth.

Table 18. MIC of Rosemary and Clove Leaf essential oils (OE) with their respective microbial reductions.

Essential oil	EO concentration (mg L^{-1})*	EO concentration (µL L^{-1})	Inhibition growth of *L. fermentum* (%)	Inhibition growth of *S. cerevisiae* (%)
Rosemary EO	137	5900	100	97.5
	103	4430	100	40
	35	1500	26	32
	8.22	1866	100	100
Clove Leaf EO	5.48	1244	41	100
	4.11	933	38	100
	2.74	622	10	100
	1.37	311	10	36

* Oil concentration on a dry basis. Source: The author.

1.1.2 Fed-batch fermentation with Rosemary essential oil

Due to the Rosemary essential oil guaranteeing a better efficiency in the inhibition of the lactic bacteria *L. fermentum* and only a partial inhibition of the yeast with a dosage of 103 mg L^{-1}, this essential oil was submitted to alcoholic fermentation tests.

1.1.2.1 Growth and cell viability

In order to obtain an estimate of the total and percentage of yeast cell viability, live and dead cells were observed under optical microscope during the fermentation

the number of yeasts had a normal variation during fermentation, since some cycles (2nd and 3rd) there were more yeasts in the treatment and others (1st, 4th and 5th) in the control. Therefore, the results reveal that there was no inhibition of yeast growth.

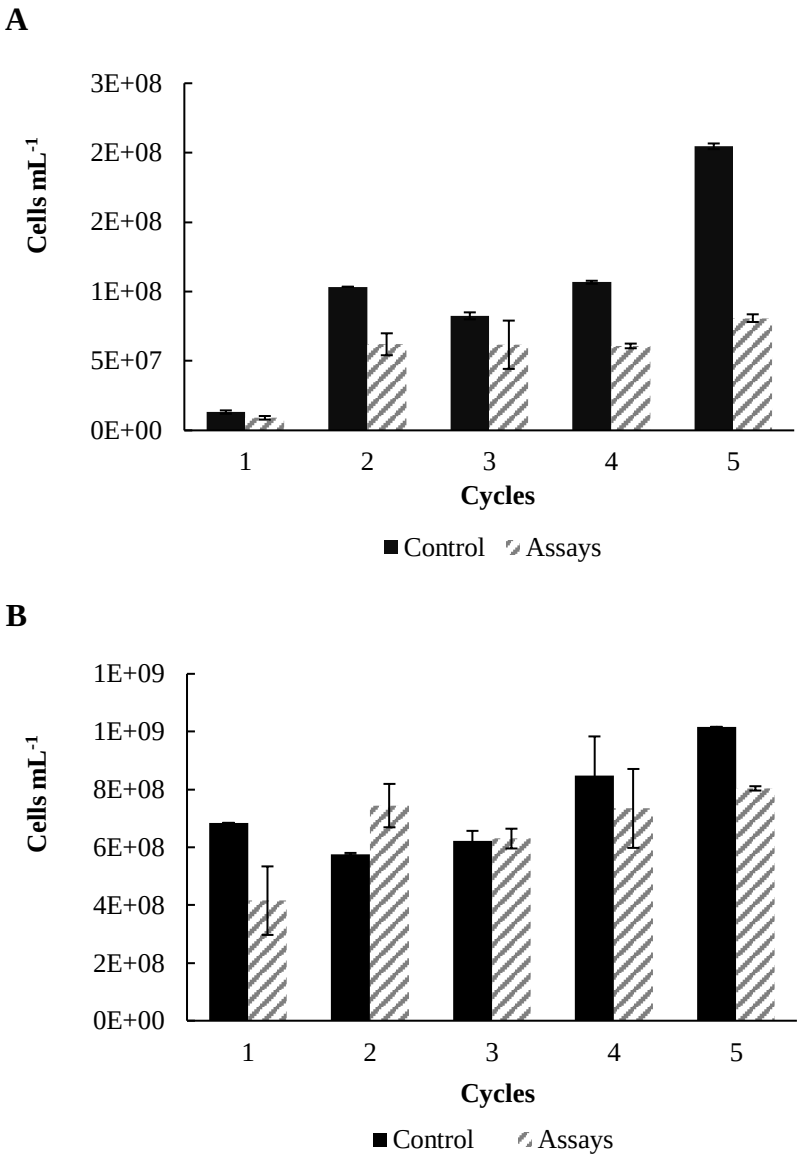
Table 19. Growth and cell viability of *S. cerevisiae* in mixed culture fermentation with Rosemary essential oil (EO).

Clycle	Assay	Yeast growth		Yeast viability (%)
1	Control	6.84E+08		100
	Rosemary EO	4.15E+08	±1.19E+08	100
2	Control	5.76E+08		100
	Rosemary EO	7.44E+08	±7.51E+07	100
3	Control	6.23E+08		100
	Rosemary EO	6.30E+08	±3.42E+07	99.95
4	Control	8.47E+08		100
	Rosemary EO	7.34E+08	±1.36+08	100
5	Control	1.02E+09		100
	Rosemary EO	8.04E+08	±7.49E+06	100

Source: The author.

The Rosemary EO was effective in the control of bacteria, showing a strong inhibition of *L. fermentum* when compared to the positive control. A marked decrease of the bacterial population during the cycles was verified. In the 5th cycle there was a high bacterial growth in the control, while in the treatment with essential oil the value was reduced by more than half. The results are shown in Figure 18.

Figure 18. Effect of Rosemary essential oil in the mixed culture alcoholic fermentation with cell recycle. *L. fermentum* (A) and *S. cerevisiae* (B).



Source: The author.

25.2.3.2 Total acidity

The acidity of the wort in the control decreased with the recycles from the 1st (1,011 mg L^{-1}) to the 5th cycle (1,477 mg L^{-1}) after 12 hours. On the other hand, the treatment with Rosemary EO dropped from the 971 mg L^{-1} (1st cycle) to 679 mgL^{-1} (5th cycle) (Table 20). Thus, the Rosemary EO showed control of bacterial contamination with 57.78% of decrease in acidity observed in cycle 5, compared to the control. It is

worth nothing that the pH of the fermented wort increased in the final cycle, being also another way to show the decrease in bacteria population.

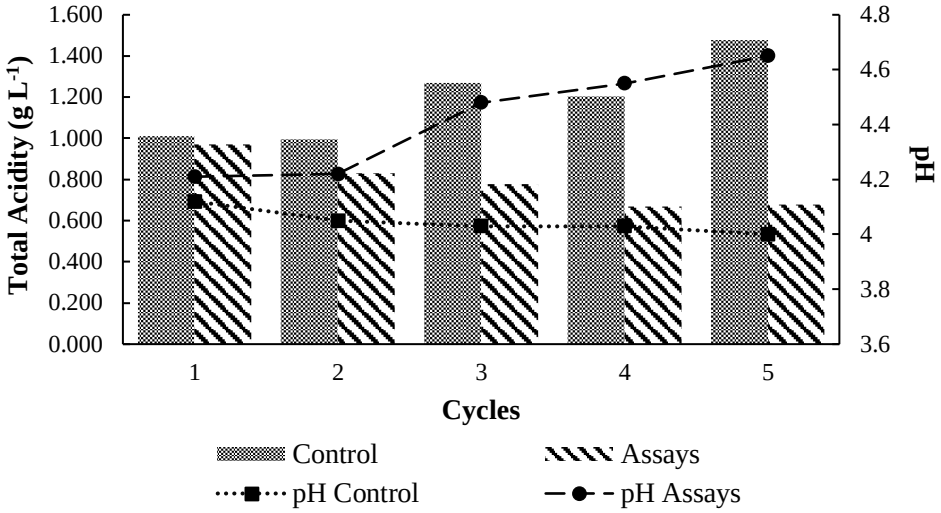
Table 20. Evaluation of total acidity during the mixed alcoholic fermentation by Fed-batch process with cell recycle (*L. fermentum* and *S. cerevisiae*) with Rosemary EO.

Clycle	**Assay**	**0 hour (g L^{-1})**	**12 hours (g L^{-1})**	**Reduction (%)**	**pH 12 hours**
1	Control	81.84	1011.00	4.06	4.12
	Rosemary EO	79.43	970.88		4.21
2	Control	84.25	994.96	17.42	4.05
	Rosemary EO	77.02	829.13		4.22
3	Control	115.54	1267.77	42.06	4.03
	Rosemary EO	110.72	778.31		4.48
4	Control	113.13	1203.581	47.51	4.03
	Rosemary EO	96.28	668.65		4.55
5	Control	101.10	1476.93	57.78	4.0
	Rosemary EO	98.69	679.35		4.65

Source: The author.

In order to prove a significant level of acidity variation among the analyzed samples, the Student T Test was performed using the Bioestat 5.0 program. It was found that there was a statistical difference between the means of triplicates and controls of the 5 cycles. For this procedure, a significance level of 5% was considered, as well as a Confidence Interval (CI) of 95%, reinforcing this hypothesis (Figure 19).

Figure 19. Analysis of total acidity (g L^{-1}) and pH of the fermented wort throughout the cyclical fermentation with the addition of Rosemary essential oil.



Source: The author.

This figure reinforces the idea that Rosemary EO was effective in controlling the *L. fermentum* bacteria, showing the disparity in the cycles of the groups analyzed. Thus, we can infer that there was a slight decline in the control pH and an increase in total acidity, while in the tests, where there is the presence of Rosemary EO, a reduction in acidity levels and an increase in pH was observed as of the cycle 2, with the gradual increase in the disparity of the groups analyzed over the cycles, especially in the last cycle.

25.2.3.3 Total reducing sugars and ethanol production analyses

The quantification of total reducing sugars (RS) and total residual reducing sugars (TRRS) was important to assess sugar consumption by yeasts and the calculation of ethanol efficiency. In the first two cycles the difference in consumed sugar was minimal, but at 5^{th} cycle there was a difference with lower consumed sugars in the essential oil treatment in relation t the control (Table 21).

Table 21. Quantification of total reducing sugars (TRS), total residual reducing sugars (TRRS) and consumed sugars (CS) in the mixed alcoholic fermentation by *L. fermentum* and *S. cerevisiae* using Rosemary essential oil (EO) as antibacterial.

Clycle	**Assay**	**TRS (g L^{-1})**	**TRRS (g L^{-1})**	**CS (g L^{-1})**
1	Control	181.34	11.70	169.64
	Rosemary OE	176.12	10.93	165.18
2	Control	181.13	11.49	169.64
	Rosemary OE	178.62	11.42	167.13
3	Control	166.93	12.53	154.39
	Rosemary OE	175.28	12.74	162.54
4	Control	166.93	12.95	153.97
	Rosemary OE	167.76	13.72	154.04
5	Control	176.74	13.58	163.16
	Rosemary OE	169.64	13.30	156.34

Source: The author.

The results obtained from the quantification of ethanol are related to the alcohol content obtained from the calculations by hydrometer and the consumption of sugar in each cycle, from these data the values found are described in Table 22.

Table 22. Ethanol quantification, yield and productivity in the mixed fermentation with *L. fermentum* and *S. cerevisiae* in cell recycle Fed-batch with Rosemary essential oil as antibacterial.

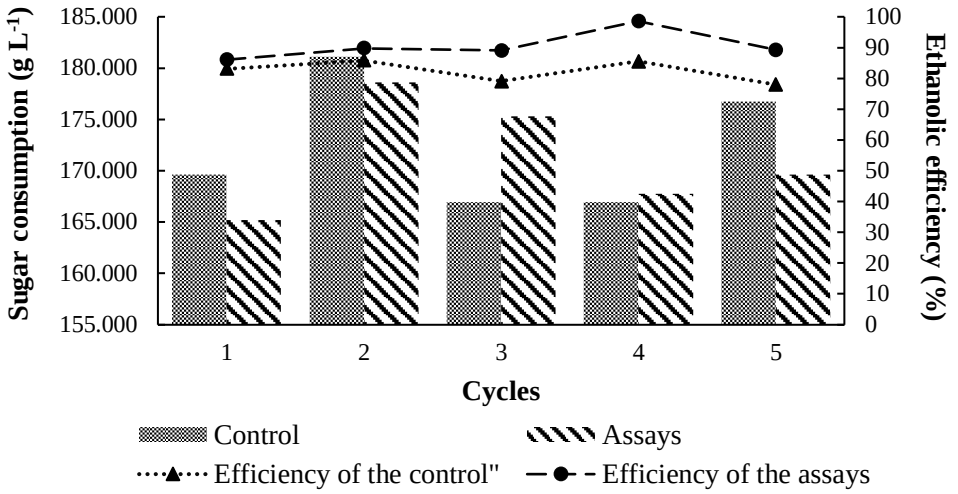
Clycle	Assay	Ethanol Efficiency (%)	Ethanol Yield (g of ethanol/100g of sugar)	Ethanol Productivity (g L^{-1} h^{-1})
1	Control	83.153	42.491	5.500
	Rosemary EO	86.059	43.976	5.583
2	Control	85.787	43.837	5.715
	Rosemary EO	89.731	45.853	5.755
3	Control	79.038	40.388	5.003
	Rosemary EO	89.029	45.494	5.589
4	Control	85.471	43.676	5.016
	Rosemary EO	98.572	50.370	5.756
5	Control	77.969	39.842	5.335
	Rosemary EO	89.237	45.600	5.523

Source: The author.

These table shows that the triplicates containing Rosemary EO always maintained a higher ethanol production compared to the control, especially from cycle 2 onwards, in which the control ethanol production remained at a lower concentration. It can be said that the best cycle was the fourth, as the ethanol yield of the triplicates reached 98.57% with a yield of 50.37 g for every 100 g of sugar consumed, and a productivity of 5.756 g L^{-1} h^{-1}. In the last cycle, the control had a great loss in ethanol production, which can be explained by the high growth of bacteria contained in the medium, causing competition and flocculation.

Thus, the ethanolic efficiency data of the samples were placed in the Bioestat 5.0 program for statistical analysis in T Student Test. In this analysis, a significance level of 5% was used and the test revealed that there was a significant difference ($p<0.05$) between triplicates and controls, reinforcing the effectiveness of microbial control by Rosemary essential oil. Analysis of CI at the 95% level also found statistical variation. The relationship between consumed sugars and ethanolic efficiency are shown in Figure 20.

Figure 20. Sugar consumption (g L^{-1}) and ethanolic efficiency during cyclic alcoholic fermentation.

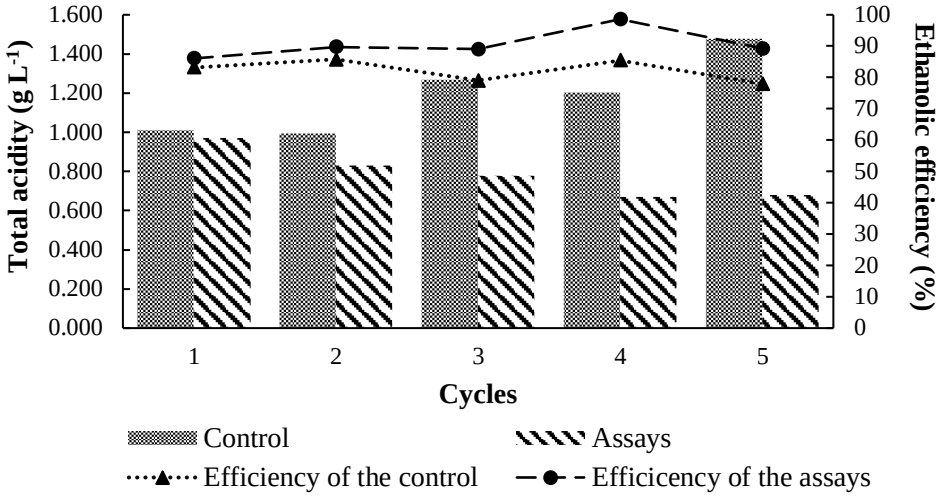


Source: The author.

It is possible to observe in Figure 20 that in the first two cycles, ethanol production remained close, even with the difference in the amount of sugar consumed. From the second cycle onwards, the dispersion of ethanol production in comparison to the groups becomes clear, although the control showed a good consumption of sugars, its ethanol production always remained below the triplicates containing Rosemary essential oil. This can be explained by the exacerbated number of bacteria present that were also consuming the sugars.

The relationship of ethanol production with the number of bacteria present and total acidity is shown in Figure 21. According to results, the high concentration of acidity is related to the decrease in ethanol efficiency. This is because from the second cycle there was a dispersion of acidity in relation to triplicates and control, and the ethanolic efficiency decreased due to the high level of acidity in the control, but the triplicates with the EO treatment always remained with a significantly higher value ethanolic efficiency.

Figure 21. Total acidity (g L^{-1}) and ethanolic efficiency during cyclic alcoholic fermentation with addition of Rosemary essential oil.



Source: The author.

26 DISCUSSION

From the analysis comparing the antibacterial action of cell lysate, whole cell and cell supernatant was obtained high, moderate and low antimicrobial actions, respectively. As the yeast cell supernatant showed less antimicrobial activity, there was indications that the antimicrobial compounds are not extracellular, but rather, linked to the cell (FAKRUDDIN; HOSSAIN; AHMED, 2017). It corroborates the results obtained at this study with no antibacterial action against *L. fermentum* by the strain tested. This may also have to do with the concentration used, as studies conducted with the fungus *S. pararoseus* had the action of a bio controller against *Botrytis cinerea*, responsible for the disease of the gray strawberry fungus (HUANG et al., 2012). Furthermore, studies revealed an increase in the production of an exopolysaccharide from the fungus *R. mucilaginosa*, when co-cultivated with *E. coli*, where it was able to inhibit the formation of biofilm in *E. coli*, *P. aeruginosa* and *S. aureus*, indicating that it has potential applications for inhibiting bacterial colonization (VAZQUEZ-RODRIGUEZ et al., 2018). In addition, the glycosidic carotenoids present in the yeast *R. mucilaginosa* were extracted and showed antibacterial activity against antibiotic resistant bacteria (YOO; ALNAEELI; PARK, 2016). Studies pointed out that *S. cerevisiae* secretes antimicrobial peptides (AMPs) during alcoholic fermentation, and are active against a wide variety of wine-related yeasts and bacteria. fragments of the protein glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) (BRANCO et al., 2014). However, further studies on antimicrobial activity of the yeast against Gram-positive bacteria are needed since in the present study there is no antimicrobial growth inhibition.

The focus of the present work was to find bacterial inhibition compounds, but there were also interesting results against yeast. The best result obtained against *Saccharomyces cerevisiae* was shown by *Agave americana* which have potential inhibiting power over M-26 at 10,000 mg L^{-1}. Researchers showed that *A. americana* did not have action on any bacteria tested, but showed strong inhibition against *Candida albicans* with only 390 mg L^{-1} (SANTOS et al., 2009), and other studies indicated inhibition against the *S. aureus* with 50,000 mg L^{-1} (PANDEY et al., 2019). Tests performed with 1,000 mg L^{-1} of the extract of the plant *A. crassiflora* showed 61% inhibition against the Gram-positive bacteria *S. aureus*, but the form of sterilization of the extract was not reported (LAGE, 2011).

Concentrations of up to 72,600 mg L^{-1} of *P. venusta* plant extract were tested on Gram-negative/positive bacteria and some fungi but did not show any antimicrobial activity against these microorganisms (FERNANDES et al., 2011), but in concentrations in range of 30,000 mg L^{-1} as tested in this research, it was no antimicrobial action against *S. cerevisiae* and *L. fermentum*.

Studies with the extract of *S. campanulata*, showed inhibition against *Enterococcus faecalis* with a MIC of 39,100 mg L^{-1} (TEINKELA et al., 2016), and MIC value of 0.78 and 7.81 μg mL^{-1} against *Staphylococcus epidermidis* and *Candida albicans*, respectively (MAGNIBOU et al., 2021). Furthermore, it was demonstrated a MIC of 300 mg L^{-1} and 1,250 mg L^{-1} against *S. aureus* and *Listeria monocytogenes* bacteria, respectively (RASHED et al., 2013). These values are very different from the concentration of 13,500 mg L^{-1} applied in this study, which was not sufficient to inhibit microbial growth.

The concentration tested on *T. procumbens* against *L. fermentum* was not efficient at a concentration of 10,000 mg L^{-1}. However, the literature highlights that in slightly larger quantities it is already possible that there is action as the same as the Gram-positive bacteria *Mycobacterium smegmatis* and *S. aureus* that were submitted to tests using the same plant extract proving the effectiveness of the extracts with a MIC of 14,000 and 9,000 mg L^{-1}, respectively (TADDEI; ROSAS-ROMERO, 2000). Also, antibacterial activity of *T. procumbens* was tested on Gram positive (*E. coli* and *P. mirabilis*) and negative (*S. aureus* and *B. subtilis*) bacteria. The MIC of inhibitory effects of *T.*

procumbens fractions on bacteria was about 15,000 to 25,000 mg L^{-1} (ANDRIANA et al., 2019). In this way, the plant extracts evaluated in this study are not discarded, because their potential still needs to be better studied as well as elaborating a technique that enables the availability of bioactive compounds.

The reduction in the lactic acid bacteria population was evaluated in two consecutive fermentation cycles, using hops as a natural biocide, noting that it may have equal or superior efficacy to conventional antimicrobials (PRADO; VENTURINI FILHO, 2014) and this means that there are several plant sources with antimicrobial properties to be explored. An example showed by researches are the mutually beneficial action between *Bacillus sp.* and *E. urograndis*, which was evidenced by the stimulation and growth of both the bacteria and the plant when one in the presence of the other. Yeast cell growth was below control; however, viability was high, indicating that the extract has low or almost no toxicity to yeasts (MOREIRA; ARAÚJO, 2013).

In the same context, studies with other *Eucalyptus* species, such as *E. grandis* and *E. urophylla*, not only showed plant toxicity on *S. cerevisiae*, but also showed that yeast is a promising alternative for the control of *Eucalyptus* rust caused by the *Puccinia psidii* bacteria (BOAVA et al., 2010). With the increase in bacteria in the assay compared to the control at this work, the likely bacterial stimulant hypothesis also holds. In study with *E. microcorys* and *E. cloeziana* the *Escherichia coli* and *Salmonella choleraeseus* were also not inhibited by the *Eucalyptus* extracts in their growth, but unlike the result obtained here, they did not suffer any apparent stimulus (ESTANISLAU et al., 2001). These data show the antimicrobial potential of *Eucalyptus* and present a diversified way, even if we compare them to the present work.

Studies have shown that there may be greater sensitivity of Gram-positive microorganisms to the action of *Eucalyptus* because essential oils generally act on the microbial cell wall that has a selective membrane. The study used dried leaves of *Eucalyptus staigeriana* to inhibit *S. aureus, Staphylococcus epidermidis* and *Bacillus cereus* (CORREA et al., 2019). In the present work *L. fermentum* may not have been inhibited due to the reduced number of cycles and another hypothesis may be related to the inappropriate way of extracting bioactive compounds. Another studies with essential oils of *Eucalyptus camaldulensis* and *Eucalyptus globulus* in quantities of 1 and 2 μL did not affect *S. aureus* and *Escherichia coli*, but when increasing to 5 to 20 μL, they obtained an inhibition zone for both bacteria, using 1 mL of inoculum in a petri dish for all assays (GHALEM; MOHAMED, 2008). Other researchers tested the activity of oils from *E.*

globulus and *E. urograndis* against the *Streptococcus mutans* and obtained positive results for both plants. Furthermore, when used together, an increase in antimicrobial activity was observed (GOLDBECK et al., 2014). Essential oils from the leaves of twenty *Eucalyptus* species were also tested, finding varied antimicrobial activities and, in particular, a good result against the Gram-positive bacteria *Staphylococcus aureus* (ELAISSI et al., 2011). In addition, studies have suggested that different fractions of essential oil of the *Eucalyptus* dives species are inhibited by the bacteria *Pseudomonas fragi, E. coli, Salmonella typhimurium, Listeria monocytogenes* and *S. aureus*, in addition to the yeast *S. cerevisiae*. Of the tested fractions, two of them were more effective for yeast. In one of them, the mixture was heterogeneous, with a predominance of alpha-phelandrene, alpha-terpinolene and alpha-turjene, and in the other, alpha-turjene represented almost 90% of the fraction (DELAQUIS et al., 2002).

From the data obtained during this work, it was possible to observe that the *Eucalyptus* extract was not effective and acted minimally in the control of the bacterial population, which can be observed by the high acidity of the assays compared to controls and by the high cellular concentration of bacteria. Thus, the result was not compatible with studies already carried out on the antimicrobial action of plants of the same genus. One of the hypotheses of these results is due to the mode of operation, the fermentation in Fed-batch with cell recycling, since the positive results obtained in tests at first screenings of cellular microbial growth inhibition. Furthermore, there are no studies using *Eucalyptus* under these same conditions. The species may also have lesser antimicrobial action than others of the genus and its 7,000 mg L^{-1} MIC may not be the same for Fed-batch and Batch operations.

In studies performed using mixed culture of *S. cerevisiae* M-26 and *L. fermentum* CCT 1407, the yeast cell viability had an observable decrease of up to 15% over the course of 6 cycles, with a statistical difference determined for the increase in contamination by the bacteria every cycle (CHERUBIN, 2003). These results were not associated with any antimicrobial treatment, this drop in yeast viability occurs due to toxins and organic acids excreted in the medium by the *L. fermentum* bacteria, such as lactic acid, butyric acid, acetic acid and fatty acids, which also make the medium more acidic, in addition to competition for nutrients and amino acids, resulting in reduced yield and productivity of fermentation (BASSI et al., 2018; BREXÓ; SANT'ANA, 2017). That indicated that the presence of *Eucalyptus* extract in the present work did not inhibit

bacterial growth. This was proven in the fifth and last cycle of the present study, which showed that yeast viability had a slight decline when there was bacterial growth.

Although this inhibition by the *L. fermentum* could occur, before fermentation, the yeast M-26 was activated repeatedly through the platinum loop, leading it to acquire a high resistance and selection of the strongest strains. Furthermore, studies carried out expressed that the strains of the yeast *S. cerevisiae* M-26 isolated by the author, compared with other 60 strains, showed a greater inhibitory capacity against *L. fermentum* growth, due to having natural antibacterial compounds (OLIVA NETO; FERREIRA; YOKOYA, 2004). This data can be explained by the secondary metabolism that produces complex substances and the release of succinic acid from this strain, ensuring an antibacterial potential.

Added to this, physiological adaptations contribute to the maintenance of viability during fermentation with recycles (CHERUBIN, 2003), ensuring the stability and resistance of yeasts. Other factors that must be considered for cell viability are the number of bacteria and fermentation cycles. This is because the contamination may not have been enough to affect the yeast viability, preventing the bacteria from causing a shift in ethanol formation, as well as deficient fermentations. It is also worth noting the advantage observed in the strain used, which is more resistant to low pH and high concentrations of ethanol, resulting in greater cell survival. In view of this, the selective advantage observed in the *S. cerevisiae* M-26 strain corroborates the industrial applicability in distilleries (GOMBERT; VAN MARIS, 2015). These facts may explain the delay in expression in the antimicrobial activity of eucalyptus throughout fermentation cycles.

With more extensive studies that cover other species, the use of plant extracts can be a solution, and this alternative has been widely studied as an antimicrobial in the agricultural, food, cosmetic and medical areas to obtain more natural products with fewer side effects (SILVEIRA et al., 2012), which can be an effective, economically viable and sustainable option for the sugar-alcohol industry. It is noteworthy that at higher concentrations of these natural compounds could present antibacterial efficiency in this study, but for large scale fermentations present in distilleries, high would not be viable, due to the exacerbated amount of extract that would be necessary, increasing the cost of the process of production.

The antimicrobial activity of Rosemary essential oil was evidenced proving strong antimicrobial activity against *Acinetobacter baumannii* and *C. albicans* with MIC

of 500 mg L^{-1} and 600 mg L^{-1}, respectively (LORENZO-LEAL et al., 2019). Studies point out the efficacy of Rosemary oil against the bacteria *Escherichia coli*, *Bacillus cereus*, *S. aureus*, *Clostridium perfringens*, *Aeromonas hydrophila* and *Salmonella choleraesuis*, also emphasizing that the inhibitory effect of Rosemary is interaction with the cell membrane that results in loss of functionality and changes in its structure, also causing changes in genetic material and nutrients, altering electron transport, leakage of cellular components and alterations in the production of fatty acids (NIETO; ROS; CASTILLO, 2018). The main constituents of Rosemary are monoterpenes like 1,8-cineole, α-pinene, camphor and borneol (BORGES et al., 2019), and these are responsible for characterizing its antibacterial activity (PORTE; GODOY, 2005). The literature highlights that the main agent of the bactericidal action of essential oil is 1,8 cineole (HUSSAIN et al., 2010).

Furthermore, there is a high antifungal efficacy of Clove Leaf essential oil at low concentrations, and some of the fungi tested as *Candida albicans*, *Candida glabrata* and *Colletotrichum* sp with total inhibition (AL-ZUBAIRI; AL-MAMARY; AL-GHASANI, 2017; GUCWA et al., 2018). One explanation for this fungicidal effect was the damage caused to cell membranes, influences the flow of potassium ions and the ability to inhibit the transition of fungi to the mycelium form, its main constituents being eugenol, eugenyl acetate and β-caryophyllene, to which eugenol is in greater amounts and has been shown to be the most potent active principle, causing breaks in the DNA strands of cells (BATISTA, 2016).

According the literature, much higher concentrations of lactic acid can reduce the pH of the medium and alter the activity of the enzyme H^{+}-ATPase, which is responsible for controlling the yeast's intracellular pH, through the pumping of protons (NARENDRANATH; THOMAS; INGLEDEW, 2001; ZABED et al., 2014). In this way, at pH 1, the solution is highly acidic so that the yeast could not survive at all. As the pH is increased, it was possible to observe an increased production of ethanol and at pH 4, ethanol production attained maximum. The pH is increased further, the ethanol production decreased and reached minimum at the pH value of 8 (REDDY et al., 2021). Therefore, it is possible to see that the levels of total acidity increased when there was an increase in the number of bacteria that are quantified and described in this research, corroborating an experiment carried out by researches who found that the fermentation process is pH sensitive (KHANDAKER et al., 2020) and the acidity of the must varies as a function of bacterial contamination (LISBOA et al., 2015; OLIVEIRA; SILVA; NETO, 2020).

As already mentioned, the M-26 yeast strain has greater resistance at low pH and high concentrations of ethanol and, therefore, its applicability is superior. However, studies have shown that at pH below 3.6 there is a strong inhibition of *S. cerevisiae* strains, given that these acidity levels promote yeast stress during fermentation which, consequently, negatively affect the alcoholic yield (DORTA et al., 2006). In this work, it was also possible to observe in fermentation cycle 3 using Rosemary essential oil that there was a large concentration of foams formed in the positive control without Rosemary oil treatment, this was probably due to the high concentrations of acids present in the control and the decrease in pH from the cycle 2. Also, foaming can be justified by the production of intense carbon dioxide, as there are enough cells to consume the fermentable sugars in the wort. Thus, there is a decrease in the density of the wort and an increase in acidity, which promotes the formation of foams by releasing carbon dioxide, requiring the use of defoamers in some cases (BASSO et al., 2008; BLASCO; VIÑAS; VILLA, 2011; FIGUEIREDO et al., 2021).

Despite the results showing a considerable antibacterial capacity, the effectiveness of the oil used is still much lower than that of other conventional antibiotics (VENTURA, 2015) showed that from a mixture of 3 mg L^{-1} concentration, composed of 40% of virginiamycin and 60% of salinomycin, there was a reduction of 85.24% of lactic acid in the first cycle and 91.83% in the second cycle, in addition to monensin at 3 mg L^{-1}, reduced lactic acid by 77.13% in the first cycle and 91.94% in the second cycle. However, as Rosemary essential oil is a natural compound, its effectiveness is considerably relevant at 103 mg L^{-1} as presented in that study, with the possibility of purification and isolation of its active principles responsible for its antibacterial action.

Studies carried out using cultures of bacteria from the genus *Bacillus* (*B. subtilis*, *B. coagulans* and *B. stearothermophilus*) and *L. fermentum* and *Lactiplantibacillus plantarum*), proved that *L. fermentum* presented higher values of total acidity, moreover, it presented lower pH values during all tests of the fermentation cycles, differing statistically from the control treatment, so the study showed that this bacterium was the most efficient producer of organic acids (NOBRE; HORII; ALCARDE, 2007). Furthermore, other studies using propolis at a concentration of 30% showed an efficiency in controlling acidity, but did not show better results when compared to conventional antibiotics such as Lactrol and Biotran (RODRIGUES; DANTAS; FINZER, 2009).

The relationship of reducing sugars is closely related to the production of ethanol, being a relevant factor in the performance of fermentation, as it shows how much

ethanol was produced as a function of the sugars contained in the feed wort (SANTOS, 2016). Furthermore, we can state that cell growth is directly related to sugar consumption, since yeasts produce ethanol by metabolizing these sugars (DASHKO et al., 2014).

Studies carried out in 33 hours of fermentation using *S. cerevisiae* Y904 at a concentration of 189.5 g L^{-1} of TRS, obtained an ethanol yield of 87.5%. The same strain was reused in a second fermentation, resulting in a residual sucrose amount of 2 g L^{-1} and an ethanol yield of 90.8 (CRUZ, 2015). Compared to this study, the best efficiency was 98.57%, with a consumption of 154.042 g L^{-1} of sugars. In addition, researchers obtained an ethanol yield of 83.1%, using 6 mg L^{-1} of Kamoran for bacterial control, during 9 hours of fermentation with a non-flocculant *S. cerevisiae* strain. Under these conditions, at the end of fermentation the number of bacteria was reduced to the order of 10E+05 cells/mL and the yeasts had 10E+08 cells/mL (LEITE et al., 2013). At this research, it was possible to had a high ethanol production using natural compounds as antibacterial sources from 100 mg L^{-1}.

27 CONCLUSIONS

There was no antibacterial activity in the free-cells cultured broth of yeasts *S. japonicus, S. pararoseus, R. mucilaginosa* and *S. cerevisiae* against *L. fermentum* at the concentrations tested.

In preliminary tests with plant extracts, it was possible to find antimicrobial activity of the extracts of *Eucalyptus urograndis* against the growth of *L. fermentum* and *Agave americana* against the growth of *S. cerevisiae*, both in concentrations of 10,000 mg L^{-1}. Although the *Eucalyptus* extract is not toxic to *S. cerevisiae*, it was not so effective in a Fed-batch process under the conditions applied at this study as expected and perhaps at lower concentrations, the extract of *E. urograndis* could have a more effective antibacterial action. Thus, further research and recycling would be needed to access this trend.

Based on the present study, it can be concluded that only Rosemary essential oil met the criteria for application in cyclic Fed-batch fermentation tests at a concentration of 103 mg L^{-1}, being the antibacterial action of this oil evidenced in the control of lactic bacteria *L. fermentum* from viability and total acidity tests, showing great efficacy in relation to control. Regarding ethanol production, the control had a great decrease in the last cycles, and the tests containing Rosemary essential oil always maintained high

alcoholic yields, suggesting that this oil should be further studied for industrial applications.

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29 GENERAL CONCLUSIONS

This research found chemical and natural molecules with great antibacterial potential acting in synergy, free or immobilized and in Fed-batch processes. The antibacterial potential the dyes gentian violet (GV) and methylene blue (MB) was proven to control the growth of *Limosilactobacillus fermentum* with MIC of 20 and 60 mg L^{-1}, respectively. As a new alternative, these same molecules can act in synergy with sodium monensin and/or chlorine dioxide. The synergy verified in GV and/or MB with monensin and/or chlorine dioxide can drastically decrease the individual chemical dosage required on an industrial scale with benefits for its use, since they can inhibit the metabolism of *S. cerevisiae*, especially when the yeast viability is low or when there are some physiological stress factors involved.

From the studies carried out, no antibacterial activity was found in the supernatants of the bleached fungi *S. japonicus, S. pararoseus, R. mucilaginosa* and *S. cerevisiae* against *L. fermentum* at the concentrations tested. In preliminary tests with plant extracts, it was possible to find antimicrobial activity of the extracts of *Eucalyptus urograndis* against *L. fermentum* and *Agave americana* against *S. cerevisiae*, both at concentrations of 10,000 mg L^{-1}. Moreover, only the Rosemary essential oil met the criteria for application in cyclic Fed-batch fermentation tests at a concentration of 103 mg L^{-1}, being the antibacterial action of this oil evidenced in the control of the lactic bacteria *L. fermentum*.

The immobilization technique of GV in Al/Av beads was efficient and proved an antibacterial action against *L. fermentum* without affecting the viability of *S. cerevisiae* in in vitro cultures. This study shows that the best bacterial inhibition results are obtained by GV, both free and encapsulated at pH 5.0-6.0. There was a high potential to reuse these gel beads, reducing the cost of the process and applying the GV dye, avoiding chemical contamination with dye residues and damage to the environment.

Finally, the test using Al/Av dye-free beads also inhibited bacteria in the last step of the study, demonstrating that industrial tests can be carried out in the future to further evaluate the potential of this technique.