Evaluation of the viability of the use of *Lactobacillus casei* in the removal of organic contaminants in waste from the craft brewing industry

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Abstract: Brewers' spent grains (BSGs) are the most abundant waste generated from the craft brewing process, accounting for approximately 85% of the total byproduct obtained. The need to develop beneficial alternatives for the contribution of the industrial sector and sustainable development has increased interest in the fermentation processes used to produce biomass, using probiotic microorganisms that provide health benefits for those who consume it, obtaining byproducts rich in nutrients. Therefore, this research aimed to evaluate the growth of *Lactobacillus casei* in Mar, Rogosa and Sharpe broth (MRS) and to evaluate the feasibility of growing *L. casei* in craft beer residues. To achieve this goal, a 10% v/v inoculum of probiotic bacteria was used in both media. The process consisted of monitoring the biotransformation process at 37°C and 120 rpm for 72 hours and evaluating carbohydrate consumption and cell growth. At the end of 52 h, the carbohydrate concentration in combination with BSG was completely consumed, considering that the initial value was 16.49 g/L. In the case of the MRS medium, a value of 3.42 g/L was obtained at 72 h. Regarding the pH range with the MRS broth and with BSG, the values were 6.89 - 5.43 and 5-4.41, respectively. Due to the acidity of the synthetic medium, the pH of the synthetic medium was greater than that of BSG. However, *L. casei* managed to develop in a similar way since quite similar cell growth values were obtained in both media, so it is feasible to use BSG as a culture medium for the development of probiotic species.

Keywords: *Lactobacillus casei*; Brewers' spent grains; MRS broth.

Introduction

Currently, the craft brewing industry is in constant growth, as producers have the possibility of modifying ingredients and raw materials to their own liking. However, this leads to the generation of significant amounts of waste that is not properly disposed of because it has a high moisture content of more than 80% w/w, making processing difficult (Medina-Saavedra *et al*., 2018). Many studies have shown that organic wastes from different surfaces, including commercial, residential and industrial surfaces, are primary materials with different proportions of lignin, cellulose and hemicellulose and are used in the production of biofuels for the development of new energy sources. In addition, they are materials generated in manufacturing and consumption activities that have not generated financial costs (Chávez *et al*., 2021).

Most of the organic waste generated by the agro-industry is disposed of in an inadequate way by dumping it directly into the environment, burning it uncontrollably or leaving it on the ground; over time, this waste can end up in the sewer system, causing it to enter the aquifers or cause foul odors, contributing to increasing environmental pollution as a result of this inadequate disposal (Fiallos, 2022). In Mexico, approximately 76 million tons of organic waste are generated annually, 79% of which is primary waste (generated during harvesting), such as the leaves and stalks of corn, wheat, barley and beans (Caltzontzin-Rabell *et al*., 2022).

Brewer's spent grains (BSGs) are the most common byproduct of the craft beer brewing process. This view is supported by Chetrariu and Dabija (2020), who reported that approximately 85% of the total byproducts generated are estimated to be from the production of 100 liters of brewed beer that generates approximately 20 kg of wet BSG. Approximately 2,400 million kg of wet waste was produced in Mexico in 2018. Although it is composed of spent grain, this residue contains high amounts of proteins and minerals; contains high amounts of proteins and minerals; and is considered a fibrous, lignocellulosic material that is a lignocellulosic fibrous material. The consumption of this material is also beneficial to human health because it accelerates intestinal transit.

Although this byproduct is considered spent grain, it contains significant amounts of nutrients, as it consists of lignocellulosic substances rich in proteins (20%), fibers (70%), vitamins and minerals. Its chemical composition can vary depending on various factors, such as the quality of the barley or other cereals used for brewing (Lalić *et al*., 2023).

The fermentation process is considered an alternative treatment for municipal waste due to the nutrients it contains. With this process, the environmental impact can be reduced, and various value-added products, such as organic acids, amino acids, volatile fatty acids, enzymes, vitamins and second-generation biofuels, can be generated (Davila *et al*., 2016 and Guarda *et al*., 2021) through the fermentative action of lactic acid bacteria (LAB), which are currently known for their diverse health associations and potential role as probiotics in fermented foods (Insuasti *et al*., 2023).

Lactobacillus casei species (LcS) is a probiotic strain that belongs to the major group of LAB; it is considered safe and provides many health benefits to the host by balancing the gut microbiota, improving gastrointestinal dysfunction, preventing infection and cancer, and modulating inflammatory and immune responses (Katao-Kataoka *et al*., 2016). In this work, we propose to investigate the viability of BSG as a medium for the adaptation, cultivation and propagation of *L. casei* in terms of the possibility of generating nutritious biomasses and to compare microbial development in MRS medium using 10% v/v in both cases and monitoring the biotransformation process for 72 hours.

Materials and Methods

Extraction and processing of organic brewery waste

The craft beer waste was provided by the Altas Cervezas Brewing Co. brewery in the city of Orizaba, Veracruz. After collection, the waste was dried in a drying oven at a temperature of 105°C for 24 hours. The samples were subsequently crushed and sieved (50 mesh) to reduce the particle size. After sieving, dilution was carried out to achieve a concentration of 3 to 5% total solids (TS), which is the appropriate amount to maintain greater homogenization during the biotransformation process (Romero-Mota *et al*., 2023), as shown in Figure 1.

Figure 1. Proposed methodology for conditioning brewing waste obtained from the fermentation of craft beer.

Characterization of the brewery waste

The physicochemical characterization of the SGW was performed to determine its composition, properties and nutrient contents. In this characterization, the total solids (TS) and total volatile solids (TVS) contents were determined using the standard method (APHA, WPCF and AWWA, 2017); pH, using a Science MED SM-3BW potentiometer; total nitrogen (4500-NTK C SM); total nitrogen, using the micro-Kjeldahl method (Mæhre *et al*., 2018); and total carbohydrate content, using the anthrone-sulfur method (López-Regarda *et al*., 2017).

Conditions for the growth and multiplication of L. casei.

The *Lactobacillus casei* strain isolated by streaking on MRS agar (Mar, Rogosa and Sharpe), which is suitable for the development of *L. casei* and other LAB, was used. The agar was previously prepared and sterilized in an autoclave at 121°C for 15 minutes; after seeding into the Petri dish, the mixture was incubated at 37°C for 24 hours. The colonies that formed were then placed in test tubes containing MRS broth for seeding to propagate the bacteria. The plates were incubated in the same way at 37°C for 24 hours; after the incubation period, the test tubes were stored in the refrigerator at 4°C until use.

Evaluation of the cell growth of L. casei in BSG and MRS broth.

For the cell development process, two Erlenmeyer flasks were prepared with a useful volume of 200 mL using a 10% concentration of probiotic bacteria inoculum, in a medium of MRS broth and craft brew residue, respectively, were placed in the incubator with a period of 72 hours at a temperature of 36°C and an agitation of 110 rpm as shown in Figure 2, factors such as pH, carbohydrate consumption were carefully monitored every 4 hours using the Antrona-Sulfuric method (Chang & Murillo, 2017) taking a sample to be analyzed during the process until the end of the incubation period.

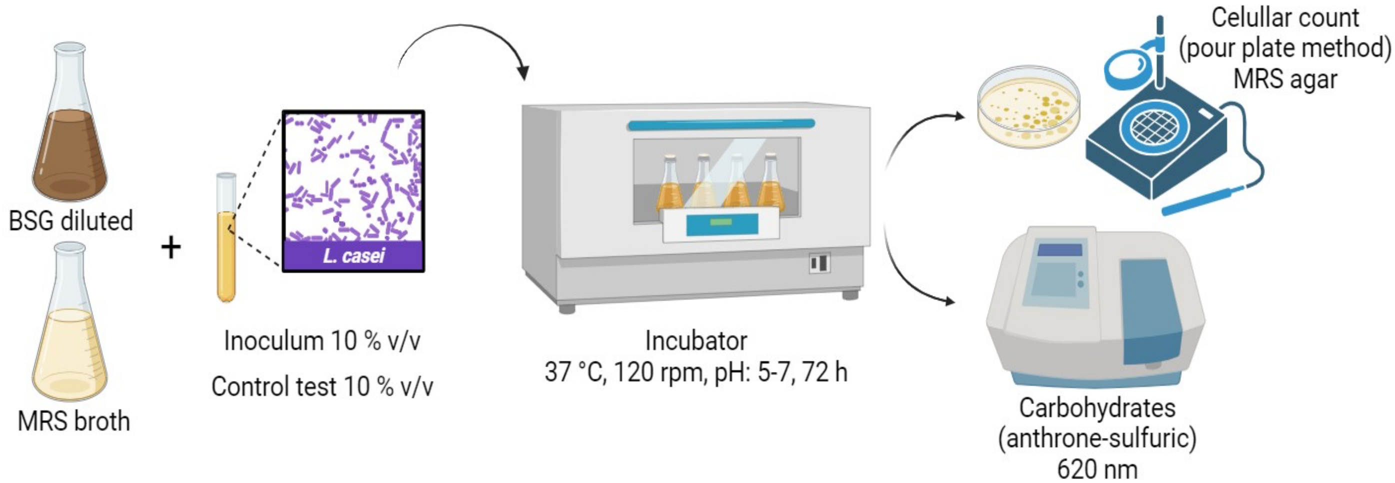


Figure 2. Sequence of plating and incubation of *L. casei* in BSG and MRS broth.

The counting procedure was performed using the pour plate method to determine the bacterial density in colony forming units (CFU) (Angeleri *et al*., 2017). Serial dilutions of base 10 of each sample with 4.5 mL of distilled water and 0.5 mL of the sample were performed to facilitate counting. MRS agar was used as the culture medium. In previously sterilized Petri dishes, 1 mL of the dilution agent and 10 mL of the agar medium were added to the culture medium over a period of 24 hours. The removal efficiency was determined by calculating the percentage of carbohydrates consumed in both media (BSG and MRS) using Equation 1 below (Chohan *et al*., 2020).

$$\%\ Carbohydrate\ consumption\ \frac{initial\ carbohydrates - final\ carbohydrates}{initial\ carbohydrates} * 100 \qquad \textbf{(1)}$$

Results and Discussion

Table 1 shows ST and STV values of 4.43% and 92.83%, respectively, which are similar to those of Glowacki *et al*. (2022), who obtained ST and STV values of 3.91% and 77.70%, respectively, for the case studied. A pH of 5 was determined, which is within the optimal range for the development of *L. casei* (Orozco *et al*., 2018); however, the MRS broth presented a higher pH of 7, but this genus is characterized by its wide pH tolerance range, as it has been reported that some strains can grow at pH 3.2 and up to 9.6 (Bevilacqua *et al*., 2019). The presence of the three main components of organic matter, carbohydrates, proteins and nitrogen, was determined as part of the chemical characterization. A carbohydrate concentration of 14.80 g/L was determined, followed by protein at 11.29%, which is within the range indicated by (Milew *et al*., 2022) for this substrate, and nitrogen at 1.80 mg/kg ST.

Table 1. Physicochemical characterization of the BSG residue on a dry basis.

Parameter	Unit	BSG
Total solids	%w/w	4.43
Volatile total solids	% w/w	92.83
Carbohydrates	g/L	14.80
Proteins	%	11.29
Nitrogen	mg/kg TS	1.80
pH	----	5.00

The fermentation process lasted 72 hours, after 8 hours the cell concentration increases considerably, for the medium containing MRS broth the initial concentration was 12.32 Log (CFU/mL), ending the fermentation process with a concentration of 13.60 Log (CFU/mL). With respect to the craft beer residue, obtaining an initial concentration of 12.25 Log (CFU/mL) and a final concentration of 13.71 Log (CFU/mL). These results of the growth kinetics of *L. casei* and carbohydrate consumption developed with the two substrates (BSG and MRS broth) are presented in Figure 3. The results of the growth kinetics of *L. casei* and carbohydrate consumption of the two substrates (BSG and MRS broth) are presented in Figure 3. The data obtained are similar to those obtained by Sanchez-Valeriano *et al.,* (2022), who used corn residue. On the other hand, the carbohydrate concentration in the BSG treatment was completely reduced at hour 52 at a value of 0 g/L, considering that the initial value was 16.49 g/L; in the case of the MRS medium, a value of 3.42 g/L was obtained at the end of 72 hours, which was similar to that reported by Rocafuerte-Peña *et al*., (2022).

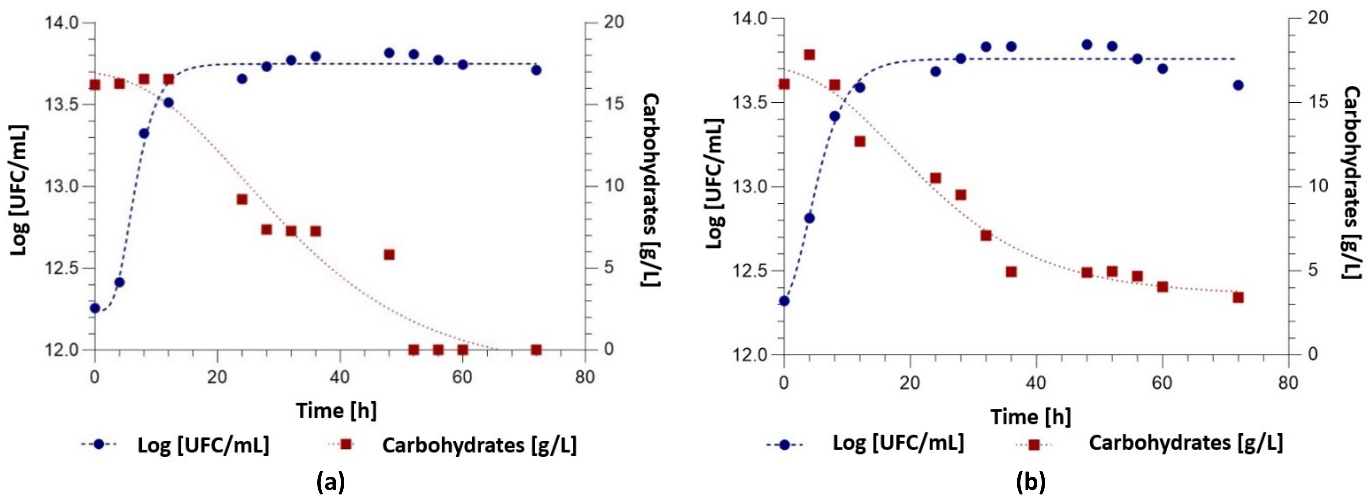


Figure 3. Growth kinetics of *L. casei* and carbohydrate consumption (a) BSG, (b) MRS broth.

With respect to the pH, Figure 4 shows the analysis of the graph of this factor, where differences were observed between the two-substrate media throughout the fermentation process and between MRS and BSG broth. Both media obtained different initial pH values; for the MRS broth, an initial value of 6.89 was obtained, and for the BSG medium, a value of 5 was obtained. These significant differences are due to the nutritional composition of both media. In the case of the MRS broth, a slight decrease in pH occurred, from an initial value of 6.89 to a final pH of 5.43 at the end of 72 hours. This decrease occurred because the MRS medium is composed of large nutritional components that are effective at promoting the development of different types of LAB; due to these conditions, an acidic medium can be obtained; however, this medium is appropriate for the growth of *L. casei* and is also maintained by Orozco *et al*., (2018). On the other hand, the pH of the BSG medium decreased from 5 to 4.41, and the pH of the medium containing the BSG organic residue was lower than that of the MRS medium. *L. casei* managed to develop similarly to MRS medium throughout the fermentation process. This result supports what was reported by Mirmohammadi *et al*., (2021), who reported that acid tolerance is an important probiotic characteristic of this species for survival during fermentation in a food media. On the other hand, it has been reported that if the pH decreases below 4.6, cell development and, in turn, the production of lactic acid may decrease or stop (Rosero *et al*., 2020), which is the reason why the medium acidifies; however, this topic is not the object of study for this case.

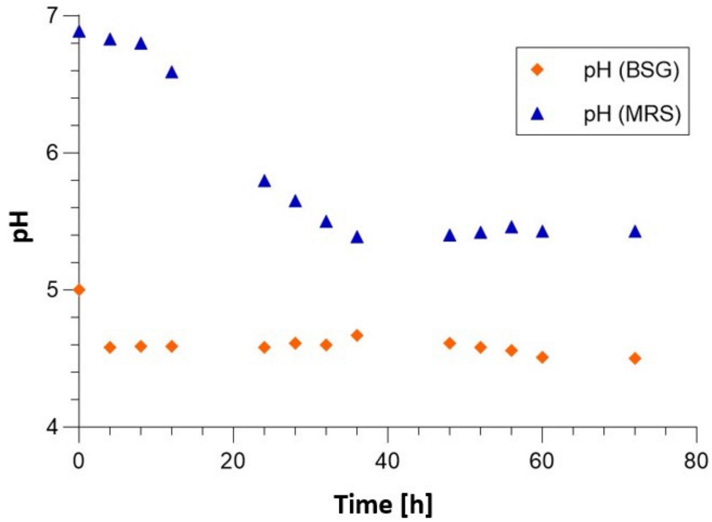
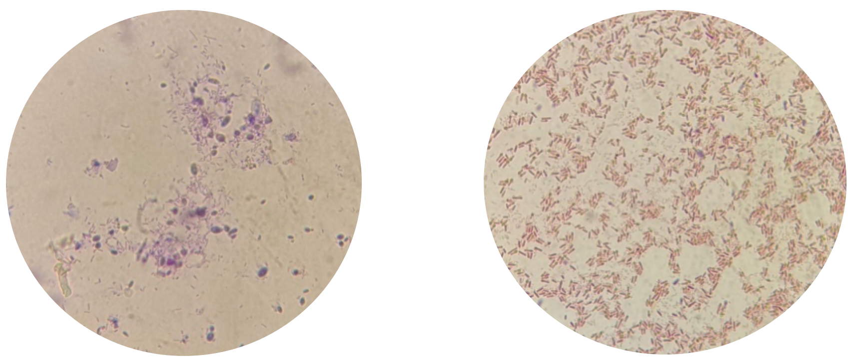


Figure 4. pH variation during the development of the process using BSG and MRS broth.

Figure 5 shows the Gram staining results for each substrate (BSG and MRS media), which revealed gram-positive staining characteristic of the *L. casei* species due to the blue or violet hue, in addition to the observation of an elongated rod or rod-like shape (da Silva *et al*., 2022). Clearly, in the MRS medium or in the broth, there was a greater distribution of microorganisms and, apparently, a greater concentration of bacteria (Figure 5b). However, when we compared the values obtained by counting on a cast plate, there were no major differences between the residue used (BSG) and the synthetic medium.



(a) **(b)**

Figure 5. Gram stain of the species *L. casei* (gram-positive) in: (a) BSG and (b) MRS media.

Significant yields were achieved in terms of carbohydrate intake. *L. casei* showed a remarkable ability to metabolize the carbohydrates present in both media, with different results in terms of carbohydrate elimination. Exceptional performance in terms of carbohydrate absorption was observed on the BSG medium. *L. casei* was able to eliminate all the carbohydrates present in the BSG media, and this process achieved 100% efficiency. This finding suggested that the strain has a high affinity for and ability to utilize the carbohydrates present in the medium as a source of energy and for growth. On the other hand, *L. casei* showed a significant ability to consume carbohydrates in MRS medium, although 78.70% degradation was achieved in this case.

The removal of carbohydrates under anaerobic fermentation conditions in this study showed that reactions related to the hydrolysis of organic compounds, as reported in conventional anaerobic digestion studies, involve consortia of microorganisms (Pasalari *et al*., 2021). However, since *L. casei* is a single microorganism that has been exclusively studied, this finding provides an indication that the same mechanism of decomposition of organic macromolecules occurs; therefore, similar high removals from conventional hydrolysis proteins can be expected and lipids (Wang *et al*., 2021). This behavior generally indicates that it is possible to use *L. casei* for future organic compound work in the craft brewery industry, and the removal of the organic compounds in this study and other organic compounds can be fully evaluated.

Conclusions

The feasibility of using BSG as a culture medium for *L. casei* was investigated by comparison with that of synthetic MRS media. The carbohydrate concentration in the BSG treatment group reached a level at which it was exhausted after 52 hours. The final carbohydrate concentration at the end of the process (72 hours) was 3.45 g/L in the MRS broth because BSG is a byproduct of the craft beer industry. These residues are hydrolyzed, which weakens cell walls and facilitates the absorption of carbohydrates by probiotic microorganisms. The pH range was greater in MRS broth, with a value of 6.89-5.43, than in BSG broth, which reached a range of 5-4.41 due to the acidity of the synthetic medium. In comparison, a greater pH range was achieved in MRS broth than in BSG broth. *L. casei* managed to develop in a similar manner in both media, as cell development was similar in both media (BSG and MRS broth), and final concentrations of 13.71 log (CFU/ml) and 13.60 log (CFU/ml) were achieved. With *L. casei*, carbohydrate consumption yields of 100% for the BSG medium and 78.70% for the MRS medium were achieved; thus, the BSG residue has the potential to develop and stimulate the growth of these probiotic bacteria because its composition enriches and favors the development of *L. casei*.

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Author contributions: I.D.-G., J.E.-G.: data collection, writing, analysis and interpretation; J.M.M.-C.: Conceptualization and data analysis; and E.H.-A., A.A.-L, J.M.M.-C: Administration, project supervision and data analysis.

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