

**PRODUCTION OF FIBROLYTIC YEASTS FROM
AGROINDUSTRIAL WASTE AND THEIR APPLICATION
IN ANIMAL NUTRITION**

JOYCE FARIA DE SOUZA

Thesis submitted to Bioenergy Research Institute, São Paulo State University "Júlio de Mesquita Filho", UNESP, Rio Claro – SP, Brazil, as part of the requirements for obtaining a Doctor of Science degree.

Advisor: Prof. Dr. Pedro de Oliva Neto

Co-advisor: Prof. Dr Adibe Luiz Abdalla

**Rio Claro -SP
2024**



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"



BIOENERGY RESEARCH INSTITUTE

PHD PROGRAM IN BIOENERGY (USP, UNICAMP and UNESP)

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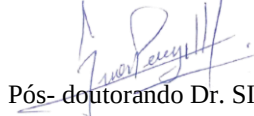
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*To my parents, Rosangela and Luis, and my
sister Julia, who have always supported,
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Somewhere, something incredible is waiting to be known

Carl Sagan

ABSTRACT

Currently, there is a growing search for new alternatives that maximize animal nutrition in livestock, favoring fattening and reducing carbon release by animals. The present research aims to develop a bioprocess focused on the production of fibrolytic yeasts with probiotic potential from agro-industrial residues applied to animal nutrition in order to develop a possible new co-product. Assays focused on cultivation, fractionation, preservation and application of yeasts were carried out with four different strains, *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2 and *Saccharomyces cerevisiae* M26. Different fractions of biomass were produced and tested for animal nutrition. Initially, a general introduction is presented on the topic to be addressed in this work. In Chapter 1 describe a brief bibliographic review on the topic addressed in the following chapters. The Chapter 2 analyze the influence of different residues on yeast production, as well as the ability to produce enzymes. Chapter 3 consisted of substrate-based scaling that was highlighted in Chapter 2, as well as evaluation of different parameters to improve fermentation that were evaluated in both single and fed-batch processes. Chapter 4 sought to evaluate the influence of different reagents for maintaining the viability of microorganisms. Chapter 5. Present the results obtained from the *in vitro* experiment with a diet based on hay and concentrate. Chapter 6 describes the results of the performance experiment, methane release, and *in vivo* metabolism. In Chapter 7, a characterization of the yeasts and an evaluation of the autolytic potential were carried out based on a defined methodology, aiming at obtaining different fractions of the cells. The research encompasses microorganisms that are little studied and described in the literature, seeking to demonstrate their biotechnological potential in comparison with *Saccharomyces cerevisiae* M26, in order to demonstrate their applicability in animal nutrition as a way of favoring the sector in accordance with the goals of maintaining a sustainable standard of production and consumption. *In vitro* gas production tests demonstrate greater degradability of the tested diets, in addition to greater diet utilization observed by the lower intake in tests with live animals that reached the same weight as the control, which is in accordance with the proposed objectives.

Keywords: animal nutrition, probiotics, submerged cultivation, yeasts, agro-industrial residues.

RESUMO

Atualmente, é crescente na pecuária a busca por novas alternativas que maximizem a nutrição animal, favorecendo a engorda e reduzindo a liberação de carbono pelos animais. A presente pesquisa tem como objetivo desenvolver um bioprocesso com foco na produção de leveduras fibrolíticas com potencial probiótico a partir de resíduos agroindustriais aplicadas à nutrição animal com o intuito de desenvolver possíveis novos co-produtos. Ensaio focados no cultivo, fracionamento, preservação e aplicação de leveduras foram realizados com quatro diferentes linhagens, *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2 e *Saccharomyces cerevisiae* M26. Foram produzidas diferentes frações de biomassa as quais foram submetidas a testes para a nutrição animal. Inicialmente, é apresentada uma introdução geral sobre o tema a ser abordado neste trabalho. No Capítulo 1 temos uma breve revisão bibliográfica sobre o tema abordado nos capítulos seguintes. No Capítulo 2 buscou-se analisar a influência de diferentes resíduos na produção das leveduras, assim como a capacidade de produção de enzimas. O Capítulo 3 consistiu no aumento de escala baseado no nutriente que se destacou no Capítulo 2, assim como avaliação de diferentes parâmetros para melhorar a fermentação que foi avaliada nos processos de batelada simples e alimentada. O Capítulo 4 buscou avaliar a influência de diferentes reagentes para a manutenção da viabilidade dos microrganismos. O Capítulo 5 apresenta os resultados obtidos a partir do experimento *in vitro* com uma dieta baseada em feno e concentrado. O Capítulo 6 descreve os resultados do experimento de desempenho, liberação de metano e metabolismo *in vivo*. No Capítulo 7 foi realizada uma caracterização das leveduras e avaliação do potencial autolítico baseado em uma metodologia definida, visando a obtenção de diferentes frações das células. A pesquisa engloba microrganismos pouco estudados e descritos na literatura, buscando demonstrar o potencial biotecnológico dos mesmos em comparação com *Saccharomyces cerevisiae* M26, a fim de demonstrar a aplicabilidade na nutrição animal como forma de favorecer o setor em acordo com as metas de manter um padrão sustentável de produção e consumo. Os testes de produção de gás *in vitro* demonstram maior degradabilidade das dietas testadas, além de maior aproveitamento observado pelo menor consumo nos testes *in vivo* e mesmo peso do controle, fato que está de acordo com os objetivos propostos.

Palavras-chave: nutrição animal, probióticos, cultivo submerso, leveduras, resíduos agroindustriais.

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1 GENERAL INTRODUCTION

Brazil is one of the largest exporters of meat in the world, and projections indicate that it will be the leading exporter by 2027, followed by India (USDA, 2018; MAPA, 2018). In 2018, the Brazilian Gross Domestic Product (GDP) reached R\$8.7 trillion for the year. Many factors are associated with GDP growth, one of which is the development of livestock (IBGE, 2021). The livestock sector was a highlight in Brazilian agribusiness in 2019, showing a growth of 23.7%. This development is mainly attributed to the increase in exports of pork (16%), beef (15%), and poultry (4%) (CEPEA, 2019).

The development of the livestock sector is primarily aligned with supply and demand, available hectares, and the quality of animal nutrition. According to Brazilian Association of Meat Exporting Industries (ABIEC, 2022), in 2021, Brazil had a cattle herd of 196.47 million heads with 39.14 million slaughters. There is an estimated increase in the herd to 201.5 million heads in 2026 with 45.8 million slaughters, which is positive; however, a reduction in pasture area is expected (ABIEC, 2022). The increase in the number of herds occurs parallel to the decrease in pasture area, as has been observed since 2001 according to ABIEC (2022), indicating an increase in demand in this sector as a consequence of population growth.

The population growth is putting pressure on the livestock sector to increase its production and develop new strategies to meet the demand. Research is focused on strategies that enhance the integration of crop-livestock-forest systems, alternatives for pasture recovery or renewal, as well as changes in animal nutrition for fattening purposes (Kichel et al., 2019; Lambo et al., 2021).

Optimizing animal nutrition contributes to greater carbon fixation and, consequently, animal fattening, which may or may not reduce greenhouse gas emissions in the atmosphere, particularly methane. Studies focused on this strategy, aimed at reducing methane release, benefit both increased productivity for the livestock sector and combating climate change and its impacts (Detroy, 2018; Malafaia et al., 2019).

Given the importance of the livestock sector, technological development to enhance animal nutrition becomes a great strategy. Currently, research focused on integrating the sugar and alcohol industry with the livestock sector is a promising way to mutually benefit these industries, aligning with the concept of biorefinery and bioeconomy (Liu et al., 2019; Bueno & Torres, 2022).

The integration of these segments adds value to the waste, benefiting both sectors considering the different residues generated by industries and crops, especially fibrous residues that are produced on a large scale and often lack proper disposal or reuse (Tripathi et al., 2019; Cho et al., 2020).

Agroindustrial waste increases according to the production of consumer goods that aim to meet the growing demand due to population growth. The main agroindustrial wastes generated include citrus pulp, brewery spent grain, corn steep water, sugarcane straw, sugarcane bagasse, cassava bagasse, among others (Tasic, 2017; De Corato et al., 2018; Cervbrasil, 2020).

It is estimated that the global annual production of waste generated in the agricultural sector reaches 140 gigatonnes (Gt) of dry waste, considering an agricultural production of 7.26 Gt. The large quantity of waste generated makes it important to consider alternatives to minimize management problems regarding the disposal of materials. China produces around 716 million tonnes (Mt), the United States of America about 682 Mt, India 605 Mt, and Brazil 451 Mt of waste (Bentsen & Felby, 2010; Tripathi et al., 2019; Vaz Junior, 2020).

As millions of tonnes of agroindustrial waste are generated in Brazil, this fact makes the country a target for applications of these residues aimed at developing new products in various industrial sectors. Waste represents a loss of energy and raw materials from a chemical perspective, indicating the need to reconsider their use in secondary processes in order to obtain products with added value. Utilizing agroindustrial waste for microbial cultivation allows for cost reduction in the process, generating new products such as xylan, enzymes, organic acids, among others, and can also add a nutrient load to the fermented waste, as is the case with yeast utilization (Vaz Junior, 2020; Guedes et al., 2021).

The increasing demand for animal-derived food products makes it important to improve nutritional and management conditions in order to achieve greater productivity in cattle and other livestock. Food additives are one of the strategies used to pursue this objective; however, the use of antibiotic-based growth promoters has been questioned and prohibited in various international markets due to the promotion of resistant microorganism proliferation. In 2006, the European Union banned the use of antibiotic-based growth promoters, leading to a growing search for alternatives. As a result, research on probiotics, prebiotics, synbiotics, and immunomodulators has increased. Among them,

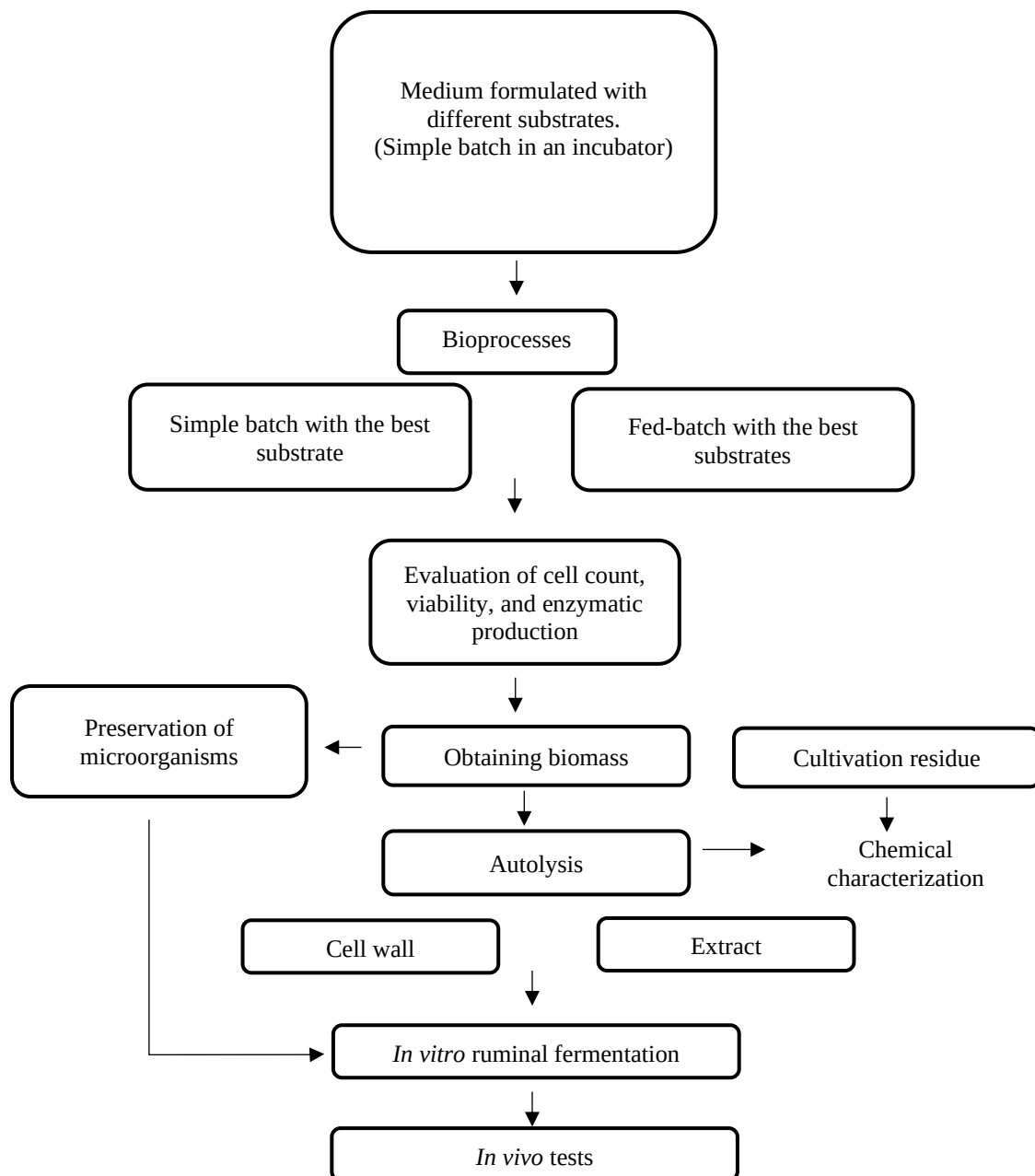
probiotics have shown great potential, considering the benefits obtained for cattle (Markowiak & Śliżewska, 2018; Lambo et al., 2021).

In light of the foregoing, the present study aims to develop a bioprocess that utilizes different residues, process parameters, and novel microbial cultures to evaluate the best combination of these parameters for the economical production of new biotechnological inputs aimed at animal supplementation, allowing for greater carbon fixation and reduced methane release. Thus, parameters such as substrate feeding type, cultivation time, microbial contaminations, temperature, brix (soluble solids), enzyme production, and formulation of nutritional media using low-cost residues, production and evaluation of special yeast biomass and its respective fractionation, chemical characterization, as well as application tests of these products in “*in vitro*” ruminal fermentations were studied in this work, aiming to contribute to the generation of new inputs for the livestock sector integrated with the agro-industrial sector.

The present work presents in Chapter 1 a literature review on the theme developed in the study. Chapter 2 presents the use of different agro-industrial residues for yeast cultivation and the enzymatic capacity in the best-selected residue. In Chapter 3, yeast undergoes scale-up with subsequent *in vitro* ruminal application. Chapter 4 describes the use of different reagents for the preservation of probiotics focused on product commercialization. Chapter 5 present the results obtained from the *in vitro* experiment with a diet based on hay and concentrate. Chapter 6 describes the results of the performance experiment, methane release, and *in vivo* metabolism. The Chapter 7 present the characterization of the studied yeasts and autolysis parameters to maximize protein extraction, as well as evaluations of *in vitro* gas production.

The proposal of this research is aligned with the sustainable development goals outlined by the UN in 2015 with a target until 2030, as well as with the concept of biorefinery and agricultural engineering for bioenergy. The flowchart in Figure 1 outlines the stages developed in the research in a summarized manner.

Fig 1. Flowchart of the stages and analyses developed with sugarcane bagasse, treated and untreated cassava bagasse, as well as analyses conducted at each stage.



2 GENERAL OBJECTIVE

The general objective of this work was to develop a new additive for animal supplementation based on less-known and unconventional yeast strains through a bioprocess using agro-industrial residues.

2.1 Specific objectives

- Evaluate carbon sources for the growth of the yeasts *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LAB2, and *Saccharomyces cerevisiae* QM 26. Among these sources are agro-industrial residues: non-hydrolyzed cassava bagasse, hydrolyzed cassava bagasse, sugarcane bagasse, and hydrolyzed sugarcane cake, corn steep liquor, and others wastewater. Evaluation of xylanase, amylase, and cellulase production with the most prominent formulation.
- Study the acid and enzymatic hydrolysis of cassava bagasse aiming for its better utilization by the studied yeasts as a carbon source.
- Produce yeast biomass and enzymes through batch and fed-batch processes by evaluating different cultivation parameters: °Brix, pH, cell viability, live sprouts, total sprouts, enzyme production, and TRS (total reducing sugars), with the selected medium using residues.
- Analyze the viability of the biomass of four less-known yeasts produced through a simple batch process. Preservation was evaluated under the conditions of: lyophilization, freezing, maintenance at 26°C, and refrigeration (4°C) using chemical compounds such as trehalose, sucrose, milk powder, glycerol, mineral oil, vegetable oil, and agricultural vegetable oil.
- Evaluate the autolysis of the yeasts to obtain yeast extract and cell wall, using technologies already patented by the team, and apply them in *in vitro* ruminal fermentation tests.
- Conduct *in vitro* ruminal fermentation with a diet based on roughage (Tifton-85 hay) and concentrate using ruminal fluid from sheep to evaluate the effect of live yeasts on gas production and total substrate degradability, aiming to stimulate animal production.

- Conduct a performance and metabolism experiment in sheep with the two most promising microorganisms selected in the *in vitro* test, in order to evaluate weight gain rate, diet degradability, nitrogen excretion, water intake, among other factors.

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CHAPTER 1: LITERATURE REVIEW: THE POTENTIAL OF NEW INOCULANTS AND BIOPROCESSES USING AGROINDUSTRIAL WASTES FOR ANIMAL NUTRITION AND HEALTH.

SUMMARY

The increase in demand for animal-derived products highlights the need to improve nutritional and management conditions to boost productivity in livestock farming. Optimized animal nutrition not only contributes to greater carbon fixation and increased productivity but also reduces emissions of greenhouse gases such as methane and nitrogen compounds released by feces and urine, which also contribute to environmental pollution. The use of food additives, such as probiotics, has shown great potential in providing benefits for cattle, especially after the prohibition of antibiotic-based growth promoters in various international markets. However, the selection and cultivation of microorganisms require an understanding of the characteristics of the selected microorganisms and their potential, as well as the characteristics of the bioreactor, chosen cultivation type, and variations in scale-up, particularly regarding the specificities of the rumen, which pose a challenge for the selected microorganisms. The integration between the sugar and alcohol industry and the livestock sector, through the utilization of residues for microorganism cultivation, emerges as a promising strategy to optimize animal nutrition and reduce agroindustrial waste. Products derived from autolytic processes, such as yeast extract and cell wall composed of β -glucan and mannanoligosaccharides, in addition to the inactive yeast itself, can benefit livestock farming in various ways. In the face of the growing demand for alternatives that maximize animal nutrition and promote weight gain while reducing carbon emissions from animals, this review provides insight into the integration of sectors and an understanding of the process chain aimed at the production and application of microorganisms in animal nutrition, especially in ruminants, highlighting three microorganisms with high potential for application in the sector.

Keywords: probiotics, fermentation, animal nutrition, methane, ruminants.

1 AGROINDUSTRIAL WASTE

The generation of agroindustrial waste is on the rise alongside food consumption and population growth. These wastes possess a rich chemical composition, presenting an opportunity for the development of new technologies to harness their potential and add value. They can serve as a carbon source for microorganisms aimed at producing various compounds of interest such as enzymes, organic acids, pharmaceuticals, or even for the production of second-generation ethanol, bioplastics, animal nutrition, among others (Tasic, 2017; De Corato et al., 2018; Cervbrasil, 2020). In this review some of these promise residues were explored.

Sugarcane bagasse, a residue from the sugarcane industry, is characterized by its fibrous nature, containing approximately 41% non-fibrous carbohydrates and 56% fibers. For every ton of sugarcane processed, around 280 kilograms of sugarcane bagasse are produced (Morais et al., 2007; Tana et al. 2016; Ahmed et al., 2018). The estimated amount of sugarcane for the 2024-2025 harvest season in Brazil was 685.85 million tons (Conab, 2024).

Cassava bagasse, a residue from starch factories, accounts for an estimated weight of around 10 to 20% of the weight of the roots, characterized by approximately 37% fibers and 54% adhered starch. For every ton of cassava root processed, approximately 928 kg of wet cassava bagasse and 257 kg of starch are produced (Leonel et al., 1998; Silva et al., 2014). Cassava production numbers have been steadily increasing, reaching 636 thousand tons in Brazil in 2022 (Cepea, 2022). Residues from the cassava industry generally have a good nutritional composition, which is why they are widely used in animal feed (Cowley et al., 2020). Cassava pulp, for example, contains a significant amount of amino acids such as arginine, aspartic acid, glutamic acid, and peptides that are available for the animal's organism or for microbial growth (Yeoh & Truong, 1996).

During the process of obtaining cassava-derived products, especially starch production, a large quantity of solid waste is generated, such as cassava peel, bagasse, bark, bran, among others, as well as residual liquids such as wash water, pressing water, and cassava wastewater (Silva et al., 2018; Martinez-Burgos et al., 2021).

Cassava wastewater, also known as cassava vinasse, can be used in biogas production as it contains organic materials that can be converted into methane by methanogenic microorganisms (Zekarias et al., 2019; Chorukova et al., 2022). Cassava wastewater is produced at a rate of 0.25 liters per ton of cassava processed and it is a toxic

and polluting residue due to the presence of hydrogen cyanide and chlorogenic acid (Magalhães et al., 2013; Silva et al., 2018). Cyanide is a colorless and volatile substance with high toxicity to any oxygen-requiring life form, as it has the ability to block the respiratory chain and ATP synthesis, leading to the death of organisms. The toxic dose of hydrogen cyanide (HCN) for cattle is 2 to 4 mg per kg of body weight per hour (Amorim et al., 2005; Chisté et al., 2010; Bosak et al., 2017; Silva et al., 2018). The wide variety of cassava can present different concentrations of cyanide, as well as the residues generated from its processing. Cassava can be classified as sweet with cyanide concentrations of 50 mg HCN.kg⁻¹, moderately poisonous with 50 to 100 mg HCN.kg⁻¹, and poisonous or bitter with values above 100 mg HCN.kg⁻¹ (Chisté & Cohen, 2008).

Cyanide can evaporate at 25.6°C if subjected to heating processes, as occurs during microbial cultivation, at around 30°C (INDSC, 2020). Therefore, the use of cassava bagasse as a nutrient source for microbial cultivation appears promising.

Cassava and corn processing industries generate various types of wastewater or effluents during their production processes, resulting from cleaning and sanitation or from a single stage of the process. These wastewater streams are typically either discharged and sent for effluent treatment or reused in alternative processes such as fertigation (Ersahin et al., 2007; Einschlag, 2011).

Cassava processing begins with cassava washing (CW), followed by peeling, and ends with equipment cleaning, which carries minimal process residues, including water from starch and cassava drying floors (SWCDF) (Martinez-Burgos et al., 2021).

Similarly, the corn processing industry generates different effluents, notably corn steep water (CSW) produced from the steeping process used to soften the grains for milling. CSW has a high Chemical Oxygen Demand (COD) and is produced in smaller quantities compared to water generated in derivative processing, such as refinery water (RW). In addition to these effluents, there is also process factory water (PFW) (Einschlag, 2011).

Corn steep water, also known as corn steep liquor, is a residue from wet milling of corn, another residue with high nutritional value, rich in minerals, vitamins, and nitrogen (Liggett & Koffler, 1948). This residue can be used as a supplement for fermentative media, aiming to add value and reduce production costs (Deepak & Jayadeep, 2022).

2 LIGNOCELLULOSIC WASTE TREATMENT FOR UTILIZATION IN MICROBIAL CULTURES

Currently, the pursuit of more sustainable, cost-effective, and environmentally friendly processes is becoming a priority. Exploring alternative strategies for the utilization of lignocellulosic biomass, especially its residues or by-products, can reduce process costs, provide appropriate disposal for the material, and benefit a variety of sectors with the production of energy, products of interest such as various enzymes, carotenoids, chemicals, microorganisms, among others microbial cultures (Inyang et al., 2022).

The concept of a biorefinery aims to maximize the utilization of all components of biomass. Similar to petroleum refineries converting crude oil into a variety of products such as diesel, gasoline, and chemicals, a biorefinery is a facility that processes and transforms biomass to produce a wide range of products, including biofuels, chemicals, pharmaceuticals, among others. The processing of the raw material used varies according to the end goal but generally involves pretreatment, hydrolysis, fermentation, and product purification or recovery (Inyang et al., 2022; Ojo et al., 2023).

Biorefineries can be classified based on the biomass conversion route, and these treatments can promote the release of fermentable sugars and facilitate the access of microorganisms or enzymes to the material, favoring future applications. The two main pathways for biomass transformation extend to biochemical and thermochemical conversion, which can involve physical, chemical, physicochemical, biological, or nanotechnological methods to achieve better material conversion. Each approach will be influenced by the characteristics of the biomass, which presents varying concentrations of cellulose (40-50%), hemicellulose (20-30%), lignin (10-25%), and pectin (5%), as well as chlorophyll, waxes, and non-structural sugars representing about 1% (Ramzan et al., 2023; Ojo et al., 2023).

Thermochemical conversion involves high temperatures, pressure variations, and the use of chemicals or catalysts, including acids, bases, ionic liquids, deep eutectic solvents, among others. Acids generally break the hydrogen bonds of hemicellulose and cellulose, while alkaline solutions solubilize lignin, with the effectiveness varying according to process conditions such as reagent concentration, pressure, temperature, among other factors. The main disadvantage is related to the use of corrosion-resistant equipment and the generation of inhibitors. In general, biochemical conversion involves the application of microorganisms or enzymes to hydrolyze the material through

fermentation, hydrolysis, and digestion technologies. The disadvantage of biochemical conversion is associated with low hydrolysis efficiency and bacterial or fungal contamination. Nanotechnological processes aim to apply immobilized enzymes for subsequent recovery and reuse, strategies that have shown promise. In addition to these, the two processes can be combined, referred to as mixed or hybrid conversion, which applies thermal conversion and then biochemical conversion through fermentation (Inyang et al., 2022; Ojo et al., 2023; Chen et al., 2023).

Enzymatic hydrolysis of residues is one of the most researched biochemical processes, but the high cost of enzyme production remains a significant challenge. Various strategies are being studied to reduce costs and increase process viability, including the selection and genetic modification of microorganisms with high enzyme production capacity, especially cellulases and xylanases. Additionally, other strategies include the use of technologies such as RNAi and CRISPR/Cas9 to increase enzyme production through gene expression control. Improving enzyme production is essential and inevitable for the industry (Saini et al., 2022; Yang et al., 2023).

In terms of applying treated residues for cell cultivation, lignin, a recalcitrant three-dimensional polymer with high potential for various applications such as biofuel and chemical production, is rarely appreciated. Lignin is the material with the highest concentration of renewable aromatic compounds and is mainly produced as a by-product by the pulp and paper industry, which typically uses it as an energy source through combustion, an environmentally harmful action and a waste of raw material potential. Lignin treatment strategies extend to thermochemical, biochemical, and catalytic depolymerization methodologies, branching into specific tests such as organosolv pretreatment, hydrogenolysis, hydrogenation, hydrodeoxygenation, hydrodesulfurization, among others, especially in applications with transition metal catalysts. However, despite its potential, lignin can pose a physical barrier in processes such as cell cultivation and paper and pulp production, blocking access to cellulose and hemicellulose. In many contexts, lignin is seen as a challenge to be overcome, directly affecting the efficiency and quality of products due to its resistant nature, which hinders hydrolysis (Djajadi et al., 2018; Kang et al., 2019; Kamimura et al., 2019; Lin et al., 2023; Ramzan et al., 2023; Rabelo et al., 2023).

The biorefinery can be used as a sustainable strategy as it promotes circular economy, reducing greenhouse gas emissions and contributing to more sustainable production processes. The problems associated with tons of solid and liquid waste

generated by livestock and industrial processes, such as manure, residual yeast from beer or ethanol production, residual lignin from the pulp and paper industry, sugarcane bagasse from ethanol production, among others, which are usually burned and used as an energy source, or improperly disposed of, can be circumvented or reduced by approaches that reuse and add value to the material. This not only reduces the amount of waste disposed of but also opens up new economic opportunities and reduces pressure on natural resources. Thus, by investing in biorefinery, industries can address the challenges associated with waste generation while advancing towards more sustainable and environmentally conscious production processes (Inyang et al., 2022; Blasi et al., 2023).

3 CULTIVATION OF MICROBIAL INOCULANTS: BATCH AND FED-BATCH PROCESSES

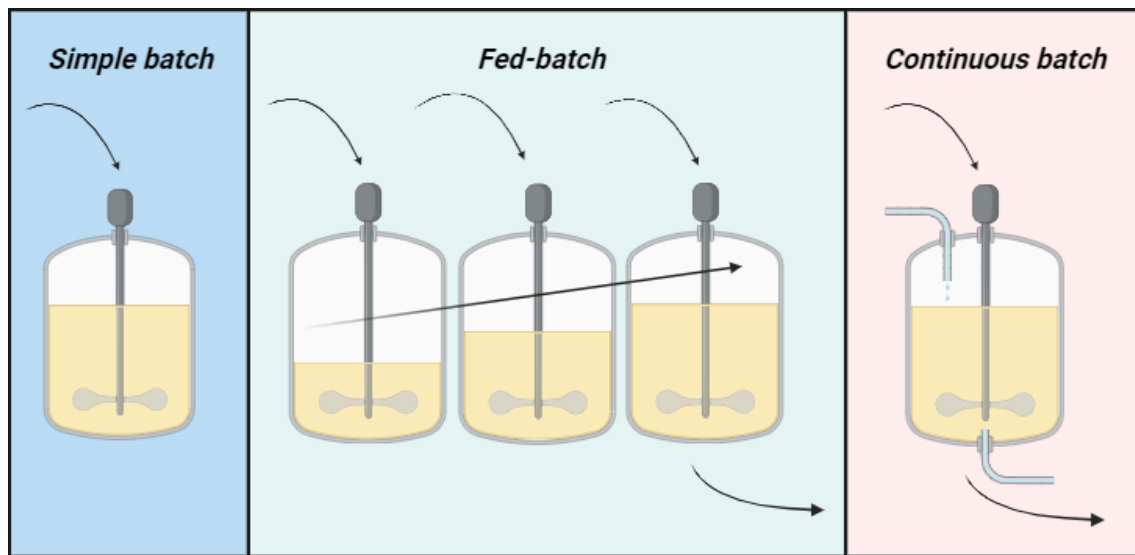
The production of biological inoculants, biofuels, beverages such as wines and beers, biopharmaceuticals, among other products, is associated with fermentative processes. Fermentation is the transformation of organic molecules used as a carbon source by microorganisms into another compound. However, in industrial processes, it is important to control fermentation conditions to achieve maximum productivity. Some parameters worth highlighting include: selection of a suitable microorganism considering process conditions and the product of interest, temperature, pH, homogenization, nutrient concentration, brix, presence of contaminants, among others (Alarcon & Shene, 2021; Hassing, 2022).

In the case of yeast biomass production, there are two phases depending on to achieve the best possible quality of the final product. First, there is cell multiplication performed by multiple metabolic cycles and budding, a phase dependent on the type of culture used and control of variables such as pH, temperature, contaminants, substrate, among others. The second phase consists of preserving the produced product, a phase that needs to prioritize conditioning the biomass considering physical and chemical factors that will interfere with the viability and, consequently, the quality of the product during processing, storage, and commercialization (Attfield, 2022).

Commercial fermentation is planned so that the supply of sugar is not excessive, to the point of inhibiting cell growth or being a limiting factor due to the lack of available nutrients (Attfield, 2022). Microbial cultivation can vary in three main types: batch, fed-batch, and continuous fermentation (Fig. 1). Batch fermentation consists of adding all the

nutrients at the beginning of fermentation and removing all the product at the end of the process. This strategy is applied to processes that require greater operational control, ensuring mainly asepsis and maintenance at the end of fermentation, and ensuring lower contamination risks. However, the process also suffers from obtaining lower concentrations of the product of interest, as a consequence of nutrient inhibition or accumulation of secondary metabolites, in addition to the cell death phase, characterized by low density of viable cells. Batch fermentation is the most widely used in the industry due to its simplicity and greater control of contaminants in cultivation (Schmidell et al., 2001; Yang & Sha, 2019).

Fig 1. Exemplification of bioreactors in simple batch, fed-batch, and continuous batch.



Source: Author, 2024. Created in BioRender.

Fed-batch fermentation is a strategy used to overcome the limitation related to nutrient concentration, characterized by the addition of substances throughout the fermentation, varying the volume linearly during cultivation. Generally, during fermentation in the stationary and cell decline phase, microbial growth inhibition occurs due to substrate concentration and production of secondary metabolites. With fed-batch fermentation, an increase in microbial growth potential is expected. Fed-batch fermentation is generally started with low substrate concentrations, which can minimize problems with catabolic toxicity. One of the drawbacks associated with this process is the subsequent equipment shutdowns related to energy expenditure and process time, as well as a higher probability of contamination, factors that can be compensated for according

to production yield (Luli & Strohl, 1990; Li et al., 2011a; Li et al. 2011b; Shuler et al., 2017).

In continuous fermentation, both substrate addition and product acquisition are done constantly and with uninterrupted fermentation, thus allowing for the constant replacement of nutrients and removal of toxic metabolites. This method can increase industry productivity, and with fewer process shutdowns, it is potentially more economically competitive. However, it poses a higher risk of contamination (Yang & Sha 2019; Teke et al., 2022).

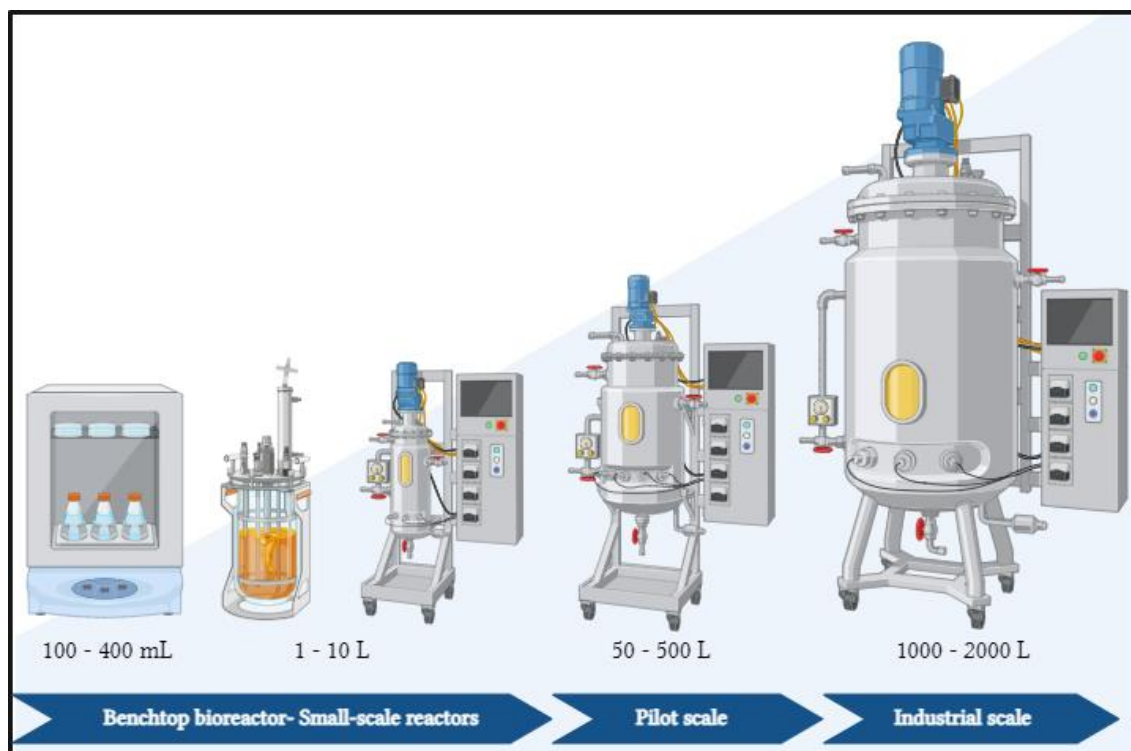
Regardless of the chosen cultivation process, cells will be subjected to physiological stress. This stress is accentuated due to the accumulation of organic acids and metals resulting from the use of molasses, as well as the accumulation of salts due to the addition of a base to control pH. Factors such as dissolved oxygen concentration, presence of contaminants, temperature control, substrate used, variations in osmotic pressure, among other variables, will directly affect the yield and quality of the process (Attfield, 2022).

Assessing operational costs and implementing strategies to reduce them are essential to make processes viable, minimizing industrial limitations and making them economically feasible and profitable. Considering these factors, studying ways to incorporate waste into specific industrial processes that allow such application is an ecological alternative that favors the reduction of operational costs (Runge, 1998).

Bioreactors are widely employed in the described processes, varying in size, shape, and transparency according to the specific needs of each process. They are also known as fermenters due to the use of fungi and bacteria. Advanced bioreactor models offer control over aeration, pH, sample collection, temperature, agitation, among other factors. The development of a process usually begins at the bench scale, ranging from cultivation in 100 mL erlenmeyer flasks to fermentation in more robust bioreactors, typically 10 liters in volume. These initial studies are crucial for determining the relevant parameters of the developing process and, consequently, for scalability. When scaling up, it is necessary to consider various aspects and adjust cultivation parameters as needed, taking into account the physical similarity of the bioreactor, oxygenation capacity due to size, higher likelihood of contamination, among other factors. More advanced tests can be conducted at pilot scale, around 500 liters, and industrial scale, with capacities of up to 2000 liters, which allows for a better understanding of the deficiencies of the developed

methodology and assists in the search for process improvements and scalability (Fig 2) (Marques et al 2010; Fernandes et al., 2016; Cytiva, 2019; Carpio 2020).

Fig 2. Comparison of bioreactors at various scales, from laboratory to industrial.



Source: Author, 2024. Based on Cytiva, 2019. Created in BioRender.

4 BIOTECHNOLOGICAL APPLICATIONS OF UNCONVENTIONAL YEASTS

Yeast are single-cell fungi with a great proliferation capacity as a biological inoculant in different bioprocesses, allowing their application in sectors such as breweries, winemaking, the sugarcane industry, as biocontrollers in agribusiness, probiotics in livestock, among others. Rich in proteins, B-complex vitamins, and amino acids, yeasts can add value to waste, while some species can act as probiotics in animal nutrition (Nandy & Srivastava, 2018; Jach et al., 2022). Furthermore, the researches encourage their application in various areas to fully exploit the capabilities of these microorganisms. Although *Saccharomyces cerevisiae* is the most studied and widely used yeast in the industry, studies demonstrate the high potential for the use of unconventional yeasts, such as *Sporidiobolus pararoseus*, *Wickerhamomyces onychis* and *Sporobolomyces japonicus*.

4.1 *Sporidiobolus pararoseus*

Sporidiobolus pararoseus, a basidiomycete with reddish colonies, has the ability to assimilate a variety of substrates, including glucose, maltose, galactose, soluble starch, cellobiose, and citrate, among others (Sampaio, 2011; Brysch-Herzberg & Siedel, 2015). Additionally, it produces enzymes such as β -glucosidases, lipases, amylase, and cellulases (Baffi et al., 2010; Zhi-Dong et al., 2017) and demonstrates biocontrol activity against various fungal pathogens, such as *Penicillium italicum*, *Botrytis cinérea*, *Rhizopus sp.*, *Fusarium fujikuroi*, among others (Huang et al., 2012; Matić et al., 2014).

Sporidiobolus pararoseus is a yeast capable of producing various carotenoids, such as β -carotene, γ -carotene, torulene, and torularhodin. Liu et al., (2023) investigated the antioxidant effect of the carotenoid torularhodin extracted from *Sporidiobolus pararoseus* ZQHL and observed a significant elimination of free radicals *in vitro*, as well as relief of RAW 264.7 cell inflammation through modulation of the TLR4 pathway. This yeast has high potential as a food additive due to its nutritional value from the production of carotenoids and lipids, in addition to the presence of β -glucan, which can bind to mycotoxins, as demonstrated by Tapingkae et al., (2022) when evaluating the possibility of yeast production (300 L bioreactor, 28 g.L⁻¹ biomass) and capacity for mycotoxin absorption in the gastrointestinal system of birds.

In addition to its antioxidant potential, this yeast is also known for its action as a biocontroller against various fungal plant pathogens, such as those affecting grapes (Xiao et al., 2022; Zhao et al., 2023). Additionally, it has demonstrated thickening, stabilizing, emulsifying effects, increased concentration of short-chain fatty acids, and decreased lipopolysaccharides in simulated gastrointestinal digestion processes (Xue et al., 2023).

Furthermore, the application of *Sporidiobolus pararoseus* as a dietary supplement for Nile tilapia improved growth performance, immune response, and disease resistance against *Streptococcus agalactiae* (Van Doan et al., 2023); it was also applied as a dietary supplement for broiler chickens, observing relief of induced toxicity compared to the control through the agglutination of mycotoxins, thus maintaining animal health (Srinual et al., 2022).

The results obtained by Liao et al., (2024) follow the same line, where they demonstrated that the application of the polysaccharide *Sporidiobolus pararoseus* in rats with induced arthritis showed significant immune stimulation and antioxidant activities, indicating that the yeast application reshaped the intestinal microbiota, increasing the

abundance of beneficial bacteria, in addition to the greater production of short-chain fatty acids, especially acetic acid, propionic acid, and butyric acid, which influenced the reduction of inflammatory response and disease symptoms. The numerous responses obtained by the application of *Sporidiobolus pararoseus* demonstrate the yeast's high biotechnological potential for applications in the food, pharmaceutical, aquaculture, poultry, and agricultural industries.

No studies were found with ruminants; however, research with fish, rats, chickens, and birds indicates a possible improvement in intestinal microbiota, increased short-chain fatty acids, immune stimulation, and antioxidant activities, which can benefit the sector, reduce the use of antibiotics and growth promoters, and promote beneficial microorganisms (Liao et al., 2024; Tapingkae et al., 2022; Srinual et al., 2022; Van Doan et al., 2023).

4.2 *Wickerhamomyces onychis*

Wickerhamomyces onychis, initially identified as *Pichia onychis* (Yarrow et al., 1965), is a poorly studied ascomycete commonly found in fermented foods like Kimchi, Lafun, or fermented beverages such as Borde, indicating potential for fermenting starchy and fibrous foods (Oh & Han, 2003; Koricha et al., 2020). *Wickerhamomyces onychis* is an ascomycete with white coloration and ability to assimilate various carbon sources, including glucose, maltose, sucrose, xylose, and 2,3-butanediol (Kurtzman, 2011). Besides being used in food, such as in the production of Kimchi, a Korean dish (Oh and Han, 2003), this yeast also exhibits high tolerance to a wide range of pH (3.0 to 9.0) and temperature (5 to 37°C). Moreover, it shows potential as a biocontrol agent against phytopathogenic fungi such as *Botrytis cinerea* and *Rhizopus stolonifer* (García et al., 2001; Zapata et al., 2015).

According to Shinya et al., (2022), the yeast has shown a high capacity to convert cassava bagasse, resulting in protein enrichment of the residue and potential applications in animal nutrition, especially for ruminants.

In addition to its fermentative potential, the yeast also demonstrates biocontrol activity against *Botrytis cinerea*, known as gray mold (Zapata et al., 2010; Moreno et al., 2012). In kiwi plantations, the main post-harvest diseases are blue mold and gray mold caused by *Penicillium expansum* and *Botrytis cinerea*, fungi that were strongly inhibited by the yeast's control action (Zhao et al., 2023). Microorganisms such as *Yarrowia*

lipolytica, *Saccharomyces cerevisiae*, *Metschnikowia bicuspidata* WCY, *Candida tropicalis*, *Candida albicans*, and *Kluyveromyces aestuarii* were also inhibited by the yeast (Guo et al., 2013). In apple orchards, the yeast showed high potential as a biocontrol agent both before and after infection by *Monilinia fructicola*, known as brown rot (Czarnecka et al., 2019). This yeast was also observed in spontaneous grape fermentations (Cioch-Skoneczny et al., 2021), indicating potential application in fermentations to maintain the sensory profile and chemical composition of the product. It was also observed in tomato fermentations, even after three weeks and high concentrations of lactic acid, indicating high resistance, in addition to being part of the fruit's microbiota (Bah et al., 2019). Studies indicate that the fungus has different biocontrol mechanisms, such as rapid surface colonization, biofilm formation, attachment to mycelial surfaces, production of volatile organic compounds with antifungal activity, among others (Zhao et al., 2023).

In studies on the interaction between microbiome, bacteriome, inflammation, and diet in people with HIV, *Pichia* was strongly correlated with various bacterial genera, including the *Faecalibacterium* genus. In the same study, the results suggest a relationship between innate and adaptive systemic immune responses with yeasts, such as the positive correlation between *Candida* species and levels of pro-inflammatory cytokines, interleukin 22 (mucosal immunity regulation), and CD8⁺ T cell counts (Gosalbes et al., 2022).

Furthermore, the yeast also demonstrates biotechnological potential for the production of bioethanol and chemicals due to its ability to ferment sugars in seawater-based media. Therefore, yeast isolated from seawater, an environment with high concentrations of salts, inhibitory compounds, low levels of soluble carbon, and nitrogen, shows high resistance and, consequently, potential for application in robust biotechnological processes (Zaky et al., 2016). Despite the low amount of research on *Wickerhamomyces onychis* yeast, it shows high resistance and fermentative capacity, as well as potential as a biocontroller of various species, highlighting its potential for application in agro-industrial sectors, the food industry, animal nutrition, and possibly in the pharmaceutical sector.

4.3 *Sporobolomyces japonicus*

Sporobolomyces japonicus, a basidiomycete, exhibits the ability to assimilate various carbon sources, such as glucose, maltose, sucrose, galactose, and cellobiose

(Hamamoto et al., 2011). Despite the lack of detailed information about this yeast in the literature, studies such as that of Khanh et al., (2012) have observed phytase production. The reddish coloration of *S. japonicus* colonies is associated with the production of pigments such as γ -carotene and β -carotene, which have antioxidant potential and strengthen the immune system (Hamamoto et al., 2011; Maldonado et al., 2012; Mata-Gómez et al., 2014).

There is limited information in the literature about the microorganism; however, based on its production of carotenoids such as torulene, torularhodin, γ -carotene, and β -carotene, there is a likely production of vitamin A, enhancement of the immune system, dye properties, and antioxidant action, indicating high biotechnological potential and relevance (Kurtzman et al., 2011; Mata-Gómez et al., 2014; Kot et al., 2021). Additionally, the yeast demonstrates good fermentative capacity in cassava bagasse, a material rich in starch and fibers, which increases the protein content of the substrate and suggests its application in animal nutrition (Shinya et al., 2022).

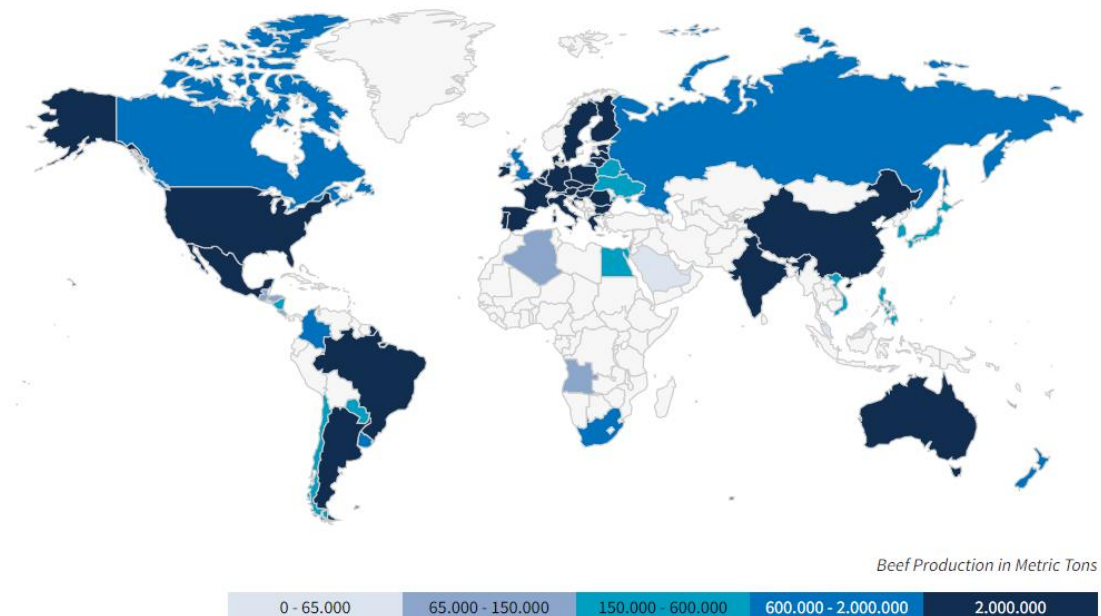
Saccharomyces cerevisiae is a traditional and important yeast, an ascomycete widely used in sectors such as baking, brewing, and ethanol production from sugarcane juice, is responsible for generating large amounts of biomass in the sugarcane industry, reaching around 870 thousand tons of yeast. These yeasts, considered process residues, are often marketed at low prices as nutritional supplements in animal feed formulations (Oliveira & Oliva-Neto, 2011; Ramos et al., 2011). *Wickerhamomyces onychis* and *Sporobolomyces japonicus* have shown potential to develop in materials with high concentrations of starch, such as cassava bagasse (Shinya et al., 2022). On the other hand, *Sporidiobolus pararoseus* demonstrated better development capacity in more fibrous materials, such as sugarcane bagasse (Shinya, 2017; Shinya et al., 2022).

5 LIVESTOCK

Currently, United States and Brazil lead in cattle production, followed by China in third place (USDA, 2023). The global average beef production is expected to grow, with an average increase of 1% per year, reaching approximately 46.5 million tons of calves and 192 million heads of cattle by 2024 (USDA, 2023). Besides high production, Brazil is considered the largest exporter of beef, with 2.29 million tons exported in 2023 (ABIEC, 2023; Malafaia & Biscola, 2023). The significant beef production and export are aligned with global consumption, which reached 58.22 million tons in 2023, with the

United States being the country with the highest consumption rate, about 12.6 million tons, a fifth of the global consumption (Fig. 3). China and Brazil follow with 11 and 8.1 million tons of consumed beef, respectively (USDA, 2023). The increasing population exerts significant pressure on the livestock sector, leading it to increase production and implement new strategies to meet this growing demand.

Fig. 3 Beef Production (2023/2024). (USDA, 2023).



In addition to the high consumption of beef, there is also the production of milk and dairy products, which are also highly consumed, although they have a higher consumption rate in developed countries. Milk is currently one of the most relevant agricultural commodities globally, ranking among the top five most traded products in the world. Brazil had an average annual consumption of 166 liters per inhabitant in 2018, while in developed countries consumption can reach 250 to 300 liters per inhabitant (Siqueira, 2019).

Therefore, the importance of livestock farming for the world population is evident, justifying the great interest in improving production systems to increase quality, yield, and productivity of processes. Ruminant nutrition is just one aspect aimed at improving production processes, especially focused on optimizing ruminal fermentation (Mcmichael et al., 2007; Pulina et al., 2017).

Global warming is a serious environmental problem that has been the subject of discussions in many international institutions. In livestock farming, the three main

greenhouse gases emitted are methane (CH₄), nitrous oxide (N₂O), and carbon dioxide (CO₂). Nitrous oxide has a warming potential 265-298 times higher than carbon, while methane is 28-36 times more potent than carbon over a hundred-year horizon (Pachauri et al., 2007; Usepa et al., 2022; Tian et al., 2023). Methane is one of the gases of greatest concern to the scientific community because of its ability to increase temperature 80 times more than CO₂ over 20 years, with a pronounced short-term impact due to its rapid decomposition in the atmosphere (IPCC, 2023). Despite methane's lower warming potential compared to nitrous oxide, it is the second most abundant greenhouse gas and decomposes in just a decade, resulting in a short-term impact and contributing more rapidly to reducing the effects of global warming (USEPA et al., 2022; IPCC, 2023).

In ruminants, methane is produced during the fermentation process by methanogenic bacteria that use released hydrogen to generate CH₄. According to the Southeast New England Program (EPA, 2024), each cow emits approximately 69.8-119.7 kg of methane annually (Leytem et al., 2011), while the annual emission from a dairy farm corresponds to about 1,301 kg.h⁻¹, with a maximum methane emission of 3.51 kg.h⁻¹ (Nowakowicz-Dębek et al., 2020), which is concerning considering the increasing consumption and production of beef worldwide. Additionally, beef and dairy cattle significantly contribute to methane emissions, accounting for about 62% of total emissions observed in livestock farming (FAO, 2023).

6 ANIMAL NUTRITION SUPPLEMENTS

In general, supplements can be defined as a mixture composed of ingredients (components of any combination or mixture) or additives (substance, microorganism, or formulated product), included in the formulation according to the consumption needs and nutritional balance of animals (INSTRUÇÃO NORMATIVA 15/2009/MAPA). The development of products for cattle must comply with minimum levels, values found in annex II of IN 12/2004 from the Ministry of Agriculture. Supplements can be classified based on the concentration of macro and micro minerals as well as crude protein. Considering mineral supplements, phosphorus is one of the most relevant, prioritizing values exceeding 40 g per kilogram for beef cattle or 73 g per kilogram for lactating cows. The calcium/phosphorus balance should be up to 7:1 (INSTRUÇÃO NORMATIVA 12/2004/MAPA).

Mineral protein supplements in their formulation include ingredients or additives, macro and micronutrients containing at least 20% crude protein, with the minimum recommended dose being 30 grams of crude protein per 100 kilograms of live animal weight. Energy-protein mineral supplements must include the aforementioned, plus 100 grams of total digestible nutrients (TDN) per 100 kilograms of live animal weight (INSTRUÇÃO NORMATIVA 12/2004/MAPA). Considering the current law that defines minimum concentrations of crude protein, in this case 20%, only 85% of this must be synthetic protein (INSTRUÇÃO NORMATIVA 12/2004/MAPA). Therefore, cellular autolysis emerges as a quick and cost-effective alternative to meet protein demands, while also providing a portion of vitamins and minerals in its composition.

In general, population growth coupled with the consumption of meat and milk and the limited pasture area indicate the need to rethink practices to increase the yield and productivity of the sector, while also reducing the release of greenhouse gases (GHGs) (UNFCCC, 2021; Cardoso et al., 2020). According to the "Fourth National Communication of Brazil to the UNFCCC (2021) "GHG emissions reached 1,467 teragrams (Tg) CO₂e (carbon dioxide equivalent) in 2016, with enteric fermentation accounting for 19% of the total. Despite variations in methane release among ruminant animals in livestock, it is known that cattle are responsible for 97% of these emissions. Environmental movements are growing, particularly around livestock, which releases a high rate of GHGs into the atmosphere.

In 2015, the UN established global sustainable development goals, with a deadline of 2030, including climate actions where reducing greenhouse gas emissions stands out (UNDP, 2023).

The growing demand for animal-derived food products underscores the importance of improving nutritional and management conditions to achieve higher productivity in livestock and other breeding animals. Food additives are one of the strategies employed toward this goal; however, the use of antibiotic-based growth promoters has been questioned and prohibited in several international markets due to promoting the proliferation of resistant microorganisms. In 2006, the European Union banned the use of antibiotic-based growth promoters, leading to an increased search for alternatives. This development has spurred expectations and interest particularly in probiotics, prebiotics, synbiotics, and immunomodulators. Among these, probiotics have shown significant potential considering the benefits obtained for livestock (Markowiak & Śliżewska, 2018; Lambo et al., 2021). The goals of the United Nations are aligned with

increased research in the livestock sector aimed at reducing methane emissions, among various studied strategies, the use of probiotics has proven promising (UNDP, 2023).

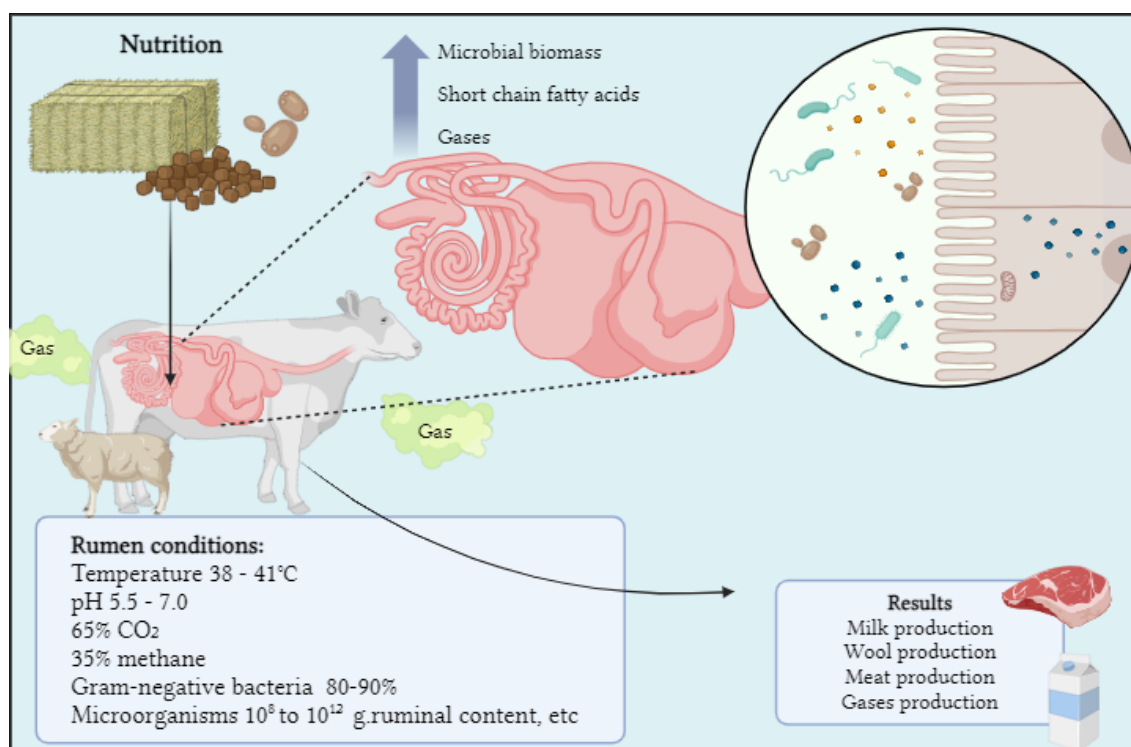
7 CHARACTERISTICS OF THE RUMEN

Ruminal fluid is a complex mixture of fermentative microorganisms, including bacteria, bacteriophages, protozoa, and fungi. The pH of the rumen varies between 5.5 and 7.0 under normal conditions, with the lowest value typically observed two to six hours after feeding. The optimal temperature for the survival of rumen microorganisms ranges between 38°C and 41°C. To ensure the survival of rumen microorganisms, maintaining anaerobic conditions is crucial, as most of these microorganisms are anaerobic, although some are facultative. The rumen consists of approximately 65% CO₂ and 35% methane, despite having low concentrations of O₂, H₂, N₂, among other gases. Acid neutralization is facilitated by the high buffering capacity of bicarbonate from saliva and blood, which helps maintain ruminal pH (Hungate et al. 1975; Choudhury et al., 2015; Nagaraja et al., 2016).

The quantity of microorganisms in the rumen is extremely high, with estimates ranging from 10⁸ to 10¹² microorganisms per gram of ruminal content. Gram-negative bacteria make up approximately 80-90% of the total population in the rumen, either free in the ruminal fluid or adhered to substrate fractions. Substrates are fermented by the action of microorganisms present in the rumen, resulting in the production of propionate, acetate, and butyrate, which are rapidly absorbed energy sources for the rumen. Lactate and propionate are the primary energy sources for methanogenic bacteria, which release methane as a byproduct of the process (Hungate et al., 1975; Nagaraja et al., 2016; WEI et al., 2022). Considering the characteristics of the rumen will allow for the selection of the most appropriate strategy to ensure optimal animal performance.

The Figure 4 summarizes the main aspects of the rumen, the consequences of ruminal fermentation, and its products.

Fig 4. Summary of some characteristics of the rumen, as well as the products of ruminal fermentation of the offered diet and final products obtained.



Source: Author, 2024. Created in BioRender.

8 MICROORGANISMS IN ANIMAL FEED

The use of microorganisms for animal feed adds nutrients if the microorganism is inactive, or serves as a probiotic if it surpasses rumen barriers and proliferates. Probiotics are food products consisting of live microorganisms that benefit health. In animal nutrition, yeasts with probiotic potential are typically applied in dry or paste form, and when concentrated and active, they demonstrate benefits related to increased nutrient assimilation, immune modulation, competition with undesirable microorganisms, reduced diarrhea, decreased methane release, maintenance of ruminal pH, among other advantages, in addition to adding nutritional value when inactive (Vohra et al., 2016; Noschang et al., 2018; Lambo et al., 2021).

Probiotics are also known as biotherapeutics, bioprotectors, and bioprophylactics, and they are widely used to prevent gastrointestinal and enteric infections. The intestinal microbiome harbors many bacterial genera across different phyla, with notable ones being *Bifidobacterium* sp. and *Lactobacillus* sp. (Puniya et al., 2015; Reuben et al., 2022). Probiotics added to the diet also contribute to nutrient synthesis and increase their bioavailability in the body. Cellulolytic microorganisms directly affect fiber degradation

and influence digestibility and animal production. They exhibit antioxidant, anticarcinogenic, and antimicrobial activities, preventing or reducing pathogen multiplication, producing vitamins, lowering cholesterol, improving lactose digestion, activating the immune system, and are part of diarrhea treatment and prevention (Kassich et al., 2020; Ashaolu, 2021; Linde et al., 2022).

The viability of probiotics can be affected by cell damage caused by oxygen-intolerant or oxygen-sensitive species, as well as incorrect storage and humidity. Furthermore, for a probiotic to be effective, it must be used in the correct dose to achieve the desired effect. Generally, the minimum acceptable concentration of probiotics is 10^6 CFU.kg⁻¹ (colony-forming unit) for daily intake. The major challenge for manufacturers is to ensure that these effective concentrations remain until the expiration date. It is essential that the quantity of microorganisms and viable cells remain stable throughout the probiotic storage process. Additionally, each product must specify the strain, CFU count, and its function for the consumer (Hotel et al., 2001; Bajagai et al., 2016; Bhagwat et al., 2020).

Probiotic additives for ruminants can be marketed as a single microorganism, a combination of microorganisms, or even associated with minerals, vitamins, and amino acids considering adequate levels of the nutrient. The nutrients commonly added include vitamin E, chelated amino acid iron, chelated amino acid cobalt, amino acid selenium complex, methionine, yeast cell wall, along with different microorganisms such as *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Bifidobacterium bifidum*, *Lactobacillus lactis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, among others (Bajagai et al., 2016; Reuben et al., 2022).

Probiotics face conditions in the rumen including temperatures ranging from 39°C to 41°C, pH around 5 to 7, competition with different microorganisms, among other factors, as well as varying conditions from production to product application such as cell damage during storage and transportation, which can reduce the effectiveness of the marketed product (Alzahal et al., 2008; Alzahal et al., 2009).

The fibrolytic enzymes produced by microorganisms enhance the degradation process of residual fibers and alter fermentative parameters such as the acetate/propionate ratio and short-chain fatty acids, which are products of bacterial fermentation of carbohydrates (Chung et al., 2012; Sakita et al., 2022). These short-chain fatty acids, available in large quantities, can provide more energy and improve animal health by reducing ruminal acidosis and hepatic abscesses. Additionally, studies show that animals

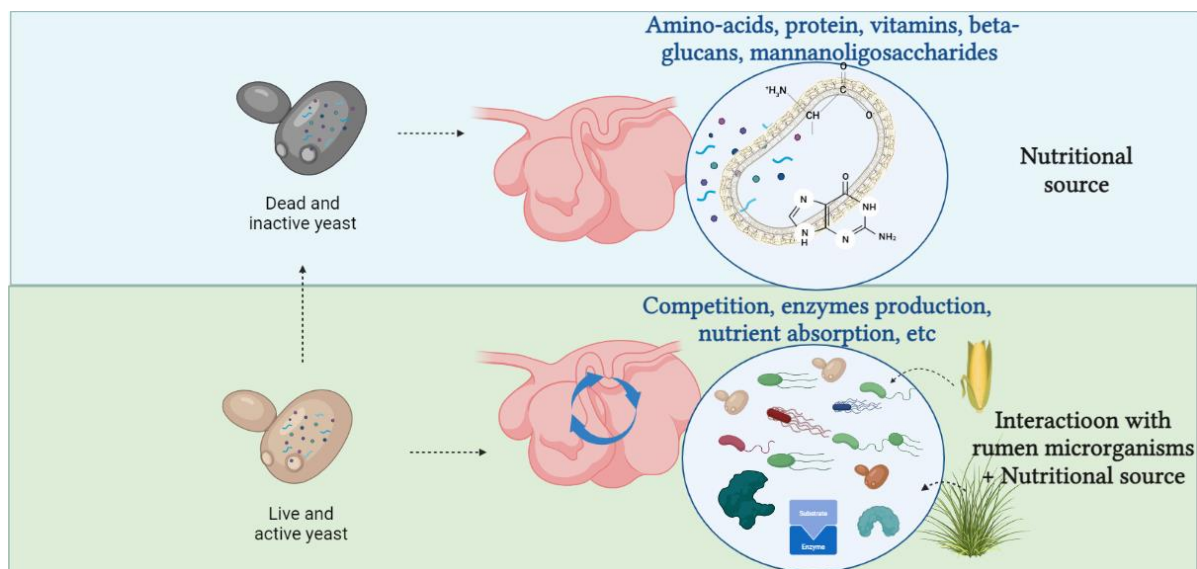
fed with residues enriched with microbial biomass (probiotics) emit less CO₂ and CH₄ during the digestive process, contributing to greenhouse gas reduction (Zeoula et al., 2011; Issakowicz et al., 2013).

The scientific community has recognized yeast as a viable alternative to antibiotic growth promoters (Bilal et al., 2022; Alagawany et al., 2023). Dead yeast can be marketed in various forms, such as dry form using methods like lyophilization or spray drying, or in a low-moisture paste form, among others, requiring less care compared to maintaining live microorganisms. Dead yeast retains all the chemical compounds present in live yeast, such as proteins, β -glucans, mannanoligosaccharides, vitamins, amino acids, and others, detailed in section 10. An important point is that once dead, the microorganism will not perform activities like enzyme production, release of secondary metabolites, nutrient consumption, proliferation, or competition with other microorganisms. Its main function is to be incorporated into the diet as a source of nutrients, including vitamins, minerals, and other essential elements, which can benefit other microorganisms. Additionally, in the case of β -glucans present in the yeast cell wall, there is interaction with the rumen wall of animals, positively influencing the immune system of ruminants, especially in the context of animal nutrition (Shurson, 2018; Alagawany et al., 2023).

When considering the use of residual yeast from breweries and ethanol industries applied to animal nutrition, it is important to consider the chemical and physical condition, as well as the treatments to which the biomass has been subjected, such as antibiotic treatment, which may render this strategy unfeasible (Costa et al., 2018; Elghandour et al., 2020).

The figure below illustrates in a simplified manner the action of active and inactive yeast in the rumen (Fig 5).

Fig 5. Summary of the main influences of active and inactive yeast in the rumen.



Source: Author, 2022. Creator in BioRender

9 ANTIBIOTICS

The use of antibiotics is nearly unavoidable for controlling bacterial infections, whether in humans or animals. In livestock farming, antibiotics are widely used for treating mastitis, gastrointestinal infections, arthritis, among other infections; however, they can end up as residues in products such as meat, milk, and eggs, leading to consequences for consumers (Arsène et al., 2022; Jia et al., 2024).

Antibiotics used to promote weight gain and feed efficiency are considered growth promoters and can alter intestinal microbiota. Monensin is an ionophore antibiotic commonly used in beef cattle farming to improve feed efficiency and promote cattle growth; however, restrictions on antibiotic use by government institutions, along with concerns about resistance development and microbiota dysbiosis, encourage the search for alternative products (Khan et al., 2016; Mcgrath et al., 2018; Reuben et al., 2022).

Regulations on antibiotic administration have been improving and becoming stricter over the past decade; however, consumption remains high, with countries such as China, Brazil, India, the USA, Australia, and countries in the European Union being among the largest users of antibiotics. The European Union has banned the use of antibiotics as growth promoters since 2006 (Mulchandani et al., 2023; Schmerold et al., 2023). The European Council 1996 (Directive 81/602/EEC) prohibited the use of compounds with thyrostatic, estrogenic, androgenic, or gestagenic action in beef cattle (Council et al., 1981), as well as the use of hormones for growth or therapeutic purposes (Directive 96/22/EC). In Italy, Germany, and Denmark, the use of hormones is also

prohibited; furthermore, Greece and Belgium have never allowed the use of growth promoters (Passantino et al., 2012).

The Food and Drug Administration (FDA) in the USA, through the Federal Register (2015), banned the use of important antibiotics such as oxytetracycline, chlortetracycline, macrolides, tylosin, among others (Drouillard et al., 2018). China has established antibiotic use only by prescription, as well as not using antibiotics for disease prevention and implementing a drug resistance containment plan by 2025 (Schmerold et al., 2023). Australia has banned the use of important antibiotics for human medicine such as fluoroquinolones, colistin, fourth-generation cephalosporins (Australian Government 2023), while Brazil has eliminated many but not all antibiotics listed as critically and critically important by the World Health Organization (WHO) for growth promotion (Da Silva et al., 2023).

Despite increased attention and regulation of antibiotics, control is uneven worldwide, with countries following their own regulations such as Brazil with the regulatory agency MAPA (Ministry of Agriculture and Livestock), or considering specifications defined by recognized organizations like the Food and Agriculture Organization (FAO) / World Health Organization (WHO) Expert Committee on Food Additives (JECFA), among others (WHO, 2020).

In the work by Da Silva et al. (2023), the level of development of legislation in five South American countries—Brazil, Chile, Argentina, Colombia, and Uruguay—was evaluated. The study considered countries "strong" if they followed regulations similar to those proposed by OIE, FAO, WHO, or more advanced, "intermediate" if they met half of the proposed standards from these organizations, and "weak" if they met less than 50%. Regarding the use of growth promoters, weight gain additives, and feed efficiency enhancers, Brazil and Uruguay were considered "intermediate," while the other countries were classified as "strong." The use of antibiotics can be classified into three types—therapeutic, metaphylactic, and preventive—considering all forms of administration (injectable or oral, through feed or water) (World Organisation for Animal Health OIE, 2020). None of the countries received a "strong" classification for preventive antibiotic use, but for therapeutic and metaphylactic use, Brazil and Chile were the only countries classified as "strong" (Da Silva et al., 2023).

The report "Intensive Industrial Livestock: Factory of Multidrug-Resistant Bacteria," released in November 2022, highlights the relationship between antimicrobial resistance and intensive industrial livestock farming. Research revealed the presence of

multidrug-resistant bacteria in meat products in several countries, including Australia, Brazil, Indonesia, Kenya, Spain, Thailand, the United Kingdom, and the USA. Resistance extends to two or more antibiotics in meat, and resistance genes spread to aquatic ecosystems and the environment, indicating the dissemination of resistant bacteria from animal production through the food chain, which has direct implications for consumers (Stavroulaki et al., 2022).

It is important to emphasize that animal health and welfare not only affect climate and the environment but also have significant implications for public health and food safety (Arsène et al., 2022). Aquaculture systems, for example, show high rates of antimicrobial resistance in 40 countries, accounting for 93% of global production (Reverter et al., 2020). About 80% of antibiotics consumed by these animals are excreted in urine and feces, contributing to the selection and dissemination of resistant microorganisms along the food chain (Oliveira, 2019).

The nine countries analyzed and described in the report "Intensive Industrial Livestock: Factory of Multidrug-Resistant Bacteria" showed higher resistance to various classes of antibiotics, notably fluoroquinolones (21.3%), third-generation cephalosporins (12.8%), aminoglycosides (11.7%), tetracyclines (8.5%), and trimethoprim-Sulfonamide combinations (7.4%), among others. Many of these antimicrobials are considered critically important for human medicine.

Research addressing these facts is crucial given estimates of 1.27 million deaths annually caused by drug-resistant microorganisms, with projections alarming predicting up to 10 million deaths per year by 2050 (The Lancet, 2022; Jim O'Neill, 2014). Antibiotic residues can have consequences for human health such as antibiotic resistance, allergies, anaphylactic shock, cancer, reproductive disorders, bone marrow toxicity, mutagenic effects, among others (Arsène et al., 2022).

The spread of resistant microorganisms along the food chain contributes to this scenario, which has raised significant concerns from the World Health Organization (WHO), encouraging the search for alternatives that promote the replacement or reduction of these microorganisms (The Lancet, 2022; Jim O'Neill, 2014). This underscores the importance of products like probiotics, which are accepted as a safe alternative to antibiotics in animal feed (Khan et al., 2016; Mcgrath et al., 2018; Reuben et al., 2022).

10 CHEMICAL CHARACTERIZATION OF YEAST BIOMASS

Saccharomyces cerevisiae is a widely used microorganism in industrial processes, thus greater understanding of its physical, chemical, and metabolic characteristics has been attained. In order to extrapolate the potential constitution of other yeasts to be studied in this research, we will detail some characteristics of *S. cerevisiae*.

S. cerevisiae is a non-pathogenic yeast-like fungus used in industries such as ethanol production, baking, brewing, among others, which has led to the development of strains more resistant to robust industrial processes. The yeast cell wall of *S. cerevisiae* is composed of glucan (48-60%), a polymer consisting of glucose units with β (1-3) and β (1-6) linkages, manoproteins (20-23%), chitin (0.6-2.7%) composed of β (1-4) N-acetylglucosamine, and a small portion of lipids (Fleet, 1985; Shurson, 2018).

The β -glucans, which constitute the major portion of yeast, belong to a class of substances known as biological response modifiers, as they alter the host response by stimulating the immune system. They activate the immune response via the complement system, directly or with the assistance of antibodies, and produce chemotactic factors that induce the migration of leukocytes to the site of infection. This ability to activate host defense mechanisms, along with other effects such as antitumor, anti-inflammatory, antimutagenic, hypocholesterolemic, hypoglycemic, protection against infections, and enhancement of vaccine response, have been extensively evaluated and proven (Bobade, 2022).

Another component of yeast cell membrane extensively studied and employed in animal feed is MOS (mannanoligosaccharides), which has proven natural growth-promoting action. It has the ability to agglutinate pathogenic bacteria such as *Escherichia coli* and *Salmonella* sp., serves as a substrate for beneficial intestinal bacteria stimulating their growth, thus beneficially altering the intestinal microbiota of the animal. MOS induces positive effects on intestinal immunity and improves zootechnical results such as weight gain and feed conversion (Diaz & Branco, 2019). The mode of action of MOS is not fully elucidated; however, it is believed that the positive charge in its structure induces attraction towards the negative charges present in toxins and mycotoxins, generating electrostatic action between them and consequently preventing their absorption by the digestive tract (Lei et al., 2013). Another hypothesis aligns with this, indicating MOS binding affinity for type 1 fimbriae lectins located on the surface of pathogenic bacteria walls, thereby preventing their adherence to the intestinal wall (Heinrichs, 2003).

Yeast cells contain various types of vitamins, amino acids, proteins, and other components that participate in their chemical composition and metabolism. The use of yeast in animal nutrition should consider its chemical composition to avoid consequences such as copper poisoning. Copper is an essential trace element for many biological processes; however, high concentrations can cause toxicity and ultimately death, especially in ruminants, particularly sheep, which are more sensitive compared to monogastric animals (Canção et al., 2020; Borobia et al., 2022). Tolerance levels vary among animals, with bovines tolerating up to 700 ppm and sheep only tolerating up to 20 ppm, despite both species having a nutritional requirement of only 5 ppm (Rosa & Gomes, 1982). Although treatments based on chelating agents or copper antagonists exist, attention should be paid to dietary inclusions to prevent occurrences (Canção et al., 2020; Borobia et al., 2022).

Below is the chemical composition of *S. cerevisiae* based on three different authors, as presented in Table 1.

Table 1. Chemical composition of *Saccharomyces cerevisiae*.

Components %	Czech et al., 2016	Sacakli et al., 2013	Vidakovic et al., 2016
Dry Matter	93.30±2.5		
Crude Protein	46.50±4.3	44.35	46.6
Ether Extract	3.90±1.1	5.90	1
Starch	1		
Crude Fiber		4.80	
Neutral Detergent Fiber	6.2±8.7		
Acid Detergent Fiber	1.8±2.4		
Ash	7.10±1.1	4.70	6.30
Arginine	2.06		2.24
Cysteine	0.29		0.98
Histidine	0.98		1.04
Isoleucine	1.99		2.28
Leucine	2.76		3.21
Lysine	2.85	3.30	3.47
Methionine	0.70	0.90	0.97
Phenylalanine	1.61		1.93
Threonine	2.01		2.29
Tryptophan	0.49		
Valine	2.20		2.81
Calcium	0.32±0.28	0.53	
Phosphorus	1.16±0.16	1.38	
Potassium		1.38	
Magnesium		0.32	
Alanine			2.57
Aspartic Acid			4.54
Glutamic Acid			6.67
Glycine			2.21
Vitamins (mg.kg⁻¹ amino acid)			
B1		18	
B2		40	
Niacin		132	
B4		3300	
B5		518	
B6		34	
B12		0.44	
Biotin		2.2	
C		14.1	

11 MICROORGANISM PRESERVATION

Microorganism preservation has gained prominence due to the increasing biotechnological applications spanning a wide range of industries, including ethanol production, baking, bioethanol, biomethane, pharmaceuticals, animal feed, probiotics, breweries, and even biological pest control, among others. The diverse applications require the use of microorganisms capable of withstanding the rigors of these processes, necessitating enhanced preservation strategies for the utilization of bioinputs. With this in mind, understanding the bio-physical and chemical characteristics, as well as the influence of fluctuations in water content, nutrition, temperature, osmolarity, and oxidation, can facilitate the biotechnological application of strains while prolonging the shelf life of microbiological products (Câmara Jr et al., 2021; Attfield, 2022).

The most well-known preservation methodologies focus on preserving strains in the laboratory; however, microbiological preservation for commercial purposes such as probiotics must consider the characteristics of the biological material and the intended use. Quality control of bioinputs aims to implement preventive actions to ensure the identity of the microorganism used, safety of the final product, effective quantity considering efficacy and viability, efficiency of production steps, preservation quality, storage conditions, use of protective solutions, among other variables up to its application. Preservation methodologies vary especially according to the preservation time frame, including short, medium, and long-term preservation (Sola et al., 2012; Iwanicki et al., 2022).

One of the short-term preservation methodologies is the culture preservation at low temperatures, 4 to 10°C, such as the continuous subculture method. It is recommended that the subculturing be performed before depletion or desiccation of the medium. Despite being simple and low-cost, this technique is not suitable for the commercialization of microorganisms in large quantities that need to remain in contact with the nutrient medium and stored in a refrigerator, with a short preservation period of about three months (Sola et al., 2012; Kumar et al., 2013).

Medium-term preservation techniques typically involve refrigeration of cultures in a refrigerator with mineral oil, thereby limiting the available oxygen concentration and favoring preservation for up to seven years. Oil layers thicker than 1 cm are not recommended as they can lead to complete oxygen exhaustion by microorganisms and consequently reduce viability. However, contact of the microorganisms with air leads to

culture dehydration due to water evaporation, making oil usage favorable. Additionally, common freezing (-4 and -20 °C) can preserve cultures for periods ranging from three months to two years due to a considerable reduction in the cellular metabolism of microorganisms. Despite the advantages, this method results in reduced viability due to cell damage caused by ice crystal formation (Goldman & Green, 2008; Sola et al., 2012).

Long-term preservation methods include lyophilization, ultra-freezing (-196 °C), and cryopreservation (-80 °C). Microorganisms are stored at ultra-low temperatures for cryopreservation in freezers at -80 °C or in liquid nitrogen at -196 °C, which does not align with the objective of this study focusing on ease of storage and transport for commercialization (Heckly, 1978). Lyophilization is a more practical alternative among long-term techniques.

Lyophilization is considered one of the most efficient techniques, preserving cultures for typically over 17 years while maintaining the viability of the agents. Lyophilization involves the removal, by sublimation, of intracellular water from frozen cultures to prevent ice crystal formation (Sola et al., 2012). Although widely used for microbial preservation, the freezing, primary dehydration, and secondary dehydration steps can cause cellular damage or alterations in the cell membrane, reducing viability. These damages can be mitigated by using protective substances called cryoprotectants. This method is highly favored because it offers high stability of preserved material, requires minimal monitoring or constant maintenance, and occupies little storage space. Additionally, lyophilized cultures can be transported over long distances without refrigeration. The drawback is that the method is costly, requiring expensive equipment for dehydration (Heckly, 1978; Bjerketorp et al., 2006).

Cryoprotectants are organic solvents characterized by low molecular weight, high solubility in aqueous medium, and low cellular toxicity. They are classified based on their ability to penetrate cells and protect against temperature-induced damage. Penetrating cryoprotectants can permeate cells due to their low molecular weight, whereas non-penetrating cryoprotectants remain outside the cell, and the effectiveness depends on the species being preserved. The most widely used cryoprotectants include glycerol, dimethyl sulfoxide (DMSO), blood serum, serum albumin, skimmed milk, sucrose, yeast extract, glucose, trehalose, chitosan, methanol, sorbitol, malt extract, dextran, and ethylene glycol. It is important to note that high concentrations of cryoprotectants can be toxic to cells, altering biochemical composition and inducing osmotic stress, resulting in low survival rates (Barbosa et al., 2009; Freitas et al., 2020).

In addition to choosing preservation methods like lyophilization and adding cryoprotectants to microbiological products such as probiotics, it is crucial to consider the process costs and shelf life. Paste-form products tend to cause less cellular damage compared with microorganisms subjected to rigorous processes like lyophilization. Furthermore, these paste products have low moisture content, which can lead to dehydration. One effect of cell dehydration is the accumulation of large amounts of trehalose, a disaccharide that acts as a protector against cellular stress. This promotes water molecule accumulation and the formation of a vitreous state, allowing the cell to maintain its native conformation even in the absence of a hydration layer. Additionally, trehalose is associated with thermal resistance and is considered a reserve carbohydrate (Eleutherio et al., 2015; Ribert et al., 2021).

12 AUTOLYSIS AS A METHOD FOR RELEASE OF MICROBIAL NUTRIENTS

Autolysis is the process of cellular lysis, resulting in the release of cytoplasmic contents through the lytic action of endogenous enzymes. Endogenous enzymes act upon reaching the stationary phase, due to natural cell aging. The autolytic process leads to changes in cellular characteristics, affecting the porosity of the cell wall and the permeability of the cytoplasmic membrane, and is responsible for reducing respiratory enzyme activity and gradually hydrolyzing the cytoplasm (Šuklje et al., 2016; Dimopoulos et al., 2018).

Yeast autolysis is a process that involves subjecting intact yeasts to changes in physical and chemical factors that lead to cell lysis, such as temperature variation, addition of acids and bases to alter pH, salts to increase osmotic pressure, enzymes, detergents, among others, resulting in the production of two products: cellular extract and cell wall. Each fraction exhibits a distinct chemical composition, with the cell wall being the insoluble portion rich in beta-glucans and MOS (mannanoligosaccharides), compounds that induce immunomodulation and enhance the innate immune system's response capability. The cellular extract, on the other hand, is the soluble portion containing higher levels of proteins, vitamins, nucleic acids, and amino acids in its composition (Alexandre, 2011; Shurson, 2018).

The yeast extract is used by the food industry as a flavor enhancer, similar to monosodium glutamate (MSG), and also as a stimulant for microbial growth (Alves et al., 2020).

Carotenoids, liposoluble pigments, have a broad spectrum of action and are associated with high-density lipoprotein (HDL) and conversion into vitamin A. Vitamin A is crucial for maintaining the mucous surfaces of the eyes and respiratory, urinary, and intestinal tracts. Additionally, it plays an important role in bone growth, reproduction, immunity, cell division, and differentiation (Álvarez et al., 2015).

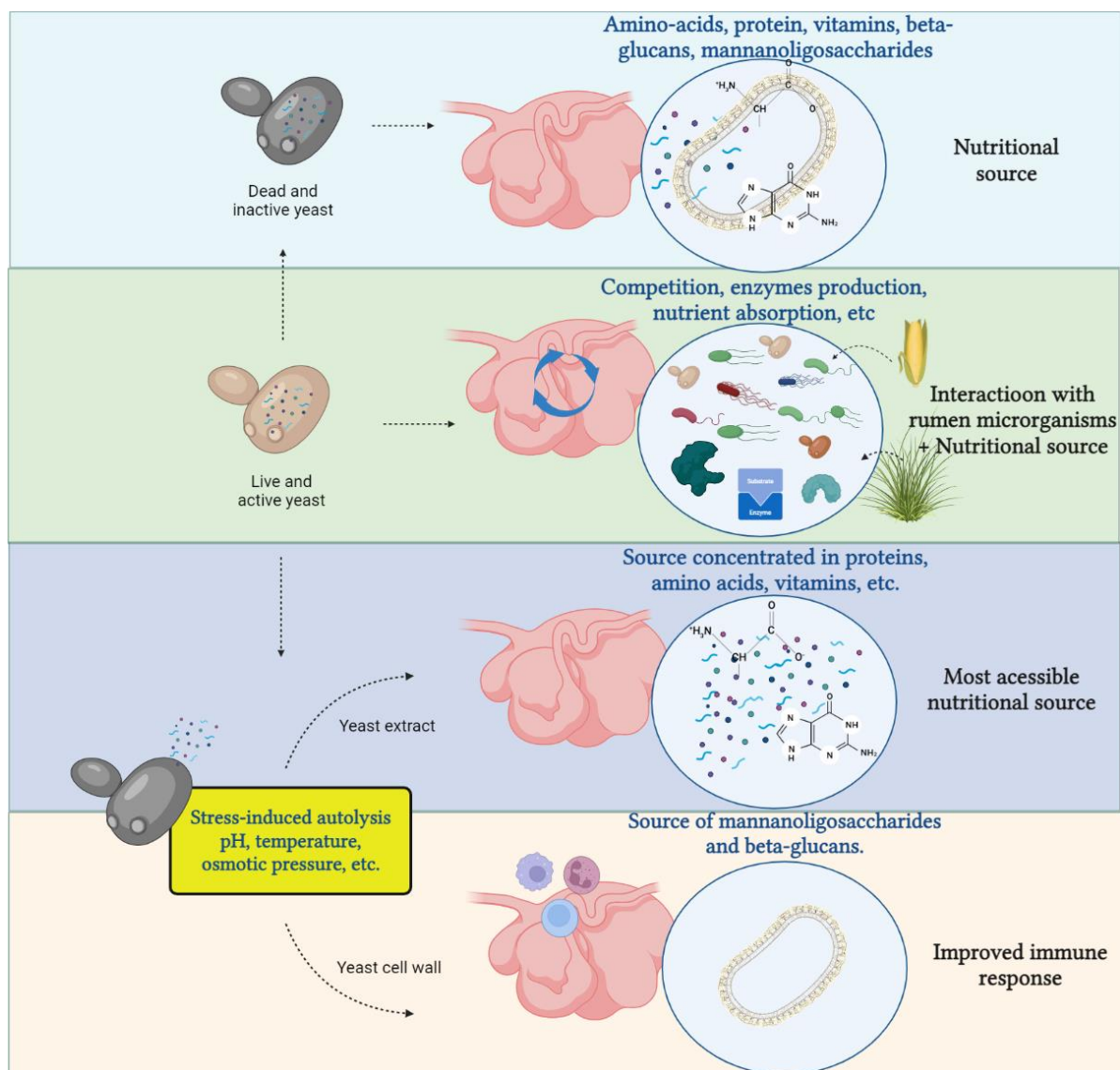
Proteins are crucial for animal nutrition and development and can be found in various forms in bovine feed, including: rumen-degradable protein (RDP), which are proteins available for consumption by rumen microorganisms; non-protein nitrogen (NPN), all nitrogen not in polypeptide form, incorporated by rumen microorganisms with the transformation of NPN into high-quality microbial protein; and true protein, crude protein minus NPN and ADICP (acid detergent insoluble protein), necessary for bacteria that degrade non-structural carbohydrates, along with ammonia, which is also important (Medeiros & Marino, 2015).

RNA, nutritionally speaking, is not essential since it is synthesized by the body using amino acids as precursors. However, in specific situations, if available, it can be utilized for nucleotide synthesis, enabling a quicker response against diseases, limited nutrient intake, or endogenous disturbance, for example. Additionally, RNA participates in various activities essential for organism maintenance, such as cell division, cell growth, and immune system modulation (Rossi et al., 2007).

Finally, vitamins are organic molecules necessary for proper organism function and are essential as enzyme cofactors or regulatory elements, as metabolic processes are triggered or controlled by them. Despite the small amount required to be consumed, the right concentration can optimize animal performance (Medeiros & Marino, 2015).

The figure 6 illustrates in a simplified manner the products obtained from autolysis and their action in the rumen, as well as for inactive and active yeast.

Fig 6. Products obtained from the autolysis of live and active yeast and their action in the rumen, along with the effects in the rumen of inactive and active yeast.



Source: Author, 2024. Creator in BioRender.

13 CONCLUSION

The additives of the biological inoculants category are crucial for food production and are expected to increasingly replace or assist in the reduction of other products such as antibiotics. Biological inoculants exhibit nutritional potential and competitive capacity against pathogens in the intestinal tract. They also stand out as modulators of the immune system, favoring disease prevention. Additionally, they reduce the release of harmful compounds into the environment, such as methane gas, as well as nitrogen released by feces and urine, thus contributing to decreased contamination potential and increased sustainability within the sector. Furthermore, the use of biological inoculants will directly

impact the decrease in resistance of pathogenic microorganisms to antibiotics, improve nutrient absorption by the animal, reduce nutritional costs and undesirable chemical compounds, decrease medication usage, among other factors. This fact can lead to the production of safer food. Therefore, further research on the use of microorganisms should be conducted to explore new microorganisms such as special yeasts, and to gain a better understanding of concentration variations, maintenance of probiotic or utilized microorganism viability, storage methods, application techniques, the influence of animal age, the application of biological inoculants in healthy or unhealthy animals, among other factors. These efforts will strengthen research outcomes and make the beneficial effects of microorganisms as additives in livestock undeniable, thus favoring the production of healthier animals and food, revolutionizing traditional methods where chemical products, often toxic or harmful to humans, were of vital importance for animal production.

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CHAPTER 2: PRODUCTION OF FIBROLYTIC YEASTS THROUGH AGROINDUSTRIAL WASTES

SUMMARY

The study aimed to develop a culture medium using agroindustrial wastes capable of stimulating the growth of fibrolytic yeasts and enzyme production, in addition to evaluating the costs of the best culture and the application of the microorganisms cultured *in vitro* ruminal fermentation. The selected microorganisms were *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2, and *Saccharomyces cerevisiae* M26, cultivated in liquid fermentation using sugarcane bagasse, hydrolyzed sugarcane cake, cassava bagasse, hydrolyzed cassava bagasse, and corn steep water (CSW). Additional tests were carried out using four wastewater streams from starch and corn industries to evaluate the potential for effluent reuse compared to deionized water in microorganism propagation, aiming to reduce costs and provide proper disposal. Subsequent tests with the best medium were carried out for the production of amylase, cellulase, and xylanase. The production costs of the best media were evaluated, as well as the application of the microorganisms cultured in *in vitro* ruminal fermentation to assess the potential degradability and methane reduction of each strain. The results showed that the best microorganism growth was achieved with the medium formulated with cassava bagasse and CSW, with highlights for *S. japonicus* Sia 70a (2.4×10^8 cells. mL⁻¹) and *S. pararoseus* Sia 33.1 (1.2×10^8 cells. mL⁻¹). However, with hydrolyzed cassava bagasse and CSW, the highlight was *W. onychis* LABI2 (2.37×10^8 cells. mL⁻¹). The potential of sugarcane bagasse was found to be low, with maximum growth for *W. onychis* LABI2 (3.9×10^7 cells. mL⁻¹) and for hydrolyzed sugarcane cake (*S. japonicus* 70a 3.99×10^7 cells. mL⁻¹). Substituting deionized water with wastewater in the medium with cassava bagasse and CSW did not increase the growth of *S. japonicus* 70a (1.2×10^7 cells. mL⁻¹) and *S. pararoseus* Sia 33.1 (7.7×10^6 cells. mL⁻¹). *W. onychis* LABI2 stood out in the cultures with the four wastewater streams without CSW, reaching at least 2×10^8 cells. mL⁻¹ for all cultures. Therefore, for the enzymatic production test, the medium with hydrolyzed cassava bagasse and CSW was chosen, where the highlight was the production of xylanases for *W. onychis* LABI2 with 0.44 U. mL⁻¹ and amylases for *S. japonicus* 70a (0.42 U. mL⁻¹) and *W. onychis* LABI2 (0.63 U. mL⁻¹). No microorganism showed cellulase production under the tested conditions. The economic analysis demonstrated that media with cassava bagasse and sugarcane bagasse associated with corn maceration water stood out. *In vitro* ruminal fermentation showed great potential for *S. japonicus* 70a and *W. onychis* LABI2, which exhibited higher degradability compared to the Control. These microorganisms are proving to be valuable as probiotics for animal nutrition, considering the enzyme production and observed growth potential. Additionally, the higher degradability *in vitro* tests may increase over the fermentation period, improving weight gain and likely methane production per animal weight, however, *in vivo* tests with animals are required to confirm these findings.

Keywords: agroindustrial wastes, yeasts, enzymes, animal nutrition.

1 INTRODUCTION

Agroindustrial waste increases in accordance with the production of consumer goods that seeks to meet the growing demand due to population growth. The main agroindustrial wastes generated are urban waste, citrus pulp, brewer's spent grain, corn steep water, sugarcane straw, sugarcane bagasse, cassava bagasse, among others. Agroindustrial wastes have a rich chemical composition, which makes it possible to develop new technologies that harness their potential and add value to the material (Tasic, 2017; De Corato et al., 2018; Cervbrasil, 2020).

Solid waste can be reused and utilized in the feeding of ruminants and also other animals, such as pigs, sheep, and poultry, through silage. Furthermore, such waste can be used as a carbon source for the development of microorganisms like fungi and bacteria, reducing the production costs of compounds of interest such as enzymes, probiotics, prebiotics, organic acids, among others. This reuse is not solely due to being a good alternative source of animal nutrition, but also to reduce waste and utilize the nutrients present in it. Despite the nutritional potential, these byproducts, coproducts, and residues used in the food industry may have antinutritional factors that hinder their inclusion in human and animal nutrition as well as the growth of microorganisms. The antinutritional factors that may be present in the materials are chlorogenic acid, cyanogenic acid, tannins, phytates, and non-starch polysaccharides, which can impair nutrient absorption (Silva et al., 2018; Abbas et al., 2020; Selo et al., 2021).

As for liquid waste, it can be used in fertigation since it presents several nutrients that provide soil fertilization and help control pests, such as calcium, potassium, nitrogen, magnesium, phosphorus, and sulfur. However, there is a lack of interest and investment from industries for more adequate waste reuse, which often leads to environmental contamination (Silva et al., 2018; Zekarias et al., 2019).

Therefore, considering the application of microorganisms as a supplement for animals, it is important for cultivation to evaluate the physical and chemical characteristics of the waste and select non-conventional microorganisms capable of consuming waste as a carbon source, aiming for maximum cell proliferation (Radecka et al., 2015).

Thus, this research aims to answer the question: is it possible to use sugarcane bagasse, cassava bagasse, hydrolyzed cassava bagasse, hydrolyzed sugarcane cake, and corn maceration water for the cultivation of yeasts, aiming for good microorganism

growth? Does the replacement of deionized water with four residual waters from cassava and corn processing washing equal the growth obtained in the previous cultivation stage with the microorganisms of interest? Is there enzymatic production by the selected culture medium for the evaluated microorganisms? The proposed approach is promising as it adds value to the material, provides an appropriate destination for agroindustrial waste, and benefits the livestock sector by providing a new strategy that includes waste and enzymes to improve the quality of animal nutrition. This sustainable practice integrates the sugarcane-cassava-livestock sectors (Monção et al., 2018).

2 OBJECTIVE

The present work aimed to evaluate the selected agroindustrial waste materials, such as corn maceration water, cassava bagasse, hydrolyzed cassava bagasse, sugarcane bagasse, and hydrolyzed sugarcane pulp, as well as other wastewater sources, as a nutritional source for the growth of some fibrolytic yeasts and enzymatic production.

3 MATERIALS AND METHODS

3.1 Preservation and isolation of microorganisms.

The research was carried out with four yeasts, *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, and *Saccharomyces cerevisiae* M26. *Wickerhamomyces onychis* LABI2 was isolated from rice bran at the Laboratory of Bioenergy (IPBEN) - UNESP - FCL Assis. The yeast strain *Saccharomyces cerevisiae* M26 was previously isolated from a bioethanol distillery (OLIVA-NETO et al., 2004). *Sporidiobolus pararoseus* Sia 33.1 and *Sporobolomyces japonicus* Sia 70a were provided by Prof. Dr. Fernando Carlos Pagnocca, responsible for the Microbiology Laboratory of the Center for the Study of Social Insects (CEIS) at the São Paulo State University (UNESP) - Rio Claro/SP, isolated from litter by professionals from the Microbiology laboratory - UNESP - IB/Rio Claro.

The maintenance of the yeasts was carried out by monthly transfers in a specific liquid culture medium here called Formulated Without Residue (FWR), according to Oliva-Neto et al., (2004) with 2.0% glucose, 0.5% yeast extract, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% de $(\text{NH}_4)_2\text{SO}_4$, 0.031% de K_2SO_4 , 0.0028% de ZnSO_4 , 0.0012% of MnSO_4 and 0.024% of MgSO_4 (w.v⁻¹), pH 5.5 and 2% agar in inclined tubes.

The cryopreservation was carried out using microtubes (Eppendorf, Kasvi, São Paulo/SP, Brazil) stored in liquid nitrogen (Volta20, Nitropec, Garça/SP, Brazil). For this, a suspension containing 1.5 mL of the concentrated microorganism grown in FWR medium (Oliva-Neto et al., 2004) in 15% (v.v⁻¹) sterile glycerol was used. The preservation in mineral oil was carried out every three months by subculturing on solid Formulated medium (in slant tubes), with addition of sterile mineral oil, and stored in a refrigerator (Ecoplus 430, Bosch, Joinville/SC, Brazil). Lyophilization was used only for *Wickerhamomyces onychis* LABI2 and *Saccharomyces cerevisiae* M26, which were able to tolerate the process better and be reactivated later.

3.2 Cultures formulated with agroindustrial residues

Agricultural wastes, sugarcane bagasse (Destilaria Água Bonita, Tarumã, SP, Brazil) and cassava bagasse (Tereos Amido e Adoçantes, Palmital, SP Brazil) and wet sugarcane press mud (Enersugar, Palmital, SP, Brazil), 70% humidity, were used to select the most suitable substrate combined with corn steep water (CSW) for the production of each yeast strain. The cassava bagasse residue was previously dried in an oven at 50 °C (Tecnal, mod. TE-394/1-MP, Piracicaba, SP, Brazil), crushed, and then stored, while the sugarcane bagasse was previously crushed and stored in a cold room until use. The two residues were ground in an industrial grinder (Spolu - model SPL-048, Itajobi, SP, Brazil) and sieved (Tamis mesh 7-20, Bertel, Caieras, Brazil) according to their particle size of 0.8 and 2.8 mm to standardize the substrate used and facilitate the access of microorganisms to nutrients.

Fermentation was carried out using 5% (w.v⁻¹) of each of the following solid substrates, in a total volume of 100 ml in a 250 mL Erlenmeyer flask, for each substrate: cassava bagasse (CB), hydrolyzed cassava bagasse (HCB - enzymatic hydrolysis), sugarcane bagasse (SB), and hydrolyzed sugarcane press cake (HSPC - alkaline hydrolysis), each mixed with 0.5% (v.v⁻¹) of corn steep water, initially adjusted to pH 5.5 (Tecnal - model R-TEC-7-MP), and no salt was added in this culture. The control culture medium used was the Formulated Without Residue (FWR) medium according to Oliva-Neto et al., (2004), as described previously. The cultures were autoclaved (SP215-26, Phoenix, Araraquara, SP, Brazil) at 121°C for 20 minutes, and then inoculated aseptically with 1x10⁶ cells. mL⁻¹. The cultures were maintained at 30°C for 48 hours at 180 rpm in

a refrigerated orbital shaker (Tecnal - model TE 421, Piracicaba, SP, Brazil). The inoculum was previously prepared in FWR medium at an initial pH of 5.5 for 48 hours at 30°C at 180 rpm.

Subsequently, the deionized water used in the selected medium of the previous step, cassava bagasse medium, was replaced with wastewater from corn or cassava industries to observe the potential of each of them in yeast growth. Among them are: corn factory process water (CFP), cassava starch drying floor wash water (DFW), refinery water (Refin), and cassava washing water (CWW). In another test with non-hydrolyzed cassava bagasse, each of the four wastewater was individually evaluated for the influence of adding 0.5% (v.v⁻¹) of CSW, aiming at yeast growth.

The production of xylanase, amylase, and cellulase was evaluated in three different substrates, namely, non-hydrolyzed cassava bagasse, hydrolyzed cassava bagasse with 1.2 U.mL⁻¹ of amylase at 55 °C for 24 hours and by hydrolyzed cassava bagasse with amylase associated with a set of salts: 2.0% glucose, 0.5% yeast extract, 0.13% of (NH₄)₃PO₄, 0.10% of (NH₄)₂SO₄, 0.031% of K₂SO₄, 0.0028% of ZnSO₄, 0.0012% of MnSO₄ and 0.024% of MgSO₄ (w.v⁻¹). The crops were carried out at an initial pH of 5.5 and 30 °C at 180 rpm for 72 hours. Samples were taken at 0, 8, 24, 48, and 72 hours.

After all the cultures were carried out, the fermented broth was filtered using a reusable porous polypropylene filter paper (Paulista Filter, 100% polypropylene, dimensions 161 x 22 x 205, Brazil, SP), and the filtered extract obtained was then used for counting of live, dead, and total cells present in the liquid fraction and for determining cell viability using a Neubauer chamber (Dorta et al., 2006).

3.3 Enzyme and acid treatment of waste

Some residues were previously treated before being used in fermentation, namely sugarcane press cake and cassava bagasse. 25% (w.v⁻¹) of wet sugarcane press cake was crushed and pre-treated with 4% KOH at 75 °C for five hours, named hydrolyzed sugarcane press cake (HSPC). The hydrolyzed cake was washed with running water for 20 minutes, followed by washing with distilled water for an additional 5 minutes. The cassava bagasse (CB) was dried at 50 °C and stored in a cold room until its use, being subsequently hydrolyzed (HCB) with amylolytic enzymes according to Shinya et al., (2017) prior to application in fermentation. Enzymatic hydrolysis was carried out by

adding 10% w.v⁻¹ of cassava bagasse in a bench-scale bioreactor (Tecnal - model Tec-Bio Plus) in 0.05 mol acetate buffer at pH 5.0 (Tecnal - model R-TEC-7-MP). The amylases were added to initiate the hydrolysis (1.2 U.mL⁻¹) carried out at 55°C for 24 hours. Amylase production was carried out by solid-state fermentation with 1x10⁶ spores.g⁻¹ of substrate of *Rhizopus oligosporus* in 10 g of wheat bran supplemented with 1.25% (NH₄)₂SO₄, 0.25% KH₂PO₄, and 1.25% urea, with 55% humidity for 72 hours at 30 °C. After enzymatic hydrolysis, the material was filtered for enzyme extraction and dried at 50 °C followed by storage at 6 °C (Ecoplus 430, Bosch, Joinville, SC, Brazil) until application in batch fermentation (Escaramboni et al., 2018).

3.4 Characterization of wastewater

The four wastewater samples (CFP: corn factory process water; Refin: refinery water; CWW: primary cassava washing water; DFW: cassava starch drying floor wash water) were characterized for dry matter content, reducing sugars, total reducing sugars, protein, and pH. Protein quantification and determination (NT 415 Nitrogen Distiller, NOVATEC, Piracicaba, Brazil) were performed according to the classical Kjeldahl methodology described by AOAC, (2000). The quantification of released and total reducing sugars was carried out by the DNS (3,5-dinitrosalicylic acid) method as described by Miller, (1959). The pH was measured with a pH meter Tecnal - model R-TEC-7-MP. The analysis of sample dry matter was performed with 40 mL of each wastewater sample in a forced air oven at 90 °C (Tecnal - model TE-394/1-MP, Piracicaba, SP, Brazil) for 72 hours.

3.5 Analytical procedures

3.5.1 Cell concentration

The cell concentration was quantified, in triplicate from different samples, using a Neubauer counting chamber (7301-1B, New Optics) according to Lee et al., (1981). The cell quantity was determined according to the equation:

$$\text{n}^{\circ} \text{ cell. mL}^{-1} = \sum \text{ of quadrants} \times 5 \times 10^4 \times \text{DF} \quad \text{Eq. (1)}$$

Where:

Σ of quadrants $\times 5$ = sum of the number of cells presents in five middle quadrants times 5 represents the 25 middle quadrants of the large central squared grid of the chamber; 10^4 = volume adjustment in the chamber to express the number of cells per mL, considering the area and height of each chamber quadrant; DF = sample dilution factor.

3.5.2 Cell viability

Cell viability was performed in triplicate using a methylene blue solution according to Lee et al., (1981). Quantification was carried out in a Neubauer chamber (7301-1B, New Optics).

3.6 Enzymatic activity

The total cellulase activity was quantified by the FPase technique (Mandels et al., 1976; Silva, 2016). The obtained extract (0.5 mL) after filtration of the fermentate was added to 1 mL of sodium acetate buffer (0.05 M, pH 5.6) with Whatman n°01 paper of $0.5.3 \text{ cm}^{-1}$. The reaction tubes were incubated for one hour at 50°C in a water bath (MA 127, Marconi, Piracicaba/SP, Brazil). After one hour of reaction, 3 mL of 3,5-dinitrosalicylic acid (DNS) was added, followed by reading at 540 nm on a spectrophotometer (UV-1600 PC spectrophotometer, VWR, International). One unit of enzyme activity (Filter Paper Unit - FPU) was defined as the release of 2 mg of glucose per mL of enzyme per minute and expressed in FPU.mL^{-1} (Ghose, 1987).

The xylanolytic activity was determined using 0.5% (w.v^{-1}) Birchwood xylan (Sigma Aldrich) in acetate buffer pH 5.0 (0.25 mol.L^{-1}) with addition of 0.1 mL of enzymatic extract in a water bath at 50°C for 10 minutes according to Bailey et al., (1992). The quantification of the released reducing sugar (xylan) was performed using 3,5-dinitrosalicylic acid (Miller, 1959) read at 540 nm on a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to release $1 \mu\text{mol}$ of reducing sugar per minute.

Amylase was determined by using 0.9 mL of starch 0.5% w.v^{-1} (Sigma Aldrich) in acetate buffer pH 5.5 (0.05 mol.L^{-1}) as a substrate, 0.1 mL of enzymatic extract in a water bath (MA 127, Marconi, Piracicaba/SP, Brazil) at 60°C for 10 minutes. DNS (0.5 mL) was used to determine the released reducing sugar, with reading at 540 nm on a

spectrophotometer. One enzyme unit was defined as the amount of enzyme that releases 1 μmol of glucose per minute of reaction (Miller, 1959).

3.7 *In vitro* ruminal fermentations

The experimental analyses of *in vitro* ruminal fermentation were conducted at the Laboratory of Animal Nutrition (LANA) at the Center for Nuclear Energy in Agriculture - University of São Paulo, located in Piracicaba, SP, Brazil (Protocol nº 0003/2021), under the supervision of Professor Dr. Adibe Luiz Abdalla.

The produced biomasses were used as additives in *in vitro* ruminal fermentation tests and evaluated for gas release and *in vitro* degradability (Bueno et al., 2005). The live yeasts were tested at a proportion of 0.1% (g.g^{-1} of substrate - diet on a dry matter basis). The four yeast strains were cultivated in batch culture at 30°C and pH 5.5 using the FWR medium. The commercial probiotic Levumilk (pure lyophilized *Saccharomyces cerevisiae*) was tested at the same concentration as the yeasts in the current study, 0.1% (g.g^{-1} of substrate). The tests were conducted with a concentrated diet of silage and corn, with the control being performed without the addition of additives. Table 1 presents the diet ingredients and their proximate composition.

Table 1. Diet Ingredients and Their Bromatological Composition.

Ingredients	Concentrated Diet (%)	Diet Composition	Concentrate (g.kg^{-1})
Corn silage	46.00	DM	873.18
Tifton hay	3.00	OM	944.99
Ground corn	24.1	MM	55.06
Soybean meal	21.4	NDF	556.32
Citrus pulp	4.5	ADF	419.96
Urea	0	ADL	44.31
Mineral	1.00	CP	181.20
		Cellulose	375.65
		Hemicellulose	136.36

FDN: Neutral Detergent Fiber; CP: Crude Protein; ADF: Acid Detergent Fiber; NDF: Neutral Detergent Fiber; NFC: Non-Fiber Carbohydrates; RDP: Rumen Degradable Protein; RUP: Rumen Undegradable Protein; TDN: Total Digestible Nutrients; FDNF: Forage Neutral Detergent Fiber.

The ruminal content from four adult Santa Inês sheep fitted with rumen cannulas was collected on the day of incubation before the morning feeding and used as inoculum (Lima et al., 2018). The substrate used in the diet (1 gram) was prepared and weighed inside Ankom F57 fiber determination bags (Ankom Technology Corp., Macedon, NY, USA), heat-sealed, and placed in 160 mL glass bottles of the penicillin type filled with

50 mL of Menke buffer medium (Menke & Steingass, 1988) and 25 mL of inoculum. In each bottle, 0.5 mL of the treatment diluted in deionized water was added. The bottles were properly sealed with rubber caps and kept in a forced ventilation oven at 39°C for 120 hours of fermentation. A control bottle containing only substrate without the treatment was also included. The total gas volume produced in each bottle was determined using the equation:

$$V = (6.14 \times P)(n = 321, R^2 = 0.991) \quad \text{Eq. 1}$$

Where:

V = volume of gases (mL)

P = measured pressure (psi).

Pressure was measured at 0, 2, 4, 8, 16, 24, 30, 36 and 48 hours (transducer Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil), and the gas produced was collected immediately after the pressure measurement, following the same time intervals (forming a single *pool* of gas). The pool of 2, 4, 8, 16 and 24 hours (collection of 2 mL of gases) and the second *pool* of 30, 36, and 48 hours (collection of 3.33 mL of gases).

In each pressure measurement, the gases produced during incubation were collected and stored in 10 mL vacuum tubes for the determination of CH₄ concentration using a gas chromatograph (Shimadzu GC-2010, Tokyo, Japan) equipped with a flame ionization detector and an HP-Molesieve capillary column (30m × 0.53mm × 25μm) under the chromatographic conditions described by Lima et al., (2018).

The amount of methane was calculated using the following equation:

$$CH_4(mL.gMS^{-1}) = (HS + PG) \times CH_4(\%) \quad \text{Eq. 2}$$

Where:

HS = headspace (85 mL),

PG = volume of gas produced (mL),

CH₄ % = methane concentration (mL.mL⁻¹) (Longo et al., 2006).

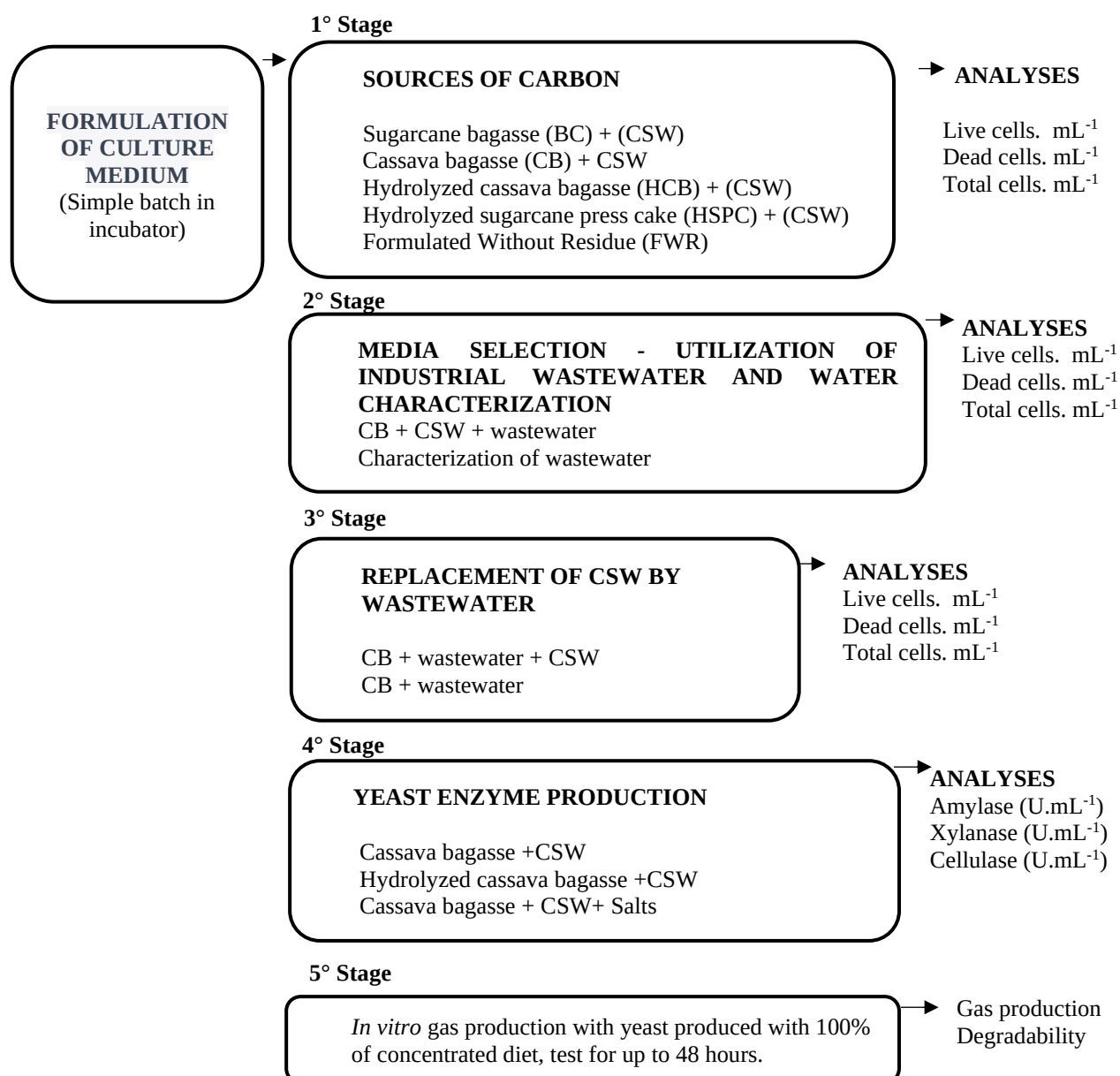
All bags underwent treatment with a neutral detergent solution at 90°C for 1 hour, followed by four 5-minute washes with deionized water at 90°C (Tecnal, mod. TE-149, Piracicaba, SP, Brazil), and finally, they were washed with acetone for 5 minutes. The bags were then placed in an oven at 105°C for 24 hours and subsequently weighed to

calculate the NDF degradability. They were then incinerated in a muffle furnace at 550°C for 5 hours (Prolab, mod. IN36, São Paulo/SP, Brazil) to determine the true degradability of organic matter. The truly degraded organic matter (MO) was calculated as the difference between the incubated MO and the non-degraded MO.

3.8 Statistical analysis

The assays were performed in triplicate in a single block, and the results were subjected to one-way analysis of variance (ANOVA), and the means were compared by the Tukey multiple sample comparison test ($p < 0.05$). Figures and tables were made using Microsoft Excel. The IBM® SPSS Statistics software was used for statistical analysis of microorganism cultivation. For the data obtained through the *in vitro* ruminal fermentation technique, R software version 4.1.2 (R Core Team, 2021) was used for statistical analysis. An analysis of variance (ANOVA) with mean comparisons was conducted using the Duncan test at a 5% significance level. Below is a simplified summary of the steps developed in this chapter (Figure 1).

Figure 1. Flowchart of the steps and analyses carried out for the cultivation of four yeast strains in media formulated with agro-industrial residues from cassava, corn, and sugarcane industries.



4 RESULTS AND DISCUSSION

4.1 Culture with residues

In this study, the selected yeasts were previously tested in media containing four different carbon sources, with Formulated Without Residue Medium (FWR) used as a comparison parameter. Table 2 presents the cell count data, including live, dead, total cells, and cell viability for each microorganism and nutrient used.

According to Tables 2 and 3, the development of each yeast varied depending on the medium. Among the evaluated residues, cassava bagasse stood out, showing cell viability above 94% for all tested yeasts, followed by hydrolyzed cassava bagasse, which obtained viability above 85% for all strains. *W. onychis* LABI2 and *S. cerevisiae* M26 achieved their best results, with 2.32×10^8 and 1.10×10^8 live cells per mL, respectively, when cultured in a medium containing 5% (w.v⁻¹) hydrolyzed cassava bagasse and 0.5% (v.v⁻¹) corn maceration water ($p < 0.05$). These findings are in line with Shinya et al., (2022), who reported higher growth for *W. onychis* LABI2 (1×10^9 cells.mL⁻¹) and *S. cerevisiae* M26 (3.2×10^8 cells.mL⁻¹) in hydrolyzed cassava bagasse. On the other hand, *S. japonicus* Sia 70a and *S. pararoseus* Sia 33.1 showed greater affinity for non-hydrolyzed cassava bagasse, reaching 2.4×10^8 and 1.2×10^8 live cells per mL, respectively, suggesting better metabolic adaptation to this non-hydrolyzed residue.

During the cultures with *S. japonicus* Sia 70a and *W. onychis* LABI2, using cassava bagasse and corn maceration water as culture media, it was observed that, after 48 hours, the number of live cells was higher compared to cultures in the Formulated Without Residue Medium (FWR). This finding was surprising, as the FWR medium contains glucose, yeast extract, and various readily available inorganic salts (Neto et al., 2004). The difference may be related to a variety of growth factors that can be present in a richer and more complex medium, even with organic residues (Marti et al., 2015).

The bromatological composition of cassava bagasse varies, generally showing high concentrations of total carbohydrates, ranging from 35.70% to 77.89%, and also total dietary fibers, from 7.39% to 47.2%. However, lipid content (0.31% to 1.95%), protein (1.36% to 1.42%), and ash (0.15% to 2.75%) are relatively low (Ronko et al., 2020). This composition is similar to what was observed by Jasko et al., (2011) with 31% dietary fiber, 18% crude fiber, 1.9% protein, and 42% starch. Additionally, cassava bagasse contains elements such as aluminum (25 mg.kg⁻¹), silicon (44 mg.kg⁻¹), sodium (370 mg.kg⁻¹), calcium (5300 mg.kg⁻¹), potassium (2297 mg.kg⁻¹), iron (84 mg.kg⁻¹), and sulfur

(567 mg.kg⁻¹) (Thuppahige et al., 2023), components that favored the cultivation of the selected microorganisms in cassava bagasse, as observed in Table 2.

Table 2. Growth of yeasts in batch cultures in incubator, with different carbon sources from agroindustrial wastes at 30°C, 180 rpm for 48 hours.

	SB (ac;ac;a;bc)	CB (a;c;b;b)	HCB (c;c;b;a)	HSPC (d;a;b;c)	FWF (a;a;a;b)
Live cells (cells.mL⁻¹)					
<i>S. pararoseus</i> (b;a;b;b;a)	1.52x10 ⁷	1.20x10⁸	6.00x10 ⁵	3.10x10 ⁷	1.01x10 ⁸
<i>S. japonicus</i> (a;b;a;a;c)	3.05x10 ⁷	2.40x10⁸	4.33x10 ⁶	3.99x10 ⁷	9.50x10 ⁷
<i>S. cerevisiae</i> (a;a;b;a;b)	5.47x10 ⁶	1.30x10 ⁷	1.10x10⁸	1.43x10 ⁷	9.10x10 ⁷
<i>W. onychis</i> (a;a;b;a;b)	3.60x10 ⁷	4.05x10 ⁷	2.32x10⁸	7.88x10 ⁶	1.94x10 ⁸
Dead cells (cells.mL⁻¹)					
	SB (a;a;a;a)	CB (a;a;b;a)	HCB (a;a;b;c)	HSPC (a;b;b;c)	FWR (a;b;b;c)
<i>S. pararoseus</i> (a;a;b;a;c)	1.79x10 ⁶	2.05x10 ⁶	1.00x10 ⁵	1.99x10 ⁶	5.00x10 ⁶
<i>S. japonicus</i> (a;a;b;c;c)	1.58x10 ⁶	2.53x10 ⁶	3.08x10 ⁵	1.28x10 ⁷	1.20x10 ⁷
<i>S. cerevisiae</i> (a;b;a;c;c)	2.67x10 ⁶	7.50x10 ⁵	1.63x10 ⁶	1.15x10 ⁷	2.50x10 ⁷
<i>W. onychis</i> (a;b;c;d;e)	3.65x10 ⁶	1.15x10 ⁶	5.15x10 ⁶	3.09x10 ⁷	9.55x10 ⁷
Total cells (cells.mL⁻¹)					
	SB (d;a;b;c)	CB (d;a;b;c)	HCB (c;c;b;a)	HSPC (d;a;b;c)	FWR (a;a;b;c)
<i>S. pararoseus</i> (a;b;c;d;e)	3.31x10 ⁷	1.22x10 ⁸	7.00x10 ⁵	3.29x10 ⁷	1.06x10 ⁸
<i>S. japonicus</i> (a;b;c;d;e)	3.21x10 ⁷	2.43x10 ⁸	4.64x10 ⁶	5.27x10 ⁷	1.07x10 ⁸
<i>S. cerevisiae</i> (a;b;c;d;e)	8.13x10 ⁶	1.38x10 ⁷	1.11x10 ⁸	2.58x10 ⁷	1.16x10 ⁸
<i>W. onychis</i> (a;a;c;d;e)	3.97x10 ⁷	4.17x10 ⁷	2.37x10 ⁸	3.88x10 ⁷	2.90x10 ⁸
Cell viability (%)					
	SB	CB	HCB	HSPC	FWR
<i>S. pararoseus</i>	45.97	98.32	85.71	93.96	95.28
<i>S. japonicus</i>	95.06	98.96	93.37	96.89	88.79
<i>S. cerevisiae</i>	67.21	94.55	98.53	55.34	78.45
<i>W. onychis</i>	90.79	97.24	97.83	20.31	66.90

SB: Sugarcane bagasse; CB: Cassava bagasse; HCB: Hydrolyzed cassava bagasse; HSPC: Hydrolyzed sugarcane press cake; FWF: Formulated Without Residue - Positive control. Analysis of variance (ANOVA) was used by the Tukey multiple sample comparison test, with a significance level of 0.05%. Letters in front of the microorganisms sequentially compare cell growth of each yeast strain among the 5 different media. Letters below the nutrients sequentially compare the growth of the four cultures in relation to each nutrient. Different letters indicate significant differences between the groups.

Table 3. Highlight of the main results of the live cell count of the four yeasts, according to the statistical analysis in Table 1.

Nutritional sources	Live cells. mL ⁻¹			
	<i>S. pararoseus</i> Sia 33.1	<i>S. japonicus</i> Sia 70a	<i>S. cerevisiae</i> M26	<i>W. onychis</i> LABI2
CB	1.20x10 ⁸	2.40x10 ⁸	-	-
HCB	-	-	1.00x10 ⁸	2.30x10 ⁸
HSPC	-	-	-	-
SB	-	-	-	-
FWR	1.00x10 ⁸	9.50x10 ⁷	9.10x10 ⁷	1.94x10 ⁸

Media formulated with 5% of the respective sources: SB: Sugarcane bagasse; CB: cassava bagasse; HCB: Hydrolyzed cassava bagasse; HSPC: Hydrolyzed sugarcane press cake; and 0.5% corn steep liquor. FWR: Formulated Without Residue - Positive Control. Live cell count according to Table 1.

The conducted cultivation revealed that the media formulated with 5% (w.v⁻¹) hydrolyzed sugarcane press cake (HSPC) or 5% (w.v⁻¹) sugarcane bagasse, even when formulated with 0.5% (v.v⁻¹) corn steep water, which contains a carbon and protein source, failed to reach the same cell density of 1x10⁸ cells.mL⁻¹ achieved in the other media.

In a previous study, Diaz et al., (2005) evaluated the production of the yeast *W. onychis* Lv027, also known as *P. onychis* Lv027, and observed that the yeast was strongly influenced by the tested nutritional and physicochemical conditions. The best results were obtained after 48 hours of liquid fermentation at 26°C with agitation at 110 rpm (with values ranging from 100 to 300 rpm), using 43.42 g.L⁻¹ of carbon source (with values tested between 0.12 and 0.5 g.L⁻¹), 0.261 g.L⁻¹ of organic nitrogen source (with values tested between 0 and 0.5 g.L⁻¹), and pH 6 (evaluated between 4.5 and 6.5), reaching a maximum production of 8.95x10⁹ cells.mL⁻¹. In the present work (Table 2), a carbon source of 50 g.L⁻¹ of residues was used, similar to the study by Diaz et al., (2005), but with higher agitation of 180 rpm and a lower pH of 5.5, resulting in a maximum production of 2.40x10⁸ cells.mL⁻¹.

In a more recent study, Shinya et al., (2022) observed that the yeasts *W. onychis* LABI2, *S. japonicus* 70a, and *S. pararoseus* 33.1 demonstrated high capacity for the absorption of glucose, maltose, and sucrose. However, none of these microorganisms were able to assimilate lactose, carboxymethylcellulose (CMC), or xylan for their development. On the other hand, Yarrow et al., (1965) assessed the development of *W. onychis* and found its ability to assimilate D-xylose, L-arabinose, succinic acid, lactic acid, citric acid, methyl α-D-glucoside, D-mannitol, D-sorbitol, among other nutrients, but not nitrate or growth in vitamin-free media, indicating a high capacity to assimilate

different substances but also the need for basic sources of vitamins for its proliferation and restriction regarding the absorption of antinutritional compounds like nitrate. Han et al., (2012) observed the development of *S. pararoseus* using glucose, sucrose, mannose, and galactose. Hamamoto et al., (2011) observed the assimilation of glucose, sucrose, galactose, soluble starch, and galactose, but not xylose, arabinose, and lactose for *S. japonicus*. However, variations in nutrient assimilation capacity may be related to differences among strains (Sampaio, 2011).

The nutrients that stood out in this study were cassava bagasse, both hydrolyzed and non-hydrolyzed, both containing glucose as the main monosaccharide after hydrolysis. Although xylose may also be available, these results demonstrate the higher affinity of these yeasts for nutrients rich in starch and monosaccharides than for those rich in fibers, such as sugarcane bagasse, despite observing cellular growth under these conditions.

In order to reuse the wastewater from the corn and starch industries and possibly reduce cultivation costs, fermentative tests and effluent characterization were performed. The waters were characterized regarding dry matter content, total reducing sugars, reducing sugars, pH, and protein content (Table 4).

Table 4. Chemical and physical-chemical characterization of cassava washing water and corn processing.

	WASTEWATER				
	CFP	DFW	Refin	CWW	CSW
pH	4.25±0.1	5.80±0.2	3.40±0.1	3.60±0.1	3.96±0.2
DM (%)	0.58±1.0	0.17±0.17	0.11 ±0.26	2.71±2.0	5.31±0.92
TRS (%)	0.58±0.09	0.50±0.04	0.45±0.005	0.02±0.03	70.71±0.8
RS (%)	0.39±0.01	0.05±0.001	0.39±0.07	0.01±0.005	21.75±0.5
Protein (%)	0.19±0.01	0.01±0.01	0.02±0.01	0.28±0.01	31.49±6.40

CSW: Corn steep liquor water; CFP: Corn factory process water; Refin: Refinery water; CWW: Primary water from cassava washing; DFW: Water from washing cassava starch drying floor, DM: Dry matter, RS: Reducing sugars, TRS: Total reducing sugars.

The CSW (Corn Steep Liquor Water) showed the highest nutritional content among the wastewater, indicating a nutritional potential for microorganism fermentation (Khan et al., 2014). The CWW (Primary Water from Cassava Washing) is the second effluent with the highest protein and dry matter content, followed by CFP (Corn Factory Process Water), Refin (Refinery Water), and DFW (Water from Washing Cassava Starch Drying Floor). The Refin and CWW residues exhibited the most acidic pH levels, with

values of 3.4 and 3.6, respectively, while the DFW and CFP had pH levels closer to the cultivation pH (5.5), with values of 5.8 and 4.25, respectively.

According to Martínez-Arcos et al., (2021), the CSW contains high levels of sugars, approximately 250 g.L^{-1} , a value similar to the one found in this study (217.58 g.L^{-1}), as well as lactic acid (6.7 g.L^{-1}), phytic acid (3.1 g.L^{-1}), acetic acid (2.8 g.L^{-1}), glucose (0.3 g.L^{-1}), arabinose (0.2 g.L^{-1}), and various ions such as chloride, phosphate, sulfate, calcium, boron, iron, potassium, magnesium, manganese, zinc, and silicon, with phosphate (0.5 g.L^{-1}) and potassium (0.8 g.L^{-1}) being the most representative. The observed pH for CSW by Martínez-Arcos et al., (2021) was 4.5 ± 0.2 , however, Choi et al., (2013) found values slightly lower (3.8 ± 0.17), closer to the values obtained in this study (pH 3.96).

Regarding the nitrogen content in CSW, Choi et al., (2013) also found the presence of nitrogen around 41.5 g.L^{-1} , equivalent to 25.94% protein. In this study, a higher value was found (31.5%), which is a perfectly acceptable variation for agro-industrial residues. The authors also identified various amino acids in CSW, including cysteine (617 mg.L^{-1}), glutamic acid (325 mg.L^{-1}), alanine (255 mg.L^{-1}), leucine (205 mg.L^{-1}), proline (969 mg.L^{-1}), aspartic acid (571 mg.L^{-1}), among others, demonstrating a high potential of CSW as a source of aminic nitrogen.

The effluents from the cassava industry contain in their composition total phosphorus (20 to 35 mg.L^{-1}), total nitrogen (80 to 150 mg.L^{-1}), total carbohydrates (330 to 400 mg.L^{-1}), among other components that can benefit the development of microorganisms in fermentative cultures (Colin et al., 2007). DFW, which is the residue from cassava washing, was not used in this study, but it is also a residue obtained from cassava processing and has a composition similar to the evaluated cassava processing effluents, containing phosphorus (160 ppm), potassium (1863 ppm), magnesium (405 ppm), zinc (4.2 ppm), manganese (3.7 ppm), sulfide (19.5 ppm), boron (5 ppm), and other essential mineral salts for microbial development (Dufour et al., 1996). The presence of mineral salts in culture formulation favors microbial development, as long as the salt concentration respects the sensitivity of the strains used in the culture (Souza et al., 2021).

Fermentation tests were carried out, replacing deionized water with water from cassava processing, in order to observe their potential for cell development (Table 5). The CSW was used to supplement the nutrient-rich fermentation medium.

Table 5. Comparison of yeast culture with medium formulation using different industrial wastewater at 30°C for 48 hours agitated at 180 rpm.

	Wastewater				Deionized water	
	CFP (a;a;b;c)	DFW (a;a;b;c)	Refin (a;b;c;d)	CWW (a;a;b;c)	DW (a,c,b,b)	FWR (c;a;a;b)
Live cells (cells.mL⁻¹)						
<i>S. pararoseus</i> (b;b;b;b;a;a)	2.13x10 ⁶	5.75x10 ⁵	6.25x10 ⁵	4.25x10 ⁵	1.20x10⁸	1.01x10⁸
<i>S. japonicus</i> (b;b;b;b;a;c)	4.08x10 ⁶	6.00x10 ⁵	6.35x10 ⁶	1.35x10 ⁶	2.40x10⁸	9.50x10⁷
<i>S. cerevisiae</i> (b;c;d;b;b;a)	3.65x10 ⁷	7.45x10 ⁶	1.53x10 ⁶	1.19x10 ⁷	1.30x10 ⁷	9.10x10⁷
<i>W. onychis</i> (e;b;d;a;d;a)	7.20x10 ⁷	4.20x10 ⁶	1.19x10 ⁷	1.39x10⁸	4.05x10 ⁷	1.94x10⁸
Dead cells (cells.mL⁻¹)						
	CFP (a;b;a;c)	DFW (a;a;b;c)	Refin (a;b;c;a)	CWW (a;b;b;b)	DW (a;a;b;a)	FWR (a;b;b;c)
<i>S. pararoseus</i> (a;b;c;d;c;a)	2.05x10 ⁶	4.25x10 ⁵	4.38x10 ⁶	7.33x10 ⁶	2.05x10 ⁶	5.00x10 ⁶
<i>S. japonicus</i> (a;b;c;d;d;c)	7.75x10 ⁵	4.75x10 ⁵	3.00x10 ⁶	1.06x10 ⁷	2.53x10 ⁶	1.20x10 ⁷
<i>S. cerevisiae</i> (a;a;a;b;b;c)	3.22x10 ⁶	3.65x10 ⁶	1.30x10 ⁶	2.02x10 ⁷	7.50x10 ⁵	2.50x10 ⁷
<i>W. onychis</i> (a;b;c;a;d;e)	3.95x10 ⁷	6.50x10 ⁵	8.03x10 ⁶	1.83x10 ⁷	1.15x10 ⁶	9.55x10 ⁷
Total cells (cells.mL⁻¹)						
	CFP (a;a;b;c)	DFW (a;a;b;c)	Refin (a;b;c;d)	CWW (a;a;b;c)	DW (d,a,b,c)	FWR (a;a;a;b)
<i>S. pararoseus</i> (ab;a;ab;b;d;c)	4.18x10 ⁶	1.00x10 ⁶	5.00x10 ⁶	7.75x10 ⁶	1.22x10 ⁸	1.06x10 ⁸
<i>S. japonicus</i> (bf;b;ab;e;d;c)	4.85x10 ⁶	1.08x10 ⁶	9.35x10 ⁶	1.19x10 ⁷	2.43x10 ⁸	1.07x10 ⁸
<i>S. cerevisiae</i> (be;b;ae;a;d;c)	3.97x10 ⁷	1.11x10 ⁷	2.83x10 ⁶	3.21x10 ⁷	1.38x10 ⁷	1.16x10 ⁸
<i>W. onychis</i> (a;b;c;d;e;f)	1.12x10 ⁸	4.85x10 ⁶	1.99x10 ⁷	1.57x10 ⁸	4.17x10 ⁷	2.90x10 ⁸
Cells viability (%)						
	CFP	DFW	Refin	CWW	DW	FWR
<i>S. pararoseus</i>	50.90	57.50	12.50	5.48	98.32	95.28
<i>S. japonicus</i>	84.02	55.81	67.91	11.30	98.96	88.79
<i>S. cerevisiae</i>	91.90	67.12	53.98	37.07	94.55	78.45
<i>W. onychis</i>	64.58	86.60	59.67	88.37	97.24	67.01

Culture medium used: 5% BM and 0.5% CSW, supplemented with wastewater. DW is the same medium (CB+CSW) but supplemented only with deionized water. Codes: CFP: Corn mill process water; DFW: Cassava starch drying floor washing water; Refin: Water from the refinery. CWW: Cassava washing water; CSW: Corn steeping water. Formulated Without Residue (FWR). Analysis of variance (ANOVA) was used by Tukey's test of multiple comparisons of samples, with a significance level of 0.05%. Letters in front of microorganisms sequentially compare the growth of each yeast cell on the five different media. Letters below the nutrients sequentially compare the growth of the four crops for each nutrient. Different letters indicate significant differences between groups.

Table 6 presents the best growths, expressed in live cells, of the cultures with the four yeasts in the formulated media containing the wastewater, results obtained from Table 5.

Table 6. Highlight of the highest yeast growths, expressed in live cells, for each medium formulated with different wastewater, based on the statistical analysis of Table 4.

Nutrients	Live cells.mL ⁻¹			
	<i>S. pararoseus</i> Sia 33.1	<i>S. japonicus</i> Sia 70a	<i>S. cerevisiae</i> M26	<i>W. onychis</i> LABI2
CFP	-	-	-	-
DFW	-	-	-	-
Refin	-	-	-	-
CWW	-	-	-	1.39x10 ⁸
DW	1.20x10 ⁸	2.40x10 ⁸	1.30x10 ⁷	-
FWR	1.01x10 ⁸	9.50x10 ⁷	9.10x10 ⁷	1.94x10 ⁸

Culture medium used: 5% CB and 0.5% CSW, supplemented with wastewater. DW is the same medium, however (CB and CSW) supplemented only with deionized water. CFP: Corn mill process water; DFW: Cassava starch drying floor washing water; Refin: Water from the refinery. CWW: Cassava washing water; FWR: Formulated Without Residue:

According to Tables 5 and 6, *W. onychis* LABI2 achieved the best results with the CWW wastewater (1.39x10⁸ cells.mL⁻¹), unlike the other strains, which were inhibited by all these wastewater sources, considering that the medium formulated with deionized water (DA) showed superior performance for the other yeasts. All the other wastewater sources behaved statistically similar in terms of low yeast growth. The results indicate the potential of the CWW wastewater for the cultivation of *W. onychis* LABI2, which could be used for a more ecological and sustainable disposal of this residue. It is also possible to infer that this yeast is more resistant to inhibitory factors that may be present in these wastewater sources.

The higher concentration of dead cells in all cultures with the CWW wastewater, combined with the fact that this water showed the highest nutrient content (Table 4), may explain the metabolic inhibition due to nutrient excess in the medium.

Table 7 presents cultures with non-hydrolyzed cassava bagasse, with and without corn steep liquor water (CSW), using different cassava industry washing waters in the medium formulation, to observe the need for CSW addition.

Table 7. Comparison of yeast cultivation using media formulated with cassava bagasse with and without corn maceration water and industrial wastewater, performed at 30°C for 48 hours at 180 rpm.

	Cassava bagasse				Cassava bagasse + CSW				
	CFP (a;a;c;b)	DFW (a;b;a;c)	Refin (a;b;a;c)	CWW (a;a;b;b)	CFP (a;a;c;b)	DFW (a;b;c;d)	Refin (a;b;c;d)	CWW (a;a;b;c)	DW (a,c,b,b)
Live cells (cells.mL ⁻¹)									
SP (a;b;c;d;e;c;d;f)	5.00x10 ⁴	2.42x10 ⁷	6.71x10⁷	4.75x10 ⁵	8.75x10 ⁵	9.83x10 ⁵	8.42x10⁷	2.50x10 ⁵	1.20x10⁸
SJ (a;a;c;a;b;a;d;a;d)	2.83x10 ⁵	2.92x10 ⁵	6.96x10 ⁶	3.50x10 ⁵	9.75x10 ⁵	3.67x10 ⁵	2.21x10⁸	4.50x10 ⁵	2.40x10⁸
SC (a;a;b;c;d;a;a;e;f)	5.87x10 ⁷	5.01x10 ⁷	6.37x10 ⁷	1.34x10⁸	3.50x10 ⁷	5.48x10 ⁷	4.70x10 ⁷	8.63x10⁷	1.30x10 ⁷
WO (a;a;a;b;a;a;c;d)	2.83x10 ⁸	2.67x10 ⁸	2.44x10 ⁸	3.30x10 ⁸	2.09x10 ⁸	2.04x10 ⁸	1.55x10 ⁸	1.15x10 ⁸	4.05x10 ⁷
Dead cells (cells.mL ⁻¹)									
SP (a;b;b;a;a;b;b;a;b)	1.67x10 ⁵	5.67x10 ⁵	5.15x10 ⁶	5.50x10 ⁵	3.67x10 ⁵	2.13x10 ⁶	8.25x10 ⁶	6.75x10 ⁵	2.05x10 ⁶
SJ (a;a;b;b;a;c;c;b;b)	1.67x10 ⁵	3.00x10 ⁵	9.83x10 ⁵	9.25x10 ⁵	2.83x10 ⁵	8.82x10 ⁶	4.80x10 ⁶	1.03x10 ⁶	2.53x10 ⁶
SC (a;a;a;b;c;c;d;e)	3.55x10 ⁷	1.20x10 ⁷	2.77x10 ⁷	1.30x10 ⁷	7.72x10 ⁷	1.55x10 ⁷	2.40x10 ⁷	4.50x10 ⁶	7.50x10 ⁵
WO (a;a;a;b;a;a;d;d)	7.48x10 ⁷	7.95x10 ⁷	4.90x10 ⁷	3.10x10 ⁷	1.30x10 ⁸	2.75x10 ⁷	8.23x10 ⁷	5.00x10 ⁶	1.15x10 ⁶
Total cells (cells.mL ⁻¹)									
SP (a;c;f;a;a;a;e;a;d)	1.49x10 ⁶	2.57x10 ⁷	6.88x10 ⁷	1.03x10 ⁶	1.33x10 ⁶	1.41x10 ⁶	8.90x10 ⁷	9.25x10 ⁵	1.22x10 ⁸
SJ (a;a;a;a;a;b;a;c)	4.50x10 ⁵	5.42x10 ⁵	8.01x10 ⁶	1.28x10 ⁶	1.30x10 ⁶	5.78x10 ⁵	3.45x10 ⁸	1.48x10 ⁶	2.43x10 ⁸
SC (c;b;e;f;c;b;c;d)	9.42x10 ⁷	6.21x10 ⁷	1.00x10 ⁸	1.47x10 ⁸	1.46x10 ⁸	9.76x10 ⁷	4.98x10 ⁷	9.08x10 ⁷	1.38x10 ⁷
WO (bf;bg;a;f;g;a;c;e;d)	3.58x10 ⁸	3.47x10 ⁸	2.97x10 ⁸	3.61x10 ⁸	3.39x10 ⁸	3.08x10 ⁸	1.58x10 ⁸	1.20x10 ⁸	4.17x10 ⁷
Cell viability (%)									
SP	93.64	95.10	93.99	46.34	65.63	96.41	95.49	27.03	98.32
SJ	60.00	88.37	89.28	27.45	72.58	92.50	94.67	30.41	98.96
SC	46.18	64.34	70.30	91.16	13.29	54.95	57.61	95.04	94.55
WO	71.03	70.51	79.07	91.41	42.53	72.30	66.85	95.83	97.24

Culture medium used: 5% CB supplemented with wastewater with or without the addition of 0.5% CSW. DW: deionized water, CFP: water from corn processing plant; DFW: water from the floor cleaning of cassava starch drying area; Refin: water from the refinery; CWW: cassava washing water; CSW: corn steep water; WO: *W. onychis* LABI2; SP: *S. pararoseus* Sia 33.1; SJ: *S. japonicus* Sia 70a; and SC: *S. cerevisiae* M26. Analysis of variance (ANOVA) was used by the Tukey's multiple comparison test, with a significance level of 0.05%. Letters in front of the microorganisms sequentially compare the cell growth of each yeast for the nine different media. Letters below the nutrients sequentially compare the growth of the four cultures for each nutrient. Different letters indicate significant differences between groups.

A summary of the best results observed for each evaluated nutrient is presented in Table 8.

Table 8. Highlight of the best yeast growth based on the evaluation of the number of live cells referring to Table 7.

Nutrients	Live cells. mL ⁻¹			
	<i>S. pararoseus</i> 33.1	<i>S. japonicus</i> 70a	<i>S. cerevisiae</i> M26	<i>W. onychis</i> LABI2
BM+ wastewater				
CFP	-	-	-	2.83x10 ⁸
DFW	-	-	-	2.67x10 ⁸
Refin	6.71x10 ⁷	-	-	2.44x10 ⁸
CWW	-	-	1.34x10 ⁸	3.30x10 ⁸
CB+CSW + wastewater				
CFP	-	-	-	2.09x10 ⁸
DFW	-	-	-	2.04x10 ⁸
Refin	8.42x10 ⁷	2.21x10 ⁸	-	-
CWW	-	-	8.63x10 ⁷	-
CB +CSW+ DW	1.20x10 ⁸	2.40x10 ⁸	-	-

Cultivation medium used: 5% CB supplemented with wastewater with or without the addition of 0.5% CSW. CB: Cassava bagasse; HCB: Hydrolyzed cassava bagasse; CFP: Corn factory process water; DFW: Cassava starch drying floor washing water; Refin: Refinery water; CWW: Cassava washing water; CSW: Corn maceration water, DW - deionized water.

Among the growth of yeasts with the mixture of CSW and industrial wastewater, previous tests are confirmed regarding both the remarkable growth of the yeast *W. onychis* LABI2 and its resistance to excess nutrients in the medium caused by the combination of CSW with each of these wastewater sources. Another important aspect is the unnecessary use of CSW in the medium formulation when any of the industrial wastewaters are used, especially BM+CWW. In this regard, the cassava washing water (CWW), present in starch factories, is the largest liquid waste generated, requiring significant resources for its biological treatment before being discharged into rivers to prevent ecological damage. Furthermore, these results demonstrate the consistency of the work, as they confirm previous tests already conducted (Tables 7 and 8).

On the other hand, *S. pararoseus* Sia 33.1 showed the highest sensitivity to excess nutrients caused by the combination of CSW and industrial wastewater, instead of deionized water, in the medium formulation. Therefore, for this yeast, the most suitable option is BM+CSW+AD (Tables 7 and 8).

The yeast *S. cerevisiae* M26 can be cultivated with BM + CWW, thus also fulfilling an ecological role by using this waste in the medium formulation, which becomes more cost-effective as there is no need for CSW addition. In reality, the best

medium for this yeast, according to the tests presented so far, is BMH + CSW or CWW, but never both waters combined.

The yeast *S. japonicus* Sia 70a also showed high sensitivity to the medium formulation with cassava bagasse, CSW, and industrial wastewaters (Tables 5 and 6). The comparison tests of the medium formulation demonstrated the yeast's sensitivity to excess nutrients, except for the medium BM+CSW+Refin, which showed the second-best result after the medium formulated with BM+CSW+AD.

Thus, the yeasts showed growth inhibition in at least three of the four evaluated industrial wastewaters, except *W. onychis* LABI2, which stood out by presenting a minimum development of 2×10^8 cells.mL⁻¹ in all cultures with wastewater + BM, indicating higher resistance to the nutrient load and/or possible toxins present in the industrial wastewaters. This inhibition is expected, given that industrial effluents commonly contain antinutritional substances or toxins that hinder or inhibit microbial development in fermentative cultures (Abbas et al., 2020).

Industrial wastewater is one of the major problems for starch factories, which need to use around 10 to 40 m³ of water per ton of starch produced (Santos et al., 2019). The characteristics of industrial wastewaters depend on the efficiency level of the machines used in the factory. A small factory producing 1 to 5 tons of cassava root consumes approximately 12 m³ of water per ton of cassava, resulting in a chemical oxygen demand (COD) of 100 kg.ton⁻¹ of root (Colin et al., 2007).

The high production of effluents is a problem for the industry, considering the high biochemical oxygen demand (BOD). Additionally, linamarin, a molecule present in all parts of the cassava plant except the seeds, can produce glucose, acetone, and hydrocyanic acid through hydrolysis, factors that increase the toxic potential in the residues, mainly because linamarin is water-soluble (Nascimento Rangel et al., 2008).

Starch factories use more water in their processes compared to cassava flour production, which reduces the pollutant load due to dilution. The main characteristics of starch extraction effluent have an average pH of 3.6 to 6.5; COD of 4200 to 7000 mg.L⁻¹, BOD of 110 to 3900 mg.L⁻¹, total carbohydrates of 330 to 400 mg.L⁻¹, lactic acid of 1200 to 2000 mg.L⁻¹, acetic acid of 330 to 400 mg.L⁻¹, total nitrogen of 80 to 150 mg.L⁻¹, total phosphorus of 20 to 35 mg.L⁻¹, cyanide of 3 to 5 mg.L⁻¹, among other factors (Colin et al., 2007).

The liquid residues from cassava contain mineral salts such as nitrogen, carbon, phosphorus, potassium, calcium, magnesium, sulfur, zinc, manganese, copper, iron, and

sodium, making them attractive for various applications to add value to the material. Different research has explored applications of these effluents as fertilizers, biogas production, and microbial growth considering their characteristics, including the presence of salts, in addition to the large quantity of effluents generated. However, due to the presence of toxic compounds such as cyanide, studies also investigate their potential as insecticides, herbicides, nematicides, among others, taking into account that minimum concentrations of certain mineral salts inhibit microbial development (Ubalua, 2007, Okunade & Adekalu, 2013; Jekayinfa et al., 2020).

4.2. Enzymatic activity

The activities of cellulases, xylanases, and amylases, produced by the yeasts in three different formulated cultures, namely, CB+CSW+DA, as well as HCB+CSW+DA and HCB+CSW+Salts, were evaluated to observe the enzymatic activity for each yeast (Figure 2 and 3).

No cellulolytic activity was observed in any of the evaluated cultures; however, according to the literature, these selected microorganisms (except *S. cerevisiae*) have the potential for cellulolytic enzyme production. Baffi et al., (2011) observed 1 U.mL⁻¹ of beta-glucosidase in the cultivation of *S. pararoseus* in 72 hours with cellobiose (YPC medium). Shinya (2017) observed cellulase activity (FPase) by *S. japonicus* 70a (0.15 FPU.mL⁻¹), *W. onychis* LABI2 (0.01 FPU.mL⁻¹), and *S. pararoseus* 33.1 (0.18 FPU.mL⁻¹) in a culture with sugarcane bagasse and whey.

The substrate used by the authors, sugarcane bagasse, is a fibrous residue with 50 to 80% fiber, according to Lazarini (2020), containing approximately 50% cellulose, 25% hemicelluloses, and 20-25% lignin (Rodrigues et al., 2020; Moura et al., 2021). On the other hand, corn cob presents around 26.5% cellulose, 25.8% hemicellulose, and 34.1% lignin (Lobo et al., 2021), higher fiber concentration compared to cassava bagasse, a fibrous-starchy residue with 45.2% starch and 35.6% insoluble fiber (Coelho, 2018), with 28.5% cellulose, 31.3% hemicellulose, and 22.2% lignin (Zanatta, 2021). Although cellobiose is not as complex a fibrous material as the residues used, it plays an important role in cellulolytic stimulation. This is because it is a repetitive disaccharide of cellulose, a product resulting from its degradation (Florencio et al., 2017).

All evaluated yeasts showed xylanolytic activity (Figure 2). The results indicate enzymatic degradation over time in cultivation, along with variation in enzymatic activity

according to the substrate used. The highest xylanase activity was observed for *W. onychis* LAB2 with 0.44 U.mL^{-1} on hydrolyzed cassava bagasse, followed by *S. cerevisiae* M26 with 0.43 U.mL^{-1} . The microorganisms *S. pararoseus* 33.1 and *S. japonicus* 70a achieved equal productions of 0.36 U.mL^{-1} , also in hydrolyzed cassava bagasse.

The results are consistent with those observed in the literature, as demonstrated by Shinya (2017), who found similar values, 0.25 U. mL^{-1} of xylanase for *S. pararoseus* 33.1, as well as 0.34 U.mL^{-1} for *W. onychis* LABI2 and 0.52 U.mL^{-1} for *S. japonicus* 70a. The yeast *S. cerevisiae* M26 does not produce amylases, although xylanases were observed (0.43 U. mL^{-1}), the enzymatic activity observed is not as significant compared to filamentous fungi such as *Trichoderma reesei*, which reached 66.93 U.mL^{-1} using the residue from the plant *Reutealis trisperma* (Riadi et al., 2019), the filamentous fungus *Aspergillus* cf. *tubingensis* LAMAI 31 produced 561.59 U.mL^{-1} with xylan as a carbon source (Dos Santos et al., 2016), among many others, as well as bacteria like *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp. However, regarding the yeast *S. cerevisiae*, there are several studies that induce enzyme activity through genetic modifications (Yeasmin et al., 2011; Sari & Wuryanti, 2013).

In previous tests (Table 2), several residues were evaluated for the production of the four studied yeasts, among which CB and HCB together with CSW were the most relevant. Among these two, HCB contains more RS and less starch and was more favorable to the production of xylanases by the studied yeasts (Figure 2b). The HCB+CSW associated with a set of salts (Figure 2c) did not increase xylanase production; on the contrary, it obtained the lowest enzymatic activity of the three residues evaluated.

Amylase enzymatic activity was observed only for *S. japonicus* Sia 70a and *W. onychis* LABI2 with 0.42 and 0.63 U.mL^{-1} of amylase, respectively, in cultivation formulated with hydrolyzed cassava bagasse and CSW (Figure 3). The results indicate that the hydrolyzed residue (HCB) presented greater potential compared to the non-hydrolyzed residue (CB).

Pham et al., (2022) observed glucoamylase activity of 0.54 U.mL^{-1} with *S. pararoseus* O1 cultivated in solid medium with cassava bagasse, similar to what was observed by Shinya et al., (2022), who reached 0.4 U. mL^{-1} of amylase activity for *S. pararoseus* 33.1 in hydrolyzed cassava bagasse, indicating the capacity for amylolytic enzyme production and degradation of starchy substrates by the microorganism, although not observed in the current study. The same authors observed amylase activity by *S.*

japonicus in cultures with non-hydrolyzed cassava bagasse (0.2 U.mL^{-1}). However, they did not observe amylolytic activity by *W. onychis* LABI2, unlike the current study, which found up to 0.63 U.mL^{-1} in hydrolyzed cassava bagasse.

The production of amylases by *W. onychis* is consistent with its applications in the production of fermented foods in some cultures, such as Lafun, a fermented cassava-based food consumed in West Africa, produced by the fermentation of a group of microorganisms, including *W. onychis*. Kimchi is another example of a fermentate that uses different microorganisms, including *W. onychis* and *S. cerevisiae*, being a Korean dish usually composed of cabbage or Chinese cabbage, rice flour, and different spices (Lee et al., 2018; Kang et al., 2019).

The use of *W. onychis* and *S. cerevisiae* in traditional foods, rich in fiber and starch, indicates the potential of these microorganisms to develop in substrates with such chemical characteristics, possibly through the production of enzymes for the hydrolysis of these materials. Therefore, it can be said that the presence of *W. onychis* in starch-based foods may be an indicator of its capacity to hydrolyze this material for its growth (Bankole et al., 2019; Ogunnaike et al., 2015).

Figure 2. Xylanolytic activity obtained through the cultivation of different yeasts using submerged fermentation with the substrate cassava bagasse (a), hydrolyzed cassava bagasse (b), and hydrolyzed cassava bagasse + Salts (c). Cultivations were carried out with 0.5% corn maceration water plus specific substrate at 180 rpm, 30°C, pH 5.5 for 72 hours

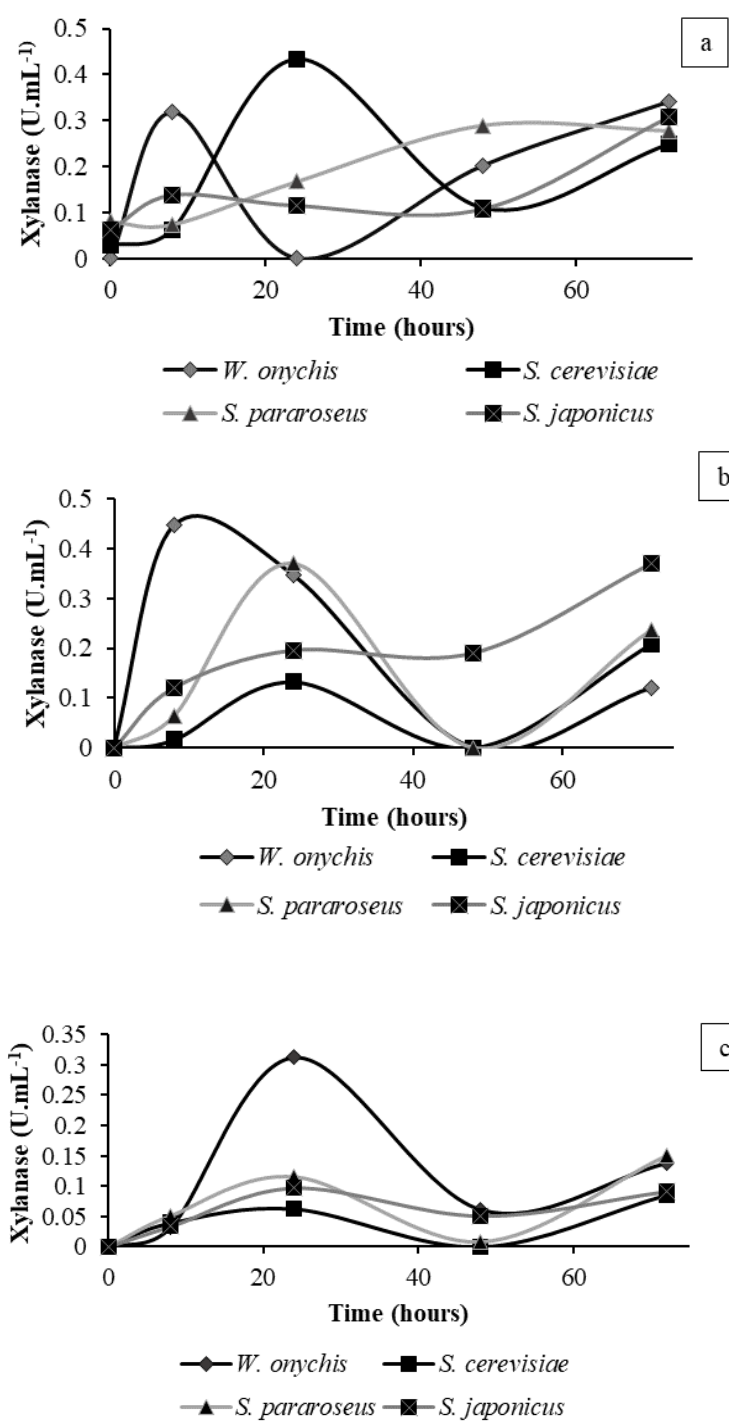
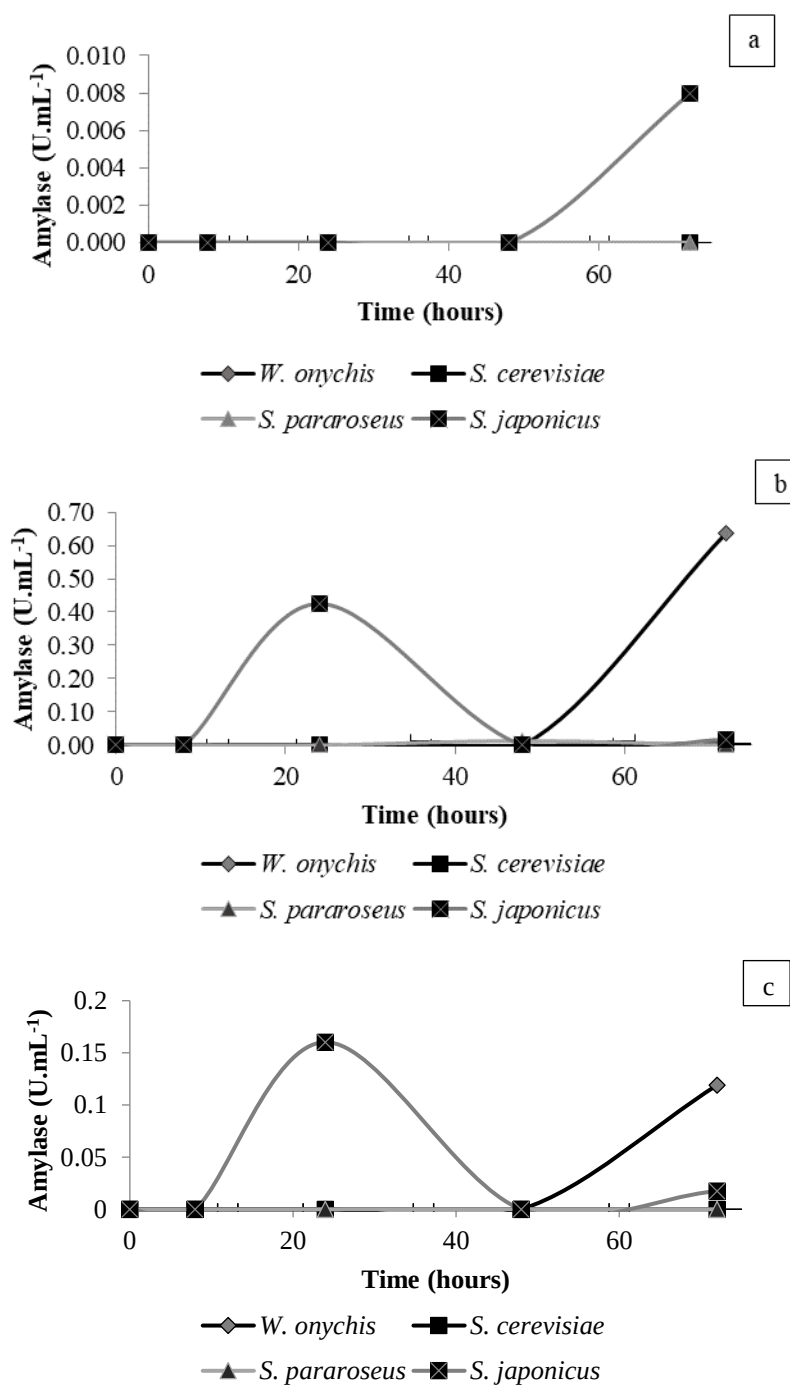


Figure 3. Amylolytic activity obtained through the cultivation of different yeasts by submerged fermentation using the substrates cassava bagasse (a), hydrolyzed cassava bagasse (b), and hydrolyzed cassava bagasse + salts (c). Cultivations were carried out with 0.5% corn maceration water plus specific substrate at 180 rpm, 30 °C at pH 5.5 for 72 hours.



5. ECONOMIC ANALYSIS

For the economic analysis, we used the substrate combinations shown in Table 2, in addition to the formulated without residue medium (FWR) and YM broth (Enzymatic digest of gelatin 5.0 g.L⁻¹, malt extract 3.0 g.L⁻¹, dextrose 10.0 g.L⁻¹ and yeast extract 3.0 g.L⁻¹), a well-known and widely used medium for yeast cultivation (Table 9). Biomass production was calculated based on the data presented in Table 2. As observed, both the residue-free formulated medium and YM broth have a high cost per liter compared to media formulated with residues. YM broth consists of only four ingredients, but with high costs (malt extract, yeast extract, dextrose, and enzymatic digestion of gelatin), while FWR contains twice as many ingredients, including essential micronutrients. Media formulated with residues showed very low costs, especially those that did not undergo prior enzyme or KOH treatments, with sugarcane bagasse and cassava bagasse supplemented with corn maceration water (SB+CSW and CB+CSW) standing out.

The costs associated with each microorganism are directly influenced by biomass production efficiency and the cost of the culture medium used. Therefore, the results revealed that the medium with cassava bagasse (CB+CSW) provided higher yield for *S. pararoseus* Sia 33.1 (14 \$.kg⁻¹) and *S. japonicus* Sia 70a (13.6 \$.kg⁻¹), while *S. cerevisiae* M26 (45.99 \$.kg⁻¹) and *W. onychis* LABI2 (121.62 \$.kg⁻¹) achieved better results in hydrolyzed cassava bagasse (HCB + CSW + Amylase). Based on the economic analysis, we can state that the production costs of the microorganism *W. onychis* LABI2, when associated with cassava bagasse and corn maceration water, are 271 times lower compared to the use of FWR or 131 times lower compared to the use of YM broth, considering the same production level as FWR.

Despite the superior production observed in hydrolyzed cassava bagasse for *S. cerevisiae* M26 and *W. onychis* LABI2, media containing cassava bagasse and sugarcane bagasse also proved to be promising. Considering the high cost associated with yeast production in commercial or formulated media, the use of low-cost residues as fermentation substrates emerges as an economically viable alternative.

It should be noted that input prices were based on quotations obtained in October 2023 from Quimicenter produtos para laboratórios LTDA, while the costs of agro-industrial residues were referenced from the MF Rural - Mercado Físico Rural website, and data on corn maceration water were obtained from the study by Tan et al., (2016).

Table 9. Economic analysis of cultivation media formulated with agro-industrial residues and standard media.

Medium composition	Input price (\$.kg ⁻¹ or \$. liter ⁻¹)	Cost of medium (\$. liter ⁻¹)	Cost.kg ⁻¹ of biomass produced			
			<i>S. p</i>	<i>S. j</i>	<i>S. c</i>	<i>W. o</i>
SB + CSW		0.07	39.73	79.55	143.25	165.63
CB + CSW		0.10	14.00	13.65	109.60	204.77
HCB + CSW +Amylase		0.33	8235.26	2412.20	45.99	121.62
HSBC + CSW + KOH		20.75	11164.23	13532.23	12607.61	47332.88
FWR		181.89	30370.68	58416.03	24577.07	55505.04
YM Broth	4205.78	88.32	14747.26	28365.39	11934.02	26951.88
Inputs for media preparation						
	Concentrat ion (%)	Input price (\$.kg⁻¹ or \$. liter⁻¹)				
SB	5.00	0.85				
CB	5.00	1.29				
SBC	5.00	2.23				
CSW	0.50	6.40				
KOH	4.00	515.23				
Amylase	1.2 U.mL ⁻¹	7.64				

Considering 1 dollar equal to 5.16 reais. SB: Sugarcane bagasse; CB: cassava bagasse; HCB: Hydrolyzed cassava bagasse; HSPC: Hydrolyzed sugarcane press cake; SBC: Sugarcane press cake; Corn steep water (CSW). FWR: Formulated Without Residue. KOH: Potassium hydroxide. Amylase (10 g of wheat bran + 1.25% (NH₄)₂SO₄, 0.25% KH₂PO₄, and 1.25% urea); Considering production of 40 U.mL⁻¹. W.o: *W. onychis* LABI2; S.p: *S. pararoseus* Sia 33.1; S.j: *S. japonicus* Sia 70a; and S.c: *S. cerevisiae* M26. Production of YM broth calculated considering production equal to FWR medium. The weight of each yeast was considered to calculate the cost per kilogram of biomass.

6. *In vitro* RUMINAL FERMENTATION WITH CONCENTRATE-BASED DIET

The actual data observed through *in vitro* ruminal fermentation conducted for up to 48 hours are presented in Table 10. The results did not show any statistical differences for the 24 hour incubation period for any of the treatments evaluated, including total gas production, methane, and organic matter degradability, a pattern also observed by Weinberg et al., (2007), who found differences only after 24 hours of fermentation with *S. cerevisiae*. The lack of statistical difference at 24 hours of fermentation may indicate yeast adaptation to the new environment or even yeast mortality.

Fermentation up to 48 hours exhibited significant differences in dry matter degraded with the W.o (575.50 g.kg⁻¹ DM) and S.p (567.16 g.kg⁻¹ DM) treatments compared to the Control (538.93 g.kg⁻¹ DM). All the other treatments numerically showed higher degradation than the Control, although they did not demonstrate statistical differences. The statistical difference observed in dry matter degradability was also noted by Mohanty et al., (2023) and Ardani et al., (2023) when using *S. cerevisiae*,

corroborating the findings. Organic matter degradability was statistically higher for the LMLK (Levumilk) treatment (558.75 g.kg⁻¹ OM), S.p treatment (558.37 g.kg⁻¹ OM), and W.o treatment (566.68 g.kg⁻¹ OM) compared to the Control (528.34 g.kg⁻¹ OM). There was a statistically greater truly degraded organic matter (TDOM), neutral detergent fiber degradability (NDFD), and organic matter degradability in the treatment with W.o compared to the Control, a fact also observed by Ardani et al., (2023) when applying *S. cerevisiae*. Organic matter degradability for the W.o treatment was 7.25% higher than the Control. Yeast in 48 hours of fermentation can serve as a nutritional source due to cell death, but also, if alive, it can help absorb oxygen, which allows for greater anaerobiosis in the ruminal environment, favoring rumen cellulolytic microorganisms (Ardani et al., 2023), which may be correlated with the improvement in dry and organic matter degradability observed in the current research.

In the study conducted by Phesatcha et al., (2020) in *in vitro* fermentations, evaluations were carried out considering the variation in the ratio of forage to concentrate (with V:C ratios of 80:20, 60:40, 40:60, and 20:80) and four different concentrations of live yeast, specifically *S. cerevisiae*, with values of 0.0, 2.0x10⁶, 4.0x10⁶, and 6.0x10⁶ CFU.0.2g⁻¹ of diet. The research results indicated that the combination of a forage to concentrate ratio of 20:80, along with the addition of 6.0x10⁶ CFU.0.2g⁻¹ of diet, equivalent to 3x10⁷ CFU.g⁻¹, resulted in significant improvements in ruminal fermentation, feed digestibility, and a reduction in CH₄ production. When comparing the concentrations defined by Phesatcha et al., (2020), it is important to note that the quantities of live yeast used in the current work varied by species, being lower for *S. cerevisiae* and *S. pararoseus* (1.57x10⁷ cells.g⁻¹ of diet) and higher for *S. japonicus* (3.4x10⁷ cells.g⁻¹) and *W. onychis* (8x10⁷ cells.g⁻¹). However, it was observed that the increase in live yeast contributed to the improvement in organic matter degradability, although it did not impact methane production.

No differences were observed in total gas and methane production at 48 hours; however, numerically, it is possible to observe a 3.44% increase in total gas production compared to the Control, as well as an increase in methane production per total gas produced of 9.96% for W.o. In 48 hours of fermentation, supplementation with W.o and S.p stood out compared to the other microorganisms and the Control, highlighting the potential of the microorganism in terms of amylase and xylanase production and its cultivation in starchy materials (Shinya et al., 2022).

Furthermore, the inclusion of yeast as probiotics in the diet of ruminants has already shown an increase in the total count of bacteria and fungal zoospores, as well as a reduction in protozoa populations ($p < 0.05$) and greater nitrogen absorption and efficiency of microbial protein production in nutrient-rich diets (Phesatcha et al., 2021). In research conducted with live *S. cerevisiae* yeast in live animals, a notable increase in final body weight gain was observed, with 39.1 kg per calf (Maamouri et al., 2022), a fact related to improved digestibility and a reduction in diarrhea (Ma et al., 2023). Future research can explore the impact of W.o in *in vivo* ruminal fermentation to examine how improved digestibility affects the live weight of animals. Additionally, a more extensive and precise investigation into the relationship between methane production and weight gain can be conducted.

Table 10. Dataset obtained through *in vitro* fermentation conducted for 24 and 48 hours with a concentrated diet for live microorganisms.

	DMD (g. kgMS ⁻¹)		OMD (g. kgMOD ⁻¹)		TDOM (g. kgMOD ⁻¹)		NDFD		pH	GP (mL. gDM ⁻¹)	CH ₄ (mL. gDM ⁻¹)	GP (mL. gMOD ⁻¹)	CH ₄ (mL. gMOD ⁻¹)	PF
24 hours														
Control	473.74		464.17		0.39		258.79		6.60	78.18	3.74	33.85	1.35	4.96
LMLK	477.80		464.81		0.39		259.67		6.60	77.64	5.07	30.62	2.37	5.05
S.c	475.08		462.03		0.39		255.83		6.58	82.80	5.83	34.75	2.71	4.66
S.j	475.52		482.02		0.40		283.48		6.54	85.21	5.57	23.01	2.66	4.72
S.p	476.50		466.80		0.39		262.41		6.59	78.41	5.17	29.93	2.42	5.01
W.o	468.44		456.55		0.38		248.24		6.60	76.56	5.41	21.85	2.50	4.98
p-Value	0.9684		0.9795		0.9796		0.9795		0.9727	0.5527	0.2837	0.9835	0.3045	0.249
48 hours														
Control	538.93	c	528.34	bc	0.44	c	347.55	c	6.44	116.58	4.92	60.71	2.61	3.80
LMLK	568.91	ab*	558.75	a*	0.47	ab	389.61	ab	6.38	124.41	4.91	52.39	2.81	3.78
S.c	542.26	bc	532.10	c	0.44	bc	352.75	bc	6.40	114.92	6.15	50.70	3.30	3.86
S.j	552.52	abc	544.24	abc	0.45	abc	369.54	c	6.34	115.14	5.13	40.42	2.95	3.98
S.p	567.16	ab*	558.37	a*	0.47	ab	389.08	ab	6.42	123.64	5.29	62.20	3.02	3.79
W.o	575.50	a*	566.68	a*	0.47	a*	400.58	a*	6.40	121.84	5.21	62.80	2.96	3.90
p-Value	0.0136		0.0088		0.0190		0.0190		0.6049	0.1096	0.6140	0.0973	0.2289	0.058

The Duncan test was used with a significance level of 0.05%. *Statistically different from the Control. Treatments: microorganisms cultured at 30°C, pH 5.5: S.j: *S. japonicus* Sia 70a, W.o: *W. onychis* LABI2, S.c: *S. cerevisiae* M26, S.p: *S. pararoseus* Sia 33.1; Control: Concentrated diet. OMD: organic matter degradability (g. kgOM⁻¹); DMD: dry matter degradability (g. kgDM⁻¹); TDOM: truly degraded organic matter (g. kgOM⁻¹); NDFD: Neutral Detergent Fiber Degradability. GP: gas production per dry matter (mL. gDM⁻¹) and per organic matter (mL. gOM⁻¹); CH₄: methane production per dry matter (mL. gDM⁻¹) and per organic matter (mL. gOM⁻¹); PF = partition factor.

7. CONCLUSION

The tested combinations revealed a high potential for utilizing residues from both cassava and corn starch industries. Notably, cassava bagasse without hydrolysis (CB), hydrolyzed cassava bagasse (HCB), and corn maceration water (CSW) stood out as suitable substrates for the growth of *S. cerevisiae* M26, *S. pararoseus* Sia 33.1, *S. japonicus* Sia 70a, and *W. onychis* LABI2 yeast strains. The industrial wastewater also showed potential for utilization, particularly cassava washing water (CWW), although it was found to be particularly beneficial for *W. onychis* LABI2 and *S. cerevisiae* M26. Cassava bagasse without hydrolysis was found to be more suitable for the growth of *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a, while hydrolyzed cassava bagasse was more recommended for *S. cerevisiae* M26 and *W. onychis* LABI2. Moreover, the studied microorganisms demonstrated xylanase activity under the analyzed conditions, and the yeasts *S. japonicus* Sia 70a and *W. onychis* LABI2 also exhibited notable amylase production. This study reveals significant potential of the selected yeasts in converting solid (CB) and liquid (CSW and CWW) residues from cassava and corn starch factories into microbial protein and other nutrients suitable for the animal feed industry. These findings hold promising implications for sustainable and economical utilization of starch industry by-products. The economic analysis of the formulated media showed costs 271 times lower with the medium formulated with cassava bagasse and corn maceration water compared to the FWR (Formulated Without Residue), indicating significant potential for cultivating using agro-industrial residues. *In vitro* ruminal fermentation tests demonstrated a positive response after 48 hours of fermentation, improving the digestibility of organic matter, dry matter, neutral detergent fiber digestibility, and truly degraded organic matter, with the treatment involving *W. onychis* LABI2 and *S. pararoseus* Sia 33.1 standing out. These tests highlighted the potential of the evaluated microorganisms in animal nutrition, as well as the low production cost and high proliferation in the used residues compared to the medium formulated with macro and micronutrients (FWR). There was no observed reduction in methane production; however, *in vivo* studies may provide a better understanding of the observed improvement in degradability concerning weight gain and, consequently, methane release per weight gain.

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CHAPTER 3 - PRODUCTION OF YEAST BIOMASS UNDER DIFFERENT CULTIVATION PARAMETERS AND YEAST ADAPTATION

ABSTRACT

The study aimed to evaluate different cultivation parameters to stimulate the growth and enzymatic production of four yeasts for application in animal nutrition. The selected microorganisms were *Sporobolomyces japonicus* Sia 70a, *Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2, and *Saccharomyces cerevisiae* M26, which were cultivated in batch and fed-batch modes for 120 hours at 30°C, using acid and enzymatic hydrolyzed cassava bagasse, molasses, and corn steep water. Evaluated cultivation parameters included viscosity, Brix, total reducing sugars (TRS), pH, viability, cell budding, and enzyme production. Microorganisms were also propagated under conditions of 37°C and pH 6.9. For maximum cell and enzymatic production, yeast cultivation viscosity needed to be maintained close to 13 mPa.s, with residual TRS near 40 g.L⁻¹ and Brix content ranging from 2 to 4. Under cultivation conditions at 30°C and pH 5.5, total biomass concentrations obtained for *S. japonicus* Sia 70a (3.81 g.L⁻¹) and *S. cerevisiae* M26 (34.5 g.L⁻¹) were higher in batch mode. Under the same conditions, *W. onychis* LABI2 produced similar values in both batch and fed-batch modes, 4.14 and 4.24 g.L⁻¹ respectively, as did *S. pararoseus* Sia 33.1, with 10.33 and 9.79 g.L⁻¹ respectively. *S. cerevisiae* M26 and *W. onychis* LABI2 adapted better to pH 6.9 and 37°C, while *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a could only adapt to pH 6.9 and necessarily needed to be cultivated at 30°C. Under the conditions of 30°C and pH 5.5, notable production of xylanases was observed for *S. pararoseus* Sia 33.1 with 0.42 U.mL⁻¹ and 0.22 U.mL⁻¹ of amylases in 96 hours of fed-batch cultivation. For batch cultivation, there was lower production for this strain, with 0.023 U.mL⁻¹ of amylases and 0.113 U.mL⁻¹ of xylanases. *S. japonicus* Sia 70a also stood out in amylase production (0.23 U.mL⁻¹) in both fed-batch and batch modes (0.24 U.mL⁻¹) after 96 hours of cultivation. With the adaptation to pH and temperature conditions and the optimization of the culture of selected microorganisms, we have significant potential for application in animal nutrition.

Keywords: Batch culture, fed-batch culture, special yeasts, enzymes, animal nutrition

1 INTRODUCTION

Fermentation processes are associated with the generation of various products such as biofuels, beverages, dietary supplements, biopharmaceuticals, among others. While fermentation occurs naturally in the presence of microorganisms and nutrients, optimizing these processes becomes necessary when considering large-scale industrial production. This optimization aims to control fermentation conditions to achieve the highest yield and efficiency possible, resulting in the maximum conversion of nutrients into the desired compounds. Key parameters evaluated in fermentation control include temperature, pH, Brix, medium viscosity, homogenization and agitation, aeration, presence of contaminants, substrate concentration, and chemical and physical characteristics of the substrate, among others, which vary according to the specificities and objectives of each cultivation (Hassing, 2022; Alarcon & Shene, 2021; Maicas, 2021; Seo et al., 2021).

The main processes used in industrial cultures for biomass (yeast) production are batch and fed-batch cultivation. Batch cultivation ensures lower contamination risk but may suffer from inhibition due to nutrient concentration or secondary metabolites. Fed-batch cultivation overcomes limitations caused by nutrient concentration and catabolic toxicity but has a higher risk of contamination, requires more energy, and takes longer to complete the process (Schmidell et al., 2001; Luli & Strohl, 1990).

Successful processes align with understanding the metabolic needs and capabilities of the microorganism used, as well as the actions required to minimize negative effects, considering the microorganism's sensitivity and the robustness of processes at an industrial scale. Thus, evaluating certain parameters at critical moments of the process is decisive for achieving maximum yield and predicted productivity, as well as ensuring product quality (Reihani & Khosravi-Darani, 2019).

Another important aspect concerns the increasing search for alternatives to make the livestock sector more sustainable, particularly focusing on greater carbon sequestration and reduction of methane release, which can be achieved by refining studies on animal nutrition (Cardoso et al., 2020). Methane (CH_4) is one of the gases that concerns the scientific community due to its ability to raise temperatures 80 times more than CO_2 over a 20-year period, a rate that increases when considering longer timeframes (IMAFLOA, 2022).

Evaluating CH₄ emissions from 1990 to 2016, a reduction of 8.2% is observed, indicating that efforts on good management practices, particularly improving animal diets, have favored herd production efficiency (UNFCCC, 2021). Recent studies have been confirming the effectiveness of probiotics in animal health and nutrition (Hassan et al., 2020), as well as the benefits of enzymatic action in the rumen (Bedford et al., 2022), evidence that encourages tests with microorganisms showing probiotic potential and the ability to degrade fiber and starch.

The strategy of this study involves the development of a bioprocess using different yeasts, including *S. cerevisiae* M26, in batch and fed-batch cultivation, for live biomass production aimed at formulating a nutritional supplement for ruminants. The study evaluates the use of agro-industrial residues, such as cassava bagasse, molasses, and corn steep water, as nutrients for converting these residues into microbial biomass, as well as enzymatic and acid treatments of cassava bagasse.

2 OBJECTIVES

The objective of the research was to evaluate different cultivation parameters of four different yeasts using agro-industrial residues in batch and fed-batch cultivation, as well as enzymatic production and enzymatic and acid treatment of residues.

3 MATERIALS AND METHODS

3.1 Preservation of microorganisms

The research was conducted with four yeasts: *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, and *Saccharomyces cerevisiae* M26. *W. onychis* LABI2 was isolated from rice bran at the Bioenergy Laboratory (IPBEN) - UNESP - FCL Assis. The *S. cerevisiae* M26 yeast strain was previously isolated from a bioethanol distillery (Oliva-Neto et al., 2004). *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a were provided by Prof. Dr. Fernando Carlos Pagnocca, who was responsible at the time for the Microbiology Laboratory of the Center for the Study of Social Insects (CEIS) at São Paulo State University (UNESP) – Rio Claro/SP, and were isolated from litter by professionals at the Microbiology Laboratory – UNESP – IB/Rio Claro.

The maintenance of the yeasts was carried out through monthly transfers to a specific culture medium called Residue-Free Formulated (RFF), according to Oliva-Neto

et al., (2004), containing 2.0% glucose, 0.5% yeast extract, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 , and 0.024% MgSO_4 (w.v⁻¹), pH 5.5, and 2% agar in slant tubes. Preservation in mineral oil was performed every three months by transferring to solid Residue-Free Formulated medium (in slant tubes), adding sterile mineral oil, followed by storage in a refrigerator (Ecoplus 430, Bosch, Joinville/SC, Brazil). Lyophilization was used only for *W. onychis* LABI2 and *S. cerevisiae* M26, which were able to tolerate the process and be reactivated later, while the others were strongly inhibited by this technique.

3.2 Enzymatic and acidic hydrolysis

The initial cultivation (experiments 1 and 3 of item 3.3.1) consisted of using cassava bagasse (previously gelatinized for 20 minutes at 80°C) hydrolyzed with enzymatic extract of *Rhizopus oligosporus* 1.2 U. mL⁻¹ at 55°C (MA 127, Marconi, Piracicaba/SP, Brazil) with 10% w.v⁻¹ of cassava bagasse in 0.05 mol acetate buffer at pH 5.0 for 48 hours. However, the added nutrient was not meeting the cultivation needs for maximum cell production.

The following hydrolysis tests were carried out with the aim of increasing the concentration of reducing sugars available in the culture, thereby reflecting in the rapid assimilation of nutrients by the microorganisms and cell proliferation. The non-hydrolyzed cassava bagasse (CB) was treated by different methodologies: (1) Hydrolysis of CB with commercial amylase; (2) Acid hydrolysis of CB at pH 0.5 and 1.5; (3) Acid hydrolysis of CB with pH varying from 1.08 to 1.92; and (4) Acid hydrolysis of wheat bran and cassava bagasse at different concentrations. The tests are detailed below.

The first test consisted of the hydrolysis of cassava bagasse (previously gelatinized for 20 minutes at 80°C) performed with commercial amylase 1.2 U.mL⁻¹ of alpha-amylase (Termamyl 120L Novozymes Latin America Ltda) at 55°C (MA 127, Marconi, Piracicaba/SP, Brazil) with 10% (w.v⁻¹) of cassava bagasse in 0.05 mol acetate buffer at pH 5.0 for 52 hours in a bench-top bioreactor (Tecnal – model Tec-Bio Plus) with a capacity of 2 L. The material was heated to 90°C after enzymatic hydrolysis to denature the enzymes in the material and sterilize it before use in bioreactor cultures.

The second test proceeded with thermo-acid hydrolysis as an alternative methodology to extract more reducing sugars from the substrate. The acid hydrolysis of cassava bagasse (10% w.v⁻¹) was initially carried out in 50 mL Falcon tubes (Conical

Tube, Labor Import, Cravinhos/SP, Brazil) containing a final volume of 40 mL of medium, composed of cassava bagasse, deionized water, and acid. Sulfuric acid P.A. (Synth) was used for pH adjustment, set to pH 0.5 and 1.5, with a pH meter (Tecnal, model R-TEC-7-MP), over a hydrolysis interval from zero to 25 hours at 97°C (Thermostatic Bath, TE-184, Tecnal, Piracicaba, Brazil).

The third test was conducted with the aim of optimizing the results found in the previous test, proceeding with hydrolysis performed over an interval of 8.59 hours to 11.41 hours, with pH varying between 1.08 and 1.92, at the same concentration of cassava bagasse and temperature (10% w.v⁻¹ at 97°C). This was now done through an experimental design using the response surface methodology - Central Composite Rotational Design (CCRD) (Design Expert software, version 11). Thus, with the design, it was possible to define the pH and hydrolysis time at the fixed temperature (97°C) for the production of acid-hydrolyzed cassava bagasse for cultivation tests. The test was conducted in 50 mL Falcon tubes with 40 mL of final medium.

The fourth and final hydrolysis test was conducted based on the previous tests, with the pH set at 1.08 for cassava bagasse at 10% (w.v⁻¹). These parameters were used for the fourth test, which evaluated different concentrations of 10, 12, 13, and 15% (w.v⁻¹) of cassava bagasse and also wheat bran, both at 97°C, taking samples at 10, 12, 14, 16, and 20 hours. The tests were conducted in 50 mL Falcon tubes with 40 mL of final medium.

For experiments 2, 3, and 4, which describe the cultivations carried out in batch and fed-batch (item 3.3), cassava bagasse (13% w.v⁻¹) hydrolyzed with sulfuric acid at pH 1.08 and 97°C for 26 hours in a bioreactor (Tecnal – model Tec-Bio Plus) was used. Regarding the material subjected to thermoacidic hydrolysis, after cooling, the pH was adjusted to 5.5 with NaOH before being used in microorganism cultures. The analysis of released reducing sugars was performed using the DNS technique for all samples obtained from the tests described above.

3.3 Simple batch and fed-batch cultivation

3.3.1 Cultivations for defining the medium formulation and feeding method for the propagation of yeast biomass: tests with *S. pararoseus* Sia 33.1

Initially, a 2.5-liter bioreactor (Tecnal – model Tec-Bio Plus) was sanitized with 50 mL of sodium hypochlorite (pure bleach – Qboa brand, 2 to 2.5% w.v⁻¹), followed by 1000 mL of hot potable water (90°C), with subsequent addition of the media described in Tables 1, 2, and 3, in order to evaluate the different process parameters for cell propagation.

Among the parameters evaluated were: a) the use of media formulated with different nutrients (CB and HCB, item 3.2), b) variation of salts, c) concentration of CSW, molasses, and sucrose; d) medium aeration, e) addition of amylases, f) nutrient feeding time, g) pH, and h) type of process - simple batch and fed-batch. The analyses monitored throughout the cultivation process include: quantification of live and dead buds, cell viability analysis, cells.mL⁻¹, Brix, total residual reducing sugars (TRRS), initial total reducing sugars (TRS), pH (adjustment), viscosity, enzymatic activity of xylanases, amylases, and cellulases. Kinetics parameters biomass yield coefficient ($Y_{x/s}$), biomass yield coefficient on initial substrate ($Y_{x/s0}$) and biomass productivity were determined according to Shuler & Kargi (2002). The analyses were carried out during the cultivations according to the identification of medium needs, in order to better understand the characteristics of the cultivation and its influence on cell proliferation.

To achieve this, five cultivations were carried out in a bioreactor using only *S. pararoseus* Sia 33.1. In these five experiments, conducted as single experiments, the strategy was to monitor the culture development (cell propagation) based on changes in the variables already described. In this way, it was possible to enhance cell growth, resulting in the 6th experiment, where the parameters were already defined and fixed, and biomass propagation occurred for the four yeasts, both in simple batch and fed-batch modes.

The cultivation conditions used in experiments 1, 2, and 3 are described in Table 1. The final volume was always 2500 mL, while the initial volume varied according to each experiment, being 2500 mL for the 1st, 2500 mL with removal and addition of 500 mL at different times in the 2nd, and 1250 mL in the 3rd experiment. The cassava bagasse (10% w.v⁻¹) used was hydrolyzed with amylase for 48 hours according to item 3.2. The

hydrolyzed and gelatinized cassava bagasse was then used for cultivation in the bioreactor (Table 1). Samples (10 mL) were taken and evaluated for cell count per mL, reducing sugars, live and total buds, among other variables.

Table 1. Description of the cultivation conditions of *S. pararoseus* Sia 33.1 in a 2.5 L bioreactor, conducted at 30°C and initial pH of 5.5 at 100 rpm, in batch mode (experiment 1), semi-continuous fermentation (experiment 2) and fed-batch mode (experiments 3).

Exper iment	Time (h)	CB (%)	HCB (%)	CSW (%)	Salt pool	Suc. (%)	Air (vvm)	Agitation (rpm)	Amyl. (U.g ⁻¹)	pH	Initial vol. (mL)	Vol. withdr awn (mL)	Vol. added (mL)	Fina l vol. (mL)
1	0	-	5.0	0.5	-	-	-	180	-	5.5	2500	-	2500	2500
2	0	2.5	-	0.5	1	1	1	100	-	4.5	2500	-	-	2500
	24	2.5	-	0.5	-	-	1	100	-	4.5	-	500	500	2500
	48	2.5	-	0.5	1	1	1	100	12	4.5	-	500	500	2500
	72	2.5	-	1.0	1	2	1	100	-	4.5	-	500	500	2500
3	0	-	2.5	0.5	2	-	1	100	-	5.5	1250	-	-	1250
	31	-	2.5	0.5	2	1	1	100	-	5.5	-	-	1250	2500

Salt 1: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%.

Salt 2: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024%; (NH₄)₃PO₄ 0.13%; Yeast extract 0.05% and ammonium sulfate 0.1%. Vol.: Volume. Suc: Sucrose; Air: Aeration – volume of air per volume of medium; Amyl: Amylase. Vol. withdrawn: corresponds to the volume of the bioreactor culture médium withdrawn. while Volume (Vol.) added corresponds to the new medium prepared and added to the bioreactor. Samples were taken and evaluated at times 0, 8, 21 and 24 hours from the start of cultivation. HCB: Hydrolyzed cassava bagasse with amylase (sector 3.2). CB: Cassava bagasse. CSW: Corn steep water. Inoculum 1x10⁶ cells.mL⁻¹. Final culture times: 1 – 48 h, 2 – 143 h, 3 – 72 h.

In contrast to experiment 1, where simple batch cultivation was used and, therefore, the carbon source (5% of HCB) was added all at once at the beginning, experiments 2 and 3 were conducted with fed-batch cultivations. In experiment 2, four feedings of 500 mL (20% of the medium) with 2.5% CB and three feedings of sucrose ranging from 1 to 2% (diluted in salts) were performed. In experiment 3, two feedings of 1250 mL of 2.5% HCB medium with larger volumes and one feeding with 1% (w.v⁻¹) sucrose were conducted. The sucrose was pre-diluted in water along with the salts for subsequent addition to the bioreactor. The feedings were carried out according to cell viability and reducing sugar content.

The conditions defined in experiments 2 and 3 regarding airflow and agitation were maintained for experiments 4 and 5. Experiment 4 (Table 2) was conducted by varying the medium composition with the addition of molasses byproduct instead of

sucrose used in the previous experiment (requiring a greater direct sugar source) and in a shorter cultivation time (54 hours). The HCB was hydrolyzed through acid hydrolysis (item 3.2) in the subsequent experiments. The criterion used for the feedings in experiment 4 was set at 4% (w.v⁻¹) of reducing sugars in the culture medium, based on the concentration of reducing sugars in each residue.

Table 2. Experiment 4. Description of the cultivation conditions in fed-batch mode with *S. pararoseus* Sia 33.1 in a 2.5 L bioreactor with air addition at 1 vvm and pH control to 5.5 at each time point, conducted at 30°C and initial pH of 5.5 and 100 rpm.

Nutrient volume added							
Time (h)	Inoculum (mL)	HCB (mL)	Molasses (mL)	H ₂ O (mL)	Total vol. (mL)	Initial vol. (mL)	Final vol. (mL)
0	125	89.82	35.17	-	250	250	-
3	-	479.04	20.958	100	850	-	-
9	-	400	22.87	177	1450	-	-
24	-	400	38.90	61	1950	-	-
28	-	400	56.02	44	2450	-	-
32	-	-	-	-	2450	-	-
48	-	-	-	400	2850	-	-
54	-	-	-	-	2850	-	2850

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% e (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1%. Addition of 0.5% (v.v⁻¹) corn steep water. Vol: Volume; Initial inoculum of 1x10⁶ cells. mL⁻¹. HCB: Hydrolyzed cassava bagasse (15% w.v⁻¹) at pH 1.08 for 26 hours at 97°C in a bioreactor. Sampling during cultivation for analyses allowed for the use of a volume of 2.8L.

Table 3 presents the cultivation conditions carried out in experiment 5, where the main goal was to reduce the viscosity of the medium and avoid inhibiting cell production due to high TRS concentrations. The criterion used for feedings was initially set at 3% (w.v⁻¹) of reducing sugars in the culture medium, based on the sugar contents of the residues used. Despite the initial criteria used high viscosity was observed due to the concentration of HCB added at the beginning of the process. As a result, dilutions were performed, leading to a reduction in the sugar concentration of the medium and the viscosity of the culture. The feeding of the sugar source was carried out while maintaining the viscosity at a maximum of 20 mPas. Analyses are described in item 3.6.

Table 3. Experiment 5. Description of the fed-batch cultivation media of *S. pararoseus* Sia 33.1 in a 2.5 L bioreactor at 30°C and initial pH of 5.5.

Time (h)	Nutrient volume added					Salts
	HCB (mL)	Molasses (mL)	H ₂ O (mL)	Inoculum (mL)	Total vol. (mL)	
0	125	-	-	290	415	+
3	160	-	85.6	-	575	+
9	160	-	87.6	-	575	-
14	320	-	173.8	-	675	-
24	-	-	60	-	735	+
27	-	-	500	-	1235	+
33	-	10.23	330	-	1565	+
50	50.8	-	350	-	1915	+
57	-	26.68	360	-	2275	+
75	-	28.38	225	-	2500	+
81	-	-	-	-	2500	-
96	-	-	-	-	-	-

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1%. Addition of 0.5% (v.v⁻¹) corn steep water. Initial inoculum of 1x10⁶ cells. mL⁻¹. HCB: Hydrolyzed cassava bagasse (15% w.v⁻¹) at pH 1.08 for 26 hours at 97°C in a bioreactor. +: Presence. - Absence. Initial inoculum of 1x10⁶ cells. mL⁻¹

After conducting the cassava bagasse hydrolysis studies (item 3.2) and the bioreactor cultivations according to experiments 1 to 5 (Tables 1, 2 and 3), the main parameters were defined and proceeded to batch and fed-batch cultivations. The sixth experiment was carried out on an orbital shaker with temperature control in 3-liter Erlenmeyer flasks, in sextuplicate, each flask containing 1 liter of culture medium, aiming to ensure culture purity (as there was contamination by bacteria in previous stages) and higher biomass production.

3.3.2 Batch and fed-batch cultivations were conducted with the four yeast strains tested.

The following tests were carried out with the four selected yeast strains: *W. onychis* LABI2, *S. pararoseus* Sia 33.1, *S. japonicus* Sia 70a and *S. cerevisiae* M26. The cultures were formulated using hydrolyzed cassava bagasse (15% w.v⁻¹, pH 1.08, 97°C. 26 hours) supplemented with (NH₄)₂SO₄ 0.1%, ZnSO₄ 0.0028%, MnSO₄ 0.0012%, MgSO₄ 0.024%, (NH₄)₃PO₄ 0.13%, K₂HPO₄ 0.087%, yeast extract 0.2%, sugarcane molasses (v.v⁻¹) and corn steep liquor (v.v⁻¹) at pH 5.5. autoclaved (SP215-26, Phoenix, Araraquara/SP, Brazil) for 20 minutes at 1 atm. based on initial experiments 1, 2 and 3.

For this purpose, 1000 mL of medium was used in Erlenmeyer flasks (3 L) in batch and fed-batch modes to determine the best production methodology for each microorganism studied.

The cultivation took place for 120 hours in a refrigerated orbital incubator (Tecnal mod. TE 421. Piracicaba. SP. Brazil) at 180 rpm and 30°C. The agitation varied from the bioreactor (100 rpm) to the cultures in the orbital incubator (180 rpm) due to the difference between agitation with paddles and on the shaker. The pH (Tecnal - model R-TEC-7-MP) was adjusted to 5.5 with each sample taken every 12 hours up to 48 hours of cultivation and every 24 hours after 48 hours because the exponential growth phase tends to occur in the first two days of cultivation, with a very slight pH change in the following days. The total cultivation time was 120 hours, both for batch and fed-batch propagation. The inoculum for experiment 6 was increased from 10^6 to 10^8 cells.mL⁻¹ to enhance culture propagation dynamics.

The samples taken (10 mL) were subjected to counting of live and dead buds, cell viability analysis, cell count per mL. Brix measurement, total residual reducing sugars (TRRS), pH adjustment, viscosity measurement and enzymatic activity of xylanase, amylase, and cellulase.

Table 4 presents experiment 6 with the defined conditions regarding the addition of medium and inoculum during cultivation in fed-batch and batch modes. In fed-batch cultivation, the criterion used for feedings in experiment 6 was set at 4% (w.v⁻¹) of reducing sugars in the culture medium, based on the concentration of reducing sugars in each residue.

Table 4. Experiment 6. Description of the cultivation media (30°C at initial pH 5.5) added at defined time intervals for conducting fed-batch cultivation for 120 hours.

Nutrient volume at the respective addition time					Total volume
Time (h)	HBM (mL)	Molasses (mL)	H ₂ O (mL)	Inoculum	(mL)
0	50	-	-	116	166
12	-	6.23	187.76	-	360
24	54	3.61	122.38	-	540
36	-	19.74	290.24	-	850
48	-	18.24	131.75	-	1000
48 -120	-	-	-	-	1000

Batch cultivation was conducted under the same conditions; however, the entire medium was added at the initial time point. Tests were performed in sextuplicate. pH adjustment of the medium was done every 12 hours for the first 48 hours, and every 24 hours thereafter. Total reducing sugar content of molasses: 88%. The cassava bagasse nutrient was hydrolyzed (HCB) at pH 1.08 for 26 hours at 97°C. HCB with pH adjusted to 5.5 was used. Inoculum of 1×10^8 cells.mL⁻¹.

After cultivation, the fermented broth was filtered using a reusable porous polypropylene filter paper (Filtro Paulista. 100% polypropylene. dimensions 161 x 22 x 205). The residual solid fraction was dried in an oven at 105°C (Tecnal. model TE-394/1-MP. Piracicaba/SP, Brazil) and characterized (section 3.7). while the liquid fraction was used for cell counting in a Neubauer chamber (Dorta et al., 2006).

3.4 Adaptation of yeast to temperature and pH through cultivation using the cutting system

In order to produce a live biomass adapted to the conditions of ruminal fermentation in sheep, the four yeast strains were adapted for growth at pH 6.9 and a temperature of 37°C. through submerged cultivation using sterilized FWR medium (SP215-26. Phoenix. Araraquara/SP, Brazil) for 20 minutes at 1 atm. For this purpose, the step-up system was used in biomass propagation. which involved removing 50% (v.v⁻¹) of the culture medium and adding 50% (v.v⁻¹) of fresh autoclaved medium (500 mL Erlenmeyer flask with 200 mL of medium) at 48-hour intervals over a 3-month period. Cultivation was maintained in a refrigerated orbital incubator at only 40 rpm (Tecnal mod. TE 421. Piracicaba/ SP, Brazil). intentionally to reduce oxygen supply and induce anaerobic metabolism of the yeasts. Cell growth was monitored using a spectrophotometer at 600 nm (UV-1600 PC Spectrophotometer, VWR, International) by optical density. Tests were conducted in triplicate.

The strains that did not adapt to the imposed conditions (pH 6.9 and temperature of 39°C) were subjected to cultivation at pH 6.9 and temperatures of 30°C and 37°C without agitation in an incubator (Tecnal mod. TE 421, Piracicaba/ SP, Brazil). Initially, attempts were made to cultivate them at 39°C using the propagation system by cuts (50% v.v⁻¹), during cultures every 7 days, in 500 mL Erlenmeyer flasks with 200 mL of medium. However, this temperature inhibited all strains. The yeasts were cultivated on plates in solid Formulated Residue-Free medium (pH 6.9 at 37°C) from which the first yeasts that propagated were transferred to new plates, followed by cultivation in tubes and propagation in Erlenmeyer flasks with liquid Formulated Residue-Free medium at pH 6.9 at 37°C, through cultivation using the step-up system, where only *S. cerevisiae* and *W. onychis* were successful under these conditions. The others (*S. japonicus* and *S. pararoseus*) were propagated at 30°C and pH 6.9 because even with a gradual increase in

temperature they were inhibited. With the scaling from 200 mL to 1500 mL of cultivation, it was necessary to carry out this scaled propagation for 3 months.

Cell viability control was performed to assess the metabolic conditions of the yeast during cultivation. The obtained biomass was centrifuged (Heraeus Cryofuge 6000 Centrifuge, Thermo Scientific, Osterode, Germany) at 4000 rpm for 20 minutes at 15°C and stored in 1 L bottles at 6°C (Ecoplus 430. Bosch, Joinville/SC, Brazil) with 10% (w.v⁻¹) of milk powder (Piracanjuba).

3.5 Analytical procedures

3.5.1 Cell concentration

The cell concentration was quantified in triplicate from different samples using a Neubauer counting chamber (7301-1B. New Optics) according to Lee et al., (1981). The cell quantity was determined according to the equation:

$$n^{\circ} \text{ cells. mL}^{-1} = \sum \text{ of the quadrants} \times 5 \times 10^4 \times DF \quad \text{Eq. 3}$$

Where: $\sum$ of the 5 quadrants $\times 5$ = sum of the number of cells presents in five average quadrants times 5 representing the 25 average quadrants of the large central squared quadrant of the chamber; 10^4 = adjustment of volume in the chamber to express in number of cells per ml considering the area and height of each quadrant of the chamber; DF = sample dilution factor.

3.5.2 Cell viability

Cell viability was performed in triplicate using a methylene blue solution according to Lee et al., (1981). Quantification was carried out in a Neubauer chamber (7301-1B, New Optics).

3.5.3 Viscosity

The viscosity was measured using a rotational viscometer with concentric cylinders, providing viscosity (mPa.s) and sample reliability (%) (Brookfield digital viscometer model DV-II+ Viscometer).

3.5.4 Soluble solids (°Brix) and reducing sugars

The concentration of soluble solids was evaluated using a refractometer (Div RT - 30 ATC). A drop of the extract to be evaluated was added to the Brix refractometer (Scale 0-32%) to identify the soluble solids present in the sample.

The quantification of released reducing sugars was performed using the DNS (3,5-dinitrosalicylic acid) method as described by Miller (1959), with readings taken on a spectrophotometer (UV-1600 PC Spectrophotometer, VWR, International) at 540 nm.

3.5.5 Enzymatic activity

Total cellulase activity was quantified using the FPase technique (Mandels et al., 1976; Silva, 2016). The enzymatic extract obtained after filtration (0.5 mL) was added to 1 mL of sodium acetate buffer (0.05 M, pH 5.6) with Whatman filter paper N°. 1 of 0.5. 3 cm^{-1} . The reaction tubes were kept for one hour at 50°C in a water bath (MA 127, Marconi, Piracicaba/SP, Brazil). After one hour of reaction, 3,5-dinitrosalicylic acid (DNS) was added (0.5 mL), followed by reading on a spectrophotometer (UV-1600 PC Spectrophotometer, VWR, International) at 540 nm (Miller, 1959). One unit of enzymatic activity (Filter Paper Unit - FPU) was defined by the release of 2 mg of glucose per mL of enzyme per minute and expressed in $\text{FPU} \cdot \text{mL}^{-1}$ (Ghose, 1987).

Xylanolytic activity was performed using 0.5% Birchwood xylan substrate (Sigma Aldrich) in acetate buffer pH 5.0 at 50°C for 10 minutes according to Bailey et al., (1992). The quantification of released reducing sugar (xylan) was carried out using the acid-3,5-dinitrosalicylic method (Miller, 1959) read at 540 nm on a spectrophotometer. One unit of enzymatic activity is defined as the amount of enzyme needed to release 1 μmol of reducing sugar per minute.

Amylase was determined by using 0.9 mL of starch (Sigma Aldrich) in acetate buffer pH 5.5 ($0.05 \text{ mol} \cdot \text{L}^{-1}$) as substrate. 0.1 mL of enzymatic extract in a water bath (MA 127, Marconi, Piracicaba/SP, Brazil) at 60°C for 10 minutes. DNS (0.5 mL) was used for determining the released reducing sugar at 540 nm on a spectrophotometer. One enzyme unit was defined as the amount of enzyme that releases 1 μmol of glucose per minute of reaction (Miller, 1959).

3.6 Chemical analyses

The residue obtained after fed-batch and simple batch cultivation (Experiment 6) of the four yeasts was characterized for NDF content, ADF content, crude protein, lipids, ash, and total carbohydrates.

Protein analysis was conducted following the method outlined by the Association of Official Analytical Chemists (AOAC, 2000). The procedure involved digesting 0.1 g of dried sample in digestion tubes with 1 g of catalyst (CuSO_4 and K_2SO_4 in a ratio of 4:96) and 3 mL of analytical grade sulfuric acid at 350°C for about 2 hours until complete sample digestion (clear solution and white smoke). After digestion, the tubes were cooled, five drops of phenolphthalein (Reagen, Quimibras Indústrias Químicas S.A., Rio de Janeiro) were added, and they were connected to the distillation apparatus (TE-036/1, TECNAL, Piracicaba/SP, Brazil), NaOH 40% w.v⁻¹ (Dinâmica) was added to the digestion tubes until a pink color appeared for subsequent distillation in a 125 mL Erlenmeyer flask containing a solution of 20 mL of 0.32 M H_3BO_3 with 2 drops of methyl red (0,1% m.v⁻¹) and bromocresol green (0,1% m.v⁻¹). Distillation was stopped at 100 mL. The distillate was titrated with 0.02 M HCl solution (Anidrol) and the volume was recorded.

The determination of ash content was carried out using pre-weighed crucibles followed by the addition of 1 g of dried sample, which were then placed in a muffle furnace (Spencer, Iperó, Brazil) at 550°C for 5 hours (Instituto Adolfo Lutz, 1985).

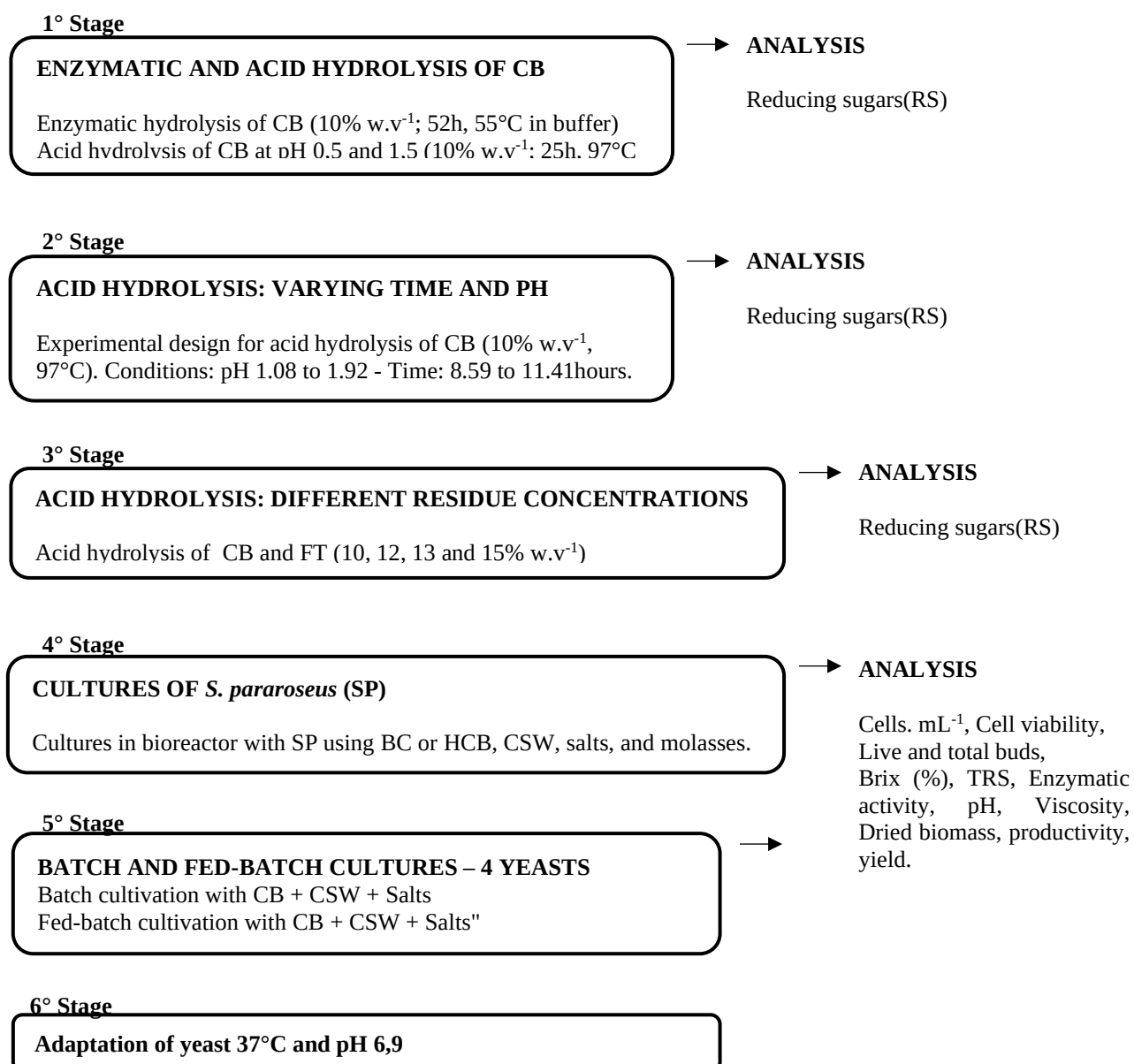
The lipid analysis was performed using a Soxhlet extractor apparatus. The Soxhlet flask was dried at 105°C (Tecnal, mod. TE-394/1-MP, Piracicaba/ SP, Brazil). weighed on an analytical balance and attached to the extractor with petroleum ether. Three grams of samples on folded Whatman N° 01 paper were added and then placed into the extractor. The apparatus was cooled with a water bath at 20°C for condensation and the flask was heated with a heating mantle. The samples were washed with ether for 10 hours. The flask was dried at 105°C . kept in a desiccator and weighed on an analytical balance. The weight of the empty flask subtracted from the flask with lipids yielded the amount of lipids per initial sample quantity (AOAC, 1990).

The analysis of crude fiber acid detergent fiber (ADF) and neutral detergent fiber (NDF) was performed according to AOAC (1990). The carbohydrate content was calculated using the difference method subtracting from one hundred the values of moisture, ash, protein, and lipids. All analyses were performed in triplicate.

3.7 Statistical Analysis

The cell culture assays were conducted in triplicate in a single block. and the results were subjected to one-way analysis of variance (ANOVA). with means compared using Tukey's multiple comparison test ($p < 0.05$). Figures and Tables were created using Microsoft Excel, IBM® SPSS Statistics software was used for statistical analysis.

Fig 1. Flowchart: Summary of the steps and analyses developed with the hydrolysis of residues and cultures formulated with cassava bagasse (CB), hydrolyzed cassava bagasse (HCB), molasses, and corn steep water (CSW). SP: *Sporidiobolus pararoseus* 33.1; FT: Wheat bran.



4. RESULTS

4.1 Enzymatically and Acid Treated Residues

Figure 2a shows the release of reducing sugars through hydrolysis using amylase from the fungus *R. oligosporus*. Hydrolysis for 24 hours resulted in the release of 10.19 g.L⁻¹ of reducing sugars, which corresponds to a yield of 19.96%, considering 51.05% starch in the residue (Souza et al., 2021), although after 11 hours of hydrolysis we had already reached 9.86 g.L⁻¹ of released sugars. Figure 2b shows the enzymatic hydrolysis of CB with a commercial amylase (alpha-amylase, Novozyme). Prior gelatinization was carried out at 80°C, a temperature of lower severity (Fig 2b.). The hydrolysis released 18.3 g.L⁻¹ of reducing sugars (RS) in 34 hours of hydrolysis, corresponding to an RS yield of 35.84%, considering 51.05% of starch in CB (10% w.v⁻¹) extracted in 32 hours. Although hydrolysis with the commercial enzyme allowed for greater sugar release compared to the enzyme from *R. oligosporus*, new tests were planned to further increase the release of sugars. Seeking a quicker and simplified extraction the subsequent tests explored sugar extraction through cassava bagasse hydrolysis via thermo-acidic means.

Fig 2. Reducing sugars released during enzymatic hydrolysis with: a. Defrosted amylase (1.2 U.mL⁻¹) produced from *R. oligosporus* (FES) hydrolyzed (10% w.v⁻¹ of CB) for 24 hours at 50°C in sodium acetate buffer pH 5.5. b. alpha-amylase (Novozymes) of cassava bagasse (10% w.v⁻¹) at an initial pH of 5.0 conducted for 52 hours at 55°C.

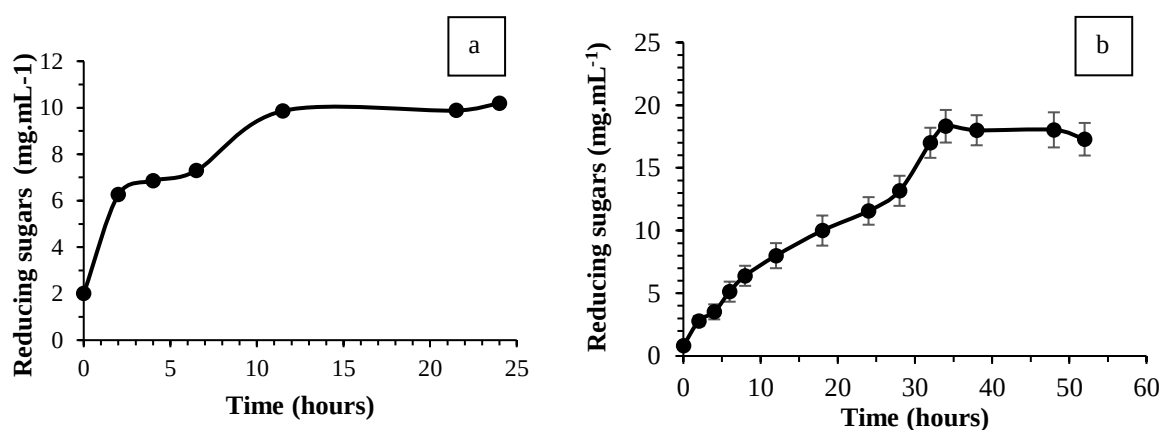


Table 5 presents the release of sugars through the hydrolysis of cassava bagasse performed over 1 to 25 hours at pH 0.50 and 1.50. Cassava bagasse (CB) at pH 0.50 achieved an average release of 54.82 g.L⁻¹ of sugars with a yield of 107% in 15 hours, considering the previously analyzed 51.05% starch, results that likely indicate the

hydrolysis of fibers present in the material. However, at pH 1.50, it did not exceed 35.68 g.L⁻¹ of sugars in 10 hours of hydrolysis with a yield of 64.89%. The thermo-acid hydrolysis of CB at pH 1.50 showed good results (35.68 g.L⁻¹), considering the use of a smaller volume of acid. The amount of concentrated sulfuric acid used was 0.15% (v.v⁻¹) to reach pH 1.50, compared to 0.48% (v.v⁻¹) to reach pH 0.50, indicating the use of 3.2 times more sulfuric acid.

Table 5. Reducing sugars released during the acid hydrolysis of cassava bagasse (10% w.v⁻¹) at pH 0.5 and pH 1.5 conducted for 25 hours at 97°C.

Time (hours)	Reducing sugars (g.L ⁻¹)			
	pH 0.50 (g.L ⁻¹)	Yield pH 0.50 (%)	pH 1.50(g.L ⁻¹)	Yield pH 1.50 (%)
0	0.55	1.08	0.65	1.27
1	5.11	10.01	2.12	4.15
2	16.13	31.60	3.41	6.68
3	19.53	38.26	3.68	7.21
4	25.71	50.36	4.67	9.15
5	27.96	54.77	6.41	12.56
6	33.99	66.58	7.81	15.30
7	36.10	70.71	9.50	18.61
8	39.55	77.47	14.79	28.97
10	41.00	80.31	35.68	69.89
12	44.07	86.33	14.81	29.01
15	54.82	107.38	13.32	26.09
20	25.26	49.48	4.44	8.70
25	21.05	41.23	5.78	11.32

CB: Cassava bagasse. Yield considering 51.05% ± 5.03 of starch present in the cassava bagasse (Souza et al., 2021).

New thermo-acid hydrolysis tests were conducted to determine the optimal pH range for the treatment of cassava bagasse (CB) over a period of 8.59 to 11.41 hours. Table 6 shows the experimental matrix carried out using a Central Composite Rotational Design (CCRD) to evaluate the best conditions for these parameters, based on the results of released reducing sugars.

Table 6. Experimental matrix of the hydrolysis of cassava bagasse (10% m.v⁻¹) based on the variation of pH and hydrolysis time for the highest release of reducing sugars.

Samples	Variable Factors		Reducing Sugars (g. L ⁻¹)	Yield of Reducing Sugars (%)
	pH	Time (h)	AR (g. L ⁻¹) or yield based on total mass	Based on 51% starch (%)
1	1.92	10.00	17.92	35.10
2	1.50	10.00	28.33	55.49
3	1.80	9.00	28.42	55.67
4	1.20	11.00	39.15	76.69
5	1.80	11.00	20.79	40.72
6	1.50	10.00	25.74	50.42
7	1.50	8.59	22.29	43.66
8	1.50	10.00	28.71	56.24
9	1.50	10.00	22.10	43.29
10	1.50	11.41	10.62	20.80
11	1.20	9.00	32.83	64.31
12	1.08	10.00	55.15	108.03

Hydrolysis of cassava bagasse (10% m.v⁻¹) performed at 97 °C. Sugar yield considering 51.05% ± 5.03 starch in the cassava bagasse.

As observed in Table 6, the concentration of released sugars varied across different intervals. Generally, when the ratio between the maximum and minimum values is greater than 10, a transformation of the response data is necessary for better model adjustments. The data transformation was carried out as needed and the mathematical modeling of the response prioritized models that maximized the values of "Adjusted R²" and "Predicted R²."

Table 7 presents a summary of the transformation used, the adjusted model that best fit the response, as well as the statistical data for R² and the precision analysis of the modified model, which considers only significant terms. The final equation in terms of actual factors for the significant terms of each modified model is also presented (Eq. 4).

Table 7. Statistical data of the model obtained for the release of reducing sugar (g.L⁻¹).

Response	Transformation	Adjusted Model	R ²	Precision	F-value	p-value
Reducing sugars	Square root	Quadratic	0.8296	8.53	5.83	<0.05

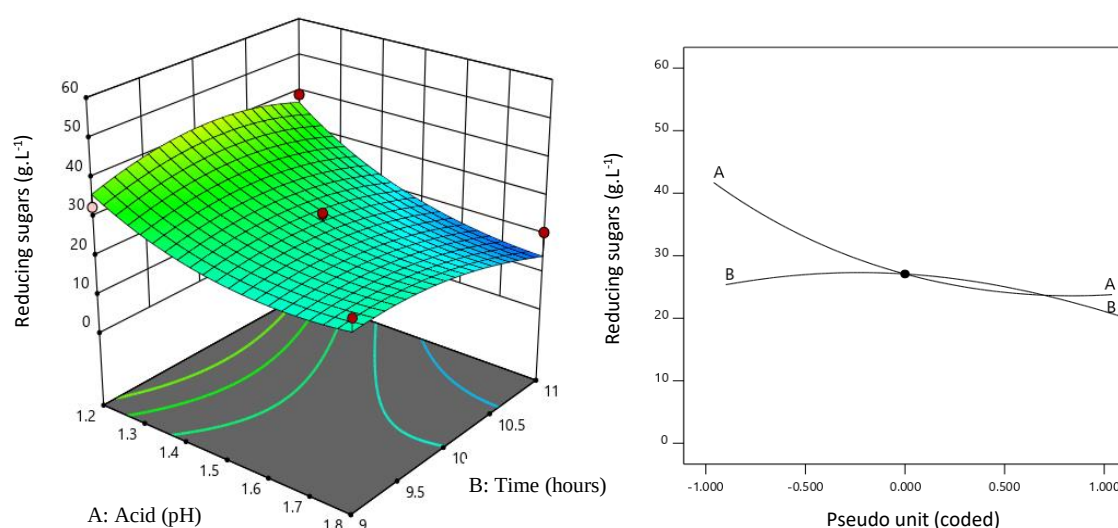
The quadratic transformation for the release of reducing sugars achieves an R² value of 0.8296, p-values less than 0.05 indicate that the terms of the model are significant.

$$\sqrt{\text{Reducing sugars}} = +5.11 - 0.8265 \times A - 0.2892 \times B - 0.3248 \times AB + 0.4978 \times A^2 - 0.4220 \times B^2 \quad \text{Eq. 4}$$

In this case, the equation in terms of actual factors can be used to make predictions about the response at specific levels of each factor A: acid and B: time. The trace plots and 3D surface plots were used to compare the effects of all components in the design space (Figures 3). Through these plots, it is possible to visualize how sensitive the response is to deviations of the reference variables for each model. The x-axis of the plot is in coded units and shows the relative position on the coded scale (0 to 1 for the treatments) and the 3D surface plot located on the left side represents the released reducing sugars.

It is possible to observe through the trace plots (Fig. 3) the greater potential of the acid to influence sugar extraction. However, the hydrolysis time, within this restricted range (8.59 to 11.4 hours), did not show a significant influence on sugar release. Thus, pH 1.08 (0.30% v.v⁻¹) was defined so that other parameters could be varied in subsequent analyses.

Fig 3. Optimization of reducing sugar release (g.L⁻¹) through the variation of pH (A) and hydrolysis time (B) using 10% (w.v⁻¹) cassava bagasse at 97 °C. The 3D surface plots represent the obtained values min max.



The model validation was performed at pH 1.08 for 10 hours of hydrolysis (Table 8). The verification showed that the model provides an adequate fit for the release of reducing sugars by hydrolysis of cassava bagasse being within the confidence interval.

Table 8. Model validation for acidic hydrolysis of cassava bagasse.

Replies (g.L⁻¹)	Predicted averages	Predicted median	Observed	Standard deviation	95% low	95% high
Reducing sugars	53.19	52.85	59.84	8.52	33.12	77.16

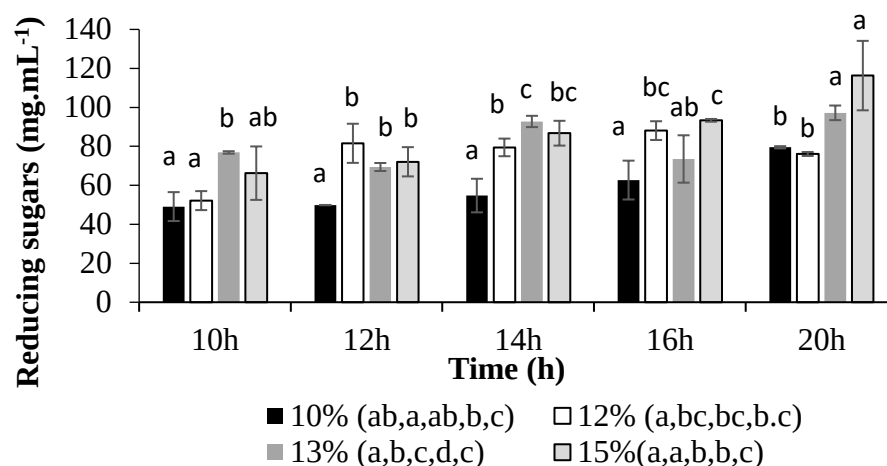
In the following tests, the reaction time was varied for two different substrates, cassava bagasse (CB) and wheat bran (WB) at different concentrations: 10, 12, 13 and 15% (w.v⁻¹). The results of released sugars (RS) between 10 and 20 hours of hydrolysis are presented for wheat bran in Figure 4, and for cassava bagasse in Figure 5. The yields achieved by acid hydrolysis for the different concentrations of wheat bran and cassava bagasse are described in Table 9, followed by the highlights of the yields in Table 10.

The results that achieved the highest yields and greatest sugar extraction were obtained with concentrations of 13% and 15% WB after 20 hours of hydrolysis at pH 1.08. The best yields remained around 70 to 79% sugar extraction, observed at 20 hours of hydrolysis with 13% WB (74.76%), 15% WB (77.53%) and 10% WB (79.51%), as well as 14 hours with 13% WB (71.35%). As the yield differences between them are relatively small, the condition of 14 hours with 13% WB might be more interesting for industrial purposes, requiring economic studies to confirm this.

For the hydrolysis of CB the highest yields and greatest extraction of reducing sugars were achieved with the hydrolysis of 13% and 15% (w.v⁻¹) CB after 20 hours at pH 1.08 (0.3% H₂SO₄), with results similar to those observed for the hydrolysis of wheat bran. However, the yield obtained with 13% (w.v⁻¹) cassava bagasse (66.71%) was numerically superior to that with 15% (w.v⁻¹) substrate (59.35%), despite no statistical differences. Similarly, the concentrations of reducing sugars, 86.73 g.L⁻¹ (for 13% CB) and 89.03 g.L⁻¹ (for 15% CB), also showed no statistical differences (Tables 9 and 10 and Figures 4 and 5). Therefore, 13% CB can be considered sufficient for good extraction via acid hydrolysis at pH 1.08 (0.3% v.v⁻¹ H₂SO₄) and 20 hours at 97°C.

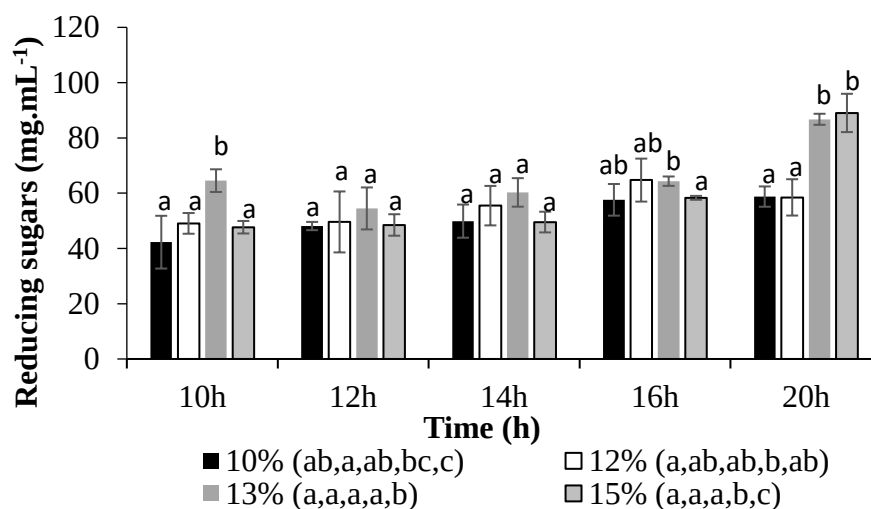
The following hydrolysis tests should consider the release of sugars and hydrolysis yield, as well as the concentration of sulfuric acid used to achieve pH 1.08 due to the increased cost of the process. The concentration of sulfuric acid was higher for wheat bran, ranging from 0.32% to 0.45%, compared to the hydrolysis of CB (0.30% to 0.40%) for the same substrate range (10% to 15% w.v⁻¹).

Fig 4. Acid hydrolysis (pH 1.08) of wheat bran conducted at different concentrations (10, 12, 13 and 15% w.v⁻¹) and at different hydrolysis times (10, 12, 14, 16 and 20 hours).



Hydrolysis was performed with sulfuric acid at 97°C at pH 1.08. Analysis of variance (ANOVA) was conducted using Tukey's multiple comparison test, with a significance level of 0.05%. Letters in front of the concentration sequentially compare the reducing sugars obtained for each time evaluated at the specified concentration. Letters above each bar compare the sugars obtained at the same time, regardless of concentration. Different letters indicate significant differences between groups.

Fig 5. Acid hydrolysis of cassava bagasse (pH 1.08) conducted at different concentrations (10, 12, 13 and 15% w.v⁻¹) and at different hydrolysis times (10, 12, 14, 16 and 20 hours).



Hydrolysis was performed with sulfuric acid at 97°C at pH 1.08. Analysis of variance (ANOVA) was conducted using Tukey's multiple comparison test, with a significance level of 0.05%. Letters in front of the concentration sequentially compare the reducing sugars obtained for each time evaluated at the specified concentration. Letters above each bar compare the sugars obtained at the same time, regardless of concentration. Different letters indicate significant differences between groups.

Table 9. Yield of reducing sugar extraction through acidic hydrolysis (pH 1.08) for the total of wheat bran and cassava bagasse (10, 12, 13 and 15% w.v⁻¹).

YIELD OF REDUCING SUGARS (%) BASED ON THE TOTAL MASS OF SUBSTRATE				
Wheat bran				
Time (hours)	10% (ab. a. ab. b. c)	12% (a. bc. bc. b.c)	13% (a. b. c. d. c)	15% (a. a. b. bc. c)
10h (a. a. b. a)	49.08±7.42	43.45±4.87	59.12±0.63	44.15±13.71
12h (a. b. c. ac)	49.85±0.07	67.96±10.04	53.37±2.05	48.05±7.50
14h (a. ab. b. a)	54.73±8.62	66.21±4.52	71.35±2.89	57.82±6.36
16h (ab. a. ab. b)	62.68±9.97	73.39±4.80	56.54±12.16	62.22±0.70
20h (a. b. c. abc)	79.51±0.56	63.39±1.00	74.76±3.74	77.53±17.81
Cassava bagasse				
Time (hours)	10% (ab. a. ab. bc. c)	12% (a. ab. ab. b. ab)	13% (a. a. a. a. b)	15% (a. a. a. b. c)
10h (abc. a. b. c)	42.26±9.54	40.88±3.74	49.64±4.10	31.78±2.26
12h (a. ab. ab. b)	48.10±1.50	41.32±11.03	41.90±7.60	32.32±3.88
14h (a. a. a. b)	49.88±6.00	46.23±7.14	46.36±5.16	33.02±3.74
16h (a. ab. b. c)	57.59±5.72	53.94±7.77	49.49±1.70	38.87±0.70
20h (a. a. b. ab)	58.76±6.57	48.72±6.57	66.71±2.00	59.35±6.92

Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with a significance level of 0.05%. Letters in front of the concentration compare sequentially the reducing sugars obtained for each time evaluated at the specified concentration. Letters in front of the time compare the yield obtained at the same time for different concentrations. Different letters indicate significant differences between groups. Yields were calculated based on the total mass of substrate used, as the hydrolysis of both starch and fiber present in the residue was considered.

Table 10. Highlight of the main results of reducing sugar extraction yield via acidic hydrolysis of cassava bagasse and wheat bran. at pH 1.08, 97°C, according to the analyses in Table 9.

YIELD OF REDUCING SUGARS (%) BASED ON THE TOTAL MASS OF SUBSTRATE				
Wheat bran				
Time (hours)	10% (ab.a.ab.b.c)	12% (a. bc. bc. b.c)	13% (a. b. c. d. c)	15% (a. a. b. b. c)
10h (a. a. b. a)	-	-	-	-
12h (a. b. c. ac)	-	67.96±10.04	-	-
14h (a. ab. b. a)	-	-	71.35±2.89	-
16h (ab. a. ab. b)	-	73.39±4.80	-	62.22±0.70
20h (a. b. c. abc)	79.51±0.56	-	74.76±3.74	77.53±17.81
Cassava bagasse				
Time (hours)	10% (ab. a. ab. bc. c)	12% (a. ab. ab. b. ab)	13% (a. a. a. a. b)	15% (a. a. a. b. c)
10h (abc. a. b. c)	-	-	-	-
12h (a. ab. ab. b)	-	-	-	-
14h (a. a. a. b)	-	-	-	-
16h (a. ab. b. c)	57.59±5.72	53.94±7.77	-	-
20h (a. a. b. ab)	58.76±6.57	-	66.71±2.00	59.35±6.92

Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with a significance level of 0.05%. Letters in front of the concentration compare sequentially the reducing sugars obtained for each time evaluated at the specified concentration. Letters in front of the time compare the yield obtained at the same time for different concentrations. Different letters indicate significant differences between groups. Yields were calculated based on the total mass of substrate used, as the hydrolysis of both starch and fiber present in the residue was considered.

The acid hydrolysis study was conducted on a 40 mL scale (Falcon tubes without agitation) and later in volumes of 2500 mL in a bioreactor with mechanical agitation at 100 rpm. only with CB. An increase in hydrolysis time of 6 hours was observed to achieve the same results under the same conditions (Table 11).

Table 11. Reducing sugars and yield (%) obtained through acid hydrolysis (pH 1.08) with 13% w.v⁻¹ cassava bagasse in a 2500 mL bioreactor at 97°C.

	REDUCING SUGARS (mg.mL ⁻¹) and YIELD (%) BASED ON THE TOTAL MASS OF SUBSTRATE			
	20 hours	22 hours	24 hours	26 hours
RS mg.mL ⁻¹	65.7±3.89	72.4±4.65	81.2±2.47	87.2±2.82
Yield (%)	50.53	55.69	62.46	67.07

The subsequent cultures were supplemented with molasses in order to increase the availability of sugars and thereby improve the development of the yeasts.

4.2 Single and fed-batch yeast cultures

The subsequent cultures were supplemented with molasses to increase the availability of sugars and thus improve the development of the yeasts.

Table 12. Overview of the results obtained in experiments 1, 2 and 3 through simple batch and fed-batch cultivation carried out in a bioreactor with *S. pararoseus* Sia 33.1 yeast at 30°C and 100 rpm.

	Experiments								
	1		2			3		4	5
Time (hours)	48	24	48	72	143	31	72	54	96
Biomass concentration (g.L ⁻¹)	0.67	0.26	0.29	0.47	1.05	0.11	1.46	8.19	10.39
Y _{X/S0} (g.g ⁻¹)	0.03	0.01	0.01	0.02	0.04	0.01	0.08	0.08	0.27
Productivity (g. Lh ⁻¹)	0.01	0.01	0.00	0.00	0.00	0.00	0.02	0.17	0.11
Cells.mL ⁻¹	1.3x10 ⁷	6.1x10 ⁶	6.7x10 ⁶	9.9x10 ⁶	2x10 ⁷	3x10 ⁶	2.7x10 ⁷	1.51x10 ⁸	1.96x10 ⁸
TRS (g)	17.16	14.12	16.75	19.57	22.59	9.08	18.16	49.24	95.11
TRRS	-	-	-	-	-	-	-	38.31	58.5

-: TRRS analysis was not performed. TRRS: Total residual reducing sugars; TRS: Total reducing sugars.

Experiment 1 (Table 1) was conducted with 5% CB and 0.5% CSW. with the nutrients added at the beginning (simple batch), while experiments 2, 3, 4. and 5 were carried out in fed-batch mode. In the second experiment. CB + sucrose + amylase were used, and in the third experiment, HCB + sucrose. In the fourth experiment, HCB + molasses were added to increase the supply of sugars. In the fifth experiment, the

viscosity and concentration of sugars were controlled with a lower addition of HCB and molasses.

The results demonstrated a gradual increase in biomass production with the changes made as indicated by the following metrics: biomass concentration increased from 0.67 g.L⁻¹ (1st experiment) to 1.46 g.L⁻¹ (3rd experiment), 8.19 g.L⁻¹ (4th experiment) and 10.4 g.L⁻¹ (5th experiment) (Table 12). Alongside the increase in biomass, there was also an increase in the biomass yield relative to the supplied sugar (Y_{x/s_0}). In the 1st culture. Y_{x/s_0} was 0.039 g.g⁻¹, in the 2nd culture it was 0.046 g.g⁻¹, in the 3rd culture it was 0.080 g.g⁻¹, in the 4th it was 0.08 g.g⁻¹ and in the 5th, it was 0.27 g.g⁻¹. Biomass productivity was also significantly higher in the 5th experiment. at 0.11 g.L.h⁻¹, which is 7.8 times higher than in the first experiment (Table 12).

The highlights for the increase in biomass production are attributed to: 1) the higher initial pH (pH 5.5) compared to the initial experiment (pH 4.5), as it was found to remain in the optimal range for a longer period. partly due to the addition of salts. 2) controlled viscosity - The initial experiments showed high viscosity (84.7 mPa.s) due to the addition of 56 mL of HCB within three hours of cultivation. which affected budding (a 24% decrease), along with high sugar concentration and high Brix (15° Brix). Therefore. the sugar source feeding was adjusted to maintain viscosity at a maximum of 20 mPa.s. The Brix level could not be directly correlated due to the presence of starch and possibly other nutrients that did not represent a direct sugar source for the yeast.

The media evaluated in the bioreactor showed contamination after 20 hours of cultivation with a wild white yeast, indicating the need to improve bioreactor sanitation and also the sterilization of the added nutrients. which were only gelatinized at high temperatures before feeding. Despite this, the initial tests helped to define the parameters for the final cultivation (Experiment 6). Therefore. in the subsequent test (Experiment N° 6). the cultivation medium was autoclaved to reduce external influences. The inoculum was increased from 10⁶ to 10⁸ cells.mL⁻¹ to enhance the culture propagation dynamics.

Based on the previous tests. the following conditions were defined for the next experiment (Experiment 6): pH 5.5, an inoculum of 1x10⁸ cells.mL⁻¹, a set of salts (Set 2) with yeast extract, an optimal Brix range of around 2 to 5%, viscosity below 20 mPa.s. and ART around 40 g.L⁻¹. These variables were adjusted and set for the following tests in both fed-batch and simple batch processes. Additionally, to minimize pH variation during cultivation a buffering solution using K₂HPO₄ and (NH₄)₃PO₄ was employed to maintain

a stable pH. Viscosity is also an important variable that can either promote or inhibit the growth of the selected yeasts.

The following tables present the cultivation results in fed-batch and simple batch processes for the four selected yeasts. In Experiment 6, the cultivation of *S. pararoseus* Sia 33.1 was conducted in fed-batch (Table 13) and simple batch (Table 14). The yeast *S. pararoseus* Sia 33.1 showed a good response in terms of sugar consumption in the fed-batch process, maintaining cell production around 1×10^8 cells.mL⁻¹. The pH of the medium decreased from 5.5 to approximately 4.6 every 12 hours of cultivation in both processes. The viscosity remained low, with a maximum of 13 mPa.s. The ARRT content did not exceed 37.92 g.L⁻¹ in the fed-batch process and 30.19 g.L⁻¹ in the simple batch process, which was desired. The highest Brix value obtained in both processes was 7.5 °Brix.

Both cultivation processes presented amylase and xylanase but not cellulase. The highest enzymatic productions were observed at the final stages of cultivation. The fed-batch cultivation reached a maximum of 2×10^8 cells.mL⁻¹ of *S. pararoseus* Sia 33.1 in 96 hours with similar results obtained in the simple batch process. achieving 1.95×10^8 cells.mL⁻¹ in 96 hours.

Table 13. Fed-batch cultivation with *S. pararoseus* Sia 33.1 in a refrigerated incubator conducted at 30°C, pH 5.5, and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹ ₁	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Vol(mL)	Visco (mPa.s)
T0h	81±0.01	12.97±1.76	16.85±0.63	1.20x10 ⁸	37.92±13.83	5.61±0.11	4.33±0.29	0±0.0	0±0.0	0±0.0	166	12.7
T12h1	84±0.06	15.61±1.52	18.88±4.34	1.63x10 ⁸	20.20±18.01	5.38±0.14	4.33±0.58	0.082±0.007	0±0.0	0±0.0	166	13.3
T12h0	99±0.01	20.43±3.18	20.61±3.41	6.55x10 ⁷	24.20±1.26	5.37±0.04	4.66±0.29	0±0.0	0.028±0.005	0±0.0	360	12.7
T24h1	95±0.03	15.38±4.08	15.93±3.72	1.52x10 ⁸	19.68±1.71	4.51±0.42	4±0.0	0±0.0	0.033±0.004	0±0.0	360	3.34
T24h0	91±0.08	16.33±3.09	17.90±2.03	1.20x10 ⁸	27.79±2.06	6.03±0.83	5±0.0	0.095±0.01	0.048±0.01	0±0.0	540	4.36
T36h1	100±0.01	13.14±6.12	13.14±6.12	2.23x10 ⁸	20.46±3.44	4.41±0.32	3.4±0.17	0.052±0.012	0±0.0	0±0.0	540	5.4
T36h0	99±0.01	12.94±4.5	13.03±4.41	1.32x10 ⁸	32.31±2.72	4.96±0.20	4.9±0.36	0.22±0.04	0.02	0±0.0	850	1.87
T48h1	99.6±0.01	14.03±4.68	14.34±4.21	1.48x10 ⁸	25.18±3.86	4.53±0.17	4.56±0.4	0.039±0.02	0.033±0.01	0±0.0	850	1.75
T48h0	99.4±0.01	11.44±4.87	11.61±4.89	1.41x10 ⁸	31.04±2.38	5.4±0.12	5.9±0.36	0±0.0	0.013±0.007	0±0.0	1000	2.58
T60h	99±0.01	20.35±7.46	20.35±7.46	1.84x10 ⁸	25.00±2.67	5.2±0.31	7.16±0.76	0.33±0.021	0.267±0.013	0±0.0	1000	1.78
T72	98.4±0.01	19.94±8.06	19.94±8.06	2.00x10 ⁸	19.86±4.22	5.0±0.75	7.5±1.0	0.22±0.1	0.048±0.01	0±0.0	1000	2.2
T96	97.46±0.01	21.92±7.95	21.92±7.95	2.16x10 ⁸	12.07±0.33	4.6±0.80	6±0.5	0.42±0.2	0.22±0.08	0±0.0	1000	2.5
T120	97.5±0.01	14.57±1.83	14.70±2.03	1.58x10 ⁸	13.38±3.61	4.7±0.14	4±0.0	0.10±0.02	0.06	0±0.0	1000	3.45

Solutions: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024%; and (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1% (w.v⁻¹). With the addition of 0.5% (v.v⁻¹) corn steep water. Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells per milliliter; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; TRRS: Total residual reducing sugars. Prior acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Time: number 1 added in front of the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition. Standard deviation used as a measure of dispersion.

Table 14. Simple batch cultivation with *S. pararoseus* Sia 33.1 in an incubator conducted at 30°C, pH 5.5, and 180 rpm.

	Via (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Visco (mPa.s)
T0h	99.5±0.01	23.89±3.95	23.89±3.95	1.30x10 ⁷	30.19±0.10	5.5 ± 0.1	7.5±0.13	0.122±0.04	0±0.0	0±0.0	2.09±0.66
T12h	99.5±0.01	20.6±0.85	20.6±0.85	2.27x10 ⁷	29.34±0.23	4.685±0.2	7.5±0.0	0±0.0	0.013±0.005	0±0.0	13.3±1.23
T24h	99.25±0.02	20.3±3.25	20.3±3.25	3.44x10 ⁷	22.79±32.23	4.35±0.24	7.25±0.35	0.056±0.01	0.023±0.01	0±0.0	3.34±0.87
T36h	98.8±0.2	14.85±1.48	14.85±1.48	8.14x10 ⁷	23.78±33.63	4.24±0.06	7±0.25	0.078±0.01	0.008±0.002	0±0.0	5.4±0.78
T48h	99.20±0.34	14.65±0.21	14.65±0.21	9.35x10 ⁷	24.03±33.99	4.25±0.01	6.5±0.71	0.061±0.01	0.033±0.01	0±0.0	1.75±0.32
T72	99±0.23	14.9±0.14	15±0.15	1.05x10 ⁸	8.68±12.29	4.185±0.06	5.5±0.71	0.069±0.02	0.0089±0.002	0±0.0	2.2±0.6
T96	97.66±0.1	15.17±0.2	15.64±0.2	1.95x10 ⁸	16.83±23.8	5.12±0.39	5.6±0.57	0.091±0.012	0±0.0	0±0.0	2.5±0.26
T120	98±0.2	11.88±0.63	12.87±0.71	1.74x10 ⁸	13.95±19.73	4.1±0.1	4.75±0.35	0.113±0.012	0.023±0.01	0±0.0	3.45±0.36

Salts K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v.v⁻¹). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. Total volume of 1L. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Standard deviation used as a measure of dispersion.

The cultivation in fed-batch and simple batch with *W. onychis* LABI2 is presented in Tables 15 and 16. *W. onychis* LABI2 demonstrated excellent cell viability for both cultivations (95 to 99%), showing increasing cell proliferation in response to the favorable conditions of the medium, especially the content of total residual reducing sugars (TRRS), Brix and viscosity, which allowed for live bud productions ranging from 21% to 29%.

The highest concentration of live buds was obtained at 36 hours for the fed-batch process (20%) and at 12 hours (29%) for the simple batch process. The highest Brix content was 6 (at 72 hours) for the fed-batch and 6.33° Brix (at 0 hours) for the simple batch. Similarly, the TRRS concentration was 24.67 g.L⁻¹ and 20 g.L⁻¹ (at 0 hours and 36 hours) for the fed-batch process and as expected, it was 61.1 g.L⁻¹ (at 0 hours) at the beginning of the simple batch cultivation.

The viscosity remained low for both cultivations, favoring cell proliferation. The results were similar, with 3.75x10⁸ cells.mL⁻¹ of *W. onychis* LABI2 for the fed-batch process and 3.90x10⁸ cells.mL⁻¹ for the simple batch process in 120 hours. Xylanase and amylase production were observed, but not cellulase production, in the fed-batch cultivation of *W. onychis* LABI2. Conversely, the simple batch cultivation did not demonstrate activity of amylase and cellulase.

Table 15. Fed-batch cultivation with *W. onychis* LABI2 in an incubator conducted at 30°C, pH 5.5 and 180 rpm

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Vol(mL)	Visco (mPa.s)
T0h	100±0.0	14.11±3.63	14.11±3.63	1.74x10 ⁸	24.67±2.82	5.50±0.06	4±0.0	0.00±0.0	0.00±0	0±0.0	166	5.82±1.6
T12h1	100±0.0	24.67±3.14	24.67±3.14	5.36x10 ⁸	17.88±1.33	4.37±0.10	4±0.0	0±0.0	0.01±0.01	0±0.0	166	13.30±1.2
T12h0	99.88±0.0	21.63±3.14	21.93±2.98	2.24x10 ⁸	18.19±4.16	5.23±0.15	4±0.0	0.21±0.013	0.03±0.01	0±0.0	360	12.70±1.8
T24h1	100±0.0	24.80±5.41	24.80±5.41	3.56x10 ⁸	8.26±0.86	4.70±0.12	3.66±0.57	0.056±0.02	0.02±0.01	0±0.0	360	3.34±0.36
T24h0	99.07±0.01	19.91±3.76	19.91±3.76	2.95x10 ⁸	16.32±1.87	5.36±0.11	4±0.0	0.095±0.02	0.02±0.01	0±0.0	540	4.36±0.65
T36h1	100±0.0	16.77±3.53	16.77±3.53	2.74x10 ⁸	14.16±0.45	4.61±0.02	3±0.0	0.07±0.03	0.0089±0.001	0±0.0	540	5.40±1.2
T36h0	99.62±0.0	20.32±0.55	20.32±0.55	2.10x10 ⁸	20.64±1.43	5.23±0.05	3.33±0.28	0.095±0.01	0.02±0.01	0±0.0	850	1.87±0.24
T48h1	100±0.0	19.06±2.59	19.06±2.59	1.99x10 ⁸	8.08±0.43	4.7±0.17	3.3±0.17	0.061±0.02	0.033±0.02	0±0.0	850	1.75±0.37
T48h0	100±0.0	12.20±4.06	12.20±4.06	2.18x10 ⁸	16.83±0.94	5.23±0.2	4.4±0.17	0.12±0.01	0.023±0.01	0±0.0	1000	2.58±0.67
T60h	98.5±0.0	16.55±0.77	16.55±0.77	2.18x10 ⁸	7.39±0.57	5.12±0.25	5.9±0.79	0.087±0.012	0.033±0.005	0±0.0	1000	1.78±0.87
T72	97.81±0.0	15.03±4.32	16.03±4.32	2.54x10 ⁸	7.14±0.51	4.7±0.39	6±0.86	0.069±0.02	0.0089±0.002	0±0.0	1000	2.20±0.46
T96	98.74±0.0	12±3.37	15.05±3.37	2.86x10 ⁸	7.04±0.15	4.62±0.29	5.33±1.15	0.091±0.02	0±0.0	0±0.0	1000	2.50±0.56
T120	99.49±0.0	9±1.27	10.5±1.27	3.75x10 ⁸	6.14±1.17	4.92±0.30	3.66±0.57	0.11±0.01	0.023±0.013	0±0.0	1000	3.45±0.24

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v/v). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells.mL⁻¹; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Time: number 1 added before the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition. Standard deviation used as a measure of dispersion.

Table 16. Simple batch cultivation with *W. onychis* LABI2 in an incubator conducted at 30°C, pH 5.5, and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	ARRT (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Visco (mPa.s)
T0h	95%±0.0	13.74±1.48	18.24±1.63	2.29x10 ⁷	61.11±2.09	5.40±0.03	6.33±0.58	0.131±0.02	0.00±0.0	0.00±0.0	10.02±0.51
T12h	97%±0.02	29.21±14.05	36.91±9.21	2.50x10 ⁸	28.37±0.41	4.76±0.01	4.33±0.58	0.000±0.0	0.00±0.0	0.00±0.0	9.36±0.58
T24h	99%±0.01	21.51±2.31	21.65±2.52	2.31x10 ⁸	17.01±1.55	4.87±0.12	4.00±0.0	0.038±0.01	0.00±0.0	0.00±0.0	9.99±0.77
T36h	98%±0.01	21.50±4.68	22.71±4.02	2.44x10 ⁸	19.66±0.72	4.99±0.0	4.00±0.0	0.000±0.0	0.00±0.0	0.00±0.0	9.57±0.54
T48h	99%±0.0	25.27±10.84	26.14±11.46	3.00x10 ⁸	19.20±0.75	4.97±0.08	4.00±0.0	0.093±0.02	0.00±0.0	0.00±0.0	9.30±0.15
T72	98%±0.01	25.62±3.81	27.95±4.26	3.44x10 ⁸	19.46±0.91	4.93±0.08	3.67±0.58	0.00±0.0	0.00±0.0	0.00±0.0	9.71±0.12
T96	96%±0.01	29.41±0.58	32.86±0.93	3.86x10 ⁸	19.96±1.05	4.88±0.15	3.67±0.58	0.075±0.02	0.00±0.0	0.00±0.0	9.81±0.43
T120	96%±0.02	26.56±3.23	28.65±2.66	3.90x10 ⁸	18.40±0.75	4.91±0.14	3.67±0.58	0.205±0.1	0.00±0.0	0.00±0.0	9.78±0.28

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v/v). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells.mL⁻¹; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%, 26h at 97°C. pH 1.08). Time: number 1 added before the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition. Standard deviation used as a measure of dispersion.

The results obtained from fed-batch and simple batch cultivation for *Saccharomyces cerevisiae* M26 are shown in Tables 17 and 18. The fed-batch cultivation exhibited a higher concentration of live buds at 36.6% (at 0 hours) from the inoculum, remaining between 6.8% and 25% throughout the cultivation. The simple batch cultivation showed live bud concentrations ranging from 4.73% to 18.2%. The highest TRRS concentration obtained was 25% (at 12 and 24 hours) for the fed-batch process and 47.73 g.L⁻¹ for the simple batch process (at 0 hours). The highest Brix content was 3.5° Brix (at 36 hours) for the fed-batch cultivation and 7° Brix (at the beginning) for the simple batch cultivation. Viscosity remained below 13.3 mPa.s for both cultivations.

The simple batch cultivation for *Saccharomyces cerevisiae* M26 showed good results in terms of viability and cell proliferation, which did not appear to be affected by the high initial substrate concentration. Within just 12 hours of cultivation, it achieved a production of 1.74x10⁸ cells.mL⁻¹. The cultivations with *Saccharomyces cerevisiae* M26 yielded 4.97x10⁸ cells.mL⁻¹ in the fed-batch process and 3.15x10⁸ cells.mL⁻¹ in the simple batch process over 120 hours.

As shown in Table 14, xylanase analyses began to show positive values starting from 36 hours in the simple batch cultivation, while the fed-batch process exhibited xylanolytic activity practically throughout the cultivation. There was no activity of cellulase and amylase in either of the cultivations.

Table 17. Fed-batch cultivation with *Saccharomyces cerevisiae* M26 in an incubator conducted at 30°C, pH 5.5 and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Vol(mL)	Visco (mPa.s)
T0h	96.65±0.02	36.65±2.51	36.65±2.1	9.63x10 ⁷	24.03±0.0	5.47±0.04	3.1±0.14	0.12±0.01	0±0.0	0±0.0	166	6.75±2.3
T12h1	99.40±0.0	12.3±0.42	12.45±0.64	2.98x10 ⁸	25.02±2.1	4.685±0.07	2.5±0.0	0	0±0.0	0±0.0	166	13.3±3.2
T12h0	96.05±0.01	14.55±2.62	14.55±2.62	5.25x10 ⁷	17.62±1.2	5.45±0.07	3±0.0	0.21±0.1	0±0.0	0±0.0	360	12.7±2.1
T24h1	99.35±0.0	18.09±0.41	18.24±0.63	3.48x10 ⁸	11.66±0.5	4.945±0.64	2.45±0.21	0.06±0.02	0±0.0	0±0.0	360	3.34±1.3
T24h0	99.60±0.01	12.63±1.23	12.63±1.23	7.15x10 ⁷	25.20±1.4	5.35±0.07	3.1±0.14	0.10±0.02	0±0.0	0±0.0	540	4.36±1.2
T36h1	99.13±0.01	15.86±0.37	15.86±0.37	1.73x10 ⁸	20.95±7.39	4.825±0.6	3.15±0.35	0.08±0.03	0±0.0	0±0.0	540	5.4±2.4
T36h0	98.85±0.0	6.8±0.0	6.8±0.0	8.70x10 ⁷	22.01±8.70	5.3±0.1	3.5±0.71	0.10±0.02	0±0.0	0±0.0	850	1.87±0.65
T48h1	99.24±0.0	14.18±1.41	14.315±1.22	1.28x10 ⁸	20.95±7.39	4.74±0.03	3±0.01	0.06±0.01	0±0.0	0±0.0	850	1.75±0.32
T48h0	99.50±0.01	15.19±2.96	15.19±2.96	1.82x10 ⁸	22.01±8.70	5.35±0.07	4±0.0	0.13±0.03	0±0.0	0±0.0	1000	2.58±0.47
T60h	99.77±0.0	19.25±3.18	19.25±3.18	3.67x10 ⁸	15.20±0.02	4.605±0.04	3.25±0.35	0.09±0.02	0±0.0	0±0.0	1000	1.78±0.23
T72	99.58±0.0	19.11±2.03	19.11±2.03	4.90x10 ⁸	11.11±0.1	4.605±0.01	3.15±0.07	0.07±0.03	0±0.0	0±0.0	1000	2.2±0.12
T96	99.70±0.0	21.7±0.99	21.7±0.99	5.57x10 ⁸	11.66±0.7	4.465±0.02	3.25±0.35	0.09±0.02	0±0.0	0±0.0	1000	2.5±1.2
T120	99.50±0.0	25.9±0.99	25.9±0.99	4.97x10 ⁸	11.30±0.02	4.335±0.04	3.15±0.21	0.11±0.03	0±0.0	0±0.0	1000	3.45±1.4

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v/v). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells.mL⁻¹; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Time: number 1 added before the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition. Standard deviation used as a measure of dispersion.

Table 18. Simple batch cultivation with *Saccharomyces cerevisiae* M26 in an incubator conducted at 30°C, pH 5.5, and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Visco (mPa.s)
T0h	82.23±0.02	12.08±1.2	16.53±1.6	9.17x10 ⁶	47.73±2.3	5.42±0.02	7.00±0.2	0±0.0	0.00±0.0	0.00±0.0	9.50±.2
T12h	99±0.01	4.73±1.4	2.57±0.25	1.74x10 ⁸	16.29±3.5	5.24±0.01	4.00±0.1	0±0.0	0.00±0.0	0.00±0.0	9.60±1.45
T24h	99±0.0	6.83±0.65	6.93±0.2	2.28x10 ⁸	15.76±2.45	5.35±0.02	4.00±0.1	0±0.0	0.00±0.0	0.00±0.0	10.79±1.32
T36h	100±0.01	10.60±1.2	11.05±0.35	2.70x10 ⁸	17.84±1.2	5.52±0.01	4.00±0.1	0.110±0.01	0.00±0.0	0.00±0.0	9.96±.5
T48h	98±0.01	11.49±0.65	11.96±1.2	2.18x10 ⁸	14.70±3.21	5.53±0.02	4.00±0.02	0.00±0.0	0.00±0.0	0.00±0.0	11.26±1.23
T72	100±1.0	15.59±1.25	16.30±2.35	2.73x10 ⁸	15.99±2.35	5.30±0.03	4.00±0.0	0±0.0	0.00±0.0	0.00±0.0	9.45±0.85
T96	100±0.0	13.98±1.2	15.02±2.35	3.11x10 ⁸	16.22±1.59	5.01±0.01	4.00±0.0	0.142±0.01	0.00±0.0	0.00±0.0	8.55±1.2
T120	97±0.01	18.25±2.3	18.42±1.2	3.15x10 ⁸	16.58±2.54	4.75±0.01	4.00±0.1	0.102±0.01	0.00±0.0	0.00±0.0	9.76±0.45

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024%; and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v.v⁻¹). Initial inoculum of 1x10⁸ cells/mL. Vi: Viability; LB: Live Buds; TB: Total Buds; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. Total volume of 1L. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 20h at 97°C. pH 1.08). Standard deviation used as a measure of dispersion.

The results of the cultivation with *S. japonicus* 70a in fed-batch and simple batch are presented in Tables 19 and 20, respectively. The fed-batch exhibited the lowest cell viability of 93%, °Brix ranging between 3.8 to 7.33 and the highest TRRS content of 84.08 g.L⁻¹ at 48 hours. The highest concentration of live buds was observed at 48 hours, reaching 34%. The simple batch showed a minimum viability of 84%, the highest TRRS content of 26.86% (at 0 hours) and a Brix level around 4%. The viscosity did not exceed 14.6 mPa.s for the fed-batch and 10 mPa.s for the simple batch, despite greater variation in the fed-batch due to nutrient addition during cultivation. the viscosity did not exceed the organism's growth capacity.

Xylanase activity was observed for the fed-batch cultivation and xylanase and amylase production were observed for the simple batch. The results obtained for the fed-batch and simple batch over 120 hours of cultivation were 1.47x10⁸ and 7.50x10⁷ cells.mL⁻¹, respectively.

Table 19. Fed-batch cultivation with *S. japonicus* 70a in an incubator conducted at 30°C, pH 5.5 and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Vol(mL)	Visco (mPa.s)
T0h	93±0.1	10±1.01	14±1.03	9.65x10 ⁷	29.91±1.2	4.69±0.2	4.33±0.1	0.00±0	0.00±0	0.00±0	166	14.60±0.90
T12h1	98±0.2	18±1.02	18±1.04	6.05x10 ⁷	21.70±2.5	5.25±0.2	4.00±0.2	0.27±0.03	0.00±0	0.00±0	166	12.90±2.5
T12h0	99±0.01	24±1.2	25±1.05	1.58x10 ⁸	55.12±3.5	5.50±0.1	4.67±0.1	0.21±0.02	0.00±0	0.00±0	360	9.74±1.2
T24h1	99±0.1	24±1.02	25±1.25	1.58x10 ⁸	20.27±3.4	5.20±0.2	3.83±0.1	0.00±0	0.00±0	0.00±0	360	10.55±1.3
T24h0	96±0.02	25±1.12	26±1.13	1.09x10 ⁸	54.30±1.25	5.50±0.2	5.07±0.3	0.04±0.03	0.00±0	0.00±0	540	10.36±1.67
T36h1	93±0.1	32±0.65	32±1.2	1.54x10 ⁸	19.69±2.5	5.13±0.1	4.50±0.2	0.22±0.02	0.00±0	0.00±0	540	12.35±2.1
T36h0	95±0.02	27±1.12	32±1.24	8.12x10 ⁷	77.88±3.5	5.50±0.1	6.33±0.3	0.19±0.03	0.00±0	0.00±0	850	10.09±1.45
T48h1	97±0.1	34±3.24	38±3.2	1.14x10 ⁸	37.72±2.5	5.15±0.1	5.83±0.1	0.13±0.01	0.00±0	0.00±0	850	10.65±1.2
T48h0	99±0.2	28±1.24	32±2.45	6.47x10 ⁷	84.08±3.5	5.50±0.1	7.33±0.2	0.00±0	0.00±0	0.00±0	1000	9.66±0.98
T72	100±0.02	19±1.02	19±1.2	1.34x10 ⁸	30.90±2.5	5.01±0.2	7.17±0.3	0.00±0	0.00±0	0.00±0	1000	10.80±1.3
T96	97±0.1	23±1.2	26±1.32	1.38x10 ⁸	31.34±2.1	4.98±0.1	6.83±0.3	0.23±0.02	0.00±0	0.00±0	1000	10.30±1.4
T120	95±0.02	28±2.03	30±2.54	1.47x10 ⁸	20.61±1.2	4.99±0.2	6.33±0.2	0.00±0	0.00±0	0.00±0	1000	10.25±1.23

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v/v). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; Cells.mL⁻¹: Cells.mL⁻¹; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Time: number 1 added before the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition. Standard deviation used as a measure of dispersion.

Table 20. Simple batch cultivation with *S. japonicus* 70a conducted in an incubator at 30°C, pH 5.5 and 180 rpm.

	Vi (%)	LB (%)	TB (%)	Cells.mL ⁻¹	TRRS (g.L ⁻¹)	pH	°Brix	Xyl (U.mL ⁻¹)	Amy (U.mL ⁻¹)	Cel (FPU.mL ⁻¹)	Visco (mPa.s)
T0h	84±0.01	13±0.80	18±1.2	2.47x10 ⁷	26.86±2.5	5.20±0.1	4.17±0.1	0.00±0	0.00±0	0.00±0	10.00±1.2
T12h	99±0.02	13±1.23	13±1.1	2.76x10 ⁷	24.88±.2	5.16±0.2	4.00±0.1	0.02±0.01	0.00±0	0.00±0	10.40±2.1
T24h	100±0.01	19.23±2.2	19.61±2.4	3.07x10 ⁷	21.80±1.8	5.26±0.1	4.00±0.2	0.00±0	0.00±0	0.00±0	10.40±2.3
T36h	84±0.01	30±2.45	30±2.54	2.75x10 ⁷	20.71±1.8	5.16±0.2	4.00±0.1	0.00±0	0.00±0	0.00±0	10.60±2.5
T48h	97±0.02	22±2.3	24±2.1	4.45x10 ⁷	21.20±2.3	5.19±0.1	3.75±0.2	0.00±0	0.03±0.01	0.00±0	10.20±2.8
T72	95±0.01	44±2.7	45±3.2	3.44x10 ⁸	18.32±1.1	5.07±0.2	3.75±0.1	0.00±0	0.00±0	0.00±0	10.20±0.8
T96	99±0.02	28±1.4	29±2.1	7.45x10 ⁷	9.80±0.9	5.09±0.1	3.00±0.1	0.30±0.02	0.24±0.02	0.00±0	10.40±1.3
T120	94±0.02	29±2.3	31±2.5	7.50x10 ⁷	9.33±0.7	5.11±0.2	3.00±0.2	0.18±0.02	0.39±0.02	0.00±0	10.10±1.2

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% e (NH₄)₃PO₄ 0.13%; yeast extract 0.05%; and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep water 0.5% (v.v⁻¹). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Buds; TB: Total Buds; C.mL⁻¹: Cells.mL⁻¹; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visco: Viscosity; Conf: Viscosity reliability. Total volume of 1L. The pH was adjusted every 12 hours. as in the fed-batch cultivation. TRRS: Total residual reducing sugars. Previous acid hydrolysis of cassava bagasse (13%. 26h at 97°C. pH 1.08). Standard deviation used as a measure of dispersion.

Table 21 presents the chemical characterization of the residual substrate from the cultivation conducted in fed-batch and simple batch for the four evaluated microorganisms.

Table 21. Chemical characterization of the residues obtained after the fed-batch and simple batch cultivations carried out with the four evaluated yeasts.

CHEMICAL CHARACTERIZATION - DRY FINAL RESIDUE OF CULTURES				
Fed-batch (%)	<i>W. onychis</i> LABI2	<i>S. pararoseus</i> Sia 33.1	<i>S. cerevisiae</i> M26	<i>S. japonicus</i> Sia 70a
Lipids	0.29±0.0	1.24±0.37	0.85±0.27	0.83±0.17
Protein	18.40±2.52	17.88±0.13	18.18±1.96	19.99±1.93
Ash	2.46±0.38	3.88±5.38	4.55±1.23	6.16±0.09
ADF	63.22±11.86	75±1.17	77.44±13.37	65.48±4.56
NDF	98.00±2.00	97.00±3.00	96.00±2.00	98.7±3.21
Total carbohydrates	15.64	1.99	1.41	7.51
Simple batch (%)				
Lipids	0.45±0.03	1.67±0.52	0.47±0.09	1.10±0.12
Protein	18.67±0.86	20.55±1.28	21.97±3.18	16.55±1.13
Ash	5.87±0.21	4.87±0.24	7.26±1.08	5.04±1.61
ADF	69.88±9.74	55.49±2.84	57.43±3.84	76.58±7.95
NDF	97.46±6.96	80.04±12.8	99±9.38	97.89±3.89
Total carbohydrates	5.11	17.4	12.94	0.71

NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber. Standard deviation used as a measure of dispersion.

The residue acts as a matrix that traps some of the yeasts, thereby increasing the protein content of the material. The results demonstrate a protein content ranging from 16 to 21% for the four yeasts. The lipid content is low as expected, as is the ash content. With prior acid hydrolysis of the material, it is believed that a good portion of the starch was hydrolyzed explaining the low total carbohydrate content. The fibrolytic material is more difficult to degrade, which justifies its higher concentration in the analyzed dry material. The material presents high fiber content and low lipid and total carbohydrate content, favorable for ruminal feeding. Below is a summary of the main results found in both simple and fed-batch fermentation for the four microorganisms (Table 22).

Table 22. General summary of the results obtained through the cultivation of the four yeasts in simple and fed-batch batches conducted at 96 and 120 hours in an orbital shaker incubator at 30°C, pH 5.5 and 180 rpm.

	<i>W. onychis</i> LABI2 (a.b.c.c)	<i>S. cerevisiae</i> M26 (a.b.c.c)	<i>S. pararoseus</i> Sia 33.1 (a.b.c.b)	<i>S. japonicus</i> Sia 70a (a.a.b.c)
Fed-batch 96 h				
Biomass concentration (g.L ⁻¹) (a.b.c.d)	2.906	34.521	11.081	3.549
Y _{X/S0} (g.g ⁻¹)	0.049	0.584	0.584	0.049
Y _{X/S} (g.g ⁻¹)	0.056	0.727	0.236	0.128
Productivity (g. Lh ⁻¹)	0.030	0.360	0.115	0.037
Xylanase (U.mL ⁻¹)	0.09	0.09	0.42	0.23
Amylase (U.mL ⁻¹)	0	0	0.22	0
Cellulase (FPU)	0	0	0	0
Fed-batch 120 h				
Biomass concentration (g.L ⁻¹) (a.b.c.a)	3.912	30.692	9.285	3.811
Y _{X/S0} (g.g ⁻¹)	0.066	0.519	0.157	0.064
Y _{X/S} (g.g ⁻¹)	0.074	0.642	0.203	0.099
Productivity (g. Lh ⁻¹)	0.033	0.256	0.077	0.032
Xylanase (U.mL ⁻¹)	0.11	0.11	0.1	0
Amylase (U.mL ⁻¹)	0.023	0	0.06	0
Cellulase (FPU)	0	0	0	0
Simple batch 96 h				
Biomass concentration (g.L ⁻¹) (a.b.a.c)	4.104	19.259	3.786	1.449
Y _{X/S0} (g.g ⁻¹)	0.069	0.326	0.064	0.025
Y _{X/S} (g.g ⁻¹)	0.105	0.449	0.090	0.029
Productivity (g. Lh ⁻¹)	0.043	0.201	0.039	0.015
Xylanase (U.mL ⁻¹)	0.07	0.142	0.09	0.3
Amylase (U.mL ⁻¹)	0	0	0	0.24
Cellulase (FPU)	0	0	0	0
Simple batch 120 h				
Biomass concentration (g.L ⁻¹) (a.b.c.d)	4.149	19.514	9.099	2.110
Y _{X/S0} (g.g ⁻¹)	0.070	0.330	0.154	0.002
Y _{X/S} (g.g ⁻¹)	0.102	0.459	0.201	0.003
Productivity (g. Lh ⁻¹)	0.035	0.163	0.076	0.001
Xylanase (U.mL ⁻¹)	0.205	0.102	0.113	0.18
Amylase (U.mL ⁻¹)	0	0	0.023	0.39
Cellulase (FPU)	0	0	0	0

Analysis of variance (ANOVA) was used with Tukey's multiple comparison test with a significance level of 0.05%. Letters in front of the microorganisms sequentially compare the biomass concentration obtained in different cultures. Letters in front of the biomass concentration in each culture compare sequentially, the biomass concentration of the four yeasts among themselves. Different letters indicate significant differences between groups.

The process parameters for evaluating the different cultures are presented in Table 22. comparatively for the four yeasts. For the culture fed with *S. cerevisiae* M26, a higher biomass

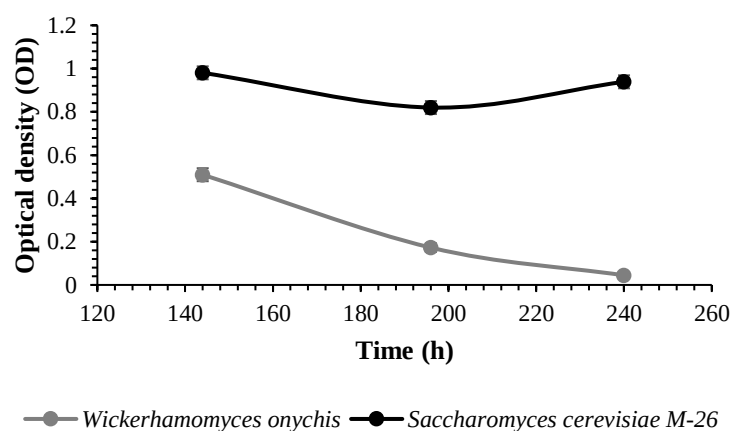
concentration was obtained (34.52 g.L⁻¹ in 96 hours). The second-best biomass concentration was obtained for *S. pararoseus* Sia 33.1 (11.08 g.L⁻¹ in 96 hours), while *W. onychis* LABI2 (3.91 g.L⁻¹ in 120 hours) and *S. japonicus* Sia 70a (3.81 g.L⁻¹ in 120 hours) achieved the lowest biomass concentrations. The low biomass concentration of *W. onychis* LABI2 may be associated with the weight of the microorganism, which is the smallest and lightest among those analyzed.

The yeast that demonstrated the highest enzymatic activities was *S. japonicus* 70a in simple batch. However, it is possible to observe that the biomass concentration and productivity in fed-batch fermentation were better. All yeasts showed xylanase activity (0.42 U.mL⁻¹ for *S. pararoseus* 33.1, 0.3 U.mL⁻¹ for *S. japonicus* 70a, 0.14 U.mL⁻¹ for *S. cerevisiae* M26, and 0.2 U.mL⁻¹ for *W. onychis* LABI2), and only *S. japonicus* 70a demonstrated amylase activity (0.39 U.mL⁻¹). Cellulase was not detected in any of the cultures.

The yeasts were also subjected to an adaptation process at pH 6.9 and 37°C, conditions present in the rumen environment. Thus, *W. onychis* LABI2 and *S. cerevisiae* M26 were the ones that best adapted to these conditions. However, in the cut system cultivation, where after cultivation 50% of the volume was removed and 50% more medium was added for a new cultivation, there was a better adaptive capacity of *S. cerevisiae* M26 compared to *W. onychis* LABI2, which took longer to produce a higher biomass concentration. *S. japonicus* Sia 70a and *S. pararoseus* Sia 33.1 did not adapt to the imposed conditions of pH 6.9 at 37°C. Attempts to impose milder temperatures of 32°C and 30°C at pH 6.9 were also tested showing slow but existent development at 30°C and pH 6.9.

S. japonicus Sia 70a and *S. pararoseus* Sia 33.1 were more sensitive to changes in pH and temperature compared to *W. onychis* LABI2 and *S. cerevisiae* M26, indicating better adaptability to adverse conditions and physiological stress by the white yeasts (*W. onychis* LABI2 and *S. cerevisiae* M26). Figure 6 shows the initial response of *W. onychis* LABI2 and *S. cerevisiae* M26 to changes in pH and temperature.

Fig 6. Optical density observed during the cultivation by batch system (50% v.v⁻¹) for *S. cerevisiae* M26 and *W. onychis* LABI2 cultivated at 37°C and pH 6.9.



Therefore, it can be stated that the *Saccharomyces cerevisiae* M26 strain showed greater adaptation capacity to pH 6.9 and a temperature of 37°C. when compared to the other strains tested in this study.

At the end of the cultivation, 13g dry weight of *Saccharomyces cerevisiae* M26 and *W. onychis* LABI2 were obtained (37°C at pH 6.9). For *S. pararoseus* Sia 33.1 15g were obtained (30°C at pH 6.9), while for *S. japonicus* Sia 70a only 9.6g was obtained (30°C at pH 6.9).

5. DISCUSSION

5.1 Enzymatic treatment of cassava bagasse

The enzymatic hydrolysis of cassava bagasse was performed after gelatinization at 80°C, aiming to avoid inhibitor formation (Kim et al., 2014), achieving a yield of 35.84% of released reducing sugars with the commercial enzyme and 19.96% for hydrolysis with the *pool* produced by *R. oligosporus*. Jasko et al., (2011) carried out enzymatic hydrolysis of cassava bagasse (12% w.v⁻¹) with alpha-amylase (90°C for 60 minutes), followed by treatment with amyloglucosidase (60°C for 30 minutes) and achieved 14.5 g.L⁻¹ of extracted reducing sugars.

The results obtained by the authors, approximately 28.40% sugar extraction, considering 51.05% starch in cassava bagasse (Souza et al., 2021), were lower than those achieved in the present study with the commercial enzyme. These values indicate that the temperature used by the authors and/or the hydrolysis time may not have been ideal and also, even when combining two commercial enzymes, the authors did not surpass the results found in the current work,

which used only alpha-amylase and performed prior gelatinization to favor sugar extraction. Mariano et al., (2020) evaluated the hydrothermal treatment (100°C for 60 minutes) of coconut pulp residue generated after milk and oil extraction (2.5% w.v⁻¹) and observed higher release of reducing sugars (26 g.L⁻¹) compared to the control (5 g.L⁻¹). indicating the benefits of gelatinization before treatment.

Gonçalves (2016) evaluated the enzymatic hydrolysis (at 50°C) of cassava bagasse at different concentrations (10, 12 and 15% w.v⁻¹). using 12 U.g⁻¹ of amylolytic extract produced by *Rhizopus oligosporus*. and achieved maximum conversion to reducing sugars (78.15%) of 39.9 g.L⁻¹ with 10% (w.v⁻¹) substrate in 10 hours of reaction, a result superior to the two tests in the present study. However, from an enzymatic hydrolysis conducted using the extract of the same fungus mentioned at a higher temperature range (55°C and 60°C) and in 10 hours of reaction, a conversion of 40% was obtained, a yield closer to that found in the present study (Escaramboni et al., 2018).

This fungus produces a *pool* of enzymes with strong amylolytic activity, breaking down starch entirely into glucose (Escaramboni et al., 2018). which may explain why the hydrolysis was superior compared to hydrolysis with commercial enzyme. However, it should have been closer to the results obtained with the hydrolysis tests using the *pool* of *R. oligosporus*. The inferior results may be a result of the prolonged storage of the enzymatic *pool* of *R. oligosporus*, which was kept in a cold chamber for two years until the tests were conducted. possibly leading to partial denaturation of the enzymes or conformational changes, compromising catalytic activity and reducing efficiency (Rozanova et al., 1985; Nardid et al., 2014; Miura et al., 2019).

Thus, strategies for hydrolysis through enzymatic association or enzyme pools produced by filamentous fungi and/or bacteria are an interesting perspective, considering the greater efficiency in sugar release achieved by the mixture of amylases and glucoamylases without prolonged storage (Chen et al., 2015). However, in other studies. greater benefits have been observed from the use of acid hydrolysis compared to enzymatic hydrolysis (Woiciechowski et al., 2002). Bearing this in mind, the thermo-acidic extraction of the bagasse was performed.

5.2 Thermo-acid treatment of cassava bagasse

The tests of acid hydrolysis performed showed a greater prominence for pH 1.50 in 10 hours of hydrolysis, considering the lower volume of sulfuric acid used and an excellent yield of released reducing sugars (69.89%).

Adeogun et al., (2018) when evaluating the acid hydrolysis of cassava bagasse (20% w.v⁻¹) at temperatures ranging from 60 to 120°C, for 30 to 60 minutes of reaction and hydrochloric acid concentration from 0.37 to 1.85% (v.v⁻¹), observed the production of 18.4 g.L⁻¹ of glucose (120°C for 60 minutes with 1.85% acid), which corresponds to a sugar extraction yield of only 15.5% (considering 51% starch in this residue). This result was inferior to those obtained in the present study with much less acid and temperature. hence much more economical: a yield of 99.6% in 15 hours with 0.48% concentrated sulfuric acid (pH 0.5) or 64.8% yield with 0.15% (pH 1.5) and 10 hours of reaction at 97°C. The findings indicate the importance of a feasible temperature and relatively low acid content, with a reaction time of 10 to 15 hours to achieve good extraction yields, thus avoiding higher acid concentrations and pressurized tanks that increase risks and equipment costs.

Through the experimental design, we observed the release of 55.15 g.L⁻¹ of sugars in 10 hours with a pH of 1.08. The results indicate something expected, that the higher the acid concentration used (lower pH), the greater the release of reducing sugars (Adeogun et al., 2018). The subsequent tests of wheat bran and cassava bagasse hydrolysis at pH 1.08. at different times (10 to 20 hours) and concentrations (10 to 15% w.v⁻¹). showed the use of acid, approximately 0.32 to 0.45% for wheat bran and 0.30 to 0.40% for cassava bagasse. Adeogun et al., (2018) conducted acid hydrolysis with HCl and cassava bagasse concentrations ranging from 1 to 20% w.v⁻¹ for 3.5 hours at 60°C with 1.85% (w.v⁻¹) pure hydrochloric acid and obtained only 20.2 g.L⁻¹ of sugars with 20% cassava bagasse, a result about 4 times lower than that obtained with six times less sulfuric acid and only 13% cassava bagasse demonstrating greater efficiency with a longer hydrolysis time (20 hours) and higher temperature (97°C). Gaewchingduang & Pengthemkeerati (2010) observed a rapid increase in released sugars through the hydrolysis of cassava bagasse at low substrate concentration (2% w.v⁻¹) in an autoclave at 121°C for 30 minutes with 0.981% (w.v⁻¹) sulfuric acid. compared to 0.98% (w.v⁻¹) phosphoric acid, a moderate acid with sugar release around 7.40 and 2.40 g.L⁻¹ respectively. very low values for the amount of acid used and high temperatures. probably due to the short reaction time (30 min). This emphasizes the results obtained in this research by acid hydrolysis despite employing a longer hydrolysis time, higher yield and lower acid concentration, which ultimately reduce process costs.

Different authors evaluate acid hydrolysis under extreme conditions of pressure and temperature (Jasko et al., 2011; Barbosa et al., 2020) in the quest for shorter hydrolysis times

ranging from 10 to 200 minutes while others assess enzymatic association and pretreatments for hydrolysis exceeding 20 hours, reaching up to 70 hours (Silva et al., 2018). However, regardless of the variations in factors the characteristics of the final product should be aligned with its likely application. Acid hydrolysis although faster compared to enzymatic hydrolysis, can inhibit microbial cultures due to the presence of inhibitors such as furfural; furthermore, it is preferable to obtain a final product more concentrated in sugars with 10% w.v⁻¹ of substrate or more being preferred considering the reduction in post-hydrolysis viscosity (Carvalho & Cândido, 2004; Zenatti et al., 2015; Escaramboni, 2019).

5.3 Cell culture in bioreactor

The cultures in bioreactors were experiencing contamination after 20 hours, which is justified given the initial strategy of only gelatinizing the material at high temperatures. Garcia et al., (2018). when assessing the microbiological composition of cassava bagasse, observed the presence of mesophilic aerobic bacteria (6.5×10^3 CFU.g⁻¹ Colony Forming Units), molds and yeasts (1.9×10^3 CFU.g⁻¹), total coliforms (2.1×10^3 most probable number $\times 100^{-1}$), coliforms at 45°C (6.8×10^3 MPN $\times 100^{-1}$). *Bacillus cereus* (1.0×10^1 CFU.g⁻¹) with the absence of *Salmonella*, sulfite-reducing *Clostridia* and *Staphylococcus aureus*, concentrations below the limits established by legislation, however, indicating the capacity for the development of strains already present in the waste utilized.

Therefore, subsequent tests had the medium autoclaved and the need for sugar and a pH around 5.5 was also considered. Moreira et al., (2015) observed better yeast performance at 15°Brix, although they also demonstrated greater sensitivity, which resulted in a decrease in viability from 12°Brix due to osmotic pressure. Additionally, when evaluating pH in yeast cultivation, the authors observed better growth at pH 5.5, the same pH selected in this study. Yeasts maintain homeostasis within this pH range, resisting acidic treatments or pH changes (Lima, Basso & Amorim, 2001).

The use of buffer solution or reagents that favor pH maintenance, such as the addition of citrate and phosphate, which were used in the conducted cultures, is also a strategy (Hahn-Hägerdal et al., 2005). Oh et al., (2000) observed variations in the growth of the bacterium *H. pylori* at pH 3. 5 and 7, obtaining higher biomass production at pH 5 despite also proliferating in acidic conditions. indicating the importance of understanding the needs of the microorganism being studied.

Viscosity was also one of the factors evaluated a condition that can either favor or inhibit cell proliferation. Humaidah et al., (2022), when assessing the cultivation of the salt-tolerant microalga *Aurantiochytrium* sp L3W in a medium containing 790by culture medium (composed of 5.0 g.L⁻¹ of D (+)-glucose, 1.0 g.L⁻¹ of polypeptone and 1.0 g.L⁻¹ of yeast extract in seawater) and CMC at viscosities of 778, 1340, 4210 and 8350 mPa.s. observed total inhibition by the strain at 8350 mPa.s. whereas at 4210 mPa.s. its growth was suppressed to 64% of that obtained at 778 mPa.s. In addition to the inhibition caused by the increase in medium viscosity, the authors evaluated the influence of conducting sterile cultures, indicating greater development under sterile conditions with a biomass production of 4 g.L⁻¹ compared to 2 g.L⁻¹.

5.4 Chemical characterization of the residue obtained after cultivation

Regarding the post-cultivation residue, it is possible to observe that acid hydrolysis of cassava bagasse released a significant portion of the starch; furthermore, the material acted as a matrix aiding in the fixation of microorganisms and increasing its protein content (Pham et al., 2022). Boonnop et al., (2009), when fermenting cassava bagasse with the yeast *Saccharomyces cerevisiae* for 132 hours at 25°C, reduced the cyanide content from 68.6 mg.kg⁻¹ to 47.3 mg.kg⁻¹ and increased the protein content from 2.8% to 18.9%. The protein contents observed by Boonnop et al., (2009) are similar to those found in the post-cultivation residue of this study, ranging from 16.55% to 21.97% protein. The authors also observed 1.26 g.kg⁻¹ of calcium and 0.69 g.kg⁻¹ of phosphate. The lipid content found in this study was lower than that found by Boonnop et al., (2009), with a maximum of 1.67% lipids compared to 3%, however, low lipid concentrations are preferable when considering animal feed to avoid disorders in the rumen and intestine that can cause reduced diet consumption and digestibility (Jenkins, 1994; Kozloski, 2017). Pham et al., (2022) using cassava bagasse in solid-state fermentation with the strain *Sporidiobolus pararoseus* O1 added to the residue β -carotene (4.8 μ g.g⁻¹), lipids (11.95 mg.g⁻¹) and protein (2.58%). It is important to highlight that carotene, especially β -carotene promotes immunity, health, reproductive success, among other benefits (Green et al., 2016). The generated residue can be provided for animal feed considering its chemical characterization, thus allowing a circular system of biomass production application of this biomass and the residue generated after cultivation especially in animal nutrition.

5.5 Cell culture in fed-batch and simple batch

S. cerevisiae M26 showed the highest biomass concentration in fed-batch cultivation demonstrating why this yeast species is the most widely used in biotechnology (Belda et al., 2019; Parapouli et al., 2020). The second-highest production was observed for *S. pararoseus* Sia 33.1 followed by *W. onychis* LABI2 and *S. japonicus* Sia 70a. Shinya et al., (2022) in fed-batch conducted for 96 hours with hydrolyzed cassava bagasse, observed the beginning of the log phase for *S. japonicus* Sia 70a, *W. onychis* LABI2, *S. cerevisiae* M26 and *S. pararoseus* Sia 33.1 within four hours of cultivation, obtaining a biomass concentration of 10.9 g.L⁻¹ for *W. onychis* LABI2 5.2 g.L⁻¹ for *S. pararoseus* 33.1 and 3.1 g.L⁻¹ for *S. cerevisiae* M26 with productivity of 0.07, 0.05 and 0.03 g.L⁻¹h⁻¹ respectively. Regarding the biomass concentrations obtained in this study, only *W. onychis* LABI2 was lower than that reported by the authors. The productivity obtained in this study over 96 hours was 0.03 g.Lh⁻¹ for *W. onychis* LAB2, lower than that achieved by Shinya et al., (2022). However, for *S. pararoseus* 33.1 (0.11 g.Lh⁻¹) and *S. cerevisiae* M26 (0.36 g.Lh⁻¹), the productivity was much higher. The latter was almost 10 times higher in biomass in the present study compared to the reference. It's worth noting that there was the presence of cane molasses along with cassava bagasse in the present study and this yeast was precisely isolated from cane molasses (Oliva-Neto et al., 2004), thus highly adapted to this substrate.

S. japonicus 70a was cultivated with non-hydrolyzed cassava bagasse in fed-batch by Shinya et al., (2022) where the authors observed biomass production of 8.1 g.L⁻¹ productivity of 0.09 g.Lh⁻¹ with Y_{x/s} of 0.19 g.g⁻¹. The results obtained in the present study for *S. japonicus* Sia 70a were lower reaching a maximum biomass production of 3.81 g.L⁻¹ in 120 h of fed-batch cultivation with a productivity of 0.032 g.Lh⁻¹. The Y_{x/s} yield obtained was 0.12 g.g⁻¹ for 96 hours of cultivation whereas Shinya et al., (2022) observed 0.19 g.g⁻¹ demonstrating lower efficiency of the cultivation performed for this yeast. The results indicate that the microorganisms *S. cerevisiae* M26 and *S. japonicus* 70a benefited more from fed-batch fermentation compared to *W. onychis* LABI2 and *S. pararoseus* 33.1. which achieved similar biomass concentrations for both simple and fed-batch cultivation. The cellular viability for all yeasts was above 94% indicating that the cultivation conditions were healthy.

Cazetta & Celligoi (2005), when cultivating *S. cerevisiae* in vinasse obtained a biomass concentration of 4.55 g.L⁻¹ whereas in cultivation with molasses they achieved 5.46 g.L⁻¹. The same authors using the yeast *R. mucilaginosa* in cultivation with molasses. obtained a biomass

concentration of 7.05 g.L⁻¹. Zhang et al., (2015). in fermentation with the yeast *S. cerevisiae* BY4742 achieved a maximum biomass concentration of 3.7 g.L⁻¹ in 96 hours a biomass concentration lower than that obtained in this study and also with a lower productivity of 0.043 g.Lh⁻¹ indicating a slower biomass production (Zhang et al., 2015), however, these authors also produced ethanol.

Chaiyaso et al., (2018) observed α -amylase activity (540 ± 0.09 U.mL⁻¹) and amyloglucosidase (23 ± 0.00 U.mL⁻¹) by *S. pararoseus*. similar to the findings of Kwon et al., (2020), who also observed amylases activity by different strains of *S. pararoseus*. Baffiet et al., (2013) noted β -glucosidase indicating the potential for cellulolytic enzyme activity by the microorganism. Amadi et al., (2020) when evaluating solid-state fermentation with *Saccharomyces cerevisiae* SCPW 17 observed 12.95 ± 0.76 U.gds⁻¹ of xylanase. Shinya et al., (2017) observed 0.2 U.mL⁻¹ of amylase by *S. japonicus* 70a and 0.26 U.mL⁻¹ for *S. pararoseus* 33.1, as well as 0.34 U.mL⁻¹ of xylanase for *W. onychis* LABI2 0.25 U.mL⁻¹ for *S. pararoseus* 33.1 and 0.52 U.mL⁻¹ for *S. japonicus* 70a. confirming the production of these enzymes by these yeasts albeit at moderate concentrations.

The low enzyme production can be explained by the low concentration of amylolytic and/or cellulolytic substrate. considering that enzymatic production requires a physicochemical association of the substrate with the microorganism to stimulate the production of specific enzymes (Polaina & Maccabe, 2007).

5.6 Yeast adaptation to pH and temperature

W. onychis and *Saccharomyces cerevisiae* M26 showed greater ease of adaptation to pH and temperature compared to *S. pararoseus* and *S. japonicus*. According to Zapata et al., (2011) *W. onychis* also known as *Pichia onychis* (evaluated strains Lv027, Lv297 and Lv315) were not affected by temperature variation from 5 to 37°C and by pH ranging from 3 to 9. Oliva-Neto et al., (2004) conducted a screening with 60 yeast strains from must and fermented juice of alcohol distilleries, where *S. cerevisiae* M26 came from an environment with high osmotic pressure and temperature, which may explain its greater thermoresistance and adaptive capacity.

The fed-batch cultivation tests demonstrate the industrial production potential of the tested yeasts. Additionally, the adaptation to pH and temperature highlights future applications in animal nutrition, especially for *W. onychis* and *S. cerevisiae*. The prominence of the evaluated yeasts increases when considering enzymatic production. The benefits of applying the

evaluated microorganisms in animal nutrition aim to improve animal health, performance, and nutrition (Hassan et al., 2020; Bedford et al., 2022).

6. CONCLUSION

The evaluated yeasts, *S. cerevisiae* M26, *S. pararoseus* Sia 33.1, *W. onychis* LABI2, and *S. japonicus* Sia 70a demonstrated good cell proliferation when cultivated in fed-batch and simple batch modes in Experiment 6, with a highlight on *S. cerevisiae* M26 and *S. pararoseus*. preferably in fed-batch mode using a medium containing hydrolyzed cassava bagasse, molasses, maize steep water and mineral salts. The yeasts also showed xylanase and amylase activities with *S. pararoseus* standing out. The main controlled variables in yeast cultivation were viscosity, pH and sugar concentration. *S. cerevisiae* M26 and *W. onychis* LABI2 demonstrated greater potential to withstand stress from temperature and pH variation compared to *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a which showed greater sensitivity at 37°C and pH 6.9. Therefore, *S. cerevisiae* M26 and *W. onychis* LABI2 demonstrate potential application in animal nutrition as live inoculants. considering their better adaptation to pH and temperature. Additionally, *S. pararoseus* also stood out for promising biomass cultivation obtained in both fed-batch and simple batch fermentation.

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8. APPENDIX

Table 23. Experiment 2. Fed-batch cultivation with *S. pararoseus* Sia 33.1 carried out in a 2.5L bioreactor at 30°C and 100 rpm.

Time (h)	Vi (%)	LB (%)	TB (%)	Cells .mL ⁻¹	Amy (U.mL ⁻¹)	RS (g.L ⁻¹)	pH
0h	93.3±2	13±1.4	13±1	1.40x10 ⁶	0.00±0	6.3±1.5	4.5±0.2
8h	93.9±1	18±2.42	18±2	3.30x10 ⁶	0.00±0	9.6±2.4	4.4±0.1
21h	92.2±0.5	33±4.58	35±2	7.10x10 ⁶	0.00±0	7±5.2	3.87±0.2
24h1	98±2.5	32±3.42	34±2	6.10x10 ⁶	0.00±0	3±1.2	3.81±0.3
24h0	100±2	39±4.68	39±3	4.60x 10 ⁶	0.00±0	26±2.5	3.83±0.2
32h	82±3	14±1.45	21±3	5.00x10 ⁶	0.00±0	20±3	3.78±0.1
45h	100±2	16±2.62	16±1	6.00x10 ⁶	0.00±0	17±4	4.45±0.3
48h1	94±1	8±2.3	11±1	6.70x10 ⁶	0.00±0	15±2.6	5±0.3
48h0	85±2.4	7±1.08	11±2	6.50x10 ⁶	0.28±0.09	21±2.1	5±0.2
56h	100±2	26.5±2	26.5±3	9.40x10 ⁶	0.27±0.1	16.7±3	4±0.1
69h	100±2.4	18±3.5	18±2	1.00x10 ⁷	0.33±0.08	23±2.70	4.08±0.04
72h1	96.9±2	15.1±2	16.1±2	9.90x10 ⁶	0.4±0.1	23±3.6	4.98±0.2
72h0	98.5±1	17.6±1.08	17.6±2	6.80x10 ⁶	0.26±0.05	13±2.4	4.88±0.05
80h	93.1±2	13.6±1.5	13.6±1	6.50x10 ⁶	0.26±0.09	11.7±3.5	4.37±0.1
143h	98.5±3	7.9±1.82	8.4±1	2.00x10 ⁷	0.26±0.1	3±2.4	4.18±0.2

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% e (NH₄)₃PO₄ 0.13%. With the addition of corn maceration water 0.5% (v.v⁻¹). Initial inoculum of 1x10⁶ cells. mL⁻¹. Vi: Viability; LB: Live Bud; TB: Total Bud; Ami: Amylase; Total volume of 2.5L. The pH was corrected every 12 hours. as well as in fed-batch cultivation. RS: Reducing sugars. Time: number 1 added in front of the time indicates sample withdrawal before feeding, while the number 0 indicates sample withdrawal after nutrient addition.

Table 24. Experiment 3. Fed-batch cultivation with *S. pararoseus* Sia 33.1 carried out in a 2.5L bioreactor at 30°C and 100 rpm.

Time (h)	Vi (%)	LB (%)	TB (%)	Cells.ml ⁻¹	Amyl (U.mL ⁻¹)	Xyl (U.mL ⁻¹)	RS (g.L ⁻¹)	pH	Vol.
0h	100±0.1	40±4	40±2	1.00x10 ⁶	0.07±0.02	0.00±0	8.61±1.2	5.5±0.2	1250
6h1	85.7±1.5	28±2	28±1	2.1x10 ⁶	0.21±0.02	0.00±0	8.35±1.3	5.5±0.2	1250
6h0	100±1.0	27±2	27±1	1.00x10 ⁶	0.09±0.01	0.08±0.01	8.29±1.7	5.5±0.1	2500
21h	95.8±0.01	16±2	16±2	2.4x10 ⁶	0.03±0.01	0.12±0.01	7.8±1.9	4.5±0.1	2500
24h	100±2	25±1	25±3	2.0x10 ⁶	0.45±0.02	0.17±0.02	7.03±2	5.5±0.3	2500
31h1	93.3±0.1	13±2	13±2	3.0x10 ⁶	0.16±0.02	0.11±0.01	6.4±2.1	4.79±0.3	1250
31h0	93±0.2	14±1	14±2	1.7x10 ⁶	0.21±0.03	0.00±0	5.99±1.2	5.5±0.2	2500
45h	98±0.1	29±3	29±3	1.00x10 ⁷	0.17±0.04	0.00±0	9.18±1.5	3.9±0.1	2500
48h	99.2±1	38±4	33±3	1.3x10 ⁷	0.15±0.03	0.01±0	6.02±1.3	3.7±0.4	2500
55h	99.2±2	9±1	9±1	1.33x10 ⁷	0.4±0.05	0.12±0.02	6.58±2.2	4.2±0.2	2500
72h	99.2±1	13±2	13±2	2.7x10 ⁷	0.5±0.07	0.02±0.01	5.03±1.5	4.5±0.1	2500

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% e (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep liquor 0.5% (v.v⁻¹). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Bud; TB: Total Bud; Xyl: Xylanase; Amy: Amylase; Vol: Volume; TRRS: Total Residual Reducing Sugars. RS: Reducing Sugars. Time: the number 1 added before the time indicates sampling before feeding, while the number 0 indicates sampling after nutrient addition.

Table 25. Experiment 4. Fed-batch cultivation with *S. pararoseus* Sia 33.1 carried out in a 2.5L bioreactor at 30°C and 100 rpm.

Time (h)	Vi (%)	LB	TB	Cells.ml ⁻¹	Amy (U.mL ⁻¹)	Xyl (U.mL ⁻¹)	RS (g.L ⁻¹)	TRRS (g.L ⁻¹)	pH	°Brix	Vol.
T0h	95.7±0.1	17.5±2	17.9±2	1.10x ⁸	0.00±0	0.00±0	26.94±3.2	47.04±4.8	5.5±0.1	4±0.5	250
T3h1	99.7±0.2	17.5±2.5	17.8±1	1.81x ⁸	0.00±0	0.00±0	27.8±2.5	64.89±6.7	5.5±0.2	5±0.3	250
T3h0	98.8±0.2	17.1±3	17.1±2	5.07x ⁷	0.00±0	0.00±0	44.85±5.2	76.85±9.5	5.5±0.1	6±0.5	850
T9h1	99.3±0.1	14.3±1.5	14.3±1	7.95x ⁷	0.00±0	0.00±0	31.39±2.8	80.3±11.2	4.8±0.2	6±1	850
T9h0	100±1	19.3±1.25	19.3±2	4.40x ⁷	0.00±0	0.00±0	78.88±12.4	91.36±15.5	5.5±0.1	10±1.5	1450
T24h1	99.7±2	25±2.45	25±2	1.40x ⁸	0.00±0	0.00±0	46.85±6.2	75.13±16.2	4.5±0.2	8.5±1	1450
T-24h0	98±0.1	12.8±1.05	12.8±1	1.20x ⁸	0.00±0	0.00±0	63.79±4.68	104.7±12.2	5.5±0.3	11±2	1950
T28h1	99±0.2	17.1±2.50	17.1±2	1.02x ⁸	0.00±0	0.00±0	31.49±2.8	132.5±14.7	4.45±0.2	11.5±1	1950
T28h0	98±0.1	12.5±1.25	12.5±4	5.60x ⁷	0.00±0	0.00±0	33.51±3.5	142.9±17.8	5.3±0.3	13.5±2	2450
T32h	99±0.2	13.9±1.25	13.9±2	1.69x ⁸	0.00±0	0.00±0	41.42±3.4	171.4±18.3	5±0.1	15±2.5	2450
T48h	100±1	7.1±1	7.1±2	1.75x ⁸	0.00±0	0.00±0	39.31±2.8	138.78±13.2	4.8±0.2	12±0.5	2450
T54h	100±2	9.2±1	9.2±1	1.51x ⁸	0.00±0	0.00±0	41.79±5.8	94.2±7.8	4.8±0.3	10±2	2850

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% and (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1% (w.v⁻¹). With the addition of corn steep liquor 0.5% (v.v⁻¹). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Bud; TB: Total Bud; Xyl: Xylanase; Amy: Amylase; Vol: Volume; TRRS: Total Residual Reducing Sugars. Time: the number 1 added before the time indicates sampling before feeding, while the number 0 indicates sampling after nutrient addition.

Table 26. Experiment 5. Fed-batch cultivation with *S. parvoseus* Sia 33.1 carried out in a 2.5L bioreactor at 30°C and 100 rpm

Time (h)	Vi (%)	LB (%)	TB (%)	Cells.ml ⁻¹	RS (g.L ⁻¹)	TRRS (g.L ⁻¹)	pH	°Brix	Volume	Visco (mPa.s)
T0h	99.3±1	35±3	35±4	8.00x ⁷	2.88±0.52	4.34±0.8	5.5±0.2	3±0.5	415	7.09±1
T3h1	100±2	44.6±4	44.6±3	1.02x ⁸	2.92±0.38	4.18±0.5	5.4±0.3	3.2±0.8	415	84.7±2
T3h0	100±1	38.8±3	38.8±5	6.95x ⁷	4.252±0.60	7.85±1.2	5.5±0.2	6±1	575	84.7±2
T9h	99.3±0.2	14.3±2	14.3±2	5.60x ⁷	6.41±1	8.79±1.5	4.8±0.2	7±1	575	72±3
T14h1	94.1±0.5	38.3±4	40±3	6.00x ⁷	5.87±1.2	8.91±1.2	5±0.3	6.5±0.5	575	60±3
T14h0	98.8±0.4	48.8±5	50±5	4.50x ⁷	3.88±0.7	7.63±1.6	5.5±0.2	5.2±0.5	675	51±2
T24h	99.3±0.8	46.4±3	46.4±4	9.25x ⁷	3.29±0.5	6.32±2.3	4.43±0.1	5.2±0.4	675	64.9±4
T-24h0	97.75±1	47.8±2	48.3±3	8.90x ⁷	2.8±0.3	5.29±2.1	5.5±0.2	5.1±0.6	735	66±4
T27h1	81.53±2	38.4±2	43.8±3	6.50x ⁷	2.68±0.32	6.19±2.4	4.48±0.3	5±0.3	735	7.26±2
T27h0	86.2±1	35±1	38.7±2	1.60x ⁷	1.02±0.2	2.30±0.8	5.5±0.4	3±0.2	1235	8.15±2.7
T31h	89±2	21.9±4	27±2	6.20x ⁷	1.39±0.3	3.04±0.7	5.3±0.2	3±0.3	1235	8.67±1
T33h1	88.9±0.8	17.1±1	24.8±2	4.60x ⁷	1.51±0.5	2.70±0.7	5.3±0.2	3±0.4	1235	10.1±2
T33h0	98.5±1.5	21.3±5	21.3±1	3.00x ⁷	2.07±0.7	3.04±0.6	5.5±0.2	4±0.5	1565	9.76±1.2
T48h	99.6±2	31.5±2	31.8±3	5.52x ⁷	1.15±0.2	2.33±0.8	5.09±0.1	3.5±0.2	1565	8.53±2.1
T50h1	99.6±2.5	31.7±1	31.7±2	6.72x ⁷	1.22±0.4	2.67±0.5	5.4±0.3	3.5±0.3	1565	6.75±2.1
T50h0	100±2	31.5±2	31.5±3	5.15x ⁷	1.13±0.3	2.54±0.3	5.5±0.3	4±0.3	1915	6±1.4
T54h	100±1	37.9±3	37.9±2	7.25x ⁷	1.21±0.56	2.33±0.9	5.09±0.2	3.8±0.2	1915	8.2±2.1
T57h	99.6±2	34±2	34±2	7.32x ⁷	1.70±0.7	2.61±0.8	5.3±0.3	4.2±0.3	1915	8.77±2.4
T57h	100±1.4	34.8±2	34.8±1	6.02x ⁷	3.71±0.9	4.58±0.4	5.5±0.2	5.2±0.3	2275	7.59±2.3
T72h	100±1.8	28.5±1	28.5±1	1.59x ⁸	1.59±0.5	2.68±0.5	5±0.1	5±0.2	2275	8.29±2.2
T75h	100±2	32.6±3	32.6±2	1.42x ⁸	2.56±0.4	4.79±0.7	5.5±0.2	7±0.5	2500	8.32±1.5
T81h	100±2.5	27.5±2	27.5±2	1.30x ⁸	1.64±0.5	2.64±1	5.2±0.3	5.5±0.2	2500	8.5±2.4
T96h	100±3	24.4±2	24.4±2	1.96x ⁸	1.30±0.4	2.34±0.4	5.35±0.2	5.8±0.4	2500	8.2±1.5

Salts: K₂HPO₄ 0.031%; ZnSO₄ 0.0028%; MnSO₄ 0.0012%; MgSO₄ 0.024% e (NH₄)₃PO₄ 0.13%; yeast extract 0.05% and ammonium sulfate 0.1% (w.v⁻¹). Initial inoculum of 1x10⁸ cells.mL⁻¹. Vi: Viability; LB: Live Bud; TB: Total Bud; Xyl: Xylanase; Amy: Amylase; Cel: Cellulase; Vol: Volume; Visc: Viscosity; ViscR: Viscosity Reliability. TRRS: Total Residual Reducing Sugars. AR: Reducing Sugars. Time: the number 1 added before the time indicates sampling before feeding, while the number 0 indicates sampling after nutrient addition.

CHAPTER 4. PRESERVATION OF YEAST CELL SUSPENSION BIOMASS USING DIFFERENT METHODS

ABSTRACT

The preservation of microorganisms through efficient and commercially applicable methodologies is strategic, especially when aiming to use them as probiotics or live biomass. This study assessed various methods to sustain the viability of four yeast genera (*Sporidiobolus pararoseus* Sia 33.1, *Wickerhamomyces onychis* LABI2, *Saccharomyces cerevisiae* M26 and *Sporobolomyces japonicus* Sia 70a) for animal nutrition tests. Yeast suspensions with 10% (v.v⁻¹) additions of powdered milk, trehalose, glycerol or sucrose were evaluated under lyophilization, freezing and at 4°C and 26°C. The microorganisms were also evaluated in three different oils, mineral oil, vegetable oil and agricultural vegetable oil, all at a concentration of 10% (v.v⁻¹) for 30 days at 26°C. Lyophilization yielded viabilities from 1.6% to 41%, while freezing resulted in 13%-62% viability with mineral and vegetable oils showing similar viabilities (13%-38%). Powdered milk (10% m.v⁻¹) alone or with mineral oil (10% v.v⁻¹) maintained above 90% viability at 26°C for 30 days and over 83% at 4°C for 3 months, except for *S. pararoseus* Sia 33.1 (50% decrease). For immediate yeast suspension use powdered milk effectively preserved cellular viability, while lyophilization was more suited for long-term preservation, particularly for *S. pararoseus* Sia 33.1 and *W. onychis* LABI2 with 36% and 41% viabilities, respectively.

Keyword: Preservation, cryoprotectants, yeasts, lyophilization, freezing.

1. INTRODUCTION

The preservation of food has played a significant role throughout human history as the pursuit of safe food has evolved. Research continues to delve into and refine various strategies to improve the preservation of products. Among these products, we have intrinsic microorganisms within them or marketed as a single product. These microorganisms play essential roles in various industrial processes such as baking, ethanol production, winemaking animal feed manufacturing, biocontrol, probiotics. It is crucial that these microorganisms are resilient, considering the robustness of biotechnological processes and their processing. Therefore, understanding the biophysical and chemical responses of relevant microorganism strains can favor the biotechnological application of these microorganisms with the aim of enhancing their preservation (Câmara Jr et al., 2021; Attfield, 2022).

The choice of the most suitable method for preserving a microorganism depends on the characteristics of the microorganism and the intended use. In the context of marketing microbiological products, it is essential to adopt a methodology that favors storage conditions, prioritizing ease, cost-effectiveness, practicality and above all the quality of the final product. To achieve these goals, it is crucial to consider the storage temperature as well as low humidity. Furthermore, the chosen preservation strategy must be capable of providing high viability of the microorganism over the storage time, ensuring the integrity and functionality of the microbiological product available in the market (Sola et al., 2012; Iwanicki et al., 2022).

Methods can be classified according to the maximum preservation time being short, medium and long-term. To ensure the maintenance of cell cultures in laboratory analyses various methodologies are employed, mainly varying in the type of protective solution cooling rate and suitable suspension medium (De Vero et al., 2019). The best preservation method is the one that meets specific usage needs, considering the species to be preserved; however, the most commonly used techniques for short, medium and long-term preservation are respectively continuous subculturing, conservation in mineral oil, and lyophilization/ freeze-drying (Goldman & Green, 2008). In research laboratories, for the purposes of maintaining culture banks, deep freezing in liquid nitrogen (-196°C) is used which includes direct storage or freezing in liquid nitrogen and storage at -80°C (Sokolski et al., 1964; She et al., 2015). In addition to these there is widespread use of spray drying of microorganisms in the biotechnological industry for baking, winemaking, brewing, among others. The method is

relatively inexpensive and allows for the preservation of large quantities of biomass in a short time (Santos et al., 2017; Aponte et al., 2016; Gélinas et al., 2019; Albagli et al., 2024).

Microorganisms intended for commercialization can be made available as dry and active with approximately 95% dry mass (AAFCO – 96.2 Active Dry Yeast; IFN 7-05-524), wet and cream with 30% and 20% dry mass, respectively (AAFCO. 2017). On the other hand, probiotics for animal supplementation are mainly available in the market as pastes or lyophilized products, although they also exist in other forms such as tablets, hard gelatin capsules, liquids and lyophilization, a process that removes moisture from the material, allows for a longer shelf life, which facilitates marketing and application, while also reducing the risk of contamination due to low humidity (Laulund, 1994; Kuo et al., 2022). However, it is important to note that the process can reduce the viability of microorganisms or even render certain strains completely nonviable, in addition to being a costly procedure (Ozerskaya et al., 2022; Freitas et al., 2020).

The viability of probiotics can be affected by various factors, such as improper storage, humidity, nutrient deficiency. For manufacturers, one of the biggest challenges is ensuring that microorganisms remain viable until the expiration date, which encourages research aimed at improving the preservation and commercialization conditions of microbiological products (Bhagwat et al., 2020; Hotel et al., 2001; Bajagai et al., 2016).

In addition to choosing the most suitable preservation method for the product considering its intended use, it is important to use different solutions that assist in maintaining cellular viability. Cryoprotectants, for example have the ability to protect cells from damage due to low temperature; however, high concentrations of these substances can be toxic, resulting in cell death (Freitas et al., 2020). The most commonly used cryoprotectants in microorganism preservation are glycerol, skim milk, sucrose, trehalose, glucose, sorbitol, serum albumin, dimethyl sulfoxide and ethylene glycol (Guo et al., 2020). Furthermore, in medium-term preservation techniques, it is common to add oils to the cell culture to limit the available oxygen concentration and prevent dehydration of the cell culture (Sharma et al., 2017). These approaches help protect and stabilize cultures contributing to the maintenance of the integrity of the preserved microorganisms (Freitas et al., 2020).

Therefore, this study was conducted with the aim of evaluating the effects of different preservation methods including lyophilization, freezing, 26°C and refrigeration temperature, combined with different cryoprotective solutions, nutrient solutions and oils, on the viability of four yeast strains. The research sought to answer the following question: which physical

approach combined with chemical solutions is the most suitable for preserving the analyzed strains? Furthermore, considering ease of transport, storage, shelf life, and application of yeast, which of the evaluated methods proved to be the most advantageous? The study aims to provide valuable information to enhance the conservation of these microorganisms considering the importance of quality and viability for practical and commercial applications. The research aimed to evaluate various techniques involving the use of chemical compounds, oils, and nutritional sources under different physical conditions, such as temperature and lyophilization, with the purpose of preserving four yeast strains.

2. MATERIALS AND METHODS

2.1. Preservation of microorganisms

The research was conducted using four distinct yeast strains: *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, and *Saccharomyces cerevisiae* M26. The *Wickerhamomyces onychis* LABI2 strain was isolated from rice bran at the Bioenergy Laboratory (IPBEN) of UNESP - FCL Assis. The *Saccharomyces cerevisiae* M26 yeast strain was previously isolated from a bioethanol distillery (Oliva-Neto et al., 2004). The *Sporidiobolus pararoseus* Sia 33.1 and *Sporobolomyces japonicus* Sia 70a strains were kindly provided by Prof. Dr. Fernando Carlos Pagnocca, responsible for the Microbiology Laboratory at the Center for the Study of Social Insects (CEIS) at São Paulo State University (UNESP) in Rio Claro/SP and were isolated from leaf litter by laboratory professionals at UNESP - IB/Rio Claro.

The monthly maintenance of the strains was carried out by culturing in a formulated medium (Oliva-Neto et al., 2004) through subculturing and agar in inclined tubes with oil. The lyophilization technique was applied only to the *Wickerhamomyces onychis* LABI2 and *Saccharomyces cerevisiae* M26 strains, as they proved to be more resistant to this process and could be reactivated later on.

2.2. Culture of microorganisms

The microorganisms *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* 33.1, *Sporobolomyces japonicus* 70a and *Saccharomyces cerevisiae* M26 were cultivated in a

formulated medium consisting of 2.0% glucose, 0.5% yeast extract, 0.13% $(\text{NH}_4)_3\text{PO}_4$, 0.10% $(\text{NH}_4)_2\text{SO}_4$, 0.031% K_2SO_4 , 0.0028% ZnSO_4 , 0.0012% MnSO_4 and 0.024% MgSO_4 (w.v⁻¹). The pH of the medium was adjusted to 5.5. and cultivation was carried out at 30°C for 48 hours of fermentation (Oliva-Neto et al., 2004) and used for subsequent preservation tests.

2.3. Characterization of commercial products and selected yeasts

Commercial products apply different solutions to optimize preservation and increase the viability of the products. Therefore, to demonstrate the characteristics of the products already available on the market the specifications are provided below (Table 1). The labels of two paste products (Product 1 and Product 2) and one lyophilized product (Product 3) were described. Products 1 and 2 underwent several evaluations including the measurement of soluble solids content using the Brix index using a portable refractometer Instrutherm, model RT-30 ATC.

Table 1. Description of three commercial probiotic products used for animals.

	Product 1	Product 2	Product 3
Description	Mineral and vitamin supplement: Paste for ruminants	Probiotic additive for animal feed – paste	Probiotic additive for animal feed – lyophilized
Quantity (g)	40	34	500
Shelf Life (months)	18	12	24
Price (\$)	4.12	13.36-7.77	7.38-9.59
Micronutrients (mg.kg ⁻¹)			
Cobalt	450	-	-
Selenium	20	-	-
Iron	750	-	-
Chromium	45	-	-
Vitamins (U.l.kg⁻¹)			
Vitamin E	4500	-	-
Amino Acids (mg. Kg⁻¹)			
Methionine	9500	-	-
Microrganisms (UFC.kg⁻¹)			
<i>Bacillus subtilis</i>	7.00x10 ¹¹	-	-
<i>Bifidobacterium bifidum</i>	4.80x10 ¹¹	-	-
<i>Enterococcus faecium</i>	4.80x10 ¹¹	-	-
<i>Lactobacillus acidophilus</i>	4.80x10 ¹¹	3.33x10 ⁶	-
<i>Lactobacillus casei</i>	4.40x10 ¹¹	-	-
<i>Lactobacillus lactis</i>	2.20x10 ¹¹	-	-
<i>Saccharomyces cerevisiae</i>	1.00x10 ¹¹	-	2.00x10 ¹³
<i>Bifidobacterium animalis</i>	—	3.33x10 ⁶	-
<i>Enterococcus faecium</i>	—	3.33x10 ⁶	-
Total (UFC.kg ⁻¹)	2.90x10 ¹²	9.99x10 ⁶	2.00x10 ¹³
Directions for use			
Cattle	5 a 10g every three days	-	10 a 20g/head/day
Sheep and Goats	2.5 a 5g every three days	-	1g/ head/day
Calves	-	4g daily	3 a 5g/ head/day
Horses	-	-	5 a 10g/ head/day

Product 2: Composition: Vegetable oil, aluminosilicates, corn starch, ethoxyquin, polysorbate 80, sucrose, silicon dioxide. Storage: Product 1, 2, and 3: Closed packaging, dry and ventilated environment, protected from sunlight and heat, temperature below 30°C. Exchange rate equivalent to 4.86 Brazilian reais (as of 08-04-2023).

In addition, the quantification of reducing sugars was performed using the DNS method (3,5-dinitrosalicylic acid), as described by Miller (1959). Cell viability was determined using the methylene blue solution, as described by Lee et al., (1981). The staining and microscopic evaluation method was chosen over plating due to its speed and ease considering that the objective was focused on producing a microorganism paste for animal supplements. The microorganisms studied may be damaged during preservation, which means that the blue staining does not necessarily indicate cell death. Despite this, the data obtained provide an indication of each reagent's ability to preserve the cells. Cell concentration was quantified using a Neubauer chamber (model 7301-1B, New Optics) and commercial dry biomass was obtained through oven drying. Microscopic and visual analyses were also carried out. The analysis of the sample dry mass was conducted according to equation 1:

$$\left(\%dry\ yeast\ mass = \frac{dry\ mass}{wet\ mass} \right) \times 100\% \quad \text{Eq.1}$$

The yeasts *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, and *Saccharomyces cerevisiae* M26 underwent analyses that assessed their surface (concave, elevated, undulated, protuberant, flat or convex), edge (smooth, lacerated, lobed, filamentous or undulated), color (colorless, white or pigmented) and cell weight (Has et al., 2020; Oliva-Neto et al., 2004).

2.4. Evaluation of different methodologies for yeast preservation

After culturing the yeasts, cultivation carried out with Formulated Without Residue medium (FWR) in 250 mL flasks with 100 mL of medium for 48 hours, pH 5.5 at 180 rpm (Oliva-Neto et al., 2004), the yeast cultures were adjusted to a concentration of 1.2×10^8 cells.mL⁻¹ (1.09×10^8 cells.mL⁻¹ after the addition of the cryoprotectant) and subjected to different methodologies, the lyophilization was done in a 2 mL microtube containing 1.5 mL of cryoprotectant associated with cell solution frozen for 48 hours at -20°C and lyophilized for 48 hours at -55°C (Liotop L101 equipment from Liobras, São Carlos/SP, Brazil). The reconstitution or rehydration of the lyophilized solution was done with 1.5 mL of autoclaved deionized water to maintain the initial concentration, refrigeration at -20°C for 168 hours (7

days), thawing was performed by leaving the microtube (2 mL microtube containing 1.5 mL of cryoprotectant associated with cell solution) at room temperature for 6 hours (Ecoplus 430 refrigerator from Bosch. Joinville/SC, Brazil) and maintaining the culture at 26°C in different solutions for 30 days (2 mL microtube containing 1.5 mL of cryoprotectant associated with cell solution). All tests were monitored for yeast cell viability before and after the application of each methodology. Yeast cell viability was assessed using a methylene blue solution as described by Lee et al., (1981). Cell concentration was quantified in a Neubauer chamber (model 7301-1B, New Optics) using an optical microscope with a 40x objective (P207BI, Precision brand – Palhoca/SC).

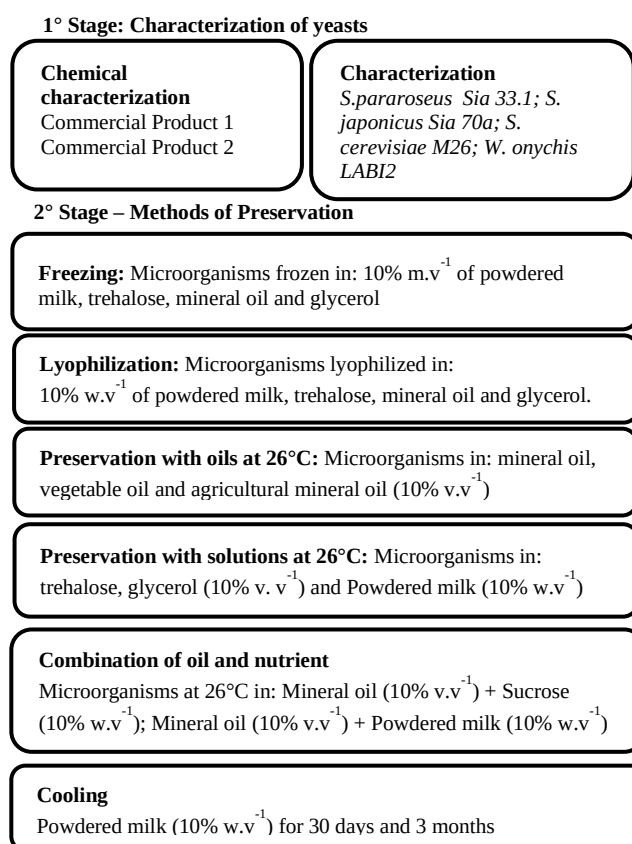
Tests were performed with autoclaved solutions containing the solid reagents, powdered milk, trehalose and sucrose that were diluted to 20% (w.v⁻¹) in deionized water for subsequent dilution to 10% (v.v⁻¹) with the cell culture (cells plus cryoprotectants). All solutions and oils used in the tests were previously prepared and autoclaved. The mixing of the cell culture with the test solution was performed in an autoclaved centrifuge microtube (Eppendorf, Kasvi, São Paulo/SP, Brazil) in a laminar flow cabinet using autoclaved pipettes with tips.

The first test involved freezing yeast cultures with the addition of one of the following products, each at a concentration of 10% (w.v⁻¹ or v.v⁻¹): sucrose (w.v⁻¹) (Êxodo), whole powdered milk (w.v⁻¹) (Piracanjuba), trehalose (w.v⁻¹) (Inlab), glycerin (v.v⁻¹) (Chemco) and mineral oil (w.v⁻¹) (Laborcun). The second experiment consisted of lyophilizing yeast cultures with the addition of 10% (w.v⁻¹ or v.v⁻¹) of one of the following ingredients: sucrose (Êxodo), whole powdered milk (Piracanjuba), trehalose (Inlab), glycerin (Chemco) and mineral oil (Laborcun). In the third experiment, three different types of oils were tested to see if any of them had advantages over mineral oil, which is traditionally used for microorganism preservation. The tested oils were mineral oil (Laborcun), almond vegetable oil (Uniphar) and agricultural vegetable oil (Quimifol), all at a concentration of 10% v.v⁻¹ in relation to yeast cultures, maintained for 30 days at 26°C. In the fourth and final experiment a 10% (w.v⁻¹) concentration of powdered milk was combined with 10% (v.v⁻¹) of mineral oil and the results were compared with the use of 10% (w.v⁻¹) of sucrose combined with 10% (v.v⁻¹) of mineral oil after 30 days at 26°C. The Brix degree (%) was evaluated for these cases. The fifth experiment involved evaluating the preservation of microorganisms in 10% (v.v⁻¹) powdered milk at 4°C after 30 days and 3 months. The initial concentration of live cells was established as 100% and used as a basis for calculating the reduction in viability over the tests.

2.5. Statistical analysis

The yeast preservation assays were conducted in triplicate. The results obtained were subjected to one-way analysis variance (ANOVA). and means were compared using the Tukey multiple sample comparison test ($p < 0.05$). Figures and tables were created using Microsoft Excel software. Statistical analyses were performed using IBM® SPSS Statistics software.

Fig. 1 Flowchart of the developed steps. regarding the evaluation of preservation of the four microorganisms.



3. RESULTS AND DISCUSSION

Table 2. Analysis of two commercial probiotic products in paste. Product 1: Analysis performed three months after manufacturing. Product 2: Analysis performed five months after manufacturing.

Observed	Product 1	Product 2
Brix (%)	30%	30%
RS (g.L ⁻¹)	39.14	25.32
TRS (g.L ⁻¹)	50.64	40.83
Dry Mass (%)	83.81	98.53
Yeast Viability (%)	33%	-
Bacteria.mL ⁻¹	1.04x10 ⁹	1.60x10 ⁸
Yeast.mL ⁻¹	4.57x10 ⁸	-
Visual Characteristic	Oil	Oil and starch
Color	Brown	Yellowish

Product 1. Evaluated after three months of manufacturing. RS: Reducing sugar; TRA: Total reducing sugar.

Table 2 displays high levels of soluble solids (brix) and reducing sugars in the evaluated products indicating the need for the addition of a nutrient source to ensure product quality over its extended shelf life. The moisture content is low, with dry matter exceeding 83% in both products. The viability of yeast in Product 1 was reduced, reaching approximately 33%, taking into account the remaining shelf life of 15 months. On the other hand, Product 2 does not contain yeast in its composition.

The packaging of Product 2 provides a more in-depth description of the preservation methods, highlighting the addition of various compounds to optimize the preservation of probiotics. This approach includes the incorporation of vegetable oil to prevent the pasta from drying out, limiting oxygen access and reducing the metabolism of the agents (Sharma et al., 2017). In addition to this, there are aluminosilicates that play a role in absorbing liquids and gases without causing significant changes in their form, preventing or minimizing the formation of clumps. Another important component is silicon dioxide, which acts as an anti-caking and anti-agglomeration agent, contributing to the stability of the medium during storage (Chan et al., 2015).

Sucrose plays a dual role, acting as a nutritional substrate and this sugar also works as a cryoprotectant for the microorganisms, contributing to their integrity (Oluwatosin et al., 2022). The presence of ethoxyquin serves as a preservative, helping maintain the product's quality over time (Costa et al., 2022). Polysorbate-80, in turn, plays a crucial role in stabilizing and emulsifying the medium, contributing to the homogeneity of the mixture. Furthermore, corn starch is believed to play a role in the robustness of the medium, promoting lower humidity, and this starch also serves as an additional substrate source for the probiotics (Chan et al., 2015).

This approach highlights the importance of exploring preservation strategies that involve the use of protective solutions, which benefit for the maintenance of probiotic viability, aiming for integrity and efficacy during storage until their application.

The choice of *S. cerevisiae* yeast (Product 1 and 3) is justified by its abundant biomass production in the ethanol and brewery industries, giving the strain distinct resistance. Studies also confirm its probiotic capacity over other microorganisms in various applications (Roy et al., 2023; Pinheiro et al., 2020; Bhima et al., 2010). Fernández-Pacheco et al., (2019) assessed the gastrointestinal resistance (pH 2 at 37°C for 2 hours under anaerobic conditions) of five yeasts selected in *in vitro* studies for their probiotic potential, with two *Saccharomyces cerevisiae* strains among them. Overall, all strains showed greater sensitivity to gastric conditions than intestinal conditions. as the former are more aggressive. Notably, *Saccharomyces cerevisiae* 3 demonstrated greater resistance to intestinal conditions compared to the other strains evaluated, with a reduced viability of only 7.9% under gastric conditions. The data confirm the ability of *S. cerevisiae* to maintain viability under adverse conditions, justifying their use as an additive in animal nutrition.

The microorganisms *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* 33.1, *Sporobolomyces japonicus* 70a, and *Saccharomyces cerevisiae* M26 were evaluated in the subsequent tests. The isolated colonies were examined with analysis of surface (concave, elevated, undulating, protuberant, flat, or convex), edge (smooth, lacerated, lobed, filamentous, or undulating), color and cell weight (g) (Has et al., 2020). The selected microorganisms are relatively unknown, except for *S. cerevisiae* M26. Thus, the characterization presented in Table 3 describes the fundamental characteristics of the evaluated microorganisms including variations in color, with two carotenoid-producing microorganisms. *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a, as well as variations in cell weight with *W. onychis* LABI2 standing out as the lightest among the four microorganisms evaluated. The evaluated microorganisms have the ability to grow under anaerobic conditions (Oliva-Neto et al., 2019).

Table 3. Morphological characterization of yeast colonies and cell weight.

Strains	Phylum*	Class*	Order*	Colony Edge	Colony Surface	Pseudohyphae	Cell Weight (g)	Color
<i>S. cerevisiae</i> M26	Ascomycota	Saccharomycetes	Saccharomycetales	Smooth	Convex	Absent	6.38x10 ⁻¹¹	Cream
<i>W. onychis</i> LABI2	Ascomycota	Saccharomycetes;	Saccharomycetales	Smooth	Convex	Absent	1.13x10 ⁻¹¹	Cream

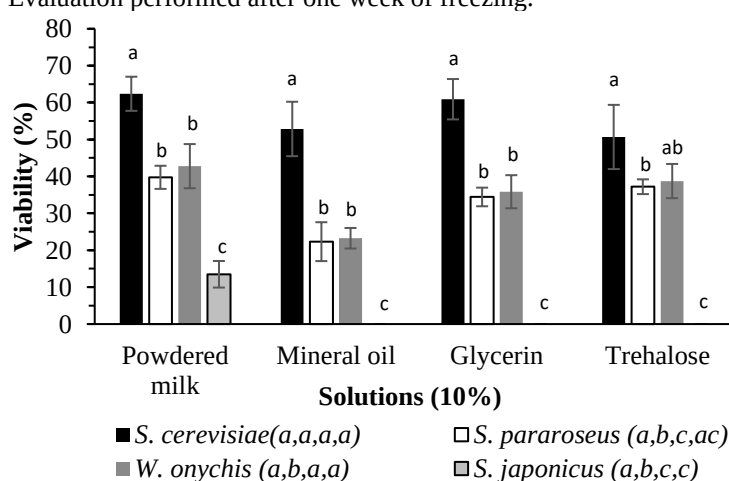
<i>S. pararoseus</i> 33.1	Basidiomycota	Microbotryomycetes	Sporidiobolales	Smooth	Convex	Absent	5.65×10^{-11}	Pigmented
<i>S. japonicus</i> 70a	Basidiomycota	Microbotryomycetes	Sporidiobolales	Smooth	Convex	Present	2.91×10^{-11}	Pigmented

* National Center for Biotechnology Information - NCBI (2014)

With the aim of preserving the cells analyses of various solutions were conducted to minimize the loss of cell viability. The solutions used in preservation may include non-permeable cryoprotectants such as milk and egg yolk, penetrating solutions like glycerol, ethylene glycol, and dimethyl sulfoxide, as well as sugars such as glucose, lactose, raffinose, sucrose and trehalose. Sugars like lactose, sucrose, raffinose, trehalose, and others do not penetrate the plasma membrane, leading to cellular dehydration due to osmotic pressure. This, in turn, reduces intracellular ice formation (Evans & Maxwell, 1987; Barbas & Mascarenhas, 2009). It is important to highlight that the specific characteristics of each cell will influence its response to different protective solutions, as well as physical preservation methodologies. For instance, freeze-drying can affect the survival of microorganisms depending on cell size, shape, and surface area, which may be related to the formation of extracellular ice crystals (Fonseca et al., 2000).

Fig. 2 illustrates the effect of freezing on the cell viability of microorganisms immersed in different solutions.

Fig. 2 Microorganisms frozen with powdered milk (10% w.v⁻¹), trehalose (10% w.v⁻¹), mineral oil (10% v.v⁻¹), and glycerin (10% v.v⁻¹). Evaluation performed after one week of freezing.



Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S.cerevisiae*, *W. onychis*, *S. pararoseus*, *S.japonicus*) compare cell viability between treatments. Letters (above each bar) compare

cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

With a focus on cell viability preservation, the freezing approach has proven to be more effective for all formulations containing *S. cerevisiae* M26. In this case, cell viability remained around 50%, reaching a maximum of 59% when using powdered milk. However, the other investigated microorganisms did not demonstrate the same resistance, regardless of the solution employed, reaching a maximum viability of only 31.2%.

A study conducted by Castro et al., (2020) examined the impact of freezing at -20°C without the use of cryoprotectants on 595 bacterial strains. Only 10.8% of these strains remained viable, highlighting the crucial need to employ protective solutions to preserve viability during freezing. It is important to note that both the freezing and thawing processes impose stress on the cell, potentially causing physiological damage, largely related to the formation of ice crystals and cell dehydration (Cabrera et al., 2020). The addition of cryoprotectants, as discussed by Cabrera et al., (2020). can mitigate these adverse effects, but as observed in Fig. 2, even with the presence of cryoprotectants, there is a general reduction in viability of at least 58%.

Cell membrane integrity is a fundamental requirement to ensure cell viability. The lipids present in the cell membrane undergo temperature and hydration changes, which can lead to membrane damage (Raju et al., 2021). Different cryoprotectants have varied interactions with the cell membrane; for example, disaccharides like trehalose and lactose are non-penetrating solutions that protect the cell membrane during dehydration, reducing the temperature of lethal phase transitions (Raju et al., 2021; Koster et al., 2000). However, the exact mechanisms are still under study.

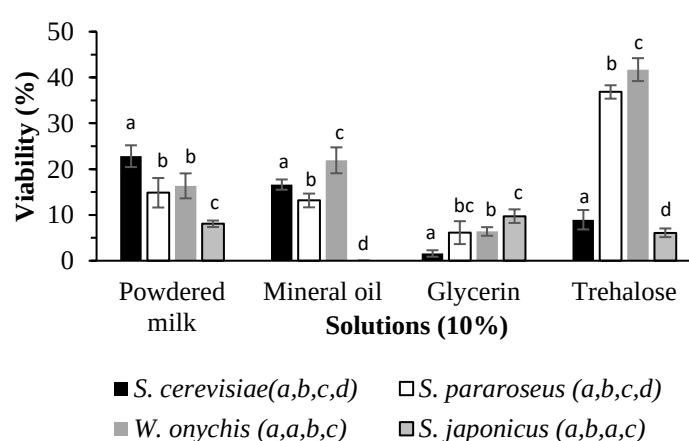
Anchordoguy et al., (1987) evaluated the preservation of unilamellar vesicles with various cryoprotectants, such as glycerol, dimethyl sulfoxide (DMSO), sucrose, trehalose, and sarcosine, and found that trehalose, sucrose, and glycerol achieved better results. These compounds interact with the membrane's phospholipids, especially with the polar heads of the phospholipids. The strength of this interaction between the cryoprotectant used and the cell's bilayer may explain the dependence on certain compounds for protection during freezing (Anchordoguy et al., 1987; Cabrera et al., 2020; Raju et al., 2021).

Another notable factor, which was not addressed in this study, is the cooling rate, a factor that must be carefully managed. Preferably, the cooling rate should be moderate, allowing for the gradual diffusion of water out of the cell, thus avoiding intracellular freezing. However,

it is crucial that this rate be fast enough to maintain thermal equilibrium and prevent ice crystal formation inside the cell (Abla et al., 2022). Dumont et al., (2004) identified through various freezing approaches that cell viability is closely linked to the cooling rate employed. Low cooling rates (5 to $180^{\circ}\text{C}.\text{min}^{-1}$) demonstrated higher viability rates, exceeding 80% for yeast. In contrast, rapid cooling rates (180 to $5.000^{\circ}\text{C}.\text{min}^{-1}$) resulted in reduced viability, reaching around 2%. This correlation was also observed by Verlhac et al., (2020), who showed a minimal loss of 0.1 log for the probiotic bacterium *Lactobacillus casei* ATCC 393 when subjected to a slower cooling rate ($0.5^{\circ}\text{C}.\text{min}^{-1}$), as opposed to the rate of $1^{\circ}\text{C}.\text{min}^{-1}$, which resulted in a loss of 0.8 log.

Each cell type has an optimal cooling rate, which is determined by factors such as cell size and shape, water permeability, and the presence of a cell wall. With this in mind, other freezing preservation approaches may prove more favorable for maximizing cell viability. However, when considering the convenience of transportation, storage and marketing of the probiotic potential, the freezing technique, which requires strict temperature control due to the adverse physiological impact resulting from freezing and thawing cycles, may not be the most practical and cost-effective strategy to adopt (Tedeschi et al., 2011; Rajam et al., 2022; Kieps et al., 2022). Fig. 3 presents the viability of lyophilized microorganisms in suspension with different solutions.

Fig. 3 Microorganisms lyophilized with powdered milk (10% w.v⁻¹), trehalose (10% w.v⁻¹), mineral oil (10% v.v⁻¹) and glycerin (10% v.v⁻¹).



Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S.cerevisiae*, *W. onychis*, *S. pararoseus* and *S.japonicus*) compare cell viability between treatments. Letters (above each bar) compare cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

Lyophilization is a widely employed method to facilitate the commercialization of various products, improving their stability and enabling the concentration of large quantities in reduced volumes, while also ensuring greater food safety. However, this process involves critical phases, such as freezing, which drastically affects cell viability, as evidenced in Fig. 3. followed by water sublimation under vacuum pressure, further exacerbating the reduction in viability. Nonetheless, the application of cryoprotectants can alleviate this decline (Abla et al., 2022; Jain et al., 2020).

Glycerin and mineral oil were tested in the lyophilization methodology, although they are not generally used for freeze-drying as they are polyols and oils respectively and consequently do not dry. The highlight of the current work was the cryoprotectant trehalose, which maintained approximately 36% and 41% viability for *S. pararoseus* Sia 33.1 and *W. onychis* LABI2, respectively, statistically differing from all other treatments. For *W. onychis* LABI2 and *S. pararoseus* Sia 33.1. lyophilized with powdered milk and mineral oil, statistical similarity was observed. In the case of *S. cerevisiae* M26, the highest preservation was achieved using powdered milk with 22.8% viability differing statistically from the other treatments. *S. japonicus* Sia 70a recorded the lowest viability among all evaluated treatments (Fig. 3) with the highest indices obtained with glycerol, around 9.7%, followed by powdered milk, with 8.0%. The lowest viability was observed in the glycerol treatment for all microorganisms evaluated except *Sporobolomyces japonicus* Sia 70a.

The need to apply cryoprotectants for microbiological preservation is emphasized by different authors, including Fernández-Pacheco et al., (2019) who observed a sharp drop in viability in the absence of cryoprotectants after lyophilization, reaching 24.9% for *S. cerevisiae* and 40.3% for *Hanseniaspora osmophila* and an even more pronounced drop for *Lactobacillus salivarius* subsp. *Salivarius*. approximately 99%, according to Zayed & Roos, (2004).

The best results were observed with the use of cryoprotectants trehalose and powdered milk (Fig. 3). Different authors have also noted the higher potential of such cryoprotectants. such as Zayed & Roos, (2004), who found that the addition of trehalose. combined with sucrose and skim milk, yielded remarkable survival rates for *Lactobacillus salivarius*, reaching approximately 83% and 85% viability, respectively. The authors also observed that immersion of the microorganism in skim milk resulted in 22.4% survival after lyophilization, results comparable to those found for *S. cerevisiae* M26 immersed in powdered milk in this study (22.82%).

Trehalose and skim milk also performed well in others researches. as observed by Archacka et al., (2019). who obtained superior results with trehalose ($67.0 \pm 1.0\%$) and skim milk ($64.5 \pm 0.5\%$) for *Lactococcus lactis*. Furthermore, Fernández-Pacheco et al., (2019) who investigated the lyophilization of *S. cerevisiae* M26 and *Hanseniaspora osmophila* with trehalose ($8\% \text{ w.v}^{-1}$), observed a reduction in viability of 15.6% and 32.2%, respectively, a low decline compared to the current study, which obtained the lowest drop of 58.29% for *W. onychis* LABI2, albeit using a lower trehalose concentration.

The use of carbohydrates often plays a crucial role in mitigating cell damage, acting to reduce both osmotic and mechanical stress faced by cells. A notable example is powdered milk, which contains lactose in its composition. Lactose, due to its size and molecular mass, provides valuable protection with satisfactory mobility. Additionally, the components present in milk, especially proteins, can establish protective layers that contribute to cell stability (Drake et al., 2018; Abla et al., 2022).

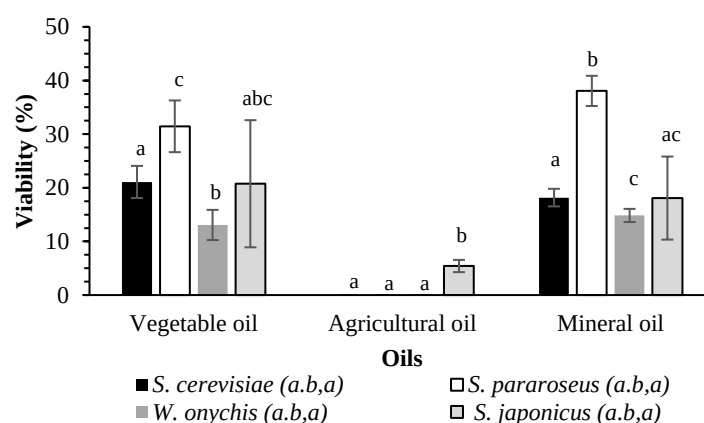
Trehalose is an important storage carbohydrate with beneficial effects for cells protection since this disaccharide induces a decrease in the speed of lipid movement, playing a crucial role in maintaining cell fluidity. Its presence provides the cell with a viscous matrix, which contributes to the stabilization of the biomolecular structure (Maiti & Daschakraborty. 2023). These findings corroborate the trend observed in the present study (Fig. 3), which obtained higher viability with trehalose and powdered milk after lyophilization.

Lyophilization presents itself as a safe and attractive strategy for the commercialization of probiotics, contributing to food safety, product stability, and consequently, an extension of the shelf life. These factors are inherently related to product costs, although lyophilization has many benefits, products can also be marketed as pastes, with low moisture content and stored at room temperature (Muzaffar et al., 2021; Abla et al., 2022; Várhidi et al., 2022). However, it is important to be aware that the viability decreases significantly after the process and this decline tends to worsen over time. The facts justify the use of the method for cultural preservation; however. when considering the commercialization of a product with high viability, cooling and freezing methods are preferable, despite the fact that drying processes are more convenient for comercilization (Abdallah et al., 2021). In addition to these, spray drying is also a viable alternative applied in different sectors, although it was not tested in the present work. However, regarding cell viability, the comparison between spray drying and

lyophilization is controversial, indicating that viability for these processes depends on the species and strain being evaluated (Silva et al., 2011; Albagli et al., 2024).

Fig. 4 presents the impact of three different oils on the cell viability of microorganisms at 26°C. The viability of microorganisms was assessed after 30 days of cells in suspension in three different oils at 26°C.

Fig. 4 Cell viability of four different yeasts applied in 10% (v.v⁻¹) vegetable oil, agricultural oil, and mineral oil for 30 days at 26°C.



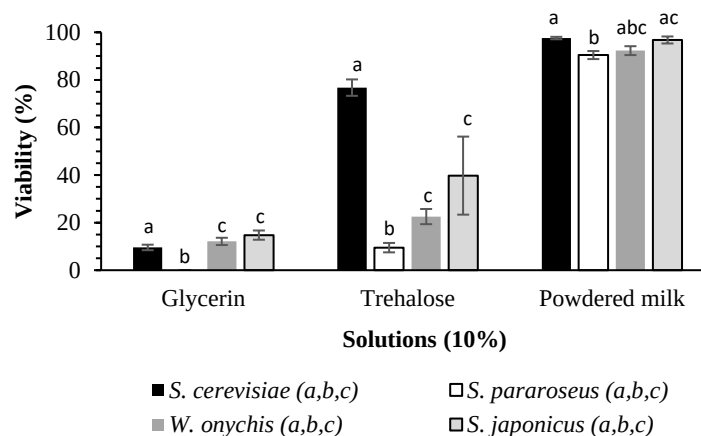
Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S.cerevisiae*, *W. onychis*, *S. pararoseus*, *S.japonicus*) compare cell viability between treatments. Letters (above each bar) compare cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

The results indicated that both vegetable oil and mineral oil showed similar levels of cell viability, encompassing all evaluated microorganisms. It is worth noting that the application of oil in cell cultures offers benefits such as reducing metabolism, preventing medium desiccation and adjusting oxygen availability (Da Silva et al., 2015). In this context, the three types of oils evaluated primarily differ in terms of their origin. Mineral oil is derived from petroleum and consists of hydrocarbons, while vegetable oil is extracted from plant sources such as seeds and almonds and contains triglycerides. Additionally, agricultural oil, used in farming, is a type of vegetable oil composed of 85.6% (w.v⁻¹) fatty acid esters derived from soybeans, along with other components in a proportion of 7% (w.v⁻¹). The agricultural vegetable oil have low toxicity according to information from Fênix Agro (2022), but for yeast the results indicated toxicity.

Although mineral oil maintained a minimum viability of 14.8%, further studies could assess the combination of oil with different nutrient or protective sources, possibly leading to

increased viability after 30 days. Fig. 5 presents the final cell viability after 30 days at 26°C immersed in glycerol, trehalose, and powdered milk (10% v.v⁻¹).

Fig. 5 Cell viability of four different yeasts with 10% (v.v⁻¹) glycerol, 10% (m.v⁻¹) trehalose and 10% (m.v⁻¹) milk powder for 30 days at 26°C.



Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S.cerevisiae*, *W. onychis*, *S. pararoseus*, *S.japonicus*) compare cell viability between treatments. Letters (above each bar) compare cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

The tests were conducted at 26°C (Fig. 5), and just like the freezing and lyophilization of microorganisms, which are directly influenced by temperature, a variable of utmost importance for cell viability, room temperature also has its effects on the viability of microorganisms. Higher temperatures, close to the optimum temperature, can result in the production of harmful metabolites, leading to cell death. On the other hand, lower temperatures, around 4°C, reduce cellular metabolism and prolong cell viability, while negative temperatures lead to freezing, causing cell damage and possibly cell death if no protective solution is used (Chan et al., 2023). Several authors, including Oluwatosin et al., (2022). have confirmed this fact, Oluwatosin et al., (2022) observed that *L. plantarum* maintained higher cellular viability stability at 4°C compared to room temperature (26°C). However, it is important to note that preserving microorganisms at room temperature has advantages, such as ease of transportation and marketing, which deserve further detailed evaluation.

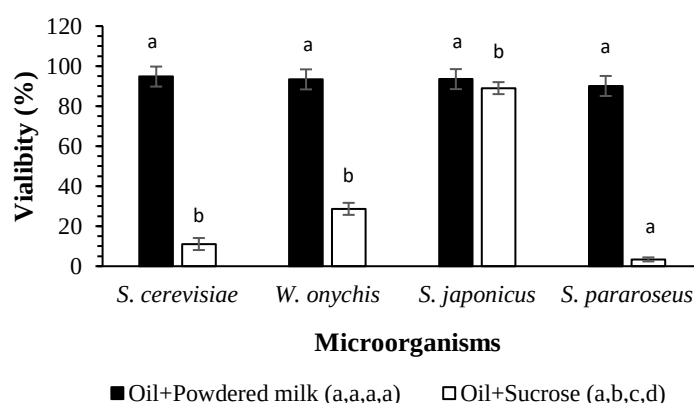
In Fig. 5 preservation performed at 26°C, it is possible to conclude that for the yeast *S. cerevisiae* M26 powdered milk and trehalose stood out, maintaining 97% and 76% of cell viability, respectively. For the other microorganisms, powdered milk also showed high potential, keeping viability above 90% (Fig. 6). A statistically significant difference was observed between all treatments for each evaluated microorganism. The results highlight the

effectiveness of powdered milk in the cell preservation of all strains studied. This is in line with the findings of Oluwatosin et al., (2022), who also observed promising results with the use of skim milk to preserve *L. plantarum* at 4°C and Neyrão et al., (2023), who evaluated fish sperm preservation with powdered milk.

Powdered milk, in addition to being similar to skim milk, is known to contain high concentrations of lactose, which serves as a carbon source for the microorganisms preserved at 26°C over time. The latter is a widely used non-penetrating cryoprotectant in cell preservation (Pugliese et al., 2017). Given this fact, the interesting possibility arises of comparing the effectiveness of preservation with sucrose, especially considering that the price of granulated sugar (trade "União") around 6.49 Brazilian Reais per kilogram, while powdered milk (Piracamjuba) costs around 45 Brazilian Reais per kilogram. Moreover, authors like Anchordoguy et al., (1987) obtained better preservation results with trehalose, glycerol, and sucrose, a fact that strengthens and encourages further research with sucrose.

Ravimannan, (2016) observed viability of 65% to 70% for four different strains of *S. cerevisiae* M26 stored in distilled water for six months, a fact that indicates the resistance of the tested microorganisms. Fig. 6 illustrates the combination of mineral oil with powdered milk, as well as mineral oil with sucrose, while the brix values of the treatments are presented in Fig. 7. The combination of powdered milk with mineral oil demonstrated high potential for maintaining the viability of the four studied microorganisms, keeping cell viability around 90% for 30 days at 26°C.

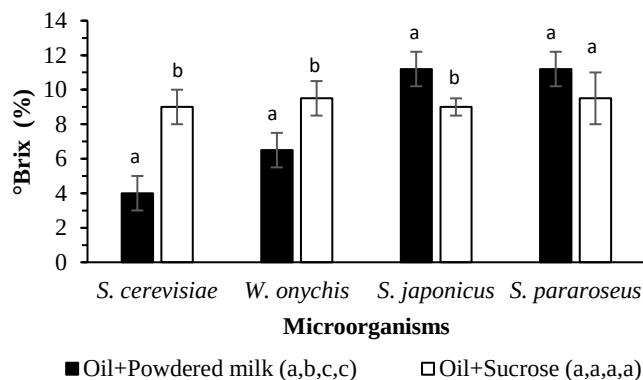
Fig. 6 Influence of mineral oil associated with sucrose (10% w.v⁻¹) and powdered milk (10% w.v⁻¹) on the cellular viability of the studied yeasts after 30 days and final °Brix (%).



Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S. cerevisiae*, *W. onychis*, *S. pararoseus*, *S. japonicus*) compare cell viability between treatments. Letters (above each bar) compare

cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

Fig. 7 Sugar consumption evaluated as residual Brix (%) in microorganisms in oil in combination with sucrose or powdered milk after 30 days at 26 °C.



Notes: Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S. cerevisiae*, *W. onychis*, *S. pararoseus*, *S. japonicus*) compare Brix between treatments. Letters (above each bar) compare Brix between microorganisms in the same treatment. Different letters indicate significant differences between groups.

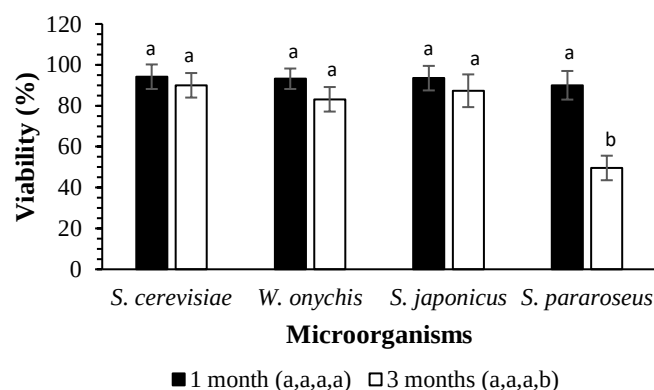
The results obtained with the combination of sucrose and mineral oil were less satisfactory, with a viability of 28% for *W. onychis* LABI2 and less than 11% for *S. cerevisiae* M26 and *S. pararoseus* Sia 33.1. *S. japonicus* Sia 70a was the only one that maintained cell viability at 89% with this combination.

The brix indicated a high consumption of powdered milk (10% w.v⁻¹) by *Saccharomyces cerevisiae* M26 and *W. onychis* LABI2, decreasing to 4% and 6.5%, respectively, which was not observed for the other microorganisms, which maintained the brix around 11.2%. Sucrose resulted in a brix of approximately 9% for all microorganisms, including *S. japonicus* Sia 70a, which showed good viability with sucrose and oil (Fig. 6). Stefanello et al., (2019) when evaluating different cryoprotectants, including 10% sucrose, 5% trehalose, 10% skimmed milk powder and 10% skimmed milk powder with 5% sodium glutamate to preserve lyophilized *L. fermentum* bacteria and *W. anomalus* 4533 yeast, found that sucrose was the least effective agent among those tested. On the other hand, trehalose and skimmed milk powder were the most efficient for yeast preservation, with skimmed milk powder standing out as the best, maintaining viability above 80% after one year at room temperature. These results obtained by the authors, although evaluated after lyophilization, explain the superiority of powdered milk compared to sucrose evaluated in this study.

The most promising results were obtained with the use of powdered milk, either in combination with mineral oil or alone. However, it is important to note that previous studies

(Fig. 4) that evaluated the use of only mineral oil demonstrated that, although it contributes to cell preservation, it is not as effective as powdered milk in preserving high microorganism viability as observed in Fig. 8 presents the viability of the four different yeasts after 1 and 3 months of refrigeration with 10% powdered milk (w.v⁻¹).

Fig. 8 Influence of mineral oil with powdered milk (10% w.v⁻¹) on the cell viability of the four yeast strains studied after 1 and 3 months under refrigeration.



Notes: Viability 100% of live cells at the initial time, before freezing. Analysis of variance (ANOVA) and Tukey's test with a significance level of 0.05%. Letters (in front of each microorganism) sequentially (*S.cerevisiae*, *W. onychis*, *S. pararoseus*, *S.japonicus*) compare cell viability between treatments. Letters (above each bar) compare cell viability between microorganisms in the same treatment. Different letters indicate significant differences between groups.

The viability results over three months (4°C) were evaluated only with the presence of powdered milk at a concentration of 10% (w.v⁻¹). and they showed a high preservation rate for all strains studied, with viabilities exceeding 83%. The exception was the *S. pararoseus* Sia 33.1 strain, which exhibited a 50% drop in viability after three months of preservation.

In a study conducted by Melin et al., (2006). the survival of the yeast *Pichia anomala* was evaluated and the results indicated high survival when the yeast was kept refrigerated at 4°C, compared to storage at temperatures of -20°C, 10°C, 20°C and 30°C. The viability rate remained above 71% when preservation was carried out with lactose and trehalose in phosphate buffer at 4°C over 12 weeks (i.e. 3 months). These results corroborate the high viability observed in the present study with the use of powdered milk and confirm the effectiveness of this approach after three months of preservation.

4. CONCLUSION

The maintenance of cells viability in yeast suspension is not easy and the choice of the method is very important for this success. Among the evaluated oils, mineral oil stood out as an efficient cryoprotectant. However, 10% powdered milk was proved to be the best method for the evaluated yeasts in suspensions, since with this method was guaranteed high viability rate of up to 90% for all tested strains after 30 days at 26°C and exceeding 83% for all strains after 3 months of storage, except for the *S. pararoseus* Sia 33.1 which retained only 49% viability after this period. On the other hand, the traditional methods of lyophilization and freezing did not yield the expected results. Although they were effective in maintaining the strains, they are not so suitable for the commercialization of products such as yeasts in a high level of viability.

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CHAPTER 5 – DOES RUMINAL SUPPLEMENTATION *in vitro* WITH DIFFERENT MICROORGANISMS AFFECT RUMINAL FERMENTATION IN HIGH-CONCENTRATE AND FORAGE DIETS?

ABSTRACT

The use of supplements in livestock farming is increasing, with probiotics standing out for their potential to enhance fiber degradation, reduce methane emissions and modulate the animal's immune system. However, it is important to note that investigations typically focus on well-known strains already applied in the industry, which differs from the present study that evaluates less-studied strains. The objective of this research was to assess the potential of applying 4 different yeast strains, as well as one bacterium and one commercial yeast (LMLK) as additives to improve ruminal fermentation patterns, particularly aimed at reducing methane emissions and increasing diet degradability. The tests were conducted using the *in vitro* gas production technique over a 96-hour incubation period at 39°C on two distinct diets: concentrate and forage (Tifton-85 hay). The strains were applied live at a dosage of 0.1% (dry matter basis) for the concentrate diet in which all strains were tested. In the second test (Tifton-85 hay), only the adapted microorganisms (pH 6.9)—specifically, 4 yeasts, a bacterium and a commercial yeast—were tested at two concentrations 10^8 and 10^9 cells per gram of diet. For the concentrate diet tests, the diet with addition of bacterium *B. breve* showed statistically higher values compared to the control diet for dry matter degradability (7.46%), organic matter degradability (5.48%), truly degraded matter (6%) and neutral detergent fiber degradability (10.17%) after 72 hours, as well as higher gas production per degraded dry matter (19.67%) after 96 hours. No significant difference was observed in methane production. For the forage-based diet, the results show higher diet degradability with the presence of *S. pararoseus* Sia 33.1 at a concentration of 10^9 cells per gram and also with the yeast *W. onychis* LABI2 at 10^8 cells per gram. Additionally, both yeasts demonstrated higher methane production per unit of organic matter. In this context, supplementation with *B. breve* shows greater potential for degradability without increasing methane production in concentrate diets. For forage-based diets, supplementation with *W. onychis* LABI2 showed greater potential for future *in vivo* applications considering the diet degradability, the lower cell concentration used, and easier cultivation. Future *in vivo* tests can emphasize the potential of this yeast and bring positive new developments for livestock farming.

Keywords: yeasts, *B. breve*, *in vitro* ruminal fermentation, methane emissions, degradability.

1. INTRODUCTION

Currently, the countries leading meat production are the United States with 20% of global production, followed by Brazil with 18% and China with 13%. Along with high production, global beef consumption reached 58.47 million tons in 2024, with 12.7 million tons consumed by the United States, accounting for one-fifth of global consumption, followed by China and Brazil with 11.13 and 8.33 million tons, respectively (USDA, 2023). High production aligns with the consumption of sector products, highlighting the importance of seeking alternatives that enhance animal performance to meet demand, as well as prioritizing alternatives that make the sector more sustainable, among which animal nutrition stands out (Jiménez et al., 2021; USDA, 2023).

Nutrition is one of the main factors associated with animal performance and methane (CH₄) emissions. The diet of confined ruminants generally consists of forage (e.g. hay and silage) and concentrate, with the proportion varying according to factors such as the financial conditions of the producer, infrastructure, desired final product such as meat and milk, and local climatic conditions (Jiménez et al., 2021; Калмагамбетов et al., 2021). Overall, production systems tend to increase the addition of concentrate in the diet to achieve higher animal performance, especially in terms of milk production (Dong et al., 2017; Ma et al., 2022). However, the careless addition of concentrate to diets does not always lead to increased production or higher product quality, which can cause digestive problems such as ruminal acidosis, stimulate inflammatory reactions, inhibit milk fat synthesis and favor the presence of saturated fatty acids in milk (Wen et al., 2021; Ma et al., 2022; Meng et al., 2022). Despite the importance of controlled feeding considering milk production and greater animal performance like the United States, which uses more intensive confinement systems (SNA, 2022), beef production in Brazil, a country with a tropical climate, is 95% pasture-based, in extensive systems, covering a total area of 167 million hectares, approximately 20% of Brazil's total land area (EMBRAPA, 2022).

Regardless of the diet provided, it is important to emphasize that the concentration of total digestible nutrients, calculated based on diet characteristics, is closely related to methane production highlighting the importance of nutrition (IPCC, 2006). Enteric fermentation is the main source of methane emissions, while nitrogen is eliminated through animal feces and urine, contributing to the formation of nitrous oxide (N₂O), compounds that contribute to environmental pollution (Jiménez et al., 2021).

Consequently, the search for economically viable strategies to minimize CH₄ release while maintaining good animal performance is increasing. This encompasses maximizing the utilization of regional conditions and characteristics such as climate, implementing different systems like integrating various crops, livestock, and forestry (Júnior et al., 2022; Hernández et al., 2022), as well as supplement additions (Markowiak et al., 2018). Many reports have demonstrated the potential of including additives in the diet of ruminants (Ma et al., 2020; Zhang et al., 2021; Muizelaar et al., 2023; Van Gastelen et al., 2024). Among these, notable examples include the addition of algae to reduce CH₄ emissions and also as a diet supplement during times of scarcity (Muizelaar et al., 2023), the addition of essential oils to mitigate CH₄ emission by inhibiting protozoa and bacteria and altering the acetate-to-propionate ratio (Van Gastelen et al., 2024). and the addition of probiotics, prebiotics and synbiotics (Markowiak et al., 2018).

Several studies have already demonstrated beneficial modifications of the ruminal environment (Diaz et al., 2018; Markowiak et al., 2018; Ogunade et al., 2019), such as increased abundance of cellulolytic bacteria in forage-based diets, improvement of pH and reduction of inflammations in ruminants supplemented with live yeast and mannanoligosaccharides in concentrate-rich diets, which reduces the chances of the animal experiencing ruminal acidosis (Diaz et al., 2018). Furthermore, reductions in lactate levels in cattle are observed improvement in nitrogen metabolism through greater retention (Zhang et al., 2021), increased digestibility of dry matter, organic matter and fibrous fraction of the diet (Silva et al., 2021). increased milk production (Ma et al., 2020; Ramirez-Garzon et al., 2024), improved nutrient intake affected by heat stress (Cai et al., 2021) and modulation of the animal's immune system (Ma et al., 2021). The most common probiotic microorganisms applied in ruminant nutrition encompass bacterial genera such as *Streptococcus*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Enterococcus*, *Bacillus* and fungal species including *Saccharomyces* and *Aspergillus* with *Saccharomyces cerevisiae* being the most commercially used (Gaggia et al., 2010; Zoumpopoulou et al., 2018; Parapouli et al., 2020).

The increasing patent production in the field globally demonstrates the importance of reconsidering industry practices and also emphasizes the potential of probiotic application in livestock farming, leading consequently to macroeconomic benefits for the entire sector (Caprarulo et al., 2022). The current study aims to evaluate the effect of different yeast strains and one bacterium in *in vitro* ruminal fermentations. The yeasts were selected based on their

production of cellulolytic and xylanolytic enzymes, as well as their development in anaerobic environments (Shinya, 2017). Studies with these microorganisms are still in their early stages. However, preliminary results have indicated that these yeasts can produce cellulolytic and xylanolytic enzymes, both responsible for fiber degradation by microorganisms in the ruminal environment (Brishketu et al., 2012). In this context, this research aimed to evaluate whether the selected microorganisms alter the fermentative pattern in the rumen and thereby increase diet degradability, whether it is forage or concentrate, without altering gas production.

2. METHODOLOGY

The experimental analyses using the *in vitro* gas production technique were conducted at the Animal Nutrition Laboratory (LANA) of the Center for Nuclear Energy in Agriculture - University of São Paulo, located in the city of Piracicaba, São Paulo, Brazil (Protocol n° 0003/2021). The cultivation and adaptation of microorganisms were carried out at the Bioenergy Laboratory (IPBEN) - UNESP - FCL Assis.

2.1 Microorganism cultivation

The four yeast strains *Wickerhamomyces onychis* LABI2, *S. cerevisiae* M26, *Sporidiobolus pararoseus* Sia 33.1 and *Sporobolomyces japonicus* Sia 70a were cultivated in fed-batch mode at 30°C and pH 5.5 for 120 hours using a medium with residues, molasses and cassava bagasse nutrient hydrolyzed (HCB - pH 1.08 for 26 hours at 97°C). The cultivation was described in Table 1. After cultivation, the fermented broth was filtered using a reusable porous polypropylene filter paper (Filtro Paulista, 100% polypropylene, dimensions 161 x 22 x 205). The biomass was centrifuged at 4500 rpm for 20 minutes at 15°C and stored refrigerated (4°C) with 10% (w.v⁻¹) powdered milk until use in the *in vitro* gas production tests with concentrate-based diet.

Table 1. Description of the cultivation media (30°C at initial pH 5.5) added at defined time intervals for conducting fed-batch cultivation for 120 hours.

Nutrient volume at the respective addition time					
Time (h)	HBM (mL)	Molasses (mL)	H ₂ O (mL)	Inoculum	Total volume (mL)
0	50	-	-	116	166
12	-	6.23	187.76	-	360
24	54	3.61	122.38	-	540
36	-	19.74	290.24	-	850
48	-	18.24	131.75	-	1000
48 -120	-	-	-	-	1000

Batch cultivation was conducted under the same conditions; however, the entire medium was added at the initial time point. Tests were performed in sextuplicate. pH adjustment of the medium was done every 12 hours for the first 48 hours, and every 24 hours thereafter. Total reducing sugar content of molasses: 88%. The cassava bagasse nutrient was hydrolyzed (HCB) at pH 1.08 for 26 hours at 97°C. HCB with pH adjusted to 5.5 was used. Inoculum of 1×10^8 cells.mL⁻¹.

The four adapted yeast at pH 6,9 and temperature of 37°C for *W. onichys* and *S. cerevisiae* and temperature of 30°C for *S. pararoseus* Sia 33.1 and *S. japonicus* Sia 70a were cultivated in formulated medium containing 2.0% glucose, 0.5% yeast extract, 0.13% (NH₄)₃PO₄, 0.10% (NH₄)₂SO₄, 0.031% K₂SO₄, 0.0028% ZnSO₄, 0.0012% MnSO₄, and 0.024% MgSO₄ (w.v⁻¹) without agitation (Oliva-Neto et al., 2004). Cultures were grown for five days in 1L erlenmeyer flasks, followed by continuous cultivation with removal of 50% (v.v⁻¹) of the medium and add 50% (v.v⁻¹) of fresh medium until sufficient biomass was obtained for testing using the *in vitro* gas production technique for concentrate-based diet and forage-based diet (Theodorou et al., 1994; Mauricio et al., 1999). The biomass was centrifuged at 4500 rpm for 20 minutes at 15°C and stored refrigerated (4°C) with 10% (w.v⁻¹) powdered milk until use in the *in vitro* gas production tests.

Two tests were conducted: one with a concentrate-based diet, referred to as Test 1, and another with a forage-based diet (Tifton-85 hay), referred to as Test 2. Test 1 involved adapted yeasts (pH 6.9) and non-adapted yeasts (pH 5.5), along with the bacterium *Bifidobacterium breve* and commercial yeast (LMLK - pure lyophilized *S. cerevisiae*, referred to as LMLK in this study) in 0,1% (g.g of diet) for all. Test 2 was conducted only with adapted yeasts at concentrations of 10⁸ and 10⁹ cells per gram of diet, along with the bacterium (10⁹ cells per gram) and LMLK.

Bifidobacterium breve cultures were grown at 37 °C for 48 hours in modified MRS medium with the addition of L-cysteine (De Man, Rogosa, & Sharp, 1960). The LMLK was lyophilized, so it was simply diluted in distilled water before use.

2.2 *In vitro* gas production technique

The produced yeast biomass was applied as an additive with the yeasts tested live at a proportion of 0.1% (w.w⁻¹ of substrate diet on a dry matter basis). The commercial probiotic Levumilk (LMLK) was tested at the same concentration as the yeasts, 0.1% (w.w⁻¹ of substrate). Similarly, the treatment with *B. breve* BB-03, referred to as *B. breve*, was also tested at a concentration of 0.1% (w.w⁻¹ of substrate). The probiotics were centrifuged at 4500 rpm for 20

minutes, weighed one day before the start of *in vitro* gas production and diluted in deionized water to facilitate the addition of 0.5 mL during *in vitro* fermentation. The assays were conducted with 1 g of concentrated diet (Table 2) and the control was performed without the addition of additives.

Table 2. Ingredients of the concentrated diet and proximate composition of the concentrated diet and Tifton-85 hay.

Ingredients	Concentrated Diet (%)	Diet Composition	Concentrate (g.kg ⁻¹)	Tifton-85 Hay (g.kg ⁻¹)
Corn silage	46.00	DM	873.18	886.18
Tifton hay	3.00	OM	944.99	941.44
Ground corn	24.1	MM	55.06	58.55
Soybean meal	21.4	NDF	556.32	813.88
Citrus pulp	4.5	ADF	419.96	432.88
Urea	0	ADL	44.31	72.52
Mineral	1.00	CP	181.20	60.60
		Cellulose	375.65	360.36
		Hemicellulose	136.36	381.00

DM: Dry Matter at 105°C. OM: Organic Matter. MM: Mineral Matter. NDF: Neutral Detergent Fiber. ADF: Acid Detergent Fiber. CP: Crude Protein. ADL: Acid Detergent Lignin; Hemicellulose calculated as the difference between NDF and ADF. Cellulose calculated as the difference between ADF and ADL (VAN SOEST et al., 1991).

The ruminal content of four adult Santa Inês sheep cannulated in the rumen with an average body weight of 61kg ± 8.84, was collected on the day of incubation before the morning feeding and used as the inoculum (Lima et al., 2018). The inoculum consisted of collecting both the solid and liquid fractions of the ruminal content using a probe attached to a 60 mL syringe (Becton-Dickson Industria Cirúrgica, Curitiba, PR, Brazil) and a forceps. The liquid material was stored in thermal bottles and the solid material was placed in plastic bags inside a styrofoam box to maintain temperature and anaerobic conditions followed by immediate transportation to the laboratory. Inoculants were prepared according to the methodology of Bueno et al., (2005) by mixing the solid and liquid fractions in equal proportions (50:50 v.v⁻¹) using a blender containing CO₂ (for 10 seconds), filtered through two layers of cotton fabric, and maintained in a water bath at 39°C with a constant flow of CO₂ until incubation.

The substrate used in the diet (1 gram) was prepared and weighed inside bags for fiber determination using Ankom F57 (Ankom Technology Corp., Macedon, NY/ USA), heat-sealed and placed in 160 mL glass bottles with 50 mL of Menke buffered medium (Menke & Steingass, 1988) and 25 mL of ruminal inoculum. In each bottle, 0.5 mL of the diluted treatment was added followed by sealing the bottles with rubber caps. They were then placed in a forced

ventilation oven at 39°C for 120 hours of incubation. Additionally, a control bottle containing only the substrate without the treatment was added, along with a blank consisting only of the empty Ankom F57 fiber bag.

For the tests with the concentrate diet, two inoculum were prepared using the content from three animals each. They were then distributed into 88 bottles for the first incubation, 88 bottles for the second and 88 bottles for the third incubation. *In vitro* gas production was conducted for 9 treatments (Wo30/5.5, Sc30/5.5, Sj30/5.5, Sp30/5.5, Wo37/6.7, Sc37/6.7, Sj30/6.7, Sp30/6.7, LMLK), with the addition of a Control and a blank for each incubation period (24, 48, 72 and 96 hours). This means there were 11 bottles (9 treatments + blank + Control) per inoculum, across 4 incubation periods. Each incubation period included one repetition per inoculum, meaning that by summing up the three incubations conducted a total of 3 results per inoculum or 6 per treatment for the given period were obtained considering the two inocula. Incubation at different periods was conducted to determine the diet's degradability.

For the tests with the forage-based diet (Tifton-85 Hay), a 96-hour gas production test was conducted with 2 inocula prepared using the ruminal content from three animals each. The production was carried out with 108 bottles, with incubation only at 24 and 96 hours. Twelve treatments were evaluated in duplicate (Wo, Sc, Sj, Sp, Bb, and LMLK at two levels. 10^8 and 10^9 cells per gram of diet), with the addition of two Controls and one blank for each incubation period (24 and 96 hours). In other words, there were 12 treatments in duplicate (24 bottles) + 1 blank + 2 Controls x 2 inocula x 2 incubation periods (24 and 96 hours). The microorganisms used in the test with the forage-based diet were only the adapted ones (pH 6.9).

The total volume of gases produced in each bottle was determined using the equation (Eq. 1):

$$V = (6.14 \times P)(n = 321, R^2 = 0.991) \quad \text{Eq. 1}$$

where:

V: volume of gases (mL)

P: measured pressure (psi)

The pressure was measured after the start of the incubation using a pressure transducer (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil) (Mauricio et al., 1999) at 0, 2, 4, 8, 16 and 24 hours, and the gas produced was collected at 2, 4, 8, 16, and 24 hours to create a composite sample for CH₄ (2 mL gas collection). at 30, 36 and 48 hours to create the second

composite sample for CH₄ (3.33 mL gas collection). at 60 and 72 hours to create the third composite sample for CH₄ (5 mL gas collection). and at 84 and 96 hours to create the fourth composite sample for CH₄ (5 mL gas collection). At each pressure measurement, the gases produced during the incubation were collected and stored in 10 mL vacuum tubes for methane concentration determination using a gas chromatograph (Shimadzu GC-2010, Tokyo, Japan) equipped with a flame ionization detector and a capillary column, following the method described by Lima et al., (2018).

The amount of methane was determined using the equation (Longo et al., 2006):

$$CH_4(mL.gDM^{-1}) = (HS + PG) \times CH_4 (\%) \quad \text{Eq. 2}$$

where:

HS: headspace (85 mL).

PG: volume of gases produced (mL).

CH₄ %: methane concentration (mL.mL⁻¹)

The degradability of organic matter (DMO) and neutral detergent fiber (DNDF) was determined according to Sakita et al., (2020). The washing of bags for determining the diet degradability was conducted at 24, 48, 72 and 96 hours for test 1 (concentrate-based diet) and only at 24 and 96 hours of incubation for test 2 (forage-based diet).

2.3 Statistics

The results were evaluated using R software version 4.3.0 (R Core Team. 2023) for statistical analyses. An analysis of variance (ANOVA) with mean comparison was performed using the Duncan test at a significance level of 5%. Treatments were considered as fixed effects, while the inoculum as random. Shapiro-Wilk normality test and Bartlett's homogeneity of variances test were performed in all cases. Where necessary, data were transformed using exponential, logarithmic or square root transformations.

3. RESULTS

3.1 Gas production, methane and *in vitro* degradability

The results of *in vitro* gas production (Table 3) showed no statistical differences for the 24-hour incubation period for any of the evaluated treatments, including total gas produced, CH₄ and OMD.

Fermentation up to 48 hours showed significant differences for degraded dry matter (DMS) for the treatment W.o 30/5.5 (575.50 g.KgMS⁻¹), which was 6.35% higher compared to the control. The OMD was statistically superior for the LMLK treatment (558.75 g.KgOMD⁻¹), S.p 30/5.5 (558.37 g.KgOMD⁻¹) and W.o 30/5.5 (566.68 g.KgOMD⁻¹), being 5.75%, 5.68% and 7.25% respectively higher than the control (528.34 g.KgOMD⁻¹). There was a significant difference for DNDF between the treatments with the yeast W.o 30/5.5, LMLK and S.p 30/5.5 compared to the Control treatment. No differences were observed for total gas production and CH₄ for any of the evaluated treatments.

For the 72-hour period higher DMS was observed for the treatment with *B. breve* BB-03 at 10⁹ cells.mL⁻¹ (658.66 g.KgDM⁻¹) compared to the Control (612.91 g.KgDM⁻¹) as well as for S.c 37/6.9 (646.58 g.KgDM⁻¹) with 7.46% and 5.49%, respectively. The same treatments showed higher DMO and DFDN compared to the Control. The SJ30/5.5 treatment showed 4.78% less gas production (141.09 g.KgDM⁻¹) although it did not demonstrate differences in diet degradation. Gas production per degraded organic matter was statistically 14.90% higher for the *B. breve* 37/6.9 treatments (101.55 g.KgOMD⁻¹) and 4.96% for the S.c37/6.9 (92.77 g.KgOMD⁻¹) compared to the Control.

The 96-hour *in vitro* fermentation showed differences only for the *B. breve* treatment in terms of gas production per degraded dry matter (192.05 g.KgDM⁻¹) and also production per degraded organic matter (132.39 g.KgOMD⁻¹) compared to the Control (160.48 g.KgDM⁻¹ and 100.04 g.KgOMD⁻¹). Additionally, statistical differences were observed for the partitioning factor, where the *B. breve* treatment demonstrated lower results than the Control and consequently lower microbial synthesis efficiency.

The *B. breve* BB-03 samples at 72 hours showed 101.55 mL.gOMD⁻¹ of total gases and 2.77 mL.gMOD⁻¹ of methane, representing 2.72% of the total gases. In contrast, the Control obtained 3.81%, which is about 1.08% less methane with the *B. breve* treatment. Furthermore, it's possible to observe 1.31% more OMD per gas production with *B. breve* compared to the Control. For the 96-hour fermentation, *B. breve* BB-03 showed 132.39 mL.gOMD⁻¹ of total

gases and $4.24 \text{ mL.gOMD}^{-1}$ of CH_4 , representing 3.20% of the total gases. In contrast, the Control obtained 3.06%, about 0.14% less methane. Despite the increase in methane concentration, it's possible to observe 4.02% more OMD per gas production with *B. breve* compared to the Control.

Table 4 presents a summary of statistically significant results in the *in vitro* fermentation tests conducted with concentrate diet, along with the difference from the treatment compared to the Control.

Table 3. Dataset obtained from the *in vitro* fermentation performed for 24, 48, 72 and 96 hours with a concentrate diet for live microorganisms

	DMD		OMD		TDOM		NDFD		pH	GPdm		CH ₄ dm	GPomd		CH ₄ omd	PF
24 hours																
Control	473.74		464.17		0.39		258.79		6.60	78.18		3.74	33.85		1.35	4.96
<i>B.breve</i> 37/6.9	457.54		444.52		0.37		231.60		6.59	87.69		4.01	39.27		1.78	4.24
LMLK	477.80		464.81		0.39		259.67		6.60	77.64		5.07	30.62		2.37	5.05
S.c30/5.5	475.08		462.03		0.39		255.83		6.58	82.80		5.83	34.75		2.71	4.66
S.c37/6.9	475.80		462.62		0.39		256.63		6.61	77.45		4.45	33.73		2.06	5.04
S.j30/5.5	475.52		482.02		0.40		283.48		6.54	85.21		5.57	23.01		2.66	4.72
S.j30/6.9	481.06		470.82		0.39		267.98		6.58	76.90		4.02	31.63		1.91	5.12
S.p30/5.5	476.50		466.80		0.39		262.41		6.59	78.41		5.17	29.93		2.42	5.01
S.p30/6.9	494.41		480.64		0.40		281.56		6.60	80.27		5.60	25.12		2.73	5.01
W.o30/5.5	468.44		456.55		0.38		248.24		6.60	76.56		5.41	21.85		2.50	4.98
W.o37/6.9	471.60		461.33		0.38		254.85		6.59	78.01		4.82	24.08		2.22	4.97
p-value	0.9684		0.9795		0.9796		0.9795		0.9727	0.5527		0.2837	0.9835		0.3045	0.2493
48 hours																
Control	538.93	cd	528.34	bc	0.44	cd	347.55	cd	6.44	116.58		4.92	60.71		2.61	3.80
<i>B.breve</i> 37/6.9	533.80	d	524.50	c	0.44	d	342.23	d	6.40	129.67		6.45	67.95		3.37	3.39
LMLK	568.91	ab	558.75	a*	0.47	ab	389.61	ab	6.38	124.41		4.91	52.39		2.81	3.78
S.c30/5.5	542.26	bcd	532.10	c	0.44	bcd	352.75	bcd	6.40	114.92		6.15	50.70		3.30	3.86
S.c37/6.9	565.21	abc	554.57	ab	0.46	abc	383.83	abc	6.47	117.39		4.75	51.70		2.84	3.75
S.j30/5.5	552.52	abcd	544.24	abc	0.45	abcd	369.54	abcd	6.34	115.14		5.13	40.42		2.95	3.98
S.j30/6.9	552.28	abcd	543.09	abc	0.45	abcd	367.95	abcd	6.40	126.55		5.88	60.20		3.23	3.62
S.p30/5.5	567.16	ab	558.37	a*	0.47	ab	389.08	ab	6.42	123.64		5.29	62.20		3.02	3.79
S.p30/6.9	551.10	abcd	541.42	abc	0.45	abcd	365.64	abcd	6.43	116.79		4.61	46.67		2.52	3.88
W.o30/5.5	575.50	a*	566.68	a*	0.47	a*	400.58	a*	6.40	121.84		5.21	62.80		2.96	3.90
W.o37/6.9	553.97	abcd	543.52	abc	0.45	abcd	368.54	abcd	6.45	116.54		5.00	61.26		2.71	3.91
p-value	0.0136		0.0088		0.0190		0.0190		0.6049	0.1096		0.6140	0.0973		0.2289	0.0582
72 hours																
Control	612.91	c	601.30	c	0.50	c	448.47	c	6.25	148.43	ab	5.61	88.38	c	3.37	3.40
<i>B.breve</i> 37/6.9	658.66	ab*	634.27	ab*	0.53	ab*	494.09	ab	6.30	159.21	a	4.37	101.55	a*	2.77	3.35

LMLK	617.77	c	608.14	bc	0.51	bc	457.94	bc	6.30	148.31	ab	5.21	70.51	c	3.18	3.42
S.c30/5.5	617.61	c	609.12	bc	0.51	bc	459.30	bc	6.28	146.10	abc	6.13	68.00	c	3.77	3.48
S.c37/6.9	646.58	a*	638.88	a*	0.52	a*	490.58	a*	6.25	142.09	ab	5.14	92.77	b*	3.23	3.70
S.j30/5.5	613.98	c	598.15	c	0.50	c	444.12	c	6.27	141.33	c*	5.58	57.54	c	3.30	3.74
S.j30/6.9	624.44	abc	615.17	bc	0.51	bc	467.66	bc	6.28	150.55	ab	4.37	86.36	c	2.71	3.41
S.p30/5.5	614.71	c	605.24	bc	0.50	bc	453.92	bc	6.29	138.37	bc	4.25	64.96	c	2.60	3.65
S.p30/6.9	624.41	abc	615.53	bc	0.51	bc	468.17	bc	6.23	146.61	abc	4.76	86.35	c	2.94	3.51
W.o30/5.5	627.82	abc	619.16	bc	0.52	bc	473.18	bc	6.22	153.52	abc	5.19	82.69	c	3.26	3.49
W.o37/6.9	621.51	bc	613.07	bc	0.51	bc	464.76	bc	6.27	150.49	ab	6.06	70.13	c	3.74	3.41
p-value	0.0294		0.0258		0.0263		0.0258		0.4850	0.0447		0.5258	0.0000		0.8207	0.0640

96 hours

Control	666.24	abc	657.81		0.55		526.64		6.34	160.48	b	4.66	100.04	b	3.07	3.43	a
<i>B.breve</i> 37/6.9	696.41	a	688.18		0.57		568.66		6.25	192.05	a*	6.14	132.39	a*	4.24	3.00	b*
LMLK	682.19	ab	673.35		0.56		548.14		6.26	164.74	b	4.91	107.02	b	3.29	3.42	a
S.c30/5.5	652.84	bc	644.79		0.54		508.63		6.28	161.90	b	5.82	104.50	b	3.78	3.33	a
S.c37/6.9	657.55	bc	648.29		0.54		513.48		6.33	158.84	b	6.35	102.97	b	4.11	3.40	a
S.j30/5.5	651.27	bc	641.83		0.54		504.54		6.31	159.52	b	4.82	102.45	b	3.09	3.37	a
S.j30/6.9	664.32	abc	656.02		0.55		524.16		6.31	163.84	b	4.76	100.00	b	3.12	3.34	a
S.p30/5.5	663.40	bc	656.46		0.55		524.77		6.28	155.86	b	4.99	102.40	b	3.27	3.52	a
S.p30/6.9	670.06	abc	660.04		0.55		529.73		6.29	161.52	b	4.19	106.53	b	2.79	3.42	a
W.o30/5.5	666.42	abc	658.09		0.55		527.04		6.26	155.07	b	5.90	102.40	b	3.90	3.56	a
W.o37/6.9	641.84	c	656.46		0.55		524.77		6.27	158.17	b	4.36	105.77	b	2.85	3.49	a
p-value	0.0334		0.0502		0.0514		0.05021		0.6170	0.0005		0.3534	0.0005		0.3393	0.0207	

The Duncan test was used with a significance level of 0.05%. *Statistically different from the Control. Treatments: microorganisms cultured at 30°C pH 5.5: SJ30/5.5: *S. japonicus* Sia 70a. WO30/5.5: *W. onychis* LABI2. SC30/5.5: *S. cerevisiae* M26. SP30/5.5: *S. pararoseus* Sia 33.1; microorganisms cultured at pH 6.9: WO37/6.9: *W. onychis* LABI2. SC37/6.9: *S. cerevisiae* M26. SJ30/6.9: *S. japonicus* Sia 70a. and SP30/6.9: *S. pararoseus* Sia 33.1. Control: Concentrate diet. OMD: Organic matter degradability (g.kgOMD⁻¹); DMD: Dry matter degradability (g.kgDM⁻¹); TDOM: Truly degraded organic matter (g.kgOMD⁻¹); NDFD: Neutral Detergent Fiber Degradability; GPdm: Gas production per dry matter (mL.gDM⁻¹); CH₄dm: Methane production per dry matter (mL.gDM); GPomd: Gas production per organic matter (mL.gOMD⁻¹); CH₄omd: Methane production per organic matter (mL.gOMD⁻¹); PF = partition factor.

Table 4. Summary of the main data obtained from the *in vitro* fermentation performed for 24, 48, 72 and 96 hours with a concentrate diet for live microorganisms and relative percentage difference (RPD)

		DMD		OMD		TDOM		NDFD		GPdm		GPomd		PF	
48 h	Control	538.93		528.34	bc	0.44	cd	347.55	cd						
	LMLK			558.75	a*	0.47	ab	389.61	ab						
	S.p30/5.5			558.37	a*	0.47	ab	389.08	ab						
	W.o30/5.5	575.50		566.68	a*	0.47	a*	400.58	a*						
72 h	Control	612.91	c	601.3	c	0.5	c	448.47	c	148.43	ab	88.38	c		
	<i>B.breve</i>	658.66	ab*	634.27	ab*	0.53	ab*	494.09	ab			101.55	a*		
	S.c37/6.9	646.58	a*	638.88	a*	0.52	a*	490.58	a*			92.77	b*		
	S.j30/5.5									141.33	c*				
96 h	Control									160.48	b	100.04	b	3.43	a
	<i>B.breve</i>									192.05	a*	132.39	a*	3.00	b*
RPD%		DMS %		OMD %		TDOM %		NDFD %		GPdm %		Gpmod %		PF%	
48h	LMLK			5.75		6.81		12.10							
	S.p30/5.5			5.68		6.81		11.94							
	W.o30/5.5	6.35		7.25		6.81		15.25							
	<i>B.breve</i>	7.46		5.48		6.00		10.17				14.90			
72h	S.c37/6.9	5.49		6.24		4.00		9.38				4.96			
	S.j30/5.5									-4.78					
96h	<i>B.breve</i>									19.67		32.33		-	12.53

The Duncan test was used with a significance level of 0.05%. *Statistically different from the Control. Treatments: microorganisms cultured at 30°C pH 5.5: SJ30/5.5: *S. japonicus* Sia 70a. WO30/5.5: *W. onychis* LABI2. SC30/5.5: *S. cerevisiae* M26. SP30/5.5: *S. paraseus* Sia 33.1; microorganisms cultured at pH 6.9: WO37/6.9: *W. onychis* LABI2. SC37/6.9: *S. cerevisiae* M26. SJ30/6.9: *S. japonicus* Sia 70a. and SP30/6.9: *S. paraseus* Sia 33.1. Control: Concentrate diet. OMD: Organic matter degradability (g.kgOMD⁻¹); DMD: Dry matter degradability (g.kgDM⁻¹); TDOM: Truly degraded organic matter (g.kgOMD⁻¹); NDFD: Neutral Detergent Fiber Degradability; GPdm: Gas production per dry matter (mL.gDM⁻¹); CH₄dm: Methane production per dry matter (mL.gDM⁻¹); GPomd: Gas production per organic matter (mL.gOMD⁻¹); CH₄omd: Methane production per organic matter (mL.gOMD⁻¹); PF = partition factor.

For Test 2 with a forage-based diet, *in vitro* ruminal fermentation did not show statistical differences compared to the control for any of the evaluated treatments after 24 hours of incubation, except for the partition factor (PF), which was higher for the Control (1.78) compared to the treatments S.c10⁹ (1.44), S.p10⁸ (1.58), S.p 10⁹ (1.53), LMLK (1.45) and B.b 10⁸ (1.52) (Table 5). The partition factor for the yeast S.j 10⁸ at 96 hours was also lower than the Control.

Contrary to the fermentation at 24 hours, fermentation for 96 hours showed differences in DMD for W.o10⁸ (421.01 g.KgDM⁻¹) compared to the Control (380.37 g.KgDM⁻¹). The organic matter degradability was statistically higher for the treatments S.p10⁹ (397.81 g.KgOMD⁻¹) and W.o10⁸ (401.02 g.KgOMD⁻¹) compared to the Control (354.24 g.KgOMD⁻¹).

In addition to the higher degradability of the diet with S.p 10⁹ as mentioned above, the yeast also showed higher gas production per dry matter, about 131.67 mL.gDM⁻¹ compared to the Control with 96.53 mL.gDM⁻¹, however, it also exhibited higher methane production per degraded organic matter.

The treatments that showed higher potential for diet degradation also exhibited higher methane production per degraded organic matter, namely S.p10⁸ (3.74 mLgOMD⁻¹), S.p 10⁹ (3.69 mLgOMD⁻¹) and W.o 10⁸ (3.44 mLgOMD⁻¹), compared to the Control (2.19 mLgOMD⁻¹). The tests also indicated a higher partitioning factor for the Control (1.41) compared to the S. j 10⁸ treatment (1.15) after 96 hours of fermentation. No statistically significant differences were observed in any of the parameters evaluated for the lyophilized commercial yeast LMLK and also for the treatment with *B. breve* bacteria. A summary of the results that showed statistical differences compared to the Control is presented in Table 6.

Table 5. Dataset obtained from *in vitro* ruminal fermentation performed for 24 and 96 hours.

24 hours	DMD		OMD		TDOM		NDFD		pH	GPdm	CH ₄ dm	GPomd	CH ₄ omd	PF			
Control	272.62		242.42		0.20		139.64		6.67	39.9	1.88	9.84	0.48	1.78	a		
S.c 10 ⁸	261.04		233.63		0.19		124.56		6.64	47.3	3.46	11.18	0.84	1.60	abc		
S.c 10 ⁹	241.33		213.43		0.17		101.49		6.64	48.42	3.2	10.51	0.72	1.44	c*		
S.p 10 ⁸	254.13		226.02		0.18		115.87		6.64	45.82	2.91	10.35	0.65	1.58	bc*		
S.p 10 ⁹	255.56		225.71		0.18		115.52		6.67	48.1	3.5	10.92	0.8	1.53	bc*		
W.o 10 ⁸	263.07		236.19		0.19		127.49		6.61	40.43	2.47	9.48	0.57	1.72	ab		
W.o 10 ⁹	250.83		225.08		0.18		114.8		6.63	38.96	1.54	8.79	0.35	1.65	ab		
S.j 10 ⁸	259.55		232.75		0.19		123.56		6.64	47.48	2.36	11.06	0.55	1.60	abc		
S.j 10 ⁹	255.52		227.79		0.18		117.9		6.62	41.42	1.32	9.44	0.29	1.64	abc		
LMK 10 ⁸	253.09		226.84		0.18		116.81		6.63	46.5	1.78	10.62	0.39	1.57	bc		
LMK 10 ⁹	244.49		217.4		0.18		106.02		6.63	51.19	2.94	11.11	0.64	1.45	c*		
B.b 10 ⁸	241.59		215.71		0.17		104.09		6.62	44.08	3.23	9.51	0.69	1.52	bc*		
B.b 10 ⁹	264.2		235.59		0.19		126.8		6.64	46.52	2.71	11.02	0.64	1.63	abc		
p- Value	0.4586		0.5394		0.5152		0.40713		0.7009	0.2803	0.0541	0.8405	0.1593	0.0123			
96 hours																	
Control	380.37	bcd	354.24	bcd	0.29	bcd	266.63	bcd	6.57	96.53	c	6.21	34.18	2.19	c	1.41	ab
S.c 10 ⁸	407.89	ab	388.05	ab	0.32	ab	300.96	ab	6.49	112.04	bc	8.42	48.16	2.83	abc	1.38	ab
S.c 10 ⁹	378.63	bcd	356.40	bcd	0.29	bcd	264.81	bcd	6.52	117.86	ab*	7.56	42.14	2.69	abc	1.3	bc
S.p 10 ⁸	394.29	abc	374.33	abc	0.31	abc	285.3	abc	6.56	117.42	abc	9.99	43.91	3.74	a*	1.36	ab
S.p 10 ⁹	417.10	ab	397.81	a*	0.33	a*	312.12	a*	6.45	131.67	a*	9.24	52.53	3.69	a*	1.37	ab
W.o 10 ⁸	421.01	a*	401.02	a*	0.33	a*	315.78	a*	6.56	109.02	bc	8.5	43.91	3.44	ab*	1.51	a
W.o 10 ⁹	394.61	abc	373.92	abc	0.31	abc	284.83	abc	6.6	103.41	bc	6.44	38.57	2.42	bc	1.44	ab
S.j 10 ⁸	349.66	d	322.05	d	0.26	d	225.57	d	6.51	105.39	bc	8.59	40.52	3.159	abc	1.15	c*
S.j 10 ⁹	389.23	abc	367.19	abc	0.30	abc	277.13	abc	6.59	99.97	bc	6.45	36.81	2.39	bc	1.44	ab
LMK 10 ⁸	379.53	bcd	357.25	bcd	0.29	bcd	265.77	bcd	6.57	103.16	bc	6.28	37.11	2.26	c	1.38	ab
LMK 10 ⁹	385.68	abcd	362.90	abc	0.30	abc	272.24	abc	6.54	107.28	bc	6.17	38.93	2.24	c	1.38	ab
B.b 10 ⁸	385.14	abcd	364.58	abc	0.30	abc	274.15	abc	6.59	99.08	bc	5.93	36.25	2.13	c	1.43	ab
B.b 10 ⁹	366.21	cd	342.39	cd	0.28	cd	248.8	cd	6.6	101.77	bc	6.13	40.00	2.09	c	1.26	bc
p- Value	0.0064		0.0035		0.0021		0.0023		0.1671	0.01987	0.0528	0.0316	0.0033	0.0101			

The Duncan test was used with a significance level of 0.05%. *Statistically different from Control. Treatments: microorganisms added at concentrations of 10⁸ and 10⁹ total cells. S.j; *S. japonicus* Sia 70a. W.o; *W. onychis* LABI2. S.c; *S. cerevisiae* M26. S.p; *S. paraseus* Sia 33.1. LMLK: Commercial Control; Control: Tifton Hay Diet. OMD: organic matter degradability (g.kgOMD⁻¹); DMD: dry matter degradability (g.kgDM⁻¹); TDOM: truly degraded organic matter (g.kgOMD⁻¹); NDFD: Neutral Detergent Fiber Degradability. GROSSGP: gross gas production (mL.gDM⁻¹); GPdm: gas production per dry matter (mL.gDM⁻¹); CH₄dm: methane production per dry matter (mL. gDM); GPomd: gas production per organic matter (mL. gMOD⁻¹); CH₄omd: methane production per organic matter (mL. gOMD⁻¹); PF = partitioning factor.

Table 6. Summary of statistically different results from the control obtained with the dataset from *in vitro* ruminal fermentation conducted for 24 and 96 hours and relative percentage difference (RPD)

		DMD		OMD		TDOM		NDFD		GPdm		CH ₄ omd		PF	
24h	Control													1.78	a
	S.c 10 ⁹													1.44	c*
	S.p 10 ⁸													1.58	bc*
	S.p 10 ⁹													1.53	bc*
	LMLK 10 ⁸													1.57	bc
	LMLK 10 ⁹													1.45	c*
	B.b 10 ⁸													1.52	bc*
96h	Control	380.37	bcd	354.24	bcd	0.29	bcd	266.63	bcd	96.53	c	2.19	c	1.41	ab
	S.c 10 ⁹									117.86	ab*				
	S.p 10 ⁸											3.74	a*		
	S.p 10 ⁹			397.81	a*	0.33	a*	312.12	a*	131.67	a*	3.69	a*		
	W.o 10 ⁸	421.01	a*	401.02	a*	0.33	a*	315.78	a*			3.44	ab*		
	S.j 10 ⁸													1.15	c*
RPD%	%	DMD (%)		OMD (%)		TDOM (%)		NDFD (%)		GPdm (%)		CH ₄ omd (%)		PF (%)	
24h	S.c 10 ⁹													-19.10	
	S.p 10 ⁸													-11.23	
	S.p 10 ⁹													-14.04	
	LMLK 10 ⁸													-11.79	
	LMLK 10 ⁹													-18.53	
	B.b 10 ⁸													-14.60	
96h	S.c 10 ⁹									22.09					
	S.p 10 ⁸											70.77			
	S.p 10 ⁹			12.29		13.79		17.06		36.40		68.49			
	W.o 10 ⁸	10.68		13.20		13.79		18.43				57.07			
	S.j 10 ⁸													-18.43	

The Duncan test was used with a significance level of 0.05%. *Statistically different from Control. Treatments: microorganisms added at concentrations of 10⁸ and 10⁹ total cells. S.j: *S. japonicus* Sia 70a. W.o: *W. onychis* LABI2. S.c: *S. cerevisiae* M26. S.p: *S. paraseus* Sia 33.1. LMLK: Commercial Control; Control: Tifton Hay Diet. OMD: organic matter degradability (g.kgOMD⁻¹); DMD: dry matter degradability (g.kgDM⁻¹); TDOM: truly degraded organic matter (g.kgOMD⁻¹); NDFD: Neutral Detergent Fiber Degradability. GROSSGP: gross gas production (mL.gDM⁻¹); GPdm: gas production per dry matter (mL.gDM⁻¹); CH₄dm: methane production per dry matter (mL. gDM); GPomd: gas production per organic matter (mL. gMOD⁻¹); CH₄omd: methane production per organic matter (mL. gOMD⁻¹); PF = partitioning factor.

4. DISCUSSION

4.1 Test 1: Concentrate diet

The hypotheses raised in this study were formulated due to the possibility of cell proliferation and enzyme production by the microorganisms, which would therefore assist in the degradation of the animal's diet (Oliva-Neto et al., 2019). However, in this initial evaluation, we chose to analyze a nutrient-rich diet (concentrate), which favors the proliferation of amylolytic microorganisms. Additionally, this diet does not hinder the action of microorganisms compared to a fiber-based diet (hay) (Ellison et al., 2017).

The conditions of ruminal fermentation such as pH (6.5), temperature (39°C), anaerobic environment and high concentrations of microorganisms in the ruminal fluid, favor microorganisms that can overcome these barriers and compete with the ruminal microbiota even in a nutrient-rich diet (Hungate et al., 1975; Nagaraja et al., 2016). The results indicate that most of the selected yeasts did not improve OMD or GP, which might be expected considering the starch-rich diet and the production of cellulolytic enzymes by the strains, along with their good development in fibrous materials (Shinya, 2017).

S. cerevisiae M26 adapted (37°C at pH 6.9) was the only yeast that showed potential for diet degradation, despite not having amylase production and therefore the ability to degrade starch (Cripwell et al., 2019). However, studies indicate benefits of microbial interactions with *S. cerevisiae*, demonstrating the strain's synergistic action in stimulating the production of amylases by other microorganisms (Fossi et al., 2014). Another microorganism that showed greater diet degradation was the bacterium *B. breve*, which was expected considering the natural presence of the *B. breve* genus in the rumen, as well as its greater abundance in starch-rich diets (Biavati & Mattarelli, 1991).

The concentration of yeast and bacterial supplementation varied with yeast cells being much larger and heavier than bacteria (Levin et al., 2015; Suh et al., 2003). Therefore, the inoculation criterion used (wet biomass, corresponding to dry biomass, 0.1% g.g⁻¹ of dry diet) directly influenced the quantity of inoculated cells favoring the response of *B. breve* compared to most yeast strains. It is believed that better results may be found by increasing the dosage of yeast inoculant relative to the current dosage (Marlida et al., 2023; Russouw et al., 2020).

In the present study, the PF was lower for the bacterium *B. breve* in 96 hours compared to the control. A low PF indicates higher gas production instead of microbial biomass,

consequently, lower efficiency in the fermentation process (Blümmel et al., 1997). However, the microorganism (*B. breve* BB-03 37/6.9) showed higher OMD of the diet compared to the control at 72 hours, as well as higher gas production per degraded dry matter at 96 hours, an interesting fact despite being a bacterium added in higher concentration, 10^9 total cells, compared to the other yeast strains evaluated, 10^7 total cells.

The yeasts LMLK, S.p30/5.5 and W.o30/5.5 at 48 hours showed higher OMD of the diet compared to the control, as well as S.c37/6.9 at 72 hours. Changes in the fermentation profile were also observed by Jiao et al., (2018) who evaluated the application of *S. cerevisiae* yeast on a diet with high grain concentrations, 10:90 (forage: concentrate), observing higher production of volatile fatty acids (VFA), greater degradability of dry matter and higher concentration of produced gases, besides favoring the greater proliferation of the ruminal microbial community. Increased competition among microorganisms, a consequence of their greater affinity for the organic matter present in higher concentrations in grain-based diets compared to hay and straw, leads to greater challenges for yeasts (Ellison et al., 2017; Shynia, 2017; Taghizadeh et al., 2021).

Despite the promising results, yeast supplementation in diets with forage appears to be more advantageous and noticeable, as observed by Opsi et al., (2012). who compared a high-fiber substrate (V: C with 60:40) and a concentrate-rich diet (20:80). This observation is also supported by Amanzougarene et al., (2021), who evaluated *S. cerevisiae*. both dry and pre-activated in fibrous substrate (beet pulp) compared to starchy substrate (barley). This preference may favor production systems based on pasture feeding, as in Brazil, although further research, particularly *in vivo* studies, can provide a better understanding of the effects of yeast on systems that prioritize concentrate feeding, such as milk production. The results demonstrate the potential of the selected microorganisms; however, it is expected that under different conditions, such as substrates with high fiber content and/or higher dosages exceeding 0.1% (g.g^{-1} of substrate) the effects of each yeast on gas production (GP) and organic matter degradation (OMD) of the substrate may be enhanced. Additionally, *in vivo* tests, even in diets with high concentrate, may indicate the effects of yeast application on the immune system, particularly on the production of IgG (Immunoglobulin G) and IgM (Immunoglobulin M) (Ma et al., 2020).

4.2 Test 2: Forage-Based Diet

The results at 96 hours of fermentation indicated a 12.29% higher degradability of organic matter in the presence of S.p10⁹ and 13.20% for W.o10⁸. Furthermore, the diet with the yeast W.o10⁸ also stood out in the degradability of dry matter, reaching 10.68% higher degradability compared to the Control, interesting results considering the lower cell concentration used (10⁸ versus 10⁹ cells per gram of diet). Russouw et al., (2020) also observed a non-linear response regarding cell concentration and effects on fiber degradability in ruminal fermentation, where 10⁶ cells.mL⁻¹ showed greater potential compared to 10⁷ cells.mL⁻¹. Mohanty et al., (2023) observed an increase in dry matter degradability, however, already at 12, 24, and 48 hours of fermentation in sorghum straw-based diets with *S.cerevisiae* (5x10⁹ CFU.kg⁻¹), a fact justified by the higher cell concentration used by the author and probably the delayed action of the W.o10⁸ selected in this study. Despite the delayed action, the yeast demonstrated the ability to degrade fibrous material a consequence of likely enzymatic production and proliferation in the rumen (Shinya, 2017). Additionally, S.c10⁹ showed only higher gas production indicating greater potential of the strain selected by the author or a delayed effect of S.c10⁹.

The yeasts *W. onychis* LABI2 (10⁸) and *S. pararoseus* Sia 33.1 (10⁹) continue to stand out, also showing good results in terms of TDOM and NDFD compared to the Control. They showed greater potential for fiber degradation also exhibited higher methane production per degraded organic matter, about 70.77% for S.p10⁸, 68.49% for S.p 10⁹ and 57.07% for W.o 10⁸. The results are surprising considering that the yeast S.p failed to grow at 37°C and showed difficulties at pH 6.9, indicating that the nutritional characteristics or interaction with the microbiota may have influenced the proliferation of cellulolytic microorganisms in the rumen.

The authors Elmasry et al., (2018) also observed similar results with the application of two different commercial strains of *S. cerevisiae*, noting higher gas and methane production in diets with high forage content. The increase in gas production through the addition of yeasts has been reported in various studies (Amanzougarene et al., 2021 and Rodriguez et al., 2021). Rodriguez et al., (2021) also observed a different response profile among similar microorganisms, such as higher gas production for the yeast *S. cerevisiae* L/25/7/13 and similar production to the control for *S. cerevisiae* (FLEISHMANN), indicating the importance of selecting appropriate strains. Additionally, the authors did not observe interference in pH,

which is consistent with the data observed in this study, a phenomenon also mentioned by other authors (Mohanty et al., 2023).

The diet based on Tifton-85 hay is low in carbohydrates compared to the concentrate-based diet (grains). The bromatological difference between the diets allows different groups of microorganisms to act more actively in substrate degradation through enzymatic production (Ellison et al., 2017). The activity of microorganisms is slower with the Tifton-85 hay-based diet, which has lower carbohydrate concentration and higher fiber concentration, thereby inducing the production of cellulolytic enzymes by microorganisms (Suberkropp et al., 1998). The inoculated microorganisms assisted in the enzymatic attack on the substrate, enhancing degradability and total gas production. However, this improvement was not observed at the beginning of fermentation. Improved nutrient digestibility is one of the main mechanisms to increase ruminant productivity (Ribeiro et al., 2015). It is believed that microorganisms may need more time to adapt to the imposed conditions and initiate enzymatic production (Hungate et al., 1975; Choudhury et al., 2015). The two concentrations of microorganisms evaluated did not necessarily show that a higher cell concentration is better (Russouw et al., 2020); however, the treatment with $Sp\ 10^9$ and $W.o\ 10^8$ in 96 hours of *in vitro* fermentation demonstrated to be statistically higher than the Control for different analyzed variables.

The samples of $Wo\ 10^8$ in 96 hours showed 43.91 mL.gOMD⁻¹ of total gases and 3.44 mL.gOMD⁻¹ of methane, which represents 7.83% of the total gases, while the Control obtained 6.42%, approximately 1.40% more methane. The microorganism *W. onychis* was more easily adapted to pH and temperature, in addition to showing greater ease of cultivation, similar to *S. cerevisiae* M26 indicating high potential for commercial applications in animal nutrition. Future tests with live animals could confirm these findings and bring about new positive developments.

5. CONCLUSION

For the concentrate-based diet (Test 1), the inclusion of yeast and bacteria affected the *in vitro* ruminal fermentation profile, increasing the organic matter degradability for *B. breve*, Levumilk, *S. pararoseus* 30/5.5 and *W. onychis* 30/5.5, while maintaining CH₄ emissions similar to the control for all evaluated treatments. Reductions in gas emissions were observed with the addition of *S. japonicus* 30/5.5. For the forage-based diet (Test 2, cultivation at pH 6,9 and temperature of 30 or 37°C), the action of *W. onychis* (10^8) and *S. pararoseus* (10^9)

demonstrated greater diet degradability. However, due to the similarities in cultivation characteristics and the minimal differences between the two strains in terms of the results obtained, *W. onychis* stands out. *W. onychis* (10^8) showed greater diet degradability and higher total gas production, although it also demonstrated higher methane emissions. This is justifiable by the characteristics of the low-quality diet, which makes methane an inevitable product of the process.

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CHAPTER 6 - SUPPLEMENTATION OF RUMINANTS WITH PROBIOTICS IMPROVES ANIMAL PERFORMANCE AND REDUCES METHANE RELEASE? AN *in vivo* TEST

ABSTRACT

The aim of this study was to investigate the potential of applying the yeast *Wickerhamomyces onychis* LABI2 and the bacterium *Bifidobacterium breve* BB-03 as ecological additives in manipulating ruminal fermentation patterns, reducing methane (CH₄) emissions and nitrogen excretions in sheep. Twenty-five crossbred Texel x Poll Dorset sheep (23.0 kg ± 1.58) were randomly divided into three groups: the control group, without probiotic application; the yeast group with application of *W. onychis* (1.43×10^{10} CFU); and the bacteria group with *B. breve* application (4.07×10^{11} CFU). Each treatment was administered for three days, followed by intervals of 14 days, totaling four applications during the experiment. The experiment lasted for 55 days, with 8 days for adaptation and 47 days for the nutrient digestibility assay and determination of enteric CH₄ emissions in respiration chambers. Lower dry matter intake was observed in the groups supplemented with microorganisms, along with higher apparent nutrient digestibility compared to the control group ($p < 0.05$). However, no difference in weight gain was observed for any of the treatments. Animals supplemented with yeast and bacteria showed lower nitrogen excretion (19.5% and 14.8%, respectively) and lower nitrogen in feces (20.38% and 10.59%, respectively) compared to the control group. Animals supplemented with yeast emitted 17.79% less CH₄ per gram of dry matter consumed and 23.72% less CH₄ per average daily weight gain compared to the control group. The discovery demonstrated that the application of the probiotic yeast *W. onychis* increased animal performance and consequently helped mitigate harmful environmental gases.

Keyword: *B. breve*, *W. onychis*, sheep, ruminal fermentation, nitrogen metabolism, methane emissions

1. INTRODUCTION

Greenhouse gases (GHGs) such as carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O) are the main contributors to climate change. Livestock farming has been identified as one of the major sources of GHG emissions and the climate crisis (Hur et al., 2024). However, there is a great complexity in this scenario, as the energy sector accounts for about 3/4 of total CO₂ emissions with 75% or 35.48 GtCO₂e, while agriculture contributes 5.8 GtCO₂e or 12% of CO₂ emissions (Climatewatch Historical GHG Emissions, 2020). Despite the energy and transportation sectors having a significant influence on CO₂ emissions, the high emissions of N₂O and CH₄ from livestock are significant and concerning as their warming potential is much higher than that of CO₂ (FAO, 2023; Reisinger, Ledgard & Falconer, 2017). It is estimated that 14.5% of global GHG emissions come from the livestock supply chain and 90% of CH₄ emissions come from ruminants, especially from enteric fermentation (FAO, 2023; Scholtz, Naser & Makgahlela, 2020).

In the global context, the food sector is responsible for about one-third of total anthropogenic emissions, approximately 30% (Clark et al., 2020). representing a significant obstacle to the goal of limiting global warming to less than 1.5°C by 2030 (Matthews et al., 2022). In this regard, it becomes crucial to rethink the production of animal-origin foods, especially ruminants, which are responsible for the majority of CH₄ emissions (Arndt et al., 2022; Parlasca et al., 2022). Emissions from livestock encompass not only enteric fermentation but also meat processing, transportation, manure management and other factors throughout the production chain, which can be analyzed and reconsidered to improve process efficiency (Parlasca et al., 2022; Gerber et al., 2013). Despite the various emission sources, it is important to emphasize that enteric fermentation accounts for a significant portion of global CH₄ emissions, approximately 64%, with 10% from dairy cattle and 87% from beef cattle according to Greenhouse Gas Emissions and Removals Estimation System (Seeg, 2024).

Strategies to reduce greenhouse gas emissions and promote sustainability in the agricultural sector point to various approaches, such as: increasing the feeding level, reducing pasture maturity and adjusting the proportion between forage and concentrate in the diet; including CH₄ inhibitors, tanniferous forages, oilseeds, among other practices; or even combining these methods simultaneously (Arndt et al., 2022). Furthermore, several studies focus on beneficial effects on animal nutrition and investigate the potential of including feed additives such as algae, probiotics, prebiotics, symbiotics and the utilization of different agro-

industrial residues as strategies to enhance animal performance (Markowiak et al., 2018; ANEE et al., 2021).

The selected yeast, *Wickerhamomyces onychis*, has not been tested *in vivo* in the literature so far whereas the bacterium *B. breve* is well-studied and applied in various sectors, besides being naturally present in animals, which may enhance its effects (Zafar et al., 2022). In this context, the present study aims to assess the impact of adding two probiotics, a yeast, and a bacterium on CH₄ emissions and animal performance. It is believed that these selected microorganisms may improve diet digestibility, resulting in higher animal weight gain, as well as reduce CH₄ emissions per kilogram of live weight.

2. METHODOLOGY

2.1 Location

The experiment was conducted between October and December 2023 at the Laboratory of Animal Nutrition (LANA) of the Center for Nuclear Energy in Agriculture (CENA) - University of São Paulo (USP), located in the municipality of Piracicaba - SP/Brazil (Protocol n° 0009/2024 and 0010/2024). The animals were provided by Fazenda União do Brasil, located in Buri/SP, Brazil.

2.2 Animals. management and treatment

The experimental trial was conducted with 25 male crossbred lambs (Pool Dorset and Texel) with an initial average body weight of 23.0 kg $\pm$ 1.58, at 5 $\pm$ 1 months of age. The animals were kept in individual pens (150 $\times$ 110 cm) with *ad libitum* access to water. The basal diet (Table 1) was formulated with a 60:40 w.w⁻¹ (Forage: Concentrate) ratio and provided twice daily in equal portions (50:50 m.m⁻¹) at 8:30 and 16:30 hours. The diet was formulated according to the NRC (2007) recommendations aiming for 200 g day⁻¹ of average daily gain (ADG) in body weight. consisting of 600 g kg⁻¹ of Tifton-85 hay (*Cynodon* spp.) and 400 g.kg⁻¹ of concentrated mixture composed of 60% soybean meal and 40% corn.

Table 1. Bromatological composition of the diet ingredients

Item	Hay Tifton-85 (g.kg ⁻¹)	Concentrate (g.kg ⁻¹)
DM	899.41	901.51
OM	934.35	966.11
MM	65.64	33.88
NDF	819.85	275.76
ADF	460.61	76.58
ADL	80.31	9.48
CP	68.32	235.27
Hemicellulose	359.24	199.18
Cellulose	380.30	67.10

DM: Dry matter at 105°C. OM: Organic matter. MM: Mineral matter. NDF: Neutral detergent fiber. ADF: Acid detergent fiber. CP: Crude protein. ADL: Acid detergent lignin determined by hydrolysis in H₂SO₄ 72%. Hemicellulose calculated as the difference between NDF and ADF. Cellulose calculated as the difference between ADF and ADL.

Hay Tifton-85 was chopped (Charger 15.0 - Menxon Agricultural Machinery, Cajuru-SP, Brazil) to obtain particles with an average size of 3 cm. The corn was ground (Nogueira - DPM2 - Nogueira S/A Agricultural Machinery, São João da Boa Vista – SP, Brazil) and mixed (M.A.P Agropecuária e Industrial Ltda., Londrina – PR, Brazil) with soybean meal for 15 minutes and stored in 100-liter plastic bags in a dry environment without exposure to sunlight. The mineral mixture (1%, containing calcium 14.3%, phosphorus 8.5%, magnesium 1%, sulfur 1%, sodium 12%, copper 1.58%, manganese 21.8%, zinc 28%, iodine 0.58%, cobalt 0.44%, selenium 0.14% and fluorine 10%) was weighed with the concentrate. Feed intake was adjusted to ensure a 10% leftover (w.w⁻¹).

The trial involved the evaluation of two treatments with microorganisms (*W. onychis* yeast and *B. breve* bacteria), with 8 replications per treatment, in addition to 9 animals monitored as controls. The additive was provided to each animal in the morning (8:30) using oral syringes. The treatments were administered for 3 consecutive days, every 14 days, totaling four times throughout the experiment.

The yeast application consisted of 1.38 grams (wet weight) per day for each animal (n=8), totaling 4.28x10¹⁰ cells over the 3-day application period. Considering the consumption of 500 grams of diet, this results in 2.86x10⁷ cells per gram per day. For the bacteria, 1.65 grams (wet weight) were applied per day for each animal (i.e. 1.22x10¹² total cells over the 3 days). Considering the daily consumption of 500 grams of diet, we observe 8.15x10⁸ cells per gram per day. The process was repeated four times with 14-day intervals. The treatments were kept refrigerated (4°C) to preserve the cells. The probiotic preparation was performed one day before application; they were centrifuged (Sorvall RC-5B PLUS, USA) for 20 minutes at 4500 rpm at

15°C in 50 mL Falcon tubes, diluted in deionized water and kept at 4°C until oral application using 20 mL syringes.

The experimental trial lasted for 55 days with an initial 8-day period for adaptation to the diet and management, followed by a 47-day period for the animal performance trial. The quantification of enteric methane, as well as the animal metabolism trial (apparent digestibility of nutrients), was conducted in semi-open respiration chambers for 6 days with two different experimental groups. Each group remained in the semi-open respiration chambers for three days, including one day for adaptation and two days for sample collection.

2.3 Animal performance trial

During the trial the animals underwent fecal egg count (FEC) and were dewormed in addition to ruminal fluid collection and weighing during the initial three days. The animals were kept in individual pens during the performance trial located in a covered environment, but with sides covered with tarpaulin to provide morning sun exposure. The average temperature during the experimental period was $26.08 \pm 5.76^{\circ}\text{C}$. with an average maximum temperature of $31.3 \pm 2.97^{\circ}\text{C}$ and minimum of $21.04 \pm 2.45^{\circ}\text{C}$.

Before the morning feeding the leftovers from each lamb were collected and weighed to determine the dry matter intake (DMI), which was done three times a week and stored to form a composite sample for that week. The weekly composite sample was stored in paper bags, dried at 50°C (Model MA 037 – Marconi, Piracicaba/SP. Brazil) for subsequent bromatological analysis. Water intake was assessed using graduated buckets.

The animals were weighed on the first day of the experiment and subsequently every seven days before the morning feeding (Days 0, 7, 14, 21, 28, 35 and 42) after a 13 hour fast to determine the feed conversion rate (total diet intake/total weight gain). The proportion of body weight gain (%BWG) was calculated using the equation (Eq. 1):

$$PBWG (\%) = \left(\frac{WBW - IBW}{IBW} \right) \times 100 \quad (\text{Eq. 1})$$

Where:

WBW (kg): weekly body weight;

IBW: initial body weight (kg);

%PBWG: percentage basal weight gain (%).

Blood samples were collected before the morning feeding with the animals fasting. Blood samples (10 ml) were collected by jugular venous puncture using vacuum tubes with EDTA anticoagulant (Becton-Dickson Indústria Cirúrgica, Curitiba, Brazil) conducted before the adaptation period (D0) and the administration of each treatment (D8, D22, D36 and D47). The vials were stored in a cooler with ice during collection, and immediately after a complete blood count (pocH-100i Diff) and blood smear for leukocyte differential count were performed. Protozoa counting (EPG) was carried out using an improved Neubauer chamber (Dehoridade, 1974; Goçmen et al., 2001) five times during the experiment to start with low egg counts and intervene if necessary. The last day of the experiment involved weighing the animals, collecting feces for OPG and collecting blood for a blood count and blood smear.

2.4 Digestibility and metabolism

After 45 days, during which the performance trial was conducted, 10 lambs were transferred to semi-open metabolic cages (157 x 71 x 167 cm) for 3 days. The animals were divided into two groups, i.e. 20 animals over 6 days with one day for adaptation and two days for sample collection. The five lightest animals were excluded from the trial conducted in the respiration chambers, Group 1 consisted of 2 Control animals, 4 for the Yeast treatment and 4 for the Bacteria treatment, Group 2 comprised 4 Control animals, 3 for the Yeast treatment and 3 for the Bacteria treatment, resulting in a total of 6 control animals and 7 animals for each treatment. The diet was provided *ad libitum* in a single morning meal (9:00 a.m.). Intake was adjusted to ensure 10% leftovers ($w.w^{-1}$). Graduated buckets were also used during the experiment to measure water consumption among the treatments and control.

The diet leftovers and feces were collected and weighed on an electronic scale (precision of 5 grams) to measure the total leftovers and feces. Subsequently, 10% ($w.w^{-1}$) was sampled to form a composite sample of feces and leftovers for each animal. The leftovers were stored in a freezer (-20°C) prior to bromatological analysis.

The collections of leftovers, feces and urine were carried out over the three days of the experiment, as well as the measurement of leftover water. All collections were performed in the morning (8:00 to 9:00 h), followed by feeding the animals. Urine was collected using basins containing 100 mL of 10% sulfuric acid (H_2SO_4) ($v.v^{-1}$) to acidify the urine and prevent ammonia volatilization. The samples were measured with graduated cylinders and then stored in 10% ($v.v^{-1}$) to form a composite sample for the three days for each animal. The containers

were stored in a refrigerator at -20°C until the analysis of total nitrogen and purine derivatives (Makkar & Chen 2004). The analyses were performed using high-performance liquid chromatography (HPLC - Agilent 1100, Agilent Technologies, Waldbronn/ Germany) to determine microbial synthesis (Verbic et al., 1990; Chen et al., 1990).

The diet and feces samples were thawed at room temperature for 12 hours, weighed, dried, ground and subjected to bromatological analysis. Diet characterization began with drying in a forced-air oven (MA 037 – Marconi, Piracicaba/ Brazil) at 55°C until a constant weight was achieved. The samples were ground in a knife mill (Marconi, Piracicaba/ Brazil) to pass through a 1 mm sieve and subsequently subjected to chemical analysis to determine dry matter (DM), organic matter (OM) and crude protein (CP) (AOAC, 2011). Fiber concentrations were determined according to Van Soest et al., (1991) with adaptations (Mertens, 2002), using a fiber analyzer (Tecnal TE-149, Piracicaba/ Brazil) and filter bags (Ankom F-57, Macedon, NY/USA). Neutral detergent fiber (NDF) was determined with thermostable amylase and expressed as ash-free NDF (aNDFom). while lignin was determined by solubilizing cellulose with sulfuric acid. Diet digestibility was calculated according to Equation 2.

$$DA = \frac{X_{consumed} - X_{excreted}}{X_{consumed}} \quad \text{Eq. 2}$$

Where: DA: Apparent digestibility (g.g⁻¹)

X: Nutrient evaluated

Nitrogen balance was determined by the micro-Kjeldahl method for the offered and excreted material (AOAC, 2011). The retained nitrogen was calculated following equation 3.

$$N_{retained} \text{ (g. day}^{-1}\text{)} = N_{ingested} \text{ (g. day}^{-1}\text{)} - N_{excreted} \text{ (g. day}^{-1}\text{)} \quad \text{Eq. 3}$$

Where:

$N_{retained}$: average amount of nitrogen retained by the animal.

$N_{ingested}$: average amount of nitrogen ingested by the animal.

$N_{excreted}$: average amount of nitrogen excreted in feces and urine by the animal.

2.5 Quantification of CH₄ emissions

The quantification of CH₄ production was carried out for two days, with one day for adaptation and two days for gas collection (44 hours in total). Ten chambers were used in this assay (157 × 71 × 167 cm - volume 1.9 m³), with water and diet provided *ad libitum* (Abdalla et al., 2012). The average temperature inside the chambers during the sampling period was 27.6°C ± 1.22 and the average humidity was 76.2% ± 3.69. measured with an internal digital thermometer. The air outflow rate was measured with an anemometer (AD-250 Digital – Instrutherm, São Paulo – SP/ Brazil) at six different times (8, 10, 12, 14, 16 and 18 hours) during the two days of gas sampling.

The cages used in the experiment are connected by tubing to an exhaust pump that allows the removal of gases from inside the cages. The gases were sampled in 7-liter balloons from 9:00 a.m. to 7:00 a.m. the next day, totaling 22 hours using a peristaltic pump. At the end of the gas collection 10 mL of this gas was collected in duplicate in 10 mL vacuum tubes with a 20 mL syringe. The sample tubes were analyzed for CH₄ concentration using gas chromatography (GC-2010, Shimadzu, Tokyo/Japan) with a flame ionization detector (FID) and a packed microcolumn Shincarbon ST 100/120. with temperatures maintained at 60°C for the column 200°C for the injector and 240°C for the detector. Helium gas was used as the carrier gas with a constant flow rate of 10 mL per minute (Lima et al., 2018).

2.6 Statistical analysis

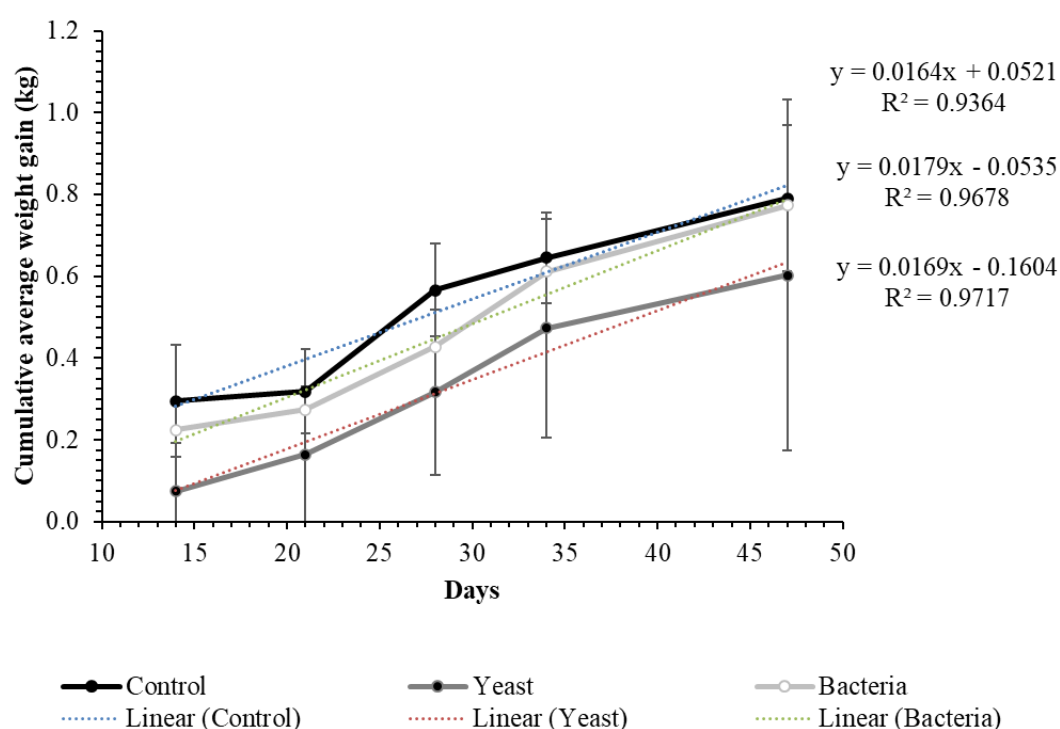
The data were analyzed using R Studio software version 4.3.0 (R Core Team, 2024) and subjected to analysis of variance (ANOVA). with mean comparisons performed by the Duncan test at 5% significance level. Normality (Shapiro-Wilk) and homoscedasticity (Bartlett) tests were conducted for all cases. Treatment was considered as a fixed effect as well as time for CH₄ evaluations and hemogram. Statistical differences were considered when observing p-values below 0.05%.

3. RESULTS

3.1 Performance assay

There was no observed evolution in the cumulative average weight gain of sheep supplemented with probiotics, bacteria and yeast according to the angular coefficient or slope compared to the control (Fig 1).

Fig 1. Evolution of average weight gain of sheep supplemented or not with probiotics. Duncan test with 5% significance. ($p = 0.804$)



The average consumption of hay, concentrate and total intake showed statistical differences between the treatments compared to the control, despite the bacterial treatment being similar to yeast and control in terms of concentrate consumption. The application of yeast was shown to induce lower feed intake by the sheep, followed by the bacterial treatment and the control, which exhibited the highest consumption among the three. The total diet intake for the bacterial and yeast treatments was 11.21% and 14.73% lower, respectively, during the performance experiment (Table 2). Water consumption did not differ between the treatments.

Table 2. DMI of hay, concentrate, and total intake, as well as water consumption of sheep supplemented or not with probiotics during the performance experiment.

	Treatments						
	Control		Bacteria		Yeast		p-value
Hay (gDM day ⁻¹)	582.60	a	528.40	b	517.40	b	0.0041
Concentrate (gDM day ⁻¹)	427.70	a	394.89	ab	376.95	b	0.0025
Total (gDM day ⁻¹)	901.73	a	808.63	b	768.88	b	0.0053
Water (L)	2.55		2.37		2.55		0.5061
Initial weight (kg)	24.71		24.71		23.90		0.7254
final weight (kg)	29.03		29.34		28.23		0.7034
TWG (kg)	4.32		4.63		4.33		0.7534
ADG (g.dia ⁻¹)	138.8		120.5		113.7		0.8043

Means with different letters in the same row differ according to the Tukey test at a 5% significance level. Average daily gain (ADG). Total weight gain (TWG)

3.2 Metabolism experiment

The average DMI and apparent digestibility of the ingested nutrients are presented in Table 3. Supplementation with bacteria did not alter the DMI compared to the control ($p>0.05$). However, the opposite was observed for yeast, which showed a statistically lower DMI with a reduction of 12.67% based on dry matter and 13.66% based on organic matter ($p<0.05$). The DMI for the bacterial treatment was similar to the control and yeast treatments ($p>0.05$). Water consumption did not show differences between the treatments. The digestibility of dry matter and organic matter was statistically higher for yeast and bacteria with an increase of 5% for both variables.

Table 3. Dry matter intake, apparent digestibility of nutrients and average daily intake by weight of sheep supplemented or not with probiotics during the metabolism experiment.

Parameters	Treatments						p-value
	Control		Bacteria		Yeast		
DMI (g/kgBW ^{0.75})	0.3931		0.3861		0.3603		0.0604
DMI % LW	0.0297		0.0296		0.0277		0.1195
Intake (g.day ⁻¹)							
DM	905.79	a	857.61	ab	790.98	b	0.0101
OM	855.60	a	800.13	a	738.66	b	0.0005
NDF	498.13	a	460.10	a	417.60	b	0.0015
ADF	264.85	a	249.26	a	221.64	b	0.0013
CP	137.25	a	131.29	a	122.49	b	0.0001
Lignin	70.51	a	65.68	a	60.28	b	0.0005
H ₂ O (L)	2.89		2.79		2.79		0.9101
Digestibility (%)							
DM	0.65	a	0.69	b	0.69	b	0.0412
OM	0.65	a	0.69	b	0.69	b	0.0405
NDF	0.48		0.52		0.51		0.2212

ADF	0.34	0.41	0.37	0.4014
CP	0.72	0.73	0.74	0.2077
Lignin	0.11	0.11	0.10	0.9631

DMI: Dry matter intake by an animal relative to metabolic weight raised to the power of 0.75. DMI % BW: dry matter intake by body weight. DMI % LW: live weight; DM: dry matter. OM: organic matter. NDF: neutral detergent fiber. ADF: acid detergent fiber. CP: crude protein. H₂O (L): water. Means with different letters in the same row differ according to the Duncan test at a 5% significance level.

3.3 Nitrogen balance

The nitrogen intake, nitrogen excreted in feces and urine as well as the nitrogen balance, retained nitrogen and their ratios to intake are presented in Table 4. No difference was observed for nitrogen excreted in urine, as well as for the ratios between the variables and intake ($p > 0.05$). Nitrogen intake was lower for the yeast treatment ($p = 0.0026$), about 10.49%. Nitrogen excretion was higher in the control compared to yeast and bacteria, which showed 19.5% and 14.8% less nitrogen excretion ($p = 0.0035$). Nitrogen excreted in feces was higher in the control compared to bacteria, which reduced by 10.59% and 20.38% for the yeast treatment ($p = 0.00014$).

Table 4. Nitrogen balance of sheep supplemented or not with probiotics.

Nitrogen balance	Treatments				p-value
	Control	Bacteria	Yeast		
N consumed (g.day ⁻¹)	21.83 _a	20.94 _a	19.54 _B		0.0002
N excreted (g.day ⁻¹)	10.00 _a	8.52 _b	8.05 _B		0.0035
N retained (g.day ⁻¹)	14.90	15.43	14.48		0.3543
N balance (g.day ⁻¹)	11.84	12.41	11.48		0.3528
N feces (g.day ⁻¹)	6.23 _a	5.57 _b	4.96 _C		0.0001
N urine (g.day ⁻¹)	3.77	2.95	3.10		0.2490
N retained/ingested (%)	68.00	74.00	74.00		0.0714
N feces/ingested (%)	28.00	27.00	25.00		0.0996
N urine/ingested (%)	17.00	14.00	16.00		0.4293

Means with different letters in the same row differ according to the Duncan test at a 5% significance level.

The results of purine derivatives, as well as the estimate of absorbed microbial nitrogen did not show statistical differences between the treatments (Table 5).

Table 5. Microbial protein synthesis by purine derivatives in sheep supplemented or not with microorganisms.

	Control	Bacteria	Yeast	p-value
PD (mmol.day ⁻¹)	7.12	5.67	6.40	0.75
PD (umol day ⁻¹ kg ^{-0.75})	551.37	461.15	517.15	0.75

MN _{absorbed} (g.N day ⁻¹)	3.07	3.00	3.01	0.75
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PD: Purine derivatives secreted in urine. MN: Microbial nitrogen. Means compared with Duncan's test at 5% significance.

3.4 Quantification of gas emissions

The methane emissions per dry matter intake, average weight gain, as well as the DMI during the respiration chamber trials are presented in Table 6. No differences were observed for the DMI. Methane (g kg DM⁻¹) and methane (g kg ADG⁻¹) were lower for the yeast treatment compared to the control and bacteria, being 17.79% and 23.72% respectively lower.

Table 6. Average enteric CH₄ production and Dry matter intake of sheep supplemented or not with probiotics in the respirometric chambers.

Parameters	Treatments						
	Control		Bacteria		Yeast		p-value
CH ₄ (g.dia ⁻¹)	20,18	a	20,43	a	14,70	b	
CH ₄ (g. kg DMI ⁻¹)	30.86	a	31.18	a	25.37	b	0.0325
DMI (kg DM. day ⁻¹)	0.90	a	0.86	ab	0.79	b	0.0101
CH ₄ (g.kg ADG ⁻¹)	2.15	a	2.12	a	1.64	b	0.0098

DMI: Dry matter intake. ADG: Average daily gain. DM: Dry matter. CH₄: Enteric methane. Means with different letters in the same row differ according to Duncan's test at 5% significance.

The hematological parameters, leukocytes, erythrocytes, hemoglobin, hematocrit and platelets are presented in Table 7. All variables are within the normal range according to the reference values used. No statistical differences were observed between the treatments and control for the variables erythrocytes and hematocrit. The leukocyte concentration showed differences between the treatments only at time five (day-47), where only the bacteria treatment was superior to the control. Hemoglobin was statistically higher at time two (day-8) for the yeast treatment compared to the control. Platelets did not show differences between the treatments at the different evaluated times. There were variations only within the yeast treatment (column), which showed fluctuations with the change in collection time.

Table 7. Hematological parameters of sheep supplemented with probiotic microorganisms or not. Time 1, 2, 3, 4 and 5; Day 0, 8, 22, 36 and 47.

Parameters	Time	Treatments						p value			
		Control		Bacteria		Yeast		Ref.*	Trat.	Time	T x T
Leukocytes (x 10 ³ .µL ⁻¹)	1	9.42	a	8.18	Ab	8.00	ab	4-12	0.3519	0.00001	0.0096
	2	9.17	ac	7.78	Ab	9.27	a				
	3	8.32	ab	7.68	Ab	7.26	ab				
	4	7.42	bc	6.29	A	6.45	b				
	5	7.00	bA	8.77	bB	7.34	abAB				
Erythrocytes (x 10 ⁶ .µL ⁻¹)	1	10.15	a	9.38	A	9.24	a	8-16	0.1458	0.0000	0.0259
	2	10.65	a	10.84	B	11.31	b				
	3	10.50	a	9.49	A	9.68	a				
	4	9.57	a	9.05	A	8.77	a				
	5	9.83	a	9.02	A	8.98	a				
Hemoglobin (g.dL ⁻¹)	1	10.71	ab	10.15	A	10.05	a	9-15	0.4239	0.0000	0.0014
	2	11.36	aA	12.15	bAB	12.65	Bb				
	3	11.2	acAB	9.93	aA	10.23	aB				
	4	9.76	b	9.68	A	9.23	a				
	5	10.02	bc	9.57	A	9.38	a				
Hematocrit (%)	1	32.18		31.5		31.5		24-50	0.5357	0.2988	0.7498
	2	30.33		31.87		30.25					
	3	31.00		30.62		29.93					
	4	31.11		31.00		30.18					
	5	31.66		30.75		29.87					
Platelets (x 10 ³ .µL ⁻¹)	1	450.11	a	409.62	A	509.87	a	100-800	0.1717	0.0000	0.8754
	2	388.00	a	293.87	A	406.37	ab				
	3	324.66	a	292.25	A	340.12	b				
	4	332.22	a	296.12	A	325.87	b				
	5	378.44	a	287.12	a	388.75	ab				

*Ref.: Reference values according to Meyer and Harvey (2004). Treat.: Fixed effect of treatment. T x T: interaction effect between treatment and day. Time: fixed effect of day. Means compared by Duncan's test at 5% significance. Lowercase letters in each treatment column indicate comparison between different times of the specified treatment, while uppercase letters indicate the difference between treatments at the specified time in the row.

4. DISCUSSION

The use of feed additives is increasing in livestock farming, aiming especially to enhance animal performance and improve or maintain the balance of the microbiota with emphasis on probiotics, prebiotics, and symbiotics (Markowiak et al., 2018). The results demonstrate that no statistical difference in weight gain was observed among the animals (Figure 1). The analyses were conducted with the average rate of weight gain during the performance experiment; besides these the data of DMI (g.kgBW^{0.75}) and DMI % LW did not

show differences compared to the control. The animals did not start with the same weight, meaning that comparing the average weight gain or initial and final weight does not reflect the findings of the experiment, only the average rate of weight gain for performance experiment. These findings are in accordance with those observed by Shoukry et al., (2023), who in tests with sheep (20.50 ± 0.65 kg) did not observe statistical differences in weight compared to the control with supplementation of the probiotic *B. amyloliquefaciens* and also, by Liu et al., (2023), who did not observe differences in metabolic weight with the application of yeast (*C. tropicalis*). Despite the authors having conducted daily supplementation the results were the same.

The DMI in the present study was lower for the treatments compared to the control a fact that indicates a change in the fermentative pattern of the animals considering that the groups did not differ in terms of weight gain. The effects of yeast supplementation on AGVs depend closely on the composition of the diet showing higher productions on diets based on forage (Liu et al., 2019), such as the diet in the present research. Supplementation with microorganisms has been shown to increase propionate production, as well as reduce the acetate to propionate (Wang et al., 2023; Cui et al., 2021), along with alterations in intestinal microflora (Cui et al., 2021; Liu et al., 2019), which may explain the higher feed efficiency of the animals.

The digestibility of DM and OM for both treatments was higher than the control, which coincides with the greater degradation of the diet (forage) by the enzymatic action of xylanases and cellulases produced by the yeast (Neto et al., 2021) and amylolytic enzymes by the bacteria (Achi et al., 2019). Additionally, yeast supplementation increases the abundance of fibrolytic bacterial genera (Wang et al., 2023), thus favoring diet digestibility. The application of microorganisms has consistently shown improvement in diet degradation as observed by Shoukry et al., (2023), who obtained higher DM (5.7% higher) and OM (0.6% higher) digestibility with the application of the bacterium *Bacillus amyloliquefaciens* and Liu et al., (2023), who also observed higher DM and OM digestibility with the use of yeast *C. tropicalis*, around 7.4% and 6%. Respectively, values similar to those observed in the present study (5% for DM and OM).

Nitrogen intake was lower for yeast while bacteria were similar to the control; regarding nitrogen excretion, both showed lower excretion. Despite the observed difference in excreted nitrogen, we did not find any variation when considering the proportion of excreted to ingested nitrogen, which demonstrates that the variation occurred due to the lower amount of food

consumed. Despite this, some authors have observed variations in nitrogen balance such as Liu et al., (2023), who found the same nitrogen pattern in feces for yeast *C. tropicalis* at concentrations of 10^8 , 10^9 and 10^{10} CFU.day⁻¹ and Abdel-Wahed et al., (2023) that observed variation in the nitrogen pattern for the different treatments, bacteria and yeast, noting lower nitrogen release in urine and higher body retention. The authors applied high concentrations of microorganisms daily. Considering this, it is possible that a more prolonged experiment or one with shorter intervals between probiotic applications could favor greater nitrogen retention per consumed diet. No difference was observed in purine derivatives and microbial nitrogen secreted in urine. Hassan et al., (2020) observed differences with the use of the probiotic *Ruminococcus flavefaciens*; however, with daily applications, which did not occur in the present study.

The release of CH₄ per unit of dry matter consumed (CH₄ g.kg DMI⁻¹) was lower for yeast compared to the control, as well as the CH₄ released per average daily weight gain (CH₄ g.kg ADG⁻¹). The treatment with bacteria showed similar results to the control. Several *in vitro* studies demonstrate the potential of probiotic application for methane reduction (Hassan et al., 2020), as well as some *in vivo* studies (Deng et al., 2018; Liu et al., 2023). Deng et al., (2018) in tests with *Bacillus licheniformis* observed statistical similarity with the control regarding CH₄ emission using a concentration of 2.5×10^{10} CFU/animal/day, while the other two concentrations (2.5×10^8 and 2.5×10^9 CFU/animal/day) showed lower CH₄ release, approximately 6.5% and 6.21% less CH₄ emitted, respectively. These findings are consistent with the data found in the present study, which also used high concentrations of bacteria, approximately 4.07×10^{11} cells.day⁻¹ and also observed similarities with the control for CH₄ emissions. Liu et al., (2023) in tests with yeast (*C. tropicalis*) also observed a reduction in CH₄ release, but at concentrations of 4×10^8 and 4×10^9 CFU.day⁻¹, achieving 11.34% and 1.77% less CH₄ per DM, respectively, whereas at a concentration of 4×10^{10} CFU.day⁻¹ an increase of 4.25% was observed. These results demonstrate the potential of yeasts in CH₄ mitigation; however, they indicate better efficiency of the yeast selected in this study, *W. onychis* compared to *C. tropicalis*. This fact may be explained by the better feed efficiency due to possible changes in the fermentative pattern and stimulation of fibrolytic bacteria (Wang et al., 2023; Cui et al., 2021; Liu et al., 2019).

The hematological parameters, leukocytes, erythrocytes, hemoglobin, hematocrit and platelets remained within the reference values used (Meyer et al., 2004), indicating the animals

health during the experiment. Responses of probiotic application to the immune system have been observed by several studies, especially regarding the increased production of IgG or IgM (Santos et al., 2021). Differences at the cellular level are rarer, usually observed in studies involving infections (Pinheiro et al., 2022). However, Mousa et al., (2022) applied a mixture of enzymes and microorganisms daily and observed improvement at the cellular level, regarding hemoglobin concentration, red blood cell count, leukocyte count and lymphocyte percentage, indicating that probiotic application affects the individual at the cellular level. Nevertheless, it is a slower response and likely requires continuous application a strategy that was not used in the present study.

5. CONCLUSION

The diet intake was lower for the animals in the treatment, yet they statistically presented the same rate of average weight gain. The reduction in diet intake observed for the probiotic treatments directly influenced the decrease in methane emissions and greater efficiency in nitrogen metabolism, with lower excretion. Yeast supplementation showed greater potential for methane reduction compared to bacteria due to lower diet intake.

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CHAPTER 7: EVALUATION OF THE AUTOLYTIC POTENTIAL OF DIFFERENT YEAST STRAINS FOR THE PRODUCTION OF A PROTEIN EXTRACT AND CELL WALL AIMED AT ANIMAL NUTRITION.

ABSTRACT

Improving the quality of animal nutrition is one of the goals of livestock farming, aiming to optimize the productivity and sustainability of the sector. One strategy to increase the quality of animal feed is to supplement with microorganisms as a protein source. With this in mind the present study aimed to evaluate the autolytic potential of four different yeast strains for protein extraction in order to produce two fractions: a protein-rich extract and a fraction with high concentrations of β -glucan and mannanoligosaccharides, which are components of the yeast cell wall. The goal was to assess the influence of both fractions on *in vitro* ruminal fermentations with concentrated diet. The study involved subjecting the yeast strains *Saccharomyces cerevisiae* M26, *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* 33.1 and *Sporobolomyces japonicus* 70a to a methodology previously defined for *Saccharomyces cerevisiae* from breweries. Autolysis was carried out for 48 hours at temperatures ranging from 45°C to 60°C at pH 2, with 7.5% (w.v⁻¹) yeast, on a dry basis. After autolysis, *Saccharomyces cerevisiae* M26 obtained 66% of extracted proteins and 5.39% of RNA. For *W. onychis* LABI2, 31% of protein and 0.87% of RNA were obtained. *S. pararoseus* Sia 33.1 reached a maximum of 15% protein and 2.7% RNA and finally for *S. japonicus* Sia 70a, 9.1% protein and 1.34% RNA were obtained. Therefore, the use of yeasts as a protein source for bovine feed supplementation is a viable option, especially when it comes to *S. cerevisiae* M26, which showed large amounts of protein and RNA extracted during autolysis. Since this yeast is widely used in the industry, its incorporation into the diet of ruminants may be easier and more economically feasible. However, it is important to note that the methodology employed was optimized for *S. cerevisiae* from brewery industrie, indicating that further testing with specialized yeasts may improve the extractions obtained. The integration of industries with the livestock sector can benefit both adding value to by-products and providing a proper destination for organic matter, while also enhancing diets by increasing protein content and other by-products such as RNA, vitamins and amino acids to improve animal nutrition. *In vitro* ruminal fermentation tests did not show statistical differences for the yeast cell wall and cellular extract tested in the concentrate diet, although interesting results have been obtained in the literature with applications in live animals, demonstrating a greater interaction than that observed in the *in vitro* tests. Future tests with live animals and also in diets with forage may better demonstrate the potential of the materials produced.

Keywords: autolysis, yeast extract, protein, nutrition, ruminants.

1 INTRODUCTION

The nutrition of ruminants has been the focus of various studies dedicated to understanding the influence of feeding and management considering the rumen ecosystem and how this reflects on the productive efficiency of the animals. The different types of feeding systems and research vary in substrate concentration, type of forage, tests with by-products, mineral and biological supplements, among others, considering the bromatological composition of each component (Mcgrath et al., 2018; Lambo et al., 2021; Markowiak et al., 2018). Supplements can be composed of live microorganisms, such as fungi and bacteria, inactive/dead ones, or even different fractions of these. They may also contain various concentrations of carotenoids, tannins, proteins, RNA, cell walls, vitamins, enzymes, among others (Copetti, 2019; Tao et al., 2023). Supplements are mainly used as a factor to stimulate rumen microbiome; however, the complexity of ruminal fermentation is even greater when analyzed in living animals (Shurson, 2018; Amin & Mao, 2021; Tirado-González et al., 2018).

Autolysis is a methodology that has been increasingly explored, especially considering the number of products of interest present in yeasts. The products obtained from cell autolysis differ according to the extraction strategies chosen and they directly influence the efficiency of product extraction (Gautério et al., 2023; Marson et al., 2019). Autolyses generally aim to extract protein, ribonucleotides, among other compounds, in order to obtain a concentrated fraction of the product of interest. The variation of factors such as pH, temperature, pressure, among others, induces stress on the cell leading to cell death without inactivating the enzymes present in the microorganism. This process results in two concentrated products: one forming the liquid fraction, rich in nutrients such as RNA and proteins and the other the solid fraction composed of the cell wall (Alves et al., 2021; Alexandre, 2011).

The economic viability of the process is mainly associated with the time and energy required, usually at least 24 hours at a fixed or ramped temperature, in addition to the costs of adding reagents to increase stress conditions and thus possibly the efficiency of autolysis (Alves et al., 2020; Arnold et al., 2018). Another challenge faced by the industry is contamination by proteolytic or pathogenic microorganisms, leading to diseases and alteration of odor and color of the autolysate, thus decreasing the quality of the yeast extract (Bataeva et al., 2020). The main challenge lies in optimizing the autolysis parameters according to the microorganism used, which directly influences the yield and productivity of the process. Finally, considering

large-scale production the separation of the fractions is also considered problematic (Arnold et al., 2018; Alves, 2020).

Autolysis mainly depends on the lytic action of endogenous enzymes, which vary for each microorganism. This variation also extends to the concentration of the product of interest in the selected microorganism (Takaloo et al., 2020; Šuklje et al., 2016; Dimopoulos et al., 2018). Thus, evaluating the autolytic potential of little-studied yeasts, as well as their chemical composition compared to a well-known yeast such as *S. cerevisiae* is interesting and can lead to new developments in the research on cell autolysis.

2 OBJECTIVES

The present study aims to evaluate the efficiency of protein extraction using a fixed methodology of acid autolysis performed on four yeast strains, as well as to assess the potential of the autolysis products, extract and cell wall, for ruminal nutrition evaluated in *in vitro* gas production.

3 MATERIALS AND METHODS

The cultivation of yeast was carried out in the bioenergy laboratory (IPBEN) at UNESP/Assis – SP, as well as the autolysis process. The *in vitro* ruminal fermentation tests were conducted in the Animal Nutrition Laboratory (LANA) at the Center for Nuclear Energy in Agriculture – University of São Paulo, located in Piracicaba – SP/Brazil (Protocol n° 0003/2021). The research involved four yeast strains: *Wickerhamomyces onychis* LABI2, *Sporidiobolus pararoseus* Sia 33.1, *Sporobolomyces japonicus* Sia 70a, and *Saccharomyces cerevisiae* M26.

3.1 Microorganism Cultivation

The microorganisms *W. onychis* LABI2, *S. pararoseus* 33.1, *S. japonicus* 70a and *S. cerevisiae* M26 were cultivated in a medium formulated with cassava bagasse, molasses and maize steep water in fed-batch mode at 30°C and pH 5.5 for 120 hours according to Table 1.

Table 1. Media added at each hour in the fed-batch fermentation in an incubator using hydrolyzed cassava bagasse (HCB) at pH 1.08 for 26 hours at 97°C. HCB was not dried; it was only pH adjusted to 5.5 and stored until use. Inoculum 1×10^8 cells. mL⁻¹.

Time (h)	HCB (mL)	Molasses (mL)	Water (mL)	Inoculum (mL)	Volume (mL)
0	50	0	0	116	166
12	0	6.23	187.76	0	360
24	54	3.61	122.38	0	540
36	0	19.74	290.24	0	850
48	0	18.24	131.75	0	1000

Tests were conducted in triplicate. pH adjustment of the medium was performed every 12 hours during the first 48 hours, and then every 24 hours thereafter. The total reducing sugar content of molasses is 88%, and for HCB it is 15%.

After cultivation, the fermented broth was filtered using a reusable porous polypropylene filter paper (Filtro Paulista, 100% polypropylene, dimensions 161 x 22 x 205). The liquid fraction was centrifuged (Heraeus Cryofuge 6000 Centrifuge, Thermo Scientific, Osterode/ Germany) at 4000 rpm for 20 minutes at 15°C and the biomass obtained was stored in 1 L bottles at 6°C (Ecoplus 430, Bosch, Joinville/SC, Brazil).

3.2 Characterization of biomass before and after autolysis

The intact yeast was characterized for RNA, protein and dry matter content before autolysis. Total RNA extraction from the intact yeast was performed prior to autolysis according to Oliveira (2008). The extraction was carried out with 1g of dry sample supplemented with 25 ml of 0.25 N HClO₄ at cold temperature, followed by 30 minutes of agitation in an ice bath and centrifugation (Heraeus Megafuge 16R Refrigerated Centrifuge, Thermo Scientific, Osterode/ Germany). The second step involved adding 10 ml of 0.3 N NaOH to the precipitate followed by incubation in a water bath at 37°C for 1 hour and centrifugation. The third step involved adding 10 mL of 1 N HClO₄ to the precipitate followed by a 20-minute ice bath and centrifugation. The third step was repeated. For the centrifugation procedures, it was set to 3500 rpm for a period of 15 minutes and all supernatants generated during the procedure were separated and reserved for analysis. The soluble fraction was assessed for RNA content using a spectrophotometer (UV-1600 PC Spectrophotometer, VWR, International) at 670 nm.

The analysis for quantification and determination of proteins (NT 415 Nitrogen Distillation Unit, NOVATEC, Piracicaba/ Brazil) was carried out according to the classical Kjeldahl methodology described by AOAC (2000).

The analysis of sample dry mass was performed according to Equation 1:

$$(\text{dry mass} \div \text{wet mass}) \times 100\% \quad \text{Eq. 1}$$

The analysis of protein, dry mass and RNA quantification were also performed on the liquid fraction after autolysis (section 3.3). RNA analysis in the liquid fraction after autolysis was performed according to Herbert et al., (1971) using the orcinol solution in spectrophotometry at 670 nm. The analysis involved preparing the solution, known as the WORK solution, mixed with the sample in a 1:3 ratio. Samples were diluted as needed. The solution was heated in a water bath (Ultrathermostatic Bath MA-184, MARCONI, Piracicaba/ Brazil) for 15 minutes at boiling temperature (Thermostatic Bath. TE-184, Tecnal, Piracicaba/ Brazil), followed by reading on a spectrophotometer (UV-1600 PC Spectrophotometer, VWR, International) at 670 nm. Results were obtained using the RNA standard curve.

3.3 Autolysis of yeasts

The cells were subjected to autolysis under controlled temperature conditions for 48 hours (Ultrathermostatic Bath MA-184, MARCONI, Piracicaba/ Brazil). The autolysis process was carried out based on the methodology described by Alves (2020) for protein extraction. Autolysis involved the addition of 7.5% yeast in water at pH 2 (H₂SO₄). Autolysis started at 45°C followed by 50°C, 55°C and 60°C with a temperature change every 12 hours. Samples were taken at the initial, final and before each temperature change, followed by centrifugation (Heraeus Megafuge 16R) at 4500 rpm, 15°C for 20 minutes. The obtained supernatant was characterized for RNA content, protein and dry mass (section 3.2). The cell wall (insoluble fraction of the sample) and yeast extract (soluble fraction) were dried in an oven at 80°C (Tecnal, model TE-394/1-MP, Piracicaba/SP – Brazil) and stored in a refrigerator (Ecoplus 430. Bosch, Joinville/SC, Brazil) until the *in vitro* ruminal fermentation tests were performed.

3.4 *In vitro* gas production tests

The produced biomass was used as a supplement in *in vitro* rumen fermentation tests to assess gas release and ruminal degradability (Bueno et al., 2005). The yeast extract and cell wall fractions were tested at a ratio of 0.1% (g.g⁻¹ of substrate). The tests were conducted using a concentrated diet for all microorganisms with the control group receiving no additive. Table 2 shows the diet composition and its bromatological composition.

Table 2. Diet ingredients and their bromatological composition.

Ingredients	Diet (%)	Diet Composition	Concentrate (g.kg⁻¹)
Corn silage	46.00	DM	873.18
Tifton hay	3.00	OM	944.99
Ground corn	24.1	MM	55.06
Soybean meal	21.4	NDF	556.32
Citrus pulp	4.5	ADF	419.96
Urea	0	ADL	44.31
Mineral	1.00	CP	181.20
		Cellulose	375.65
		Hemicellulose	136.36

MS: Dry Matter at 105°C. OM: Organic Matter. MM: Mineral Material. NDF: Neutral Detergent Fiber. ADF: Acid Detergent Fiber. CP: Crude Protein. ADL: Acid Detergent Lignin determined by hydrolysis in 72% H₂SO₄. Hemicellulose calculated as the difference between NDF and ADF. Cellulose calculated as the difference between ADF and ADL (VAN SOEST. et al. 1991).

The ruminal content of four adult Santa Inês sheep with rumen cannulas was collected on the day of incubation before the morning feeding and used as inoculum (Lima et al., 2018). The substrate (1 gram) was prepared and weighed into Ankom F57 fiber bags (Ankom Technology Corp, Macedon, NY/ USA), heat-sealed and placed in 160 mL glass bottles, type penicillin filled with 50 mL of Menke buffer solution (Menke & Steingass, 1988) and 25 mL of inoculum. In each bottle, 0.5 mL of the diluted treatment in deionized water was added. The bottles were properly sealed with rubber caps and kept in a forced ventilation oven (incubator) at 39°C for 120 hours of fermentation. A control bottle containing only substrate without treatment was also added. The total gas volume produced in each bottle was determined following the equation:

$$V = (6.14 \times P)(n = 321, R^2 = 0.991) \quad \text{Eq. 2}$$

Where:

V = volume of gases (mL)

P = measured pressure (psi)

The pressure was measured at 0, 2, 4, 8, 16 and 24 hours combining into a single pool for CH₄ and at 30, 36 and 48 hours, combining into the second pool, after the start of incubation using a pressure transducer (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil). For the pressure measured in the first 24 hours, 2.5 mL of gas produced during incubation were collected and stored in 10 mL vacuum tubes, while for the production from 30 to 48 hours, 3.33 mL were collected in vacuum tubes of 10 mL for the determination of CH₄ concentration using

a gas chromatograph (Shimadzu GC-2010, Tokyo/ Japan) equipped with a flame ionization detector and a capillary. The chromatographic conditions (HP-Molesieve -GC column 30m × 0.53mm × 25µm) were set as described by Lima et al., (2018).

The amount of methane was determined by the equation:

$$CH_4(mL.gDM^{-1}) = (HS + PG) \times CH_4(\%) \quad \text{Eq. 3}$$

Where:

HS = headspace (85 mL).

PG = volume of gases produced (mL).

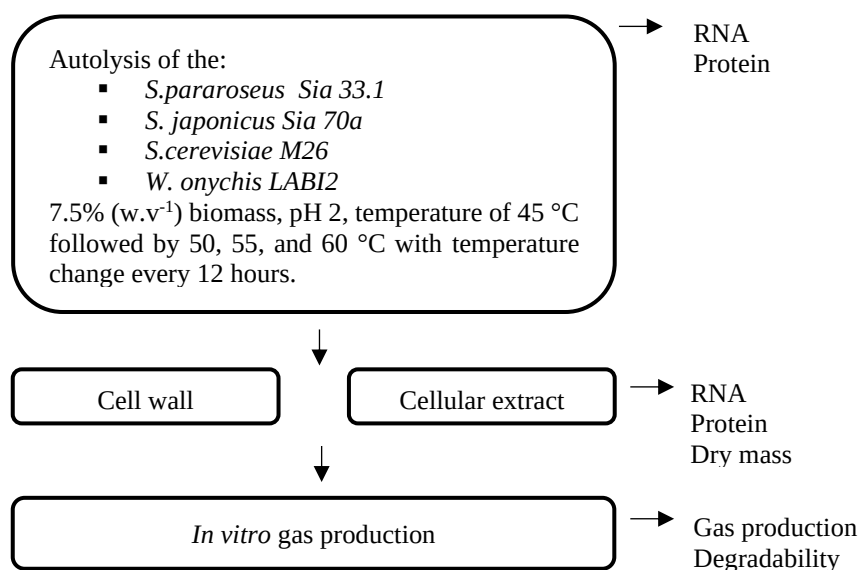
DM = Dry matter (g)

CH₄ % = methane concentration (mL.mL⁻¹) (Longo et al., 2006).

The truly degraded organic matter (TDOM) was calculated as the difference between the incubated organic matter and the undegraded organic matter. All bags were treated with neutral detergent solution for 1 hour at 90°C followed by four washes of 5 minutes with deionized water at 90°C (TE-149, Tecnal, Piracicaba – SP, Brazil) and finishing with washing the bags in acetone for 5 minutes. The bags were placed in an oven at 105°C for 24 hours and then weighed for calculation of NDF degradability, then reduced to ashes in a muffle furnace (for 5 hours at 550°C) to determine true organic matter degradability.

3.5 Statistical Analysis

The cell autolysis assays were conducted in duplicate in a single block, and the results were subjected to one-way analysis of variance (ANOVA), with means compared using Tukey's multiple comparisons test ($p < 0.05$). Figures and tables were created using Microsoft Excel. IBM® SPSS Statistics software was used for statistical analysis of the autolysis results. For data obtained from the *in vitro* rumen fermentation technique, R version 4.1.2 (R Core Team, 2021) software was utilized. Analysis of variance (ANOVA) with mean comparison was conducted using the Duncan test at a significance level of 5%.

Figure 1. Flowchart of the steps developed in the current chapter.

4 RESULTS

4.1 Centesimal composition of yeast

The composition of whole yeast, regarding characterization in terms of RNA concentration, proteins and others, is presented in Table 3.

Table 3. Centesimal composition of whole yeast. dry basis.

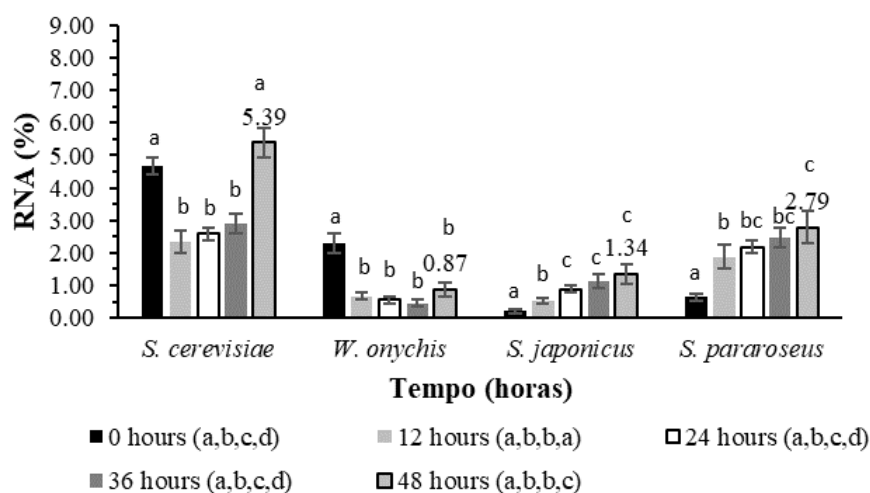
	<i>S. japonicus</i> 70a	<i>S. pararoseus</i> 33.1	<i>W. onychis</i> LABI2	<i>S. cerevisiae</i> M26
RNA (%)	4.67±0.29	5.20±0.42	12.14±0.37	9.66±1.02
Protein (%)	40.32±0.99	42.61±0.53	70.13±4.37	49.57±2.73
Others (%)	55.00	52.18	17.72	40.76

W. onychis LABI2 exhibited the highest concentrations of RNA and proteins, 12.15% and 70.13%, respectively. The yeast that showed the lowest concentrations of RNA and protein was *S. japonicus* 70a, with 4.67% and 40.32%, respectively. *S. cerevisiae* M26 exhibited expected levels for both variables: 9.66% RNA and 49.57% protein. The results indicate that the yeasts *W. onychis* LABI2 and *S. cerevisiae* M26 present the best composition in terms of RNA and protein content.

4.2 RNA quantification

The autolysis process was carried out aiming to extract the protein from the different microorganisms based on a studied and defined methodology for protein extraction from *S. cerevisiae* in the brewing industry (Alves, 2020). RNA quantification was also performed (Fig 2).

Fig 2. Percentage of RNA extracted at different autolysis times based on the dry mass of the extract.



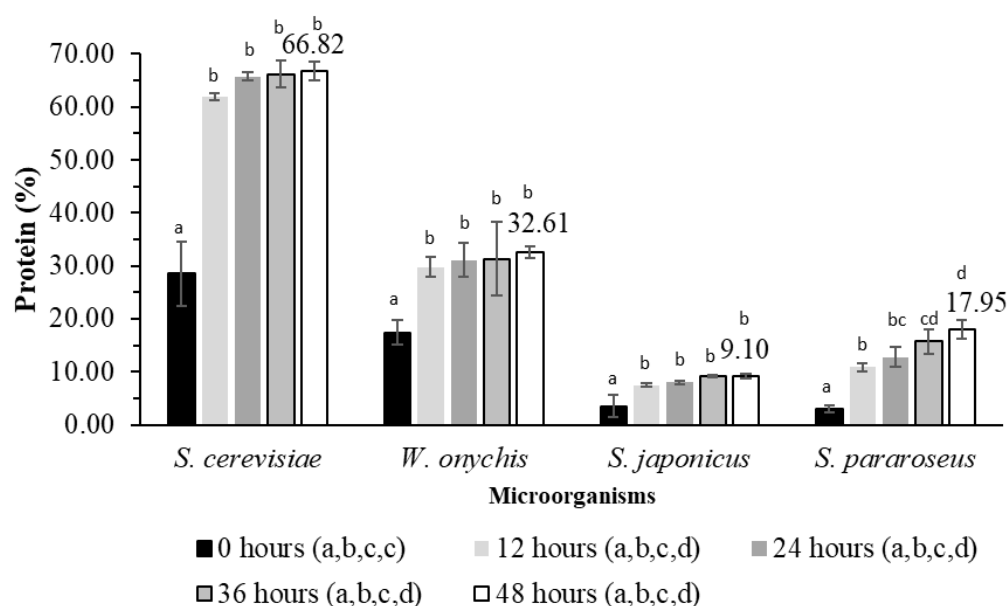
Analysis of variance (ANOVA) was used by the Tukey multiple comparison test, with a significance level of 0.05%. Letters in front of the times sequentially compare the RNA (%) for each microorganism. Letters above each bar compare the RNA (%) obtained with the same microorganism for the RNA concentration at different times. Different letters indicate significant differences between groups.

The best results for RNA extraction were obtained for *S. cerevisiae* M26 with 5.39%, followed by *S. pararoseus* 33.1 and *S. japonicus* 70a with 2.79% and 1.34%, respectively. The worst extraction was attributed to *W. onychis* LABI2, with 0.87%. These results can be explained due to the use of a protocol defined for the extraction of proteins from *S. cerevisiae*, which justifies the better performance of *S. cerevisiae* M26 and the low protein extraction by the other microorganisms. However, the strain used, *S. cerevisiae* M26 is a selected yeast from the ethanol industry and consequently presents different characteristics from brewery yeasts, yeast used for the methodology base by Alves (2020). Also, the influence of lytic enzymes and the ability of yeasts to undergo autolysis should be taken into account, which are unknown for the yeasts *S. pararoseus* Sia 33.1, *S. japonicus* Sia 70a, and *W. onychis* LABI2.

4.3 Protein quantification

The methodology of the present study was standardized to evaluate protein extraction by the microorganisms *S. pararoseus* Sia 33.1, *S. japonicus* Sia 70a, *W. onychis* LABI2 and *S. cerevisiae* M26 compared to brewery *S. cerevisiae* for which the methodology was optimized (Alves, 2020). Thus, the use of temperature ramps aimed to optimize the action of different enzymes to achieve efficient autolysis and consequently a higher concentration of extracted proteins. The results of this extraction are presented in Figure 3.

Figure 3. Percentage of extracted proteins at different autolysis times based on the dry mass of the extract.



Analysis of variance (ANOVA) was conducted using the Tukey multiple comparison test, with a significance level of 0.05%. Letters in front of the times sequentially compare the protein (%) for each microorganism. Letters above each bar compare the protein (%) obtained with the same microorganism for the protein concentration at different times. Different letters indicate significant differences between the groups.

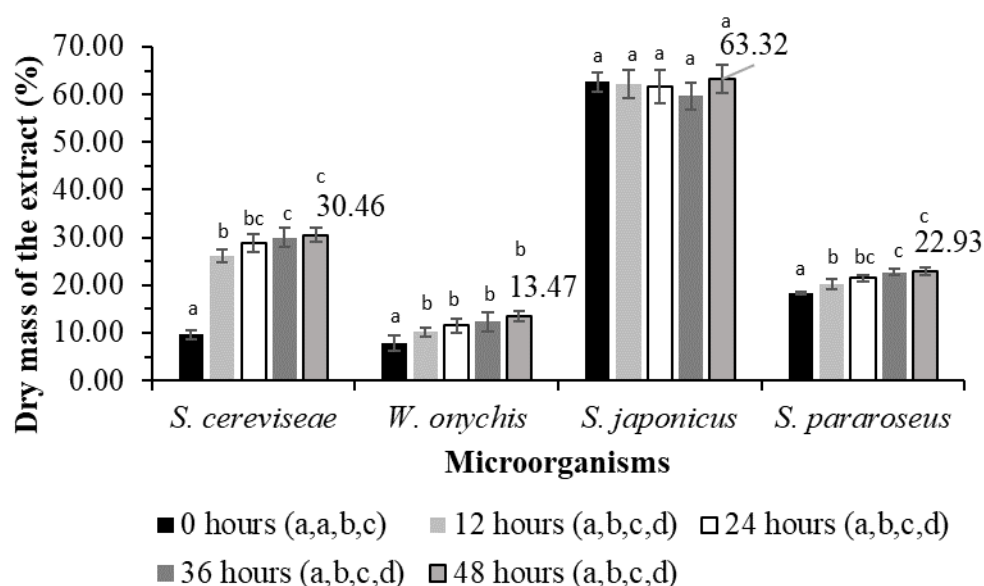
The highest protein extraction was obtained with *Saccharomyces cerevisiae* M26, as expected, reaching 66.82% of extracted proteins in 48 hours. Following this is *W. onychis* LABI2, with 32.61%, *S. pararoseus* 33.1 and *S. japonicus* 70a obtained the poorest protein extractions considering the methodology used with 17.95% and 9.10%, respectively. The results demonstrate that the protocol used was more efficient for *S. cerevisiae* M26, indicating the need for further studies for the other three yeasts, especially for *W. onychis* LABI2, which presents high concentrations of protein and RNA. The results indicate that the autolytic potential of the special yeasts is lower than for *S. cerevisiae* M26 under the tested conditions, considering that the temperatures used were not excessive. However, pH sensitivity may have

hindered their autolysis, unlike what occurs with *S. cerevisiae* M26, which increases its autolytic potential at acidic pH for protein extraction.

4.4 Dry mass

Dry mass is based on the total yeast used in the experiment (7.5% w.v⁻¹) considering the mass extracted during autolysis. The results of extracted dry mass are shown in Figure 4.

Figure 4. Percentage of dry mass extracted at 0, 12, 24, 36 and 48 hours of autolysis.



Analysis of variance (ANOVA) was performed using the Tukey multiple comparison test, with a significance level of 0.05%. Letters in front of the times sequentially compare the dry mass (%) for each microorganism. Letters above each bar compare the dry mass (%) obtained with the same microorganism for the dry mass concentrations at different times. Different letters indicate significant differences between the groups.

The best extraction of soluble solids was observed for *S. japonicus* 70a, reaching 62.65% at time 0, indicating high sensitivity to pH already at the beginning of the process. *S. cerevisiae* M26 achieved 30.46% of extracted dry mass at 48 hours. The expected result for *S. cerevisiae* M26 would be around 50% of mass extracted; however, this yeast is a selected strain from the ethanol industry, being more resistant to stress than brewery yeast. Following are *S. pararoseus* 33.1 and *W. onychis* LABI2 with 22.93% and 13.47%, respectively.

The extracts and cell walls of each tested yeast were applied in *in vitro* ruminal fermentation tests to evaluate if the material would affect fermentation with a concentrated diet. The *in vitro* ruminal fermentations are presented in Table 4 for 24 and 48 hours of incubation.

The results obtained from the *in vitro* ruminal fermentation did not show statistical differences for any of the analyzed variables.

Table 4. *In vitro* ruminal fermentation for the different treatments and control during the incubation period of 24 and 48 hours. No statistical difference was observed between the treatments.

24 hours	OMD	TDOM	NDFD	pH	GPdm	CH ₄ omd	GPomd	PF
Control	413.92	0.35	368.68	6.60	78.94	1.42	32.72	2.96
Extract Sc	423.60	0.35	374.68	6.59	75.12	1.79	31.53	3.21
Extract Sj	433.54	0.36	385.75	6.59	84.99	2.19	37.54	2.96
Extract Sp	438.06	0.37	390.46	6.60	81.41	2.03	35.93	3.10
Extract Wo	416.14	0.35	367.10	6.61	80.57	1.71	30.66	3.21
Cell wall Sc	430.42	0.36	382.31	6.59	73.32	1.73	33.44	3.16
Cell wall Sj	423.98	0.35	375.22	6.57	77.59	1.85	32.74	3.15
Cell wall Sp	431.72	0.36	383.17	6.62	78.48	1.83	33.50	3.18
Cell wall Wo	415.69	0.35	366.62	6.63	80.13	1.69	29.40	3.31
48 hours								
Control	517.46	0.43	474.18	6.43	115.10	1.64	596.50	2.86
Extract Sc	511.72	0.44	466.58	6.44	122.67	2.22	663.17	2.68
Extract Sj	535.97	0.45	494.01	6.44	124.90	2.60	668.54	2.78
Extract Sp	537.31	0.45	496.01	6.43	124.11	2.67	667.55	2.79
Extract Wo	531.21	0.44	489.90	6.39	123.05	1.94	656.33	2.79
Cell wall Sc	544.02	0.46	504.05	6.43	125.40	1.99	685.55	2.81
Cell wall Sj	546.85	0.44	506.66	6.36	121.98	1.98	638.63	2.73
Cell wall Sp	537.80	0.45	496.52	6.43	121.71	2.36	655.48	2.85
Cell wall Wo	529.82	0.44	487.25	6.43	121.87	1.75	645.66	2.80

Control: Concentrated diet. The samples are divided into cell wall and cell extract for all yeasts. Microorganisms were cultured at 30°C, pH 5.5. SJ: *S. japonicus* Sia 70a, SC: *S. cerevisiae* M26, SP: *S. pararoseus* Sia 33.1 and WO: *W. onychis* LABI2. The Tukey test was used with a significance level of 0.05%. OMD: organic matter degradability (g.kgOMD⁻¹); TDOM: truly degraded organic matter (g.kgOMD⁻¹); NDFD: Neutral Detergent Fiber Degradability. GPdm: gas production per dry matter (mL.gDM⁻¹); GPomd: gas production per organic matter (mL.gMOD⁻¹); CH₄omd: methane production per organic matter (mL. gOMD⁻¹); PF = partitioning factor.

5 DISCUSSION

The composition of the selected yeasts in terms of RNA and protein content shows a higher concentration of RNA for *W. onychis* LAB2 (12.14%), followed by *S. cerevisiae* M26 (9.66%), both also standing out in terms of protein content at 70% and 49%, respectively. The values observed in the characterization are in accordance with the literature, which predicts 40 to 60% protein and 7 to 12% RNA (Chae & In, 2001; Kuninaka, 2008). Alves (2020) found for brewery *S. cerevisiae* yeast 53.4% protein, 9.47% RNA and 37.10% other compounds. similar to the observed data (Table 3).

The best RNA extraction was observed for *S. cerevisiae* M26 (5.39%). Alves (2020) observed better protein extraction using a temperature ramp at pH 2, a strategy used in the

present work focusing on protein extraction and not RNA, justifying the low RNA extraction for *S. cerevisiae* M26 (5.39%). Zhao & Fleet (2005) evaluated autolysis at different pH and temperatures on RNA degradation, observing faster degradation at 50°C and pH 7.0. Autolysis at low temperatures such as 30 to 40°C and pH 5 to 6 favored the formation of 5' and 2' nucleotides. The temperature of 50°C (pH 7) and 40°C (pH 4) favored the release of flavor-enhancing nucleotides (5' AMP and 5' GMP). The results observed by Zhao & Fleet (2005) support the strategy of using a temperature ramp, demonstrating variation in the release of ribonucleotides with temperature variation as performed in the present study, 45°C to 60°C.

S. cerevisiae M26 and *W. onychis* LAB2 showed a higher potential for protein release with the autolysis methodology employed, reaching 66.82% and 32.61% in 48 hours. Takaloo et al., (2020) achieved a protein release of 65.8%, using a 15% (w.v⁻¹) cell suspension at pH 5.5. The authors evaluated the efficiency of plasmolysis, hydrolysis and autolysis for *S. cerevisiae*, obtaining better release of cytoplasmic content with hydrolysis and plasmolysis, indicating a similar methodology and similar results regarding the released protein content, 66.8% for the methodology applied to *S. cerevisiae* M26 and 65.8% by the authors. Alves (2020) achieved 41% protein in acid hydrolysis with a temperature ramp, with lower results indicating variation in the strain used. Vukašinović-Milić et al., (2007) evaluated the effect of enzymatic hydrolysis with papain on the protein extraction from *S. cerevisiae*, finding 56.38 mg.mL⁻¹ with high solids recovery, around 68%.

The extraction of soluble solids was higher for *S. japonicus* 70a reaching 62.65% at time 0, indicating greater sensitivity to pH or temperature used. The extraction of solids followed higher for *S. cerevisiae* M26 followed by *S. pararoseus* 33.1 and *W. onychis* LABI2. Chae et al., (2001) when using *S. cerevisiae* from breweries achieved 18.2% of extracted solids using 5'-phosphodiesterase and deaminase and also extraction of 9.25% of crude protein, results inferior to those obtained in this study for *S. cerevisiae* M26, 30.46% of solids and 66% of protein. Alves (2020) obtained a solids extraction in the range of 58%, above the results found for *S. cerevisiae* M26, demonstrating that changes in the methodology can improve the extraction for the strain used.

The *in vitro* ruminal fermentation with concentrated diet and application of yeast cell wall and extract did not show statistical differences for any of the analyzed variables. It is believed that the yeast cell wall and extract do not affect the ruminal microorganisms nutritionally considering the diet with a high amount of concentrate. However, the yeast cell

wall applied in live animals may favor an interaction with the rumen wall, allowing the activation of immune system cells and preventing the attachment of pathogenic microorganisms to the rumen wall, thus promoting the health of the system and influencing the proliferation of beneficial microorganisms and consequently greater diet degradability as observed by some authors (Sanchez et al., 2014; Zapata et al., 2021). Sanchez et al., (2014) when using *S. cerevisiae* cell wall in beef heifers, observed changes in the metabolic response to LPS challenge (lipopolysaccharide, which increases inflammation and insulin resistance in tissues), with alterations also in the concentrations of glucose, insulin, non-esterified fatty acids and urea nitrogen in the blood. An increase in energy metabolism during this immune challenge was observed through the supplementation of yeast cell wall, allowing for a faster return to homeostasis. The results obtained in this study may be due to the possibly inconsistent application of the additive and the lack of interactions of the additives with the live animal system because, as observed by Sanchez et al., (2014), the treatments favor the animal's immune system, which may possibly reduce the use of antibiotics. Zapata et al. (2021) observed that with the use of a prebiotic composed of *S. cerevisiae* Active Flora yeast cell wall, ICC, São Paulo/ Brazil (3g of mannanoligosaccharide plus beta-glucans/lamb/day, equivalent to 0.002% g of prebiotic.g⁻¹ of diet), in an 80:10 diet (concentrate: hay) for 21 days, there was an increase in apparent digestion of organic matter, NDF, digestible energy and ruminal pH. The results indicate that constant prebiotic supplementation for lambs allowed for improved total digestion and digestible energy. Despite using a lower concentration of prebiotic (0.002% g.g⁻¹), the results demonstrate that daily feeding with prebiotics stimulated the metabolism of ruminal microorganisms.

Strategies prioritizing the constant addition of the additive as performed by Zapata et al., (2021) may yield better responses, in addition to using a poorer diet such as a fiber-based one, which may allow for greater influence of nutrients (cell wall and extract) on the pool of microorganisms in the rumen fluid. The cited authors conducted *in vivo* experiments, which allowed for influences on both the nutrition of the ruminal microbiota and the immune system, even though both factors were not analyzed together (Zapata et al., 2021; Sanchez et al., 2014; Bruno et al., 2009).

Oeztuerk et al., (2016). while investigating *in vitro* ruminal fermentation conducted using the rumen simulation technique (Rusitec) over 22 days with a daily increment of 9 grams of diet (5g of meadow hay and 4g of concentrate), evaluated three different products with an

application of 0.75 g.day⁻¹ of: 1, hydrolyzed whole yeast (*Saccharomyces cerevisiae*) (HWY). 2. less hydrolyzed whole yeast (LHWY) and 3. yeast cell wall (YCW). In response to the yeast cell wall treatment (YCW) no alteration in ruminal pH was observed; however, total productions of short-chain fatty acids, acetate, propionate, butyrate and methane, as well as the concentration of ammonia nitrogen, increased significantly ($p < 0.05$). The digestibility of organic matter did not show statistical differences for hydrolyzed whole yeast (HWY) and yeast cell wall (YCW) compared to the control. No effects were observed with the use of less hydrolyzed whole yeast (LHWY), indicating that the degree of hydrolysis (low or high), in addition to the composition of the yeast used, whole cell or cell wall, has effects on the response of ruminal microbiota in the Rusitec system. The present study also did not observe alterations in pH; there was an increase in methane release compared to the control for most of the additives tested (without statistical difference), as observed by the author. However, future analyses of short-chain fatty acids, acetate, propionate, butyrate, among others, may present benefits of using such compounds in nutrition. It is also important to note that autolysis was not as effective for the yeasts *S. japonicus*, *W. onychis* and *S. pararoseus*, which possibly classifies them as less hydrolyzed yeasts.

6 CONCLUSION

S. cerevisiae stood out as it achieved the best autolysis among the four microorganisms evaluated, resulting in the highest protein extraction. Economically, *S. cerevisiae* is the most viable yeast for industrial production, considering its availability in the market and its higher autolytic potential. Given the objective of this study to produce a protein-rich extract, autolysis proved to be a quick and cost-effective alternative to supplement animal feed. The results demonstrate that *W. onychis* LABI2 showed great potential for future studies, given its high concentration of RNA and proteins. As for *S. pararoseus* 33.1 and *S. japonicus* 70a, it is believed that future studies with smaller variations in pH may favor autolysis for these yeasts. *In vitro* ruminal fermentations showed no difference compared to the control. It is believed that future studies with *in vivo* ruminal fermentations may better explain the use of these compounds in animal nutrition in addition to daily supplementation.

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GENERAL CONCLUSION

Chapter 1 discusses the main points covered in the thesis, from biomass production to rumen characteristics and applications of microorganisms in ruminant nutrition. In Chapter 2, it was observed that cassava bagasse hydrolyzed or non-hydrolyzed, as well as corn steep water, were efficient for cell development. Yeasts showed a preference for hydrolyzed or non-hydrolyzed cassava bagasse (5% w.v⁻¹) and corn steep water (0.5% v.v⁻¹); however, in larger-scale cultures (Chapter 3), the results demonstrate that the conditions defined in the *in vitro* tests (Chapter 2. Erlenmeyer flask with a final medium volume of 100 mL) for biomass production were not reproduced in a 2.5 L bioreactor (Chapter 3). In Chapter 3 alterations to the cultivation process were explored to improve cell production, in which viability, sugar concentration, pH and the association of these factors with the type of substrate used demonstrated the greatest influence on cultivation. Fed-batch cultivation showed better results in terms of cell production and enzyme production compared to simple batch cultivation. Yeasts also underwent a process of adaptation regarding the pH and temperature of cultivation (37°C and pH 6.9); *Saccharomyces cerevisiae* M26 and *W. onychis* LABI2 adapted more easily compared to *Sporidiobolus pararoseus* 33.1 and *Sporobolomyces japonicus* 70a. which showed greater sensitivity to changes in these factors, especially temperature. In Chapter 4, different methodologies, cryoprotective and nutritive solutions were investigated to preserve the biomass produced. The use of powdered milk (10% w.v⁻¹) proved to be the best cryoprotectant for maintaining cell viability at 4°C. In Chapter 5, *in vitro* ruminal fermentation was conducted with a diet based on concentrate and forage (Tifton Hay), in which better results were observed for *B. breve* BB-03 for the concentrate-based diet, showing higher degradability and gas production. For the forage-based diet, the yeast *W. onychis* LABI2 demonstrated better results. Chapter 6 addresses the application of the microorganisms selected in Chapter 5 (*B. breve* and *W. onychis*) in live sheep, where greater utilization of the diet by supplemented animals compared to the control, lower nitrogen excretion and reduced methane emission per weight gain were observed. In Chapter 7, the autolytic capacity of the four yeast strains selected in this study was explored using a standard methodology defined by other authors. Autolysis was more efficient for *Saccharomyces cerevisiae* M26; however, *W. onychis* LABI2 also showed great potential due to its high RNA and protein content in its chemical composition. *In vitro* ruminal fermentation conducted with the autolysates (cell wall and extract) did not yield results favoring degradability and reduced methane production. However, analyses of fatty acids, propionate,

among others, may provide a new perspective on the action of these additives. Furthermore, *in vivo* tests expanded the understanding of the additives' ability to improve animal metabolism. The research addresses the production of different yeast-based products, methods to preserve the material and its application in *in vitro* ruminal fermentation.