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Leveraging Milk Permeate Fermentation to Produce Lactose-Free, Low-In-Glucose, Galactose-Rich Bioproducts: Optimizations and Applications

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Abstract: Previous studies highlighted *Brettanomyces claussenii* as a versatile yeast that produces ethanol or acetic acid from lactose, or selectively metabolizes glucose while leaving behind galactose, depending on different operational conditions. This flexibility enables the production of galactose-rich bioproducts from liquid dairy residues. The purpose of this study is to: (i) optimize the anaerobic fermentation of milk permeate (MP) by *B. claussenii* to maximize the yields of galactose and ethanol and minimize leftover glucose, and (ii) combine the optimized process with distillation and drying and characterize its multiple products. Response surface methodology was used for the optimization. Three fermentation parameters were chosen as input factors: temperature (25–35 °C), inoculation level (7.0–8.5 log cfu/mL), and time (4–40 days), with three metabolites as responses: galactose, glucose, and ethanol. The optimal combination of parameters resulted in temperature, 28 °C; inoculation level, 7.6 log cfu/mL; and time, 33 days. Under these conditions, the fermented product was predicted to have 63.6 g/L galactose, 4.0% *v*/*v* ethanol, and 0 g/L residual glucose. The optimal parameters were used to run 18 L fermentations followed by distillation and freeze-drying. As a result, four product streams were obtained and characterized for relevant physicochemical and nutritional attributes. Our results show that the partial fermentation of MP by *B. claussenii* can be the first step to develop lactose-free, low-in-glucose, galactose-rich bioproducts, which improve the value of this residue and broaden its applications in the food supply chain.

Keywords: *Brettanomyces claussenii*; response surface methodology; ethanol; fermentation; distillation; freeze-drying

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1. Introduction

The rise in the consumption of high-protein-based products, such as protein drinks, protein bars, and protein supplements [1], has led to the development of processes aimed at concentrating and isolating milk proteins. These processes increase the protein content of fluid milk or separate this protein for ingredient applications, among other uses. The increase in the protein content of fluid milk is achieved through a concentration step, generally ultrafiltration, which separates milk components based on size, charge, and shape using semi-permeable membranes [2]. This process divides milk into two streams: retentate and permeate. The former is subject to further processing until it becomes a commercial product; the latter is regarded as a byproduct.

For years, the residual stream milk permeate (MP) has been explored to manufacture products such as crystalline lactose, lactulose, lactobionic acid, methane, ethanol, and single-cell protein [3–8]. Its powder has also been used for commercial applications, including the standardization of fluid milk, and in ingredient applications such as bakery products, confections, and frozen desserts [9]. Due to its rich composition of sugars, vitamins, and minerals [10], more recently, this coproduct has been the subject of research studies aiming

to take the most advantage of such components. Some applications studied as alternatives for MP include the production of functional beverages [11–14] and drinks with antibiotic properties and galactooligosaccharides [15]. All these studies have highlighted MP as a viable substrate for developing products nutritionally valuable and potentially beneficial.

Previous studies presented novel biotechnological approaches for producing foods and ingredients rich in galactose from dairy coproducts such as Greek-style yogurt whey and dairy permeates through their fermentation by *Brettanomyces claussenii* [16,17]. Recently, the *Brettanomyces* genus has been highlighted for its potential in industrial fermentations [18–20]. Mainly, *B. claussenii* has demonstrated the potential to make beverages from lactose-containing streams [21]. Furthermore, Rivera Flores, et al. [22] observed the sugar utilization profile of a commercial strain of *B. claussenii* and found that it yielded various products when cultured under different conditions of oxygen and sugar availability [22,23]. Their results showed that under anoxic conditions, it metabolized all the available lactose into ethanol, whereas it metabolized only glucose and preserved nearly all galactose when presented with the two monosaccharides [22]. This operational flexibility opened the possibility of developing lactose-free, low-in-glucose, and galactose-rich products from dairy residues.

To take advantage of this flexibility, several fermentation parameters have been studied for their effects on the final composition of the products fermented by this strain of *B. claussenii*. The impact of oxygen availability and degree of lactose hydrolysis, along with additional variables such as temperature, substrate concentration, inoculation level, pH, and time, has been modeled and optimized using response surface methodology (RSM). Jencarelli [17] studied these variables in synthetic media to simulate the scenarios of different dairy coproducts. At the same time, Rivera Flores, et al. [24] presented ethanol and galactose optimization results in cheese whey permeate (WP). The results from these studies showed that it is possible to approximate and maximize the amount of galactose and galactose-ethanol during the respective aerobic and anaerobic fermentations with this organism, respectively. In addition, distillation and freeze-drying were proposed to combine with fermentation to generate multiple food and ingredients derived from this approach [24].

Therefore, the aim of the present study is two-fold: (i) optimize the anaerobic fermentation of MP by *B. claussenii* to maximize galactose recovery, glucose consumption, and ethanol production from this stream and (ii) combine fermentation with distillation and freeze-drying to obtain and characterize various prospective products resulting from this approach.

2. Materials and Methods

2.1. Substrate and Enzyme

Ultrafiltered MP with approximately 17–19% TS was provided by a commercial dairy facility. Except for the experiments involving 18 L fermentations, upon receiving, it was adjusted to 15% TS using water filtered through a Milli-Q Advantage A10 system (MilliporeSigma, Burlington, MA, USA). Then, the adjusted MP solution was filter-sterilized using a 0.45 µm polyethersulfone vacuum membrane filter (VWR International, Radnor, PA, USA) and stored at room temperature until use.

The food-grade, commercial lactase solutions used in this study are Maxilact A4 (Koninklijke DSM N.V., Heerlen, The Netherlands) and Nola Fit 5500 (Chr. Hansen, Milwaukee, WI, USA). Unless stated otherwise, they were sterilized using 0.2 µm syringe filters (Corning Inc., Corning, NY, USA) before each use.

2.2. Microbial Culture

A commercial strain of *B. claussenii* (OYL-201, Omega Yeast Labs, Chicago, IL, USA) that had been frozen and kept at −80 °C was reactivated by streaking it onto potato dextrose agar with a supplement of chloramphenicol at 25 mg/L (PDA + CAM); this agar plate was incubated for 6 days at 30 °C. After this, a single colony was taken and inoculated into a tube containing 5 mL of dry malt extract broth (DME, Briess Malt and Ingredients

Company, Chilton, WI, USA). This tube was incubated for 1 day at 30 °C, with a constant agitation of 200 rpm.

The next step of the propagation involved scaling up the incubation in DME to a volume of starter that would yield the necessary number of yeast cells for each set of experiments (preliminary factor assessment, optimization, and validation). Each starter was tested for its concentration of cells and viability under the microscope, which was performed as follows. A sample of 500–1000 µL of starter was collected and diluted at a rate of 1:9 with 1× phosphate buffered saline solution. This was then further diluted 1:1 with a 0.1% sterile solution of methylene blue (Ward's Natural Science, Rochester, NY, USA). Upon confirming the concentration of living cells with a hemocytometer, it was used to calculate the volume required for each run in every experiment.

2.3. Preliminary Factor Assessment

The preliminary factor assessment investigated the effect of initial dissolved oxygen (DO) on the following responses: concentration of ethanol, glucose, and galactose at the end of the fermentation. It also aimed to investigate the amount of time required for the total consumption of glucose at an established inoculation level.

2.3.1. Design

A 2 × 2 factorial design was conducted in triplicate. The factors studied were DO, at levels ranging from 50 to 100% saturation (approximately 4.5–8.9 mg/L), and time, ranging from 4 to 40 days. Three center points were included to better estimate the effects, resulting in a total of 15 runs, given by $2^2 \times 3 + 3$. The run list is presented in Table 1 in the randomized order generated by the software JMP Pro 16.0.0 (SAS Institute Inc., Cary, NC, USA).

Table 1. Runs for the 2 × 2 factorial design to assess the effect of initial dissolved oxygen and time on glucose consumption, and the production of ethanol and galactose from MP. The runs were performed in triplicate with 3 center points. The Pattern column shows the combination of factor levels used in each run: high (+), low (−), and center (0), for initial dissolved oxygen and time, respectively. The randomized order was provided by JMP Pro 16.0.0.

Run	Pattern	Initial Dissolved Oxygen (% Saturation)	Time (Days)
1	−−	50.0	4
2	00	75.0	22
3	+−	100.0	4
4	−−	50.0	4
5	−+	50.0	40
6	++	100.0	40
7	+−	100.0	4
8	00	75.0	22
9	++	100.0	40
10	+−	100.0	4
11	++	100.0	40
12	−−	50.0	4
13	−+	50.0	40
14	−+	50.0	40
15	00	75.0	22

2.3.2. Setup

Before the individual fermentation bottles were prepared, the bottles containing MP at 15%TS were adjusted to 50, 75, or 100% DO saturation, as needed. For this purpose, the DO was first measured using a Hanna DO Meter (Hanna Instruments, Providence County, RI, USA). After this, the bottles with excess DO were sonicated and subjected to vacuum until reaching the required 75 or 50% saturation. The bottles needing extra oxygenation were agitated at 1000 rpm in a NuAire Biosafety Cabinet (NuAire, Plymouth, MN, USA)

for 1 to 2 h until reaching 100% saturation. After the levels of DO were confirmed for each case, 190 mL of these solutions were aliquoted in 250 mL media bottles, preventing oxygen incorporation or dissipation in the process.

The starter of *B. claussenii* and the commercial enzymes were combined in individual tubes before the inoculation, one tube corresponding to each fermentation run, as described next. First, aliquots of 1.4 mL of the starter were pipetted into 15 mL centrifuge tubes to attain an inoculation level of 6.5 log cfu/mL. This inoculation level was reported as optimal for galactose retention by Rivera Flores, et al. [24]. All tubes were centrifuged at 3220× *g* for 2 min, their DME supernatants discarded, and the cell pellets resuspended in 10 mL of MP solution. After this, 400 µL of sterile Maxilact A4 and 400 µL of sterile Nola Fit 5500 were added to each tube so that, once inoculated to the bottles, they each reached an enzyme/substrate ratio of 100 IU/g lactose. An additional tube was prepared to inoculate a surrogate bottle to verify the inoculation.

After this process, the contents of each tube were poured into the corresponding bottle of MP solution for a total volume of 200 mL. The bottles were closed using a rubber stopper and an airlock and incubated at 30 °C without agitation. The fermentation time was determined for each run by the settings in Table 1.

2.3.3. Data Collection and Analysis

On day 0, samples of the uninoculated MP were taken to record the initial density using a DMA^{TM} 35 density meter (Anton Paar, Graz, Austria), and pH using an iCinac TM analyzer (AMS Alliance, Weston, FL, USA), for verification purposes. Additionally, the inoculation level was verified in the surrogate bottle by plating appropriate dilutions onto PDA + CAM and incubating at 30 °C for 6 days. On both day 0 and final, samples were collected to determine the concentration of ethanol, lactose, glucose, and galactose via HPLC analysis, as described previously [22].

The results of glucose, galactose, and ethanol were input to the design table, excluding those considered outliers. Then, ANOVAs and *t*-tests, including individual and interaction effects, were conducted for each response, using a standard least square method. In every model, each effect was assessed for its significance using a *t*-test with a significance level of 0.05.

2.4. Optimization

2.4.1. Design

Three factors—temperature, inoculation level, and time—were studied at the levels presented in Table 2. The range of temperature included optimal conditions for this strain in WP reported in a previous study [24], and the ranges of inoculation level and time were determined by the findings of the previous section. A central composite design was built via response surface methodology, considering the following responses: galactose retention, final glucose, and ethanol production.

Table 2. Factors assessed for their effect on glucose consumption, ethanol production, and galactose retention in 15%TS milk permeate. Fermentations were carried out by *B. claussenii* OYL-201; the combination of parameters in every fermentation was obtained using a face-centered central composite design.

Factor	Axial Point −1	Minimum −1	Center Point 0	Maximum +1	Axial Point +1
Temperature (°C)	25	25	30	35	35
Inoculation Level (log cfu/mL)	7.00	7.00	7.75	8.50	8.50
Time (days) *	4	4	22	40	40

* Once the design was augmented, it included runs ending in 13 and 31 days. See Table S1 for details.

JMP Pro 16.0.0 was used to make the design table of the optimization. The established goals regarding these response variables were to maximize galactose and ethanol production and to minimize glucose retention. Each factor was set at its respective maximum and minimum levels, shown in Table 2. Then, a central composite design of 16 runs was chosen; this included 8 factorial runs, 6 axial runs, and 2 center points. After this, the axial value was set to 1 to generate a face-centered design, and the number of additional replicates was established as 2. Face-centered designs place the axial points at the same level of the maximum and minimum values for each factor, keeping 3 levels for each (Table 2). Overall, the generated table contained 48 randomized runs, which included a total of 6 center points.

Additionally, this design was augmented by including 2 added time points: days 13 and 31, which increased the power of this design regarding the effect of time on the responses. This action resulted in 8 extra runs (4 of each) that maintained temperature and inoculation level at their center points. The final design table, with a total of 56 runs, can be found in the Supplemental Material as Table S1.

2.4.2. Setup

All runs were set up on the same day. For this purpose, the uninoculated MP solution was first divided into aliquots of 115 mL and poured into 250 mL media bottles, each representing an individual run.

Meanwhile, the starter of *B. claussenii* was divided into individual centrifuge tubes; the volume of starter that went into each depended on the level of inoculation required for every bottle. Bottles expected to achieve an inoculation of 7 log cfu/mL used 2.8 mL of starter; bottles requiring 7.75 log cfu/mL, 16.8 mL; and bottles requiring 8.5 log cfu/mL, 91.2 mL. This last volume was divided into two 50 mL centrifuge tubes for better centrifugation. Additionally, extra tubes were prepared to inoculate one surrogate bottle of each inoculation level for confirmation. All tubes were centrifuged and resuspended in 10 mL of MP solution, as detailed in Section 2.3.2.

After this, the contents of each tube were poured into their corresponding fermentation bottles, achieving a total of 125 mL of substrate in all runs. Moreover, 283 µL of sterile Nola Fit 5500 was pipetted into each to achieve 100 IU/g lactose. After this, the bottles were capped with a stopper and an airlock and placed in separate incubators, depending on their fermentation temperatures. Bottles were incubated at 25 °C, 30 °C, and 35 °C, as determined by the experimental design. The fermentation time for each bottle is shown in the design table (Table S1).

2.4.3. Data Collection and Analysis

The initial cell count was confirmed for each level of inoculation by enumerating cell plate counts in each surrogate bottle. On day 0, samples of uninoculated MP solution were collected to determine the concentration of ethanol, lactose, galactose, and glucose; the same analyses were performed on the final day of each run. The ethanol concentration in % v/v was analyzed via gas chromatography, and the concentration of sugars in g/L was obtained using enzymatic assays, as reported by Rivera Flores, et al. [24].

The results were input to the design table in JMP® Pro 16.0.0, and a regression model containing linear, quadratic, and interaction effects was built for each. For this purpose, a standard least square method was followed, assigning all responses as y-variables in the software but generating individual outputs. The estimated coefficients are presented for each model to determine the significance of the effect of each term in their respective response, using a significance level of 0.05.

Additionally, a profiler combining all responses was used for the optimization. The optimum factor levels for each response were determined using the desirability function method [25], assigning a maximum theoretical desirability of 0.99 ($D_{0.99}$) and importance (I) to each variable, as follows. Ethanol, $D_{0.99}$ = 4.71% v/v and I = 0.1; galactose, $D_{0.99}$ = 72.73 g/L and I = 1; glucose, $D_{0.99}$ = 0 g/L and I = 1. Individual optimizations were

also explored using the same $D_{0.99}$ for each response but assigning the importance of 1 only to the response to be optimized and 0 to the others.

2.4.4. Validation

Experiments applying the optimized fermentation parameters were conducted in biological triplicates to verify the results predicted by the RSM model. New, sterile solutions of MP (15% TS) and starters of *B. claussenii* were prepared for each replicate. The fermentations were carried out at volumes of (i) 225 mL, (ii) 125 mL, and (iii) 75 mL; this last one served as a negative control. A 10 mL suspension of *B. claussenii* in MP was prepared for bottles (i) and (ii) to target an inoculation level of 7.6 log cfu/mL. During the inoculation, Nola Fit 5500 was added to each bottle to reach an E/S ratio of 100 IU/g lactose. Bottle (iii) received only sterile MP. The 3 bottles were capped with sterile stoppers and sanitized airlocks and were incubated for 33 days at 28 °C.

To track the changes in pH, sugar concentrations, and ethanol over time, samples were drawn from bottle (i) regularly throughout the 33 day incubation period. Bottle (ii) was sampled only on days 0 and 33 and used as a reference that used the same conditions of the RSM runs. Bottle (iii) was sampled and plated on standard plate count agar on the last day of the fermentation to assess any potential microbial contamination. The pH was measured as described previously, and the sugar concentrations and ethanol were determined as stated in Section 2.4.3.

2.5. Application

The optimized and validated fermentation was scaled up to 18 L batches to investigate its potential in combination with other food processes to develop value-added products from MP. All 18 L fermentations and the following processes were conducted in biological triplicates. All chemical analyses, including those of the dried products, were performed as reported by Rivera Flores, et al. [24].

2.5.1. Eighteen Liter Fermentations

Fresh MP was obtained for each replicate. Upon receiving, 2 L from this substrate was set apart, adjusted to 15%TS, and filter-sterilized to be used in the final step of the yeast propagation. The remaining MP was pasteurized at 70 °C for 30 min in a 35 L BrewZilla All In One Brewery with Pump Generation 3.1 (KegLand Distribution PTY LTD, Noble Park, VA, Australia) and stored under refrigeration until use.

The starter of *B. claussenii* was prepared under the same protocol described in Section 2.2. Following the second incubation in DME, which was performed in 50 mL, an appropriate volume of this starter was used to proceed with the propagation in MP. For this purpose, the starter was divided into three centrifuge tubes, each containing approximately 1.7×10^8 cfus. Upon a 2 min centrifugation at $3220\times g$, the DME solution was discarded, and the yeast pellet was used to inoculate three 600 mL flasks containing the sterile MP solution. This solution had previously undergone lactose hydrolysis for 2 h at 30 °C using Nola Fit 5500, at a level of 100 IU per gram of lactose. The inoculated flasks were incubated for 4 days at 30 °C and 200 rpm.

On the day of the fermentation, the portion of MP stored under refrigeration was adjusted to 15%TS with sterile Milli-Q water and pasteurized a second time at 70 °C for 30 min before inoculation. After cooling down to 30 °C, it was transferred into a sanitized 5 gallon plastic bucket. During this transfer, proper volumes of the starter of *B. claussenii* and Nola Fit 5500 were also added to the bucket to reach an inoculation rate of 7.6 log cfu/mL and E/S ratio of 100 IU/g lactose. After the total volume was brought to 18 L, the bucket was sealed with a lid with 4 airlocks to prevent the accumulation of gas pressure inside the bucket. The fermentation of MP underwent at 28 °C for 33 days in a Forma™ Environmental Chamber (Thermo Scientific, Waltham, MA, USA). Samples for both the unfermented MP and the fermented product were taken to analyze density, total solids, pH, turbidity, color, sugars, alcohols, organic acids, vitamins, and minerals.

2.5.2. Distillation

Upon completion of the fermentation, the product was subject to a 2-stage distillation in a 50 L Neocision ETL Lab Certified Rotary Evaporator (BVV, Naperville, IL, USA). For this purpose, approximately 16.5 L of fermented MP were transferred into the rotary flask, avoiding the yeast layer on the bottom of the fermentation bucket. The first stage of the distillation was performed for 40 min using a water bath temperature of 48 ± 1 °C and a rotation speed of 40 rpm. Additionally, the vacuum pressure was monitored throughout the process, reaching values between 29 and 27 inHg (98.2–91.4 kPa). After this, the distillate in the receiving flask was used as the feed for the second stage, which took place for 15 min under the same temperature, rotation, and pressure conditions. Samples of the first distillation bottoms were taken for the same analyses as the fermented product, and samples of the second distillate were taken to assess density, pH, turbidity, color, alcohols, and organic acids. In addition, 2.5 L of the distillation bottoms was collected for freeze-drying.

2.5.3. Freeze-Drying

Individual bottles containing the bottoms of the three distillation replicates were taken to the Cornell Food Venture Center Pilot Plant (Geneva, NY, USA) to be freeze-dried using a Harvest Right HR7000-L Freeze Dryer (Harvest Right LLC, Salt Lake, UT, USA). To achieve this, five polypropylene plastic containers (19 × 11 cm) were prepared with 300 mL of sample from each bottle; the depth of the liquid did not exceed 1 cm. Drying took place in four stages: (i) freezing at −30 °C for 9 h, (ii) conditioning until reaching 500 mTorr (66.7 kPa), (iii) drying at 52 °C for 46 h at a pressure of 66.4–80.4 kPa, and (iv) hold at 4 °C until the process was stopped. After the completion of this protocol, we noticed incomplete water removal in the samples. For this reason, the drying process was carried out a second time under the same conditions to ensure the collection of a low-moisture powder. Once the powders were obtained, they were analyzed for their moisture, water activity, color, hygroscopicity, and composition of sugars, protein, fat, vitamins, and minerals.

2.5.4. Statistical Analysis

All experiments were performed in biological replicates. They were set up on different days, using a new batch of MP and a new starter every time. Data presented in the tables include the mean ± standard deviation of all biological replicates obtained using JMP.

3. Results and Discussion

3.1. Preliminary Factor Assessment

The existing literature discusses the Custers effect exhibited by *B. bruxellensis*, where the fermentation of glucose to ethanol is blocked in conditions of full anaerobiosis and stimulated in the presence of oxygen [18]. This prompted questions about the influence of initial DO on the final galactose, glucose, and ethanol that could be obtained through the fermentation of dairy coproducts with *B. claussenii*. Earlier studies performed in WP by the present authors suggested that vigorous agitation before fermentation led to a faster conversion of glucose into ethanol but reduced the galactose yield. Thus, the following assessment aimed to investigate the effect of initial DO, time, and their interaction on the targeted products during MP fermentation to inform the forthcoming optimization.

Table 3 shows the results of the 2 × 2 factorial design. While time notably impacted the concentrations of galactose, ethanol, and glucose, initial DO at levels of 50% and 100% yielded almost the same amount of all 3 products. This indicated that under the conditions studied, the effect of initial DO was insignificant, while time was critical for all responses.

Table 3. Results of the preliminary factor assessment that investigated the effect of initial dissolved oxygen and time on galactose, ethanol, and glucose concentrations by fermenting milk permeate (15%TS) with *B. claussenii* OYL-201.

Initial Dissolved Oxygen (% Saturation)	Time (Days)	Galactose (g/L)	Ethanol (% *v*/*v*)	Glucose (g/L)
50	4	75.93 ± 2.51	0.50 ± 0.00	66.20 ± 1.22
50	40	65.70 ± 5.70	2.00 ± 0.20	31.73 ± 2.30
75 *	22	65.30 ± 4.10	1.65 ± 0.21	38.25 ± 0.35
100	4	76.77 ± 4.20	0.53 ± 0.06	66.27 ± 2.40
100	40	63.00 ± 3.210	2.07 ± 0.15	28.37 ± 0.85

Results are expressed as mean ± standard deviation for $n = 3$. * One outlier was excluded from the center point, resulting in $n = 2$.

A formal statistical analysis of the results supported these observations. Table 4 presents the significance levels in the form of p values for both initial DO and time and their interaction on these three responses. It can be noted that, aside from the intercept term, the only significant factor was time ($p < 0.001$).

Table 4. Significance, presented as p values, of the effect of different factors on the production of galactose and ethanol, and consumption of glucose from milk permeate using *B. claussenii* OYL-201. Results were obtained through a factorial experiment, analyzed via ANOVA and a *t*-test for each effect, using a significance level of 0.05.

Factors and Interactions	Significance to the Response (p Value)		
	Galactose	Ethanol	Glucose
Intercept	<0.0001	<0.0001	<0.0001
Initial Dissolved Oxygen	0.7213	0.6813	0.5315
Time	0.0008	<0.0001	<0.0001
Initial Dissolved Oxygen × Time	0.5031	0.8907	0.5154

Another observation was that galactose was successfully recovered at percentages greater than 83% of its theoretical value. However, the remaining glucose was also notably high, even after 40 days of incubation.

Based on the findings described, the optimization experiments presented in the next section did not include initial DO as a factor and included a range of higher inoculation levels to promote a more rapid glucose consumption in the already defined range of time.

3.2. Optimization

3.2.1. Overview of the Results

Table 5 presents the results of the multiple combinations of temperature, inoculation level, and time specified in the RSM design table. The experiment aimed to assess the effect of these factors on galactose, ethanol, and glucose and to optimize each of their outputs.

Galactose showed excellent stability throughout the fermentation, ranging from 61 to 67 g/L. These results are satisfactory as they demonstrate that the galactose that can be retained in fermented MP is reliable, an advantageous trait in processes aimed at recovering this sugar employing microbial fermentation. The galactose concentration results in this experiment were notably higher than those obtained in WP, presented by our previous study [24], in which they varied significantly across combinations.

On the other hand, the results for ethanol and glucose showed more variability amongst groups of runs, suggesting a greater dependence on the factors tested. The maximum amount of ethanol obtained was 3.8 ± 0.3% v/v (group 3) in runs where almost all glucose was depleted (final concentration: 0.18 ± 0.02 g/L). Regarding the groups of runs exhibiting low leftover glucose (2, 5, and 11), which resulted in ≤0.05 g/L glucose,

it is essential to highlight that they were all incubated for 40 days. This duration can undoubtedly be improved to reduce the processing time.

Table 5. Amounts of galactose, ethanol, and glucose obtained upon fermentation of milk permeate at a concentration of 15%TS with *B. claussenii* OYL-201. Each group of runs represents a different combination of the multiple fermentation factors selected for the response surface model. A total of 56 individual fermentations were run.

Group of Runs	Temperature (°C)	Inoculation Level (log cfu/mL)	Time (Days)	Galactose (g/L)	Ethanol (% *v*/*v*)	Glucose (g/L)
1	25	7.00	4	65.82 ± 3.71	0.61 ± 0.05	54.07 ± 3.12
2	25	7.00	40	63.22 ± 1.47	3.71 ± 0.12	0.05 ± 0.02
3	25	7.75	22	64.48 ± 0.25	3.84 ± 0.31	0.18 ± 0.02
4	25	8.50	4	65.69 ± 5.99	1.10 ± 0.05	46.53 ± 4.40
5	25	8.50	40	60.64 ± 1.75	3.82 ± 0.21	0.05 ± 0.00
6	30	7.00	22	64.84 ± 0.55	3.33 ± 0.27	9.84 ± 1.93
7	30	7.75	4	65.17 ± 6.24	0.95 ± 0.16	46.57 ± 5.27
8 *	30	7.75	13	66.54 ± 1.27	3.60 ± 0.05	6.62 ± 0.54
9 ♦	30	7.75	22	64.07 ± 0.52	3.76 ± 0.30	0.09 ± 0.01
10 *	30	7.75	31	62.87 ± 1.74	3.55 ± 0.20	0.08 ± 0.00
11	30	7.75	40	64.36 ± 1.55	3.78 ± 0.06	0.00 ± 0.00
12	30	8.50	22	60.44 ± 1.17	3.65 ± 0.25	0.09 ± 0.01
13	35	7.00	4	65.36 ± 6.26	0.41 ± 0.04	56.50 ± 5.54
14	35	7.00	40	66.02 ± 0.58	1.25 ± 0.05	42.97 ± 1.82
15	35	7.75	22	63.82 ± 0.73	1.93 ± 0.19	27.33 ± 1.47
16	35	8.50	4	65.69 ± 1.48	1.47 ± 0.04	40.23 ± 2.85
17	35	8.50	40	65.31 ± 1.58	2.75 ± 0.14	8.30 ± 1.83

Results are expressed as mean ± standard deviation for $n = 3$, * $n = 4$, ♦ $n = 6$.

Different from the preliminary runs, the tested inoculation levels in these experiments successfully enabled a complete consumption of glucose in some runs (group 11). Inputting results that exhibit no leftover glucose in the RSM model helped improve the predictions of the final glucose concentration. The following sections will discuss the main effects of the factors assessed on the individual models generated for glucose, ethanol, and galactose.

3.2.2. Models for Final Glucose and Ethanol

Due to the innate inverse relationship between glucose and ethanol, which was also evidenced in the construction of their models, these results are presented together. Tables 6 and 7 show the estimated coefficient of each term of the empirical models for final glucose and ethanol, respectively. Based on the individual *t*-tests performed on the terms associated with each factor, all proved significant to both responses. In addition, all terms that were significant for glucose were also significant for ethanol.

Among the terms for predicting final glucose, the intercept proved insignificant ($p = 0.23$). This result is unsurprising given that the final glucose seen on the center point runs showed, on average, <0.1 g/L (Table 5). On the contrary, the estimated intercept of the ethanol model was significant ($p < 0.0001$), which is explained by the fact that the model's response when running all factors at their respective center points is expected to be 3.67% *v*/*v*.

The primary interpretation of the linear effects in a similar model constructed for WP fermentation was described in more detail in our previous study [24]. The focus of the following discussion centers around the quadratic and interaction effects. The significance of the quadratic terms of an optimization model is arguably the most important to determine whether the experimental region included the vertex of the curve or the place where a local maximization or minimization is possible. All but inoculation levels exhibited statistically significant quadratic terms for both final glucose and ethanol. These results suggest that inoculation level only had linear effects in the range studied; Figure S1 in the Supplemental Materials provides a visual representation of the trends observed

for each factor. The results also suggest that the inoculation levels could be higher to potentially obtain more ethanol; however, given that the maximum inoculation tested in the present study was 8.5 log cfu/mL, concentrations above this level would be impractical in traditional fermentation processes.

Table 6. Estimated coefficients, standard errors, and significance of the terms included in the model for predicting final glucose constructed with the data obtained via response surface methodology. Fermentations were carried out in milk permeate (15%TS) by *B. claussenii* OYL-201.

Term	Coefficient	Standard Error	*p* Value	Significance
Intercept	1.5187	1.2474	0.2296	
Linear				
Temperature	7.4453	1.0073	<0.0001	***
Inoculation level	−6.8213	1.0073	<0.0001	***
Time	−18.4584	0.9753	<0.0001	***
Interaction				
Temperature × Inoculation level	−5.4246	1.1262	<0.0001	***
Temperature × Time	6.8788	1.1262	<0.0001	***
Inoculation level × Time	−1.3588	1.1262	0.2338	
Quadratic				
Temperature × Temperature	10.1036	1.9004	<0.0001	***
Inoculation level × Inoculation level	1.3136	1.90043	0.4929	
Time × Time	18.6852	1.9615	<0.0001	***

Asterisks denote significance *** $p < 0.001$.

Table 7. Estimated coefficients, standard errors, and significance of the terms included in the model for predicting ethanol concentration constructed with the data obtained via response surface methodology. Fermentations were carried out in milk permeate (15% TS) by *B. claussenii* OYL-201.

Term	Coefficient	Standard Error	*p* Value	Significance
Intercept	3.6732	0.0899	<0.0001	***
Linear				
Temperature	−0.5267	0.0726	<0.0001	***
Inoculation level	0.3477	0.0726	<0.0001	***
Time	1.0063	0.0703	<0.0001	***
Interaction				
Temperature × Inoculation level	0.2438	0.0811	0.0043	**
Temperature × Time	−0.4638	0.0811	<0.0001	***
Inoculation level × Time	0.0071	0.0811	0.9308	
Quadratic				
Temperature × Temperature	−0.6574	0.1369	<0.0001	***
Inoculation level × Inoculation level	−0.0457	0.1369	0.7400	
Time × Time	−1.1124	0.1413	<0.0001	***

Asterisks denote significance *** $p < 0.001$ and ** $p < 0.01$.

Regarding interactions, only two were deemed significant for both responses (Tables 6 and 7).

Temperature × Inoculation Level. At 25 °C, both low and high inoculation levels had similar final glucose and ethanol concentrations; at 35 °C, a higher inoculation level resulted in less leftover glucose and more ethanol generated. Even so, the amount of glucose transformed into ethanol at 25 °C was overall higher than at 35 °C. This general trend where lower temperature settings resulted in the production of more ethanol was observed in our previous study [24].

Temperature × Time. Four days into the fermentation, both high and low temperatures had comparable glucose and ethanol levels. However, as time progressed, we observed a clear difference between 25 °C and 35 °C, with the former exhibiting a better performance in ethanol production and glucose consumption. Previous optimization studies on alcoholic fermentation processes reported optimal temperatures between 25 °C and 29 °C [26,27]. Thus, 35 °C was likely too high to support the metabolism of this strain of *B. claussenii*.

Both models were shown to be an excellent fit for the observed values. The root mean square error (RMSE) of the model for final glucose was 5.5172 g/L, its r-squared was 0.9463,

and its adjusted r-squared was 0.9358. For ethanol, the obtained RMSE was 0.3974% v/v, its r-squared was 0.9198, and its adjusted r-squared was 0.9041. These metrics indicate that almost 95% of the variability seen in the results of final glucose and 92% of the variability in ethanol can be explained by their respective models, demonstrating good predictive power. Additionally, both RMSE results indicated that the difference between the predicted values versus measured results exhibited minor variations.

3.2.3. Model for Galactose

The significance of the terms in the galactose model is presented in Table 8. According to the results, only two terms were considered significant in the retention of galactose in MP, intercept, and time. Consequently, all terms associated with temperature and inoculation level were deemed insignificant to final galactose.

Table 8. Estimated coefficients, standard errors, and significance of the terms included in the model for the prediction of final galactose constructed with the data obtained via response surface methodology. Fermentations were carried out in milk permeate (15% TS) by *B. claussenii* OYL-201.

Term	Coefficient	Standard Error	*p* Value	Significance
Intercept	63.9488	0.6189	<0.0001	***
Linear				
Temperature	0.6360	0.4997	0.2095	
Inoculation level	−0.7483	0.4997	0.1411	
Time	−0.9963	0.4839	0.0452	*
Interaction				
Temperature × Inoculation level	0.2908	0.5587	0.6052	
Temperature × Time	0.9917	0.5587	0.0825	
Inoculation level × Time	−0.4367	0.5587	0.4385	
Quadratic				
Temperature × Temperature	0.4851	0.9429	0.6094	
Inoculation level × Inoculation level	−1.0299	0.9429	0.2804	
Time × Time	1.2451	0.9731	0.2072	

Asterisks denote significance *** $p < 0.001$ and * $p < 0.05$.

The p-value associated with time ($p = 0.0452$) was close to our defined significant level of 0.05 and corroborated the stability of this sugar during the fermentation. The intercept of this model was another term considered significant ($p < 0.0001$), and its coefficient was estimated to be 63.9 g/L.

Due to the lack of significance of 8 out of 10 terms included in the model, its r-squared was 0.2430, with an adjusted r-squared of 0.0949. Despite being low, these results are favorable from a process development perspective. They show that producers can modify their fermentations' temperatures and inoculation levels without significantly impacting galactose recovery. In addition, the resulting RMSE of the model was 2.7372 g/L, representing less than 4.3% of the mean response (64.3684 g/L), demonstrating good precision of the predictions.

The empirical models from the RSM experiments allowed us to construct continuous surfaces for each response studied. Figure S2 presents these surfaces for all possible two-way factor interactions.

3.2.4. Optimization and Validation

The three models were optimized simultaneously to obtain the combination of fermentation parameters that would yield the highest galactose and lowest glucose while maximizing ethanol production, this latter response in second order of priority. For this reason, the importance (I) of each was set to 1, 1, and 0.1, respectively. Table 9 shows the results of this combined optimization. Individual optimizations were also included to present how the process parameters would change if each response were pursued alone.

Table 9. Individual and combined optimization of final galactose, glucose, and ethanol during the fermentation of milk permeate with *B. claussenii*. Values presented in brackets correspond to the 95% confidence intervals of the predicted responses.

Factor	Galactose [64.5–69.7] (g/L)	Glucose [−14.2–−5.4] (g/L)	Ethanol [4.0–4.6] (% *v*/*v*)	Combined		
				Galactose [62.3–65.0] (g/L)	Glucose [−6.9–−1.5] (g/L)	Ethanol [3.8–4.2] (% *v*/*v*)
Temperature (°C)	25.0	28.1	28.1		28.0	
Inoculation Level (log cfu/mL)	7.5	8.5	8.5		7.6	
Time (days)	4.0	31.0	31.0		33.3	

The combined optimization resulted in the following parameters: temperature, 28 °C; inoculation level, 7.6 log cfu/mL; and time, 33 days. Together they were predicted to retain 63.6 g/L of galactose, leave no remaining glucose and produce 4.0%*v*/*v* of ethanol from MP. These results achieve a combined desirability of 93%, based on the theoretical values expected for all responses.

Following this optimization, the suggested levels of each factor were tested to determine the validity of the predictions made by the models. These results are presented in Figure 1. Galactose, glucose, and ethanol fell within the limits of their confidence intervals (dotted lines) throughout the fermentation. The predictions are presented starting on day 4, as the models did not include days 1–3 of the process.

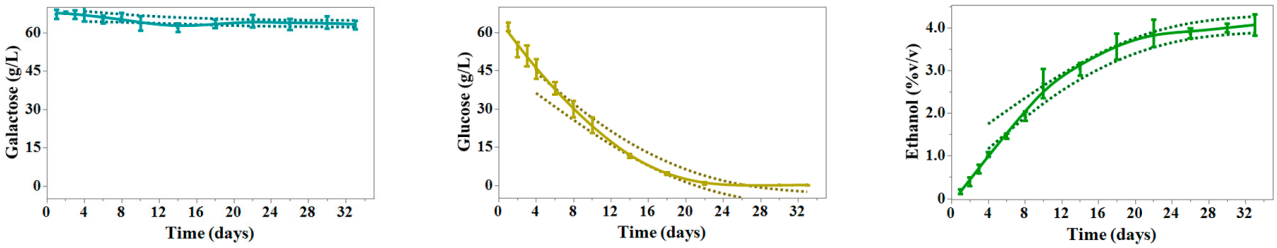

Figure 1. Concentration of galactose, glucose, and ethanol obtained during the fermentation of milk permeate (15%TS) with *B. claussenii* OYL-201. The process was carried out using the parameters recommended for the combined optimization of all responses (Table 9). Data are presented as means ± standard deviations of 3 biological replicates. The dotted lines correspond to the 95% confidence intervals of the predictions made for each response starting from day 4.

In this figure, galactose concentration dropped minimally compared to its result immediately following hydrolysis (~67.2 g/L), illustrating its stability throughout the fermentation; the final galactose concentration was 63.2 ± 1.7 g/L. On the other hand, glucose was utilized at an exponential rate, reaching values as low as 0.37 g/L on day 22; and with a final glucose concentration of 0.08 ± 0.01 g/L. Lastly, ethanol also agreed with its predictions; the final concentration achieved was 4.07 ± 0.25% *v*/*v*.

These results show that the designed RSM successfully modeled the effects of relevant process variables on the fermentation of MP with *B. claussenii* OYL-201. It also optimized and predicted the final product's galactose, glucose, and ethanol concentrations. A link to an interactive profiler showing the predictions for each of these products is publicly available at https://hdl.handle.net/1813/113351 (accessed on 5 August 2023), alongside MP's pH profile during this process (Figure S3). We believe that these estimations can serve as a reference for the expected range of fermentation products. However, the use of commercial laboratory bioreactors could provide conditions that better reflect those of industrial fermentation processes, which may include variables not accounted for in the presented study.

3.3. Applications

3.3.1. Milk Permeate Fermentate

Following the validation of the predictions, the optimized fermentations were scaled up to 18 L batches. After each, the fermented product was subjected to a two-stage distillation. Table 10 shows various quality attributes of the products resulting from these processes.

Table 10. Quality attributes of three product streams obtained upon fermenting milk permeate (MP) with *B. claussenii* OYL-201. The fermentation settings corresponded to the optimized conditions presented in Table 9. The results are shown as means ± standard deviations of three biological replicates. "MP solution": product pre-fermentation, "Fermented product": product post-fermentation, "Galactose-rich drink base": bottoms post-distillation, and "Distillate": distilled product.

Analysis	Unit	MP Solution	Fermented Product	Galactose-Rich Drink Base	Distillate
Physicochemical					
Density	g/mL	1.06 ± 0.00	1.03 ± 0.00	1.04 ± 0.00	0.93 ± 0.01
Total solids	% *w*/*w*	15.27 ± 0.07	9.60 ± 0.25	10.51 ± 0.12	-
pH		5.90 ± 0.05	4.92 ± 0.23	4.92 ± 0.26	4.94 ± 0.31
Turbidity	FTU	103.45 ± 38.91	33.34 ± 16.1	46.65 ± 17.35	0.47 ± 0.29
Color (Total transmittance)					
Lightness (L*)		84.56 ± 2.73	89.99 ± 2.27	88.32 ± 3.27	96.17 ± 0.06
Red/Green (a*)		−1.10 ± 1.22	−2.61 ± 0.43	−2.65 ± 0.46	−0.84 ± 0.02
Yellow/Blue (b*)		13.39 ± 2.65	13.77 ± 0.41	15.11 ± 0.81	−0.86 ± 0.02
Color (Regular transmittance)					
Lightness (L*)		84.09 ± 2.84	89.62 ± 2.27	88.03 ± 3.23	95.81 ± 0.06
Red/Green (a*)		−1.07 ± 1.22	−2.57 ± 0.45	−2.62 ± 0.47	−0.8 ± 0.02
Yellow/Blue (b*)		13.57 ± 2.66	13.97 ± 0.41	15.35 ± 0.81	−0.64 ± 0.03
Sugars					
Galactose	g/L	0.17 ± 0.00	65.6 ± 1.45	71.47 ± 2.64	-
Glucose	g/L	0.29 ± 0.02	0.16 ± 0.19	0.16 ± 0.20	-
Lactose	g/L	130.96 ± 2.05	0.00 ± 0.00	0.00 ± 0.00	-
Alcohols					
Ethanol	% *v*/*v*	0.00 ± 0.00	3.97 ± 0.08	2.24 ± 0.33	48.37 ± 3.82
Methanol	% *v*/*v*	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Organic Acids					
Acetic Acid	g/L	0.01 ± 0.01	0.79 ± 0.25	0.86 ± 0.26	0.03 ± 0.01
Lactic Acid	g/L	0.00 ± 0.01	1.00 ± 0.88	1.17 ± 0.92	0.10 ± 0.18

FTU: Formazin Turbidity Units.

None of the 3 responses differ significantly from their respective predictions. The average ethanol produced fell within its predicted interval (4.0 ± 0.1% *v*/*v*), galactose resulted in values on the higher end of its prediction (65.6 ± 1.5 g/L), and glucose exhibited some variability between replicates (0.16 ± 0.19 g/L). Regarding lactose, none was detected at the end of the fermentation. These findings suggest good scalability of the optimized process. Final galactose was over 97% of its theoretical value, calculated from the lactose concentration seen in the MP solution.

Regarding pH, we observed a lower mean compared to the one seen in the validation (5.08 ± 0.03). One plausible scenario for this discrepancy is the presence of lactic-acid-producing bacteria in the unfermented MP. Given the volume of this substrate, it was pasteurized before the fermentation, as opposed to filter-sterilized, as was performed for previous experiments. Lactic acid at approximately 1 g/L in the fermented product suggests that lactic-acid-producing bacteria endured pasteurization. Fritze and Claus [28] described several *Bacillus* and *Sporolactobacillus* species capable of producing lactic acid and forming spores, which would facilitate their survival during heat treatments.

There is also no indication that *B. claussenii* produced lactic acid during the fermentation. A previous study showed that this strain did not produce this acid during the

metabolism of different sugars in synthetic media [22]; in fact, it seemed to consume it. This potential intake of lactic acid by *B. claussenii* could explain the higher concentration of galactose and the variability in final glucose at the end of the fermentation.

Another quality attribute assessed before and after the fermentation was turbidity. Turbidity is a critical factor known to determine the quality of beverages; clear beverages are generally perceived as light and refreshing [29]. Our results showed notable variability of this measurement among replicates in the different stages of the process. The value observed in the MP fermentate was 33.6 ± 16.1 Formazin Turbidity Units (FTU), which can be compared to "brilliant" to "almost brilliant" beers based on the formally established beer clarity classification [30]. Commercially available clarifying agents and filtration processes can further reduce the value of this parameter for the development of beverages.

Table 11 shows the products' concentration of vitamins and minerals. While most nutrients remained unchanged after the fermentation and distillation processes, we observed a reduction in the content of niacin, thiamin, and calcium after the fermentation. All three nutrients are known to play a role in yeast metabolism during their growth and fermentation [31–33]. Niacin requirements are bound to the combination of pathways used for NAD biosynthesis [31], extracellular thiamin has been recognized as a requirement for yeast of the *Brettanomyces* genus [32], and calcium ions were concluded to play a direct and specific part in yeast flocculation [33].

Table 11. Composition of vitamins and minerals in the products obtained through the anaerobic fermentation of MP by *B. claussenii* OYL-201, followed by concentration via vacuum distillation.

Attribute	MP Solution	Fermented Product	Galactose-Rich Drink Base
Vitamins (mg/100 g)			
Niacin	0.12 ± 0.05 [1]	<0.0161	<0.0163 [2]
Pantothenic Acid	0.99 ± 0.09	1.09 ± 0.06	1.18 ± 0.14
Riboflavin	0.24 ± 0.01	0.21 ± 0.01	0.23 ± 0.01
Thiamin	0.04 ± 0.01	<0.00232 [4]	0.003 ± 0.001 [5]
Minerals (mg/100 g)			
Calcium	97.37 ± 6.37	73.47 ± 18.59	80.63 ± 20.40
Copper	<0.00996	<0.00498	<0.00500
Iron	<0.0996	<0.0498	<0.0500
Magnesium	19.13 ± 0.21	19.70 ± 0.26	21.63 ± 0.38
Manganese	<0.00498	<0.00249	<0.00250
Potassium	269.67 ± 4.16	285.00 ± 2.00	309.67 ± 9.50
Phosphorus	119.33 ± 3.79	120.33 ± 3.06	131.67 ± 4.04
Sodium	61.33 ± 1.99	65.53 ± 1.10	72.43 ± 3.06
Zinc	<0.0199	<0.00995	<0.0100 [3]

[1] $n = 2$, one replicate resulted in <0.0163 mg/100 g; [2] $n = 2$, one replicate resulted in 0.0165 mg/100 g; [3] $n = 2$, one replicate resulted in 0.0109 mg/100 g; [4] $n = 2$, one replicate resulted in 0.00284 mg/100 g; [5] $n = 2$, one replicate resulted in <0.00235 mg/100 g.

Previous studies have highlighted the potential of MP to be used as the substrate for functional and electrolyte beverages due to its high vitamin and mineral content [11–14]. Our results suggest that the fermented product and the galactose-rich drink base obtained in the present study could also serve as the substrate for the development of beverages exhibiting the same benefits regarding vitamin and mineral content, in addition to the potential functionality of galactose as a prebiotic precursor [34].

3.3.2. Lactose-Free, Galactose-Rich Drink Base, and Dairy-Based Distillate

The distillation process separated the fermented MP into two streams: a concentrated fermented product with lower alcohol content, potentially suitable as a lactose-free, galactose-rich drink base, and a dairy-based distillate.

Combining the optimized fermentation with this separation process aimed to assess if extracting the ethanol from the fermented MP using vacuum distillation could result in

another nutritionally attractive product stream. Our results show that all the components studied were concentrated during the distillation, including vitamins, indicating that these can still be present upon ethanol separation.

The obtained galactose-rich drink base exhibited, on average, 71.5 g/L galactose, representing approximately 17 g of this sugar per 240 mL serving. Given this amount, adding other ingredients, such as fruit juices or other substances, to formulate a commercial product would not undermine the presence and potential benefits of this monosaccharide.

One important point is that the distillation parameters proposed in this study resulted in a final ethanol concentration of 2.2 ± 0.3% v/v in the drink base. While this level of ethanol did not achieve the standards necessary for non-alcoholic beverages according to the FDA, further research involving the optimization of this process can minimize this remaining alcohol to be suitable for such products [35].

Another valuable stream resulting from the distillation was the dairy-based distillate. Upon completion of the distillation's first stage, the ethanol concentration of the obtained solution was 22.4 ± 2.1% v/v, which increased to 48.4 ± 3.8% v/v at the end of the process. Dairy-based distillates have been investigated for years for their volatile compounds profile, as well as for the reduced carbon emissions and water use associated with their production [36–40]. Their manufacture results in aroma profiles mainly comprised of higher alcohols, esters, and acetaldehydes, with an overall good organoleptic character [36,38]. Additionally, Risner, et al. [40] concluded that the conversion of cheese whey to a distilled product is more environmentally responsible compared to the production of white whiskey or whey disposal through landfilling, which emphasized another advantage of producing ethanol from dairy coproducts. No studies have been published on distillates obtained through the fermentation of MP streams.

3.3.3. Galactose-Rich Powder

Finally, we explored the potential of MP fermentation with *B. claussenii* to produce a galactose-rich food powder with applications in lactose-free, low glycemic index foods. This product was made from the galactose-rich drink base described in the previous section, and some of its quality attributes are presented in Table 12.

Regarding its macronutrient composition, the total sugars were approximately 66.4%, the protein content was 3.7%, and the total fat was <0.7%. A more detailed analysis of the sugars confirmed that the concentration obtained was almost all galactose, as there were no detectable levels of lactose, glucose, fructose, sucrose, or maltose. The result seen in protein content was most likely due to the presence of whey proteins. Previous studies have shown that the protein profile of MP obtained via ultrafiltration mainly consists of α-lactalbumin and β-lactoglobulin in variable ratios [10,41,42]. α-lactalbumin has been identified as an important source of bioactive peptides and essential amino acids [43]. Further characterizing the profile of these macromolecules will allow producers to take full advantage of this product's composition.

Regarding its micronutrient composition in the form of B vitamins and selected minerals, as seen previously, significant amounts of pantothenic acid, riboflavin, potassium, and phosphorous were found. Pantothenic acid plays a vital role in the synthesis of coenzyme A, cholesterol, and fatty acids; at the same time, riboflavin contributes to heme protein synthesis, the metabolism of macromolecules, and exhibits antioxidant activity [44]. Similarly, potassium and phosphorous contribute to human metabolism. Potassium is needed for nerve transmission, muscle contraction, and blood pressure regulation; phosphorous, for protein synthesis in cells and tissues and for keeping healthy bones, among other functions [45].

Regarding its physicochemical characteristics, the powder color resulted in L* = 89.8, a* = −1.2, and b = 18.5. The L* value suggests that the obtained product is lighter than previously reported dry MP (L* = 67.3) [46]. Nonetheless, this color parameter is comparable to the results of Rivera Flores, et al. [24] for the powder resulting from fermented WP using a

similar approach (L* = 90.4). The a* and b* values were within the range seen in commercial dairy powders [46].

Table 12. Physicochemical and nutritional characteristics of the freeze-dried powder made from fermented and concentrated milk permeate. The results represent the mean ± standard deviation for $n = 3$.

Attribute	Result
Moisture (%)	0.67 ± 0.06
Water activity	0.13 ± 0.01
Lactose (%)	<0.1
Glucose (%)	<0.1
Galactose (%)	66.37 ± 1.50
Total Sugar (%)	66.40 ± 1.55
Protein (%)	3.69 ± 0.03
Fat (%)	0.60 ± 0.10
Color (Reflectance Specular Included)	
Lightness (L*)	89.75 ± 0.37
Red/Green (a*)	−1.19 ± 0.36
Yellow/Blue (b*)	18.48 ± 0.67
Hygroscopicity at 75%RH (%)	16.53 ± 1.55
Vitamins (mg/100 g)	
Niacin	<0.167
Pantothenic Acid	10.66 ± 0.99
Riboflavin	1.57 ± 0.13
Thiamin ♦	0.04 ± 0.01
Minerals (mg/100 g)	
Calcium	773.67 ± 146.51
Copper	0.04 ± 0.01
Iron	<0.248
Magnesium	206.67 ± 0.58
Manganese	<0.0124
Phosphorus	1260.00 ± 17.32
Potassium	3090.00 ± 52.92
Sodium	705.00 ± 54.74
Zinc	0.07 ± 0.01

♦ $n = 2$, one replicate resulted in <0.0231 mg/100 g.

Figure 2 visually represents the product streams described in this section.

Figure 2. Appearance of the unfermented 15%TS milk permeate and the four product streams derived from the proposed approach. (**a**) Liquid products: MP solution, fermented MP, galactose-rich drink base, and distillate. (**b**) Freeze-dried galactose-rich powder.

Another important observation was the hygroscopicity exhibited by this product. The results showed that the moisture content of the powder increased from 0.7% to 16.5% upon reaching equilibrium at a relative humidity of 75%. This final moisture was over 80% higher than that reported for ultrafiltered MP (~9.0%) [46]. A classification of food powders as a function of their hygroscopicity created by the same authors categorizes this product as a "hygroscopic powder." Other foods with similar hygroscopicity include 90% *w*/*w*

whey protein isolate (~15.4%), glucose syrup with a dextrose equivalent of 39 (~15.8%), and gelatin (~17.4%) [46]. Given this hygroscopicity, manufacturers exploring this product's applications should develop proper strategies for its handling and storage to prevent its degradation or contamination.

The characteristics presented above serve as a general description of some relevant quality attributes of food powders. We expect these results to help guide initiatives to utilize this product in the future. To the best of our knowledge, no other study has obtained a galactose-rich powder from MP via fermentation. A comparable product was obtained by Rivera Flores, et al. [24] in WP using a similar approach.

4. Conclusions

The present study had two principal objectives (i) optimize the anaerobic fermentation of MP by *B. claussenii* to minimize glucose and maximize the production of galactose and ethanol, and (ii) characterize multiple products that could be obtained by combining this process with other food technologies. We found that the optimal fermentation parameters were temperature, 28.0 °C; inoculation level, 7.6 log cfu/mL; and time, 33.3 days. This combination of factors, which was predicted to yield an average of 0 g/L glucose 63.6 g/L, galactose, and 4.0% v/v ethanol, was successfully validated at benchtop and pilot-plant scales. As part of our second objective, we separated the ethanol in the fermented product to produce a distilled spirit with an average of 48% v/v and a low-alcohol drink base (2% v/v) with substantial galactose (71 g/L). This latter product was also subjected to freeze-drying, after which we obtained a galactose-rich powder (66% w/w) with significant amounts of vitamins and minerals. With these strategies, we aimed to propose innovative applications of already used technologies in the food industry to valorize MP as the precursor of lactose-free, low-in-glucose, and galactose-rich foods that could be incorporated into the human food supply chain. Further studies should focus on the systematic design of the processes proposed, the development of prototypes for relevant sensory investigations, and the feasibility of this approach from multiple perspectives, among others.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090825/s1, Table S1: Design table for the optimization of galactose retention, residual glucose, and ethanol production by *B. claussenii* OYL-201; Figure S1: Individual effects of each fermentation parameter on the final concentrations of galactose, glucose, and ethanol; Figure S2. Response surfaces representing the predicted values for: (a) glucose; (b) ethanol; (c) galactose for all possible 2-way factor interactions; Figure S3. pH profile of the anaerobic fermentation of 15%TS milk permeate with *B. claussenii* OYL-201.

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References

1. Technavio Research. High Protein-Based Food Market—37% of Growth to Originate from North America | Evolving Opportunities with Abbott Laboratories & Clif Bar & Co. Available online: https://www.prnewswire.com/news-releases/high-protein-based-food-market{-}{-}37-of-growth-to-originate-from-north-america{-}{-}evolving-opportunities-with-abbott-laboratories{-}{-}clif-bar{-}{-}co{-}{-}technavio-301548949.html (accessed on 23 February 2023).
2. Bastian, E.D.; Collinge, S.K.; Ernstrom, C.A. Ultrafiltration: Partitioning of Milk Constituents into Permeate and Retentate. *J. Dairy Sci.* **1991**, *74*, 2423–2434. [CrossRef]
3. Coton, S.G. The utilization of permeates from the ultrafiltration of whey and skim milk. *Int. J. Dairy Technol.* **1980**, *33*, 89–94. [CrossRef]
4. Kappeli, O.; Halter, N.; Puhan, Z. Upgrading of milk ultrafiltration permeate by yeast fermentation. In *Advances in Biotechnology. Vol. II. Fuels, Chemicals, Foods and Waste Treatment*; Pergamon Press: Toronto, ON, Canada, 1981; pp. 351–356.
5. Hobman, P.G. Review of Processes and Products for Utilization of Lactose in Deproteinated Milk Serum. *J. Dairy Sci.* **1984**, *67*, 2630–2653. [CrossRef]
6. Talabardon, M.; Schwitzguébel, J.-P.; Péringer, P. Anaerobic thermophilic fermentation for acetic acid production from milk permeate. *J. Biotechnol.* **2000**, *76*, 83–92. [CrossRef]
7. Villamiel, M.; Corzo, N.; Foda, M.I.; Montes, F.; Olano, A.N. Lactulose formation catalysed by alkaline-substituted sepiolites in milk permeate. *Food Chem.* **2002**, *76*, 7–11. [CrossRef]
8. Meleigy, S.A.; Khalaf, M.A. Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant Fusarium moniliforme cells immobilized on loofa sponge. *Bioresour. Technol.* **2009**, *100*, 374–379. [CrossRef]
9. Idaho Milk Products. The Emerging Market for Milk Permeate Powder. Available online: https://www.idahomilkproducts.com/the-emerging-market-for-milk-permeate-powder/ (accessed on 23 February 2023).
10. Menchik, P.; Zuber, T.; Zuber, A.; Moraru, C.I. Short communication: Composition of coproduct streams from dairy processing: Acid whey and milk permeate. *J. Dairy Sci.* **2019**, *102*, 3978–3984. [CrossRef]
11. El-Khair, A.A.A. Formulation of Milk Permeate for Utilization as Electrolyte Beverages. *Aust. J. Basic Appl. Sci.* **2009**, *3*, 572–578.
12. Atallah, A.A. Development of new functional beverages from milk permeate using some probiotic bacteria and fruits pulp. *Egypt J. Dairy Sci.* **2015**, *43*, 25–39.
13. El-Shenawy, M.; Fouad, M.T.; Hassan, L.K.; Seleet, F.L.; El-Aziz, M.A. A Probiotic Beverage Made from Tiger-nut Extract and Milk Permeate. *Pak. J. Biol. Sci.* **2019**, *22*, 180–187. [CrossRef]
14. Berry, C.W.; Murray, B.; Kenney, W.L. Scientific basis for a milk permeate-based sports drink—A critical review. *Int. Dairy J.* **2022**, *127*, 105296. [CrossRef]
15. Zokaityte, E.; Cernauskas, D.; Klupsaite, D.; Lele, V.; Starkute, V.; Zavistanaviciute, P.; Ruzauskas, M.; Gruzauskas, R.; Juodeikiene, G.; Rocha, J.M.; et al. Bioconversion of Milk Permeate with Selected Lactic Acid Bacteria Strains and Apple By-Products into Beverages with Antimicrobial Properties and Enriched with Galactooligosaccharides. *Microorganisms* **2020**, *8*, 1182. [CrossRef] [PubMed]
16. Lawton, M. Biotechnological Approaches for Combating Food Waste in the Dairy Industry. Ph.D. Thesis, Cornell University, Ithaca, NY, USA, 2021.
17. Jencarelli, K.G. Proposed Methods to Valorize Dairy Effluents Via Aerobic Fermentation with The Yeast *Brettanomyces claussenii*. Master's Thesis, Cornell University, Ithaca, NY, USA, 2022.
18. Steensels, J.; Daenen, L.; Malcorps, P.; Derdelinckx, G.; Verachtert, H.; Verstrepen, K.J. *Brettanomyces* yeasts—From spoilage organisms to valuable contributors to industrial fermentations. *Int. J. Food Microbiol.* **2015**, *206*, 24–38. [CrossRef] [PubMed]
19. Colomer, M.S.; Funch, B.; Forster, J. The raise of *Brettanomyces* yeast species for beer production. *Curr. Opin. Biotechnol.* **2019**, *56*, 30–35. [CrossRef] [PubMed]
20. Colomer, M.S.; Chailyan, A.; Fennessy, R.T.; Olsson, K.F.; Johnsen, L.; Solodovnikova, N.; Forster, J. Assessing Population Diversity of *Brettanomyces* Yeast Species and Identification of Strains for Brewing Applications. *Front. Microbiol.* **2020**, *11*, 637. [CrossRef]
21. Lawton, M.R.; deRiancho, D.L.; Alcaine, S.D. Lactose utilization by *Brettanomyces claussenii* expands potential for valorization of dairy by-products to functional beverages through fermentation. *Curr. Opin. Food Sci.* **2021**, *42*, 93–101. [CrossRef]
22. Rivera Flores, V.K.; DeMarsh, T.A.; Gibney, P.A.; Alcaine, S.D. Fermentation of dairy-relevant sugars by *Saccharomyces*, *Kluyveromyces*, and *Brettanomyces*: An exploratory study with implications for the utilization of acid whey, Part I. *Fermentation* **2021**, *7*, 266. [CrossRef]
23. Rivera Flores, V.K.; Demarsh, T.A.; Gibney, P.A.; Alcaine, S.D. Fermentation of Dairy-Relevant Sugars by *Saccharomyces*, *Kluyveromyces*, and *Brettanomyces*: An Exploratory Study with Implications for the Utilization of Acid Whey, Part II. *Fermentation* **2022**, *8*, 257. [CrossRef]

24. Rivera Flores, V.K.; Timothy, A.; Fan, X.; Alcaine, S.D. Cheese whey permeate as a precursor of lactose-free, galactose-rich bioproducts: An approach for optimization and application. *Food Bioproc. Tech.* 2023, *preprint*. [CrossRef]
25. Myers, R.H. *Response Surface Methodology Process and Product Optimization Using Designed Experiments*, 3rd ed.; Wiley: Hoboken, NJ, USA, 2009.
26. Yah, C.; Iyuke, S.; Unuabonah, E.; Pillay, O.; Vishanta, C.; Tessa, S. Temperature Optimization for Bioethanol Production from Corn Cobs Using Mixed Yeast Strains. *Online J. Biol. Sci.* **2010**, *10*, 103–108. [CrossRef]
27. Boudjema, K.; Fazouane-Naimi, F.; Hellal, A. Optimization of the Bioethanol Production on Sweet Cheese Whey by *Saccharomyces cerevisiae* DIV13-Z087C0VS using Response Surface Methodology (RSM). *Rom. Biotechnol. Lett.* **2015**, *20*, 10814–10825.
28. Fritze, D.; Claus, D. Spore-forming, lactic acid producing bacteria of the genera *Bacillus* and *Sporolactobacillus*. In *The Genera of Lactic Acid Bacteria*; Wood, B.J.B., Holzapfel, W.H., Eds.; Springer: Boston, MA, USA, 1995; pp. 368–391.
29. Berry, D. The Value of Beverage Clarity. Available online: https://www.foodbusinessnews.net/articles/3233-the-value-of-beverage-clarity (accessed on 19 January 2023).
30. Speers, R.A.; Jin, Y.-L.; Paulson, A.T.; Stewart, R.J. Effects of β-Glucan, Shearing and Environmental Factors on the Turbidity of Wort and Beer. *J. Inst. Brew.* **2003**, *109*, 236–244. [CrossRef]
31. Li, Y.-F.; Bao, W.-G. Why do some yeast species require niacin for growth? Different modes of NAD synthesis. *FEMS Yeast Res.* **2007**, *7*, 657–664. [CrossRef]
32. Smith, M.T. Chapter 25—*Dekkera* van der Walt (1964). In *The Yeasts*, 5th ed.; Kurtzman, C.P., Fell, J.W., Boekhout, T., Eds.; Elsevier: London, UK, 2011; pp. 373–377.
33. Stratford, M. Yeast flocculation: Calcium specificity. *Yeast* **1989**, *5*, 487–496. [CrossRef]
34. Torres, D.; Gonçalves, M.; Teixeira, J.; Rodrigues, L. Galacto-Oligosaccharides: Production, Properties, Applications, and Signicance as Prebiotics. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 438–454. [CrossRef]
35. Mangindaan, D.; Khoiruddin, K.; Wenten, I.G. Beverage dealcoholization processes: Past, present, and future. *Trends Food Sci. Technol.* **2018**, *71*, 36–45. [CrossRef]
36. Dragone, G.; Mussatto, S.I.; Oliveira, J.M.; Teixeira, J.A. Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. *Food Chem.* **2009**, *112*, 929–935. [CrossRef]
37. Risner, D.; Tomasino, E.; Hughes, P.; Meunier-Goddik, L. Volatile aroma composition of distillates produced from fermented sweet and acid whey. *J. Dairy Sci.* **2019**, *102*, 202–210. [CrossRef] [PubMed]
38. Gantumur, M.-A.; Sukhbaatar, N.; Qayum, A.; Bilawal, A.; Tsembeltsogt, B.; Oh, K.-C.; Jiang, Z.; Hou, J. Characterization of major volatile compounds in whey spirits produced by different distillation stages of fermented lactose-supplemented whey. *J. Dairy Sci.* **2022**, *105*, 83–96. [CrossRef]
39. Hughes, P.; Risner, D.; Meunier Goddik, L. Whey to Vodka. In *Whey—Biological Properties and Alternative Uses*; Gigli, I., Ed.; IntechOpen: London, UK, 2019.
40. Risner, D.; Shayevitz, A.; Haapala, K.; Meunier-Goddik, L.; Hughes, P. Fermentation and distillation of cheese whey: Carbon dioxide-equivalent emissions and water use in the production of whey spirits and white whiskey. *J. Dairy Sci.* **2018**, *101*, 2963–2973. [CrossRef] [PubMed]
41. Barbano, D.M.; Sciancalepore, V.; Rudan, M.A. Characterization of Milk Proteins in Ultrafiltration Permeate. *J. Dairy Sci.* **1988**, *71*, 2655–2657. [CrossRef]
42. Jahadi, M.; Ehsani, M.R.; Paidari, S. Characterization of Milk Proteins in Ultrafiltration Permeate and Their Rejection Coefficients. *J. Food Biosci. Technol.* **2018**, *8*, 49–54.
43. Layman, D.K.; Lönnerdal, B.; Fernstrom, J.D. Applications for α-lactalbumin in human nutrition. *Nutr. Rev.* **2018**, *76*, 444–460. [CrossRef] [PubMed]
44. Hanna, M.; Jaqua, E.; Nguyen, V.; Clay, J. B Vitamins: Functions and Uses in Medicine. *Perm. J.* **2022**, *26*, 89–97. [CrossRef] [PubMed]
45. Gharibzahedi, S.M.T.; Jafari, S.M. The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. *Trends Food Sci. Technol.* **2017**, *62*, 119–132. [CrossRef]
46. Schuck, P.; Dolivet, A.; Jeantet, R. *Analytical Methods for Food and Dairy Powders*, 1st ed.; John Wiley & Sons, Ltd.: West Sussex, UK, 2012; pp. 167–190.