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An Integrated Bioprocess to Recover Bovine Milk Oligosaccharides from Colostrum Whey Permeate

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Abstract

A major challenge in isolating oligosaccharides from dairy streams is to enrich oligosaccharides while simultaneously reducing the content of simple sugars (mono- and disaccharides) that do not possess the desired prebiotic functions. An integrated approach based on optimized conditions that favor maximum lactose hydrolysis, monosaccharide fermentation and oligosaccharides recovery by nanofiltration was developed. Upon complete lactose hydrolysis and fermentation of the monosaccharides by yeast, nanofiltration of fermented whey permeate from colostrum enabled the recovery of 95% of the oligosaccharides at high purity. While the number of commercially available standards has limited the quantification of only a few sialylated oligosaccharides, the application of both high performance anion-exchange chromatography with pulsed amperometric detection and mass spectrometry provided a complete profile of the final product. Approximately 85% of the oligosaccharides in the final concentrate were sialylated, with the remainder being neutral.

Keywords

bioactive oligosaccharides; nanofiltration; fermentation; lactose hydrolysis; mass spectrometry

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Chemical compounds studied in this article

3'-sialyllactose (PubChem CID: 123914), 6'-sialyllactose (PubChem CID: 643987), and 6'-sialyl-N-acetyllactosamine (PubChem CID: 16212424)

1 Introduction

Human milk oligosaccharides (HMO*) are indigestible sugars with complex structures that act as selective growth substrates for beneficial bacteria in the infant's gastrointestinal tract and possess anti-pathogenic and immunomodulatory activities (Zivkovic and Barile, 2011). Increasing interest in HMO has prompted the search for suitable sources of HMO-like oligosaccharides and for the development of economically viable scalable processes for the production of bioactive oligosaccharides for *in vitro* and human studies, and as therapeutic ingredients (Lange et al., 2014).

Our research group has demonstrated that bovine milk and dairy streams such as whey permeate contain bovine milk oligosaccharides (BMO) that are structurally similar to HMO in that both contain branched oligosaccharides with glucose, galactose, N-acetylglucosamine, sialic acid and to a certain extent, fucose decorations (Barile et al., 2009; Aldredge et al., 2013). BMO are more similar to HMO than the currently available oligosaccharides in commercial prebiotics, which possess simpler structures and may not selectively stimulate the growth of *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) in the infant gut to the same extent as HMO (Ninonuevo and Bode, 2008).

Whey permeate, a by-product of the recovery of whey protein by membrane filtration, has generally been considered a problematic and abundant stream leading to environmental pollution if disposed of inappropriately. Owing to recent research findings, whey permeate is now considered an attractive source of bioactive oligosaccharides with many applications in human nutrition. Considering the presence of bovine milk oligosaccharides (Barile et al., 2009) and the global production of whey, exceeding 200 million tons per year (Affertsholt, 2009), the development of large-scale processing techniques and high-throughput analytics will enable the production of large quantities of high value compounds that will ameliorate both environmental and economic issues associated with dairy processing (Cohen et al., 2015).

Current techniques to isolate oligosaccharides from milk rely on the combination of lactose hydrolysis and membrane filtration, typically followed by diafiltrations to increase the purity of the recovered components (Sarney et al., 2000; Nordvang et al., 2014; Altmann et al., 2015). However, isolating oligosaccharides in milk remains challenging due to the high lactose concentration and lower concentration of target oligosaccharides. This imbalance complicates recovery of the target bioactive oligosaccharides at high purity.

*HMO: Human milk oligosaccharides
BMO: Bovine milk oligosaccharides
3'-SL: 3'-sialyllactose
6'-SL: 6'-sialyllactose
6'-SLN: 6'-sialyl-N-acetyllactosamine
NeuGc: N-glycolylneuraminic acid
NeuAc: N-acetylneuraminic acid
HPAE-PAD: High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
nano-LC Chip Q-ToF MS: nano-liquid chromatography Chip quadrupole time of flight mass spectrometry
PGC: Porous graphitized carbon
CF: Concentration factor

Recently, several nanofiltration membranes were evaluated for the recovery of oligosaccharides from bovine milk (Altmann et al., 2015). Experiments at industrial scale (1000 L) using a 300 Da spiral wound membrane at 5 to 10 bar and concentration factor (CF= volume of feed/volume of retentate) of 10 have enabled the recovery of 70–97% of three oligosaccharides present in bovine milk (3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), and N-acetylgalactosaminyl-lactose), with a very low degree of purity (1.4%). The use of diafiltration increased the purity of the retained oligosaccharides from 1.4 to 10.6% but at the extent of reducing their retention from between 70 to 97% down to 32 to 56%.

Thus far, oligosaccharides have been isolated at a high recovery yield at the expense of their purity, and vice versa. High purity is critical for assessing their biological activity. In addition, owing to the low concentration of bovine milk oligosaccharides, high recovery yield corresponds with maximal process feasibility and economics.

The overall objective of this study was to develop an integrated process to maximize the recovery yield and purity of bovine milk oligosaccharides. The process relies on optimized conditions for maximum lactose hydrolysis, complete fermentation of monosaccharides released from the hydrolysis of lactose followed by concentration by nanofiltration membrane. The specific objectives of this work were to: a) optimize monosaccharide fermentation at laboratory-scale; b) scale-up monosaccharide fermentation to pilot-scale; c) evaluate the effects of transmembrane pressure on the retention of biologically important sialyloligosaccharides (3'-siallylactose (3'-SL), 6'-siallylactose (6'-SL), and 6'-Sialyl-N-acetyllactosamine (6'-SLN)) and permeate flux; and d) demonstrate the proof-of-concept of the integrated process at pilot-scale.

2 Material and methods

2.1 Bovine colostrum whey permeate

Bovine colostrum whey permeate was kindly provided by La Belle Colostrum (Bellingham, WA, USA). Industrial production of colostrum whey includes an initial defatting step via cream separators followed by the removal of caseins by enzymatic precipitation. The obtained whey was pasteurized at 63°C for 30 minutes. Whey proteins were removed by ultrafiltration (10 kDa membrane) under continuous diafiltration to produce whey permeate (de Moura Bell et al., 2016). Lactose and oligosaccharide content of colostrum whey permeate were 21.22 ± 3.91 g L^{-1} and 0.21 ± 0.05 g L^{-1} respectively. A relative standard deviation of 24.7% for the oligosaccharide composition of the starting material denotes the effect of different lactation stages on the composition of different batches of colostrum whey permeate.

2.2 Process integration to recover oligosaccharides at pilot-scale

An integrated process has been developed to enable the recovery of high purity oligosaccharides from fermented bovine colostrum whey permeate (Figure 1). This approach relies on the combination of optimized conditions that favor maximum lactose hydrolysis and fermentation of simple sugars (glucose, galactose) prior to final concentration of oligosaccharides by nanofiltration. With the goal of evaluating the biological activities of the

recovered oligosaccharides, high performance anion-exchange chromatography with pulsed amperometric detection and mass spectrometry were used to determine the purity and characterize the profile of recovered oligosaccharides.

2.2.1 β-galactosidase treatment of bovine colostrum whey permeate at pilot-scale—A food grade fungal lactase (Bio-Cat Inc., Troy, Virginia, USA) derived from *Aspergillus oryzae* was used to hydrolyze lactose into β-D-galactose and α-D-glucose. The pH of 200–300 L of whey permeate batches was adjusted to 4.5 with citric acid before adding 0.2% (w/v) active dried β-galactosidase. The slurry was stirred for 1 h at 60 rpm and 50°C. Lactose hydrolysis conditions were selected based on our previous work (de Moura Bell et al., 2016). Immediately following lactose hydrolysis, samples were pasteurized using a continuous UHT/HTST lab pasteurizer (MicroThermics, Raleigh, NC, USA) at 72 °C for 15 seconds and fermented thereafter.

2.2.2 Monosaccharide fermentation—Commercial active dry yeast *Saccharomyces cerevisiae* (UCD 522 Montrachet, Lallemand Inc., Montreal, Canada) was used to ferment hydrolyzed colostrum whey permeate. Prior to inoculation, the active dry yeast was rehydrated according to manufacturer's recommendations. A temperature of 28°C, on the higher end of the temperature range for moderate yeast activity provided by the yeast supplier (10–29°C), was used during the optimization study. For each experiment at laboratory-scale, 80 mL of hydrolyzed whey permeate was incubated at 28°C in 250 mL Erlenmeyer flaks and stirred at 150 rpm. Incubation time, amount of active dry yeast and yeast extract (Bacto™ Becton, Dickinson and Company, Sparks, MD) levels are described in Table 1.

2.2.2.1 Optimization of monosaccharide fermentation at laboratory scale: experimental design and statistical analysis: A central composite rotatable design (CCRD) was employed to investigate the interaction of crucial reaction parameters as well as the effects of individual parameters affecting monosaccharide fermentation. In order to find the optimal combination of amount of active dry yeast (g L^{-1}), incubation time (h), and amount of yeast extract (g L^{-1}), a complete 2^3 factorial design of the central rotational type was established, with three repetitions in the central point and six axial points, based on Response Surface Methodology (Rodrigues and Iemma, 2014). The effects of amount of active dry yeast (0.16 – 0.58 g L^{-1}), incubation time (6.9 – 32.1 h), and amount of yeast extract (0.62 – 12.38 g L^{-1}) on the fermentation of monosaccharides derived from lactose hydrolysis were evaluated (Table 1). The independent variables (amount of yeast, incubation time, and amount of yeast extract) and the ranges were evaluated according to coded levels ($-\alpha$, −1, 0, +1, $+\alpha$). The amount of active dry yeast was chosen to ensure an adequate initial cell concentration. According to the manufacturer's instructions, a concentration of 0.25g L^{-1} corresponds to an initial cell concentration of about 3–4 million of viable cells per milliliter. In order to obtain a fast and reliable alcoholic fermentation, a cell concentration of about 6×10^6 cells mL^{-1} of *S. cerevisiae* was chosen for the central point. Additional parameters (incubation time and amount of yeast extract) were chosen based on values commonly used in the literature and on preliminary results (not shown). Center points are the average of levels −1 and +1 and axial points were determined by interpolation for each factor. The axial distance ($\alpha = \pm 1.68$)

for the full factorial design was calculated according to the following equation: $\alpha = \sqrt[4]{2^n}$, where n is the number of factors (Rodrigues and Iemma, 2014).

Coded and uncoded levels and their corresponding independent variables are shown in Table 1. The dependent variable (i.e., evaluated response) was the extent of monosaccharide (glucose and galactose) fermentation.

Data were analyzed by the Protimiza Experiment Design Software (http://experimental-design.protimiza.com.br). The significance of the model was tested by Analysis of Variance (ANOVA).

2.2.2.2 Scale-up of monosaccharide fermentation: Experimental conditions where complete monosaccharide fermentation was observed at laboratory-scale were evaluated and modified slightly at pilot-scale to maximize fermentation rate and efficiency. Monosaccharide fermentation was performed using a 208 L jacketed stainless steel research fermenter system (Cypress, San Jose, CA, USA).

The reaction kinetics were evaluated by withdrawing samples at time intervals of one to five hours until specific gravity values stabilized. After the first exploratory trial at pilot-scale, constant density measurements were used as a rapid way to determine complete fermentation. All pilot scale fermentations were conducted in duplicate at 30°C. The first attempt to scale the monosaccharide fermentation up was performed using 0.4 g L^{-1} of dry yeast and 3.0 g L^{-1} of yeast extract, which were added into approximately 100 L of whey permeate. Two pump configurations were utilized; 13 min of stirring per hour, corresponding to 100 L per hour, as well as 29 min of stirring every 30 min. Once fermentations were complete, the slurry was cooled down to 4°C overnight to allow gravity sedimentation of the yeast. Residual yeast in the supernatant was removed by gross filtration (Millipore® Polysep™ II, Cartridge Filter, pore size 0.5 μm Nominal, Millipore Corporation, Bedford, MA, USA) followed by subsequent microfiltration.

2.2.3 Membrane concentration of fermented colostrum whey permeate—

Microfiltration experiments were carried out in a pilot-scale tangential membrane filtration system (Model L, GEA Filtration, Hudson, WI, USA) to remove residual yeast in the fermented whey permeate. The system was composed of a plate-and-frame membrane module (0.036 – 0.72 m^2 area), a 378 L jacketed stainless steel reactor, a Proline Promass 80E flowmeter for mass flow rate and density (Endress+Hauser, Reinach, Switzerland), a heat exchanger, inlet and outlet manometers, and a 7.0 HP feed pump (Hydra-Cell™, Minneapolis, MN, USA). The fermented whey permeate was microfiltered using 0.432 m^2 of a 150 kDa regenerated cellulose membrane (Mycrodyn Nadir, Austin, Texas, USA) at transmembrane pressure (TMP) of 0.8 bar, feed flow of 6.1 L min^{-1}, and temperatures ranging from 10–12°C.

The microfiltered whey permeate was concentrated by nanofiltration using the same equipment used during the microfiltration stage with a different filtration module. Depending on the experiment, approximately 70 L or 260 L of fermented whey permeate were concentrated using a 500–700 Da spiral-wound sulfonated polyethersulfone (SPES)

nanofiltration membrane with an effective surface area of 1.86 m^2 (HYDRACoRe70pHT, Hydranautics, Oceanside, CA, USA) to a CF of 10 and 20. Nanofiltrations were performed at 50°C, 9.0 L min^{-1} recirculation flow rate, and either 20 or 35 bar transmembrane pressure.

2.2.4 Membrane performance—Membrane performance indicators were evaluated according to Cohen et al. (2016). The performance of the nanofiltration membrane was evaluated with respect to permeate flux and retention of target sialyloligosaccharides (3'-SL, 6'-SL, and 6'-SLN). Permeate samples were collected and weighed in regular time intervals to determine the permeate flux (J_p), presented in terms of volume per unit area per unit time (L m^{-2} h^{-1}) (Equation 1):

$$J_p = \frac{V_p}{A * t} \quad (1)$$

where V_p is the permeate volume (l), A is the membrane area (m^2), t is the time (h) for collecting the permeate volume. The retention of major acidic oligosaccharides (3'-SL, 6'-SL, and 6'-SLN) was determined according to the following equation:

$$R = \left(1 - \frac{C_p}{C_f}\right) * 100 \quad (2)$$

where C_p and C_f are the carbohydrate concentrations in the permeate and feed, respectively.

The purity of the target oligosaccharides in relation to monosaccharides in the retentates was calculated according to the following equation:

$$\%\text{Purity} = \left(\frac{C_{\text{BMO}}}{C_{\text{BMO}} + C_{\text{Mono}}}\right) * 100 \quad (3)$$

where C_{BMO} is the summed concentration of all quantified bovine milk oligosaccharides (6'-SLN, 6'-SL, 3'-SL) and C_{Mono} is the summed concentration of all quantified monosaccharides (glucose and galactose)

The recovery yield represents the fraction of oligosaccharides originally present in the whey permeate which was retained in the retentate. The oligosaccharide yield in the retentate was calculated based on the following equation:

$$\%\text{Yield} = \left(\frac{C_{i,r} * V_R}{C_{i,f} * V_f}\right) * 100 \quad (4)$$

where $C_{i,r}$ is the concentration of a specific component in the retentate, V_R is the volume of liquid in the retentate) $C_{i,f}$ is the concentration of a specific component in the feed, and V_f is the feed volume.

2.2.5 Carbohydrate Analysis

2.2.5.1 Quantification of carbohydrates in colostrum whey permeate: The main bovine colostrum oligosaccharides (3'-SL, 6'-SL and 6'-SLN) as well as simple sugars (glucose, galactose, and lactose) were quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD, Thermo Scientific ICS-5000, Sunnyvale, CA, USA) (Lee et al., 2013). Samples were diluted as appropriate and filtered through a 0.2 μm syringe filter (Acrodisc 13 mm PES, Pall Life Sciences, Port Washington, NY, USA) into 2 mL vials with septa. Calibration curves (coefficient of determination ≥ 0.999) were prepared using 3'-SL, 6'-SL, 6'-SLN (V-Labs, Covington, LA, USA) and glucose, galactose, and lactose (Sigma, St. Louis, MO, USA) standards. 25 μL of diluted, filtered samples were injected into a CarboPac PA200 (Dionex, Sunnyvale, CA, USA) column at 0.5 mL min^{-1} flow rate using 100 mM sodium hydroxide (NaOH) and 10 mM sodium acetate (NaOAc) isocratically for acidic oligosaccharide quantification. Glucose, galactose, and lactose quantification was carried out on a CarboPac PA10 (Dionex, Sunnyvale, CA, USA) column with a flow rate of 1.2 mL min^{-1} and 10 mM NaOH for the first 12 min and a gradient from 10–100 mM NaOH for 12.5 min.

2.2.5.2 Oligosaccharide profiling using nanoHPLC/Chip quadrupole time of flight mass spectrometry (Q-ToF MS) and data analysis: Lyophilized samples were resuspended at 1 mg mL^{-1} concentration in nanopure water, diluted 1:10 in nanopure water, and filtered through 0.2-micron PES syringe filters. Filtered bovine milk oligosaccharide samples were analyzed by Q-ToF MS. Samples were injected in randomized order on the Agilent 6520 (Santa Clara, CA) Q-ToF MS with a porous graphitized carbon (PGC) micro-chip with a 40 μL enrichment column and a 43 mm × 75 um i.d analytical column, both of which had a stationary phase of 5 μm 250Å PGC. Changes to the procedure reported in Aldredge et al. (2013) include using a 325°C drying gas with flow rate of 3 L min^{-1} and a spectral acquisition rate of 1 spectra per second.

Data analysis was performed on Agilent Mass Hunter Quantitative analysis. The "Find by molecular feature" algorithm was employed to extract peaks that matched a bovine milk oligosaccharide database assembled in-house. The peaks were matched with a mass accuracy filter as low as less than 20 ppm error. Only compounds with exact mass in a range of 400 – 3000 m z^{-1} and retention between 5 min to 40 min were selected. The extraction was performed with the following parameters: absolute peak height cut-off of 1000 ion counts; Quality score over 70; ion species limited to H^+; charge state was limited to 1–3. The isotope type used was "glycan". Isotopes were grouped by peak spacing 0.025 m z^{-1} + 7.0 ppm. Each extracted ion chromatogram was examined manually and incorrect assignments and integrations were corrected manually.

3 Results and Discussion

3.1 Optimization of monosaccharide fermentation at laboratory-scale

The interaction of the reaction parameters as well as their individual effects on the fermentation of monosaccharides and their optimum levels were investigated using a central composite rotatable design. The effects of reaction time (h) and amounts of active dry yeast and yeast extract (g L^{-1}) on the fermentation of monosaccharides arising from the lactose hydrolysis step are shown in Table 2. Maximum fermentation (100%) was observed in 9 out of the 17 experimental runs (3, 4, 7, 8, 10, 12, 15, 16, 17). In general, higher amounts of active dry yeast (0.35 – 0.58 g L^{-1}) and intermediate concentrations of yeast extract (6.5 g L^{-1}) were associated with increased rate of fermentation and complete fermentation at 19.5 hours. The use of amounts of yeast extract away from the central point (6.5 g L^{-1}) increased the monosaccharide fermentation time to 27 hours, compared with 19.5 hours. The comparison of our results with those in the literature is challenging due to differences in the composition of the starting material used and experimental conditions. However, fermentation of whey permeate without supplementation containing 50 g L^{-1} lactose using a flocculating *S. cerevisae* mutant expressing lactose utilization genes was complete within 30 hours (Domingues et al., 2001). Taking into account differences in the yeast strain used, the reported fermentation rate (50 g/L of lactose in 30 h) was faster than the work presented herein (~ 20 g/L in 20 h).

3.1.1 Statistical analysis for the fermentation of monosaccharides at laboratory-scale—

The optimum level for each independent variable as well as their interaction effects evaluated was determined by multiple regression analysis of the experimental data. Within the range evaluated, all variables significantly affected the rate of monosaccharide fermentation. However, the quadratic term of the variables x1 and x3 as well as the interaction effects between all variables (x1x2, x1x3, x2x3) were not significant at $p<0.05$ (Table 3) and were excluded from the final model.

The reparameterized model and the coefficient of determination (R^2) for monosaccharide fermentation from bovine colostrum hydrolyzed whey permeate by *S. cerevisiae* were determined after excluding parameters having little or no influence (not significant at $p<0.05$) on the monosaccharide fermentation. The regression equation of second order shows the dependence of the fermentation rate of monosaccharides to the amount of active dry yeast, reaction time, and amount of yeast extract:

$$Y_1 = 100.24 + 6.43\,x_1 - 6.50\,{x_1}^2 + 22.10\,x_2 - 10.64\,{x_2}^2 - 7.67\,{x_3}^2$$

where Y_1 is the percent of monosaccharide fermentation, X_1 is the amount of active dry yeast, X_2 is the reaction time, and X_3 is the amount of yeast extract. The coefficient of determination (R^2) of the predictive model for monosaccharide fermentation from bovine colostrum whey permeate by *S. cerevisiae* was 0.92, indicating that the regression model was able to explain 92% of the total variation between the observed and predicted values and the remainder 8% being attributed to the residual values. According to the model, complete monosaccharide fermentation can be achieved using 0.37 g L^{-1} of active dry yeast (0.0), 6.5

g L^{-1} of yeast extract (0.0) and reaction time of 19.5 h (0.0) or 0.5 g L^{-1} of active dry yeast (+1.0), 3.0 g L^{-1} of yeast extract (−1.0) and reaction time of 27 h (+1.0).

The analysis of variance of the estimated regression model is presented in Table 4. The regression was significant ($F_{calculated} > F_{table}$) and could be used for predictive goals in the range of parameters evaluated as the ratio $F_{calculated}/F_{table}$ is greater than 3 (Rodrigues and Iemma, 2014). Although the F-test for the lack of fit was statistically significant ($F_{calculated} > F_{table}$), this significance is associated with a pure error that tends towards zero, due to the high reproducibility of the repetitions performed in the central point (100, 100, and 100). Indeed, the model has a very low lack of fit [(809.3/9642.8)*100 = 8.4%]. However, when calculating the F-test for the lack of fit, the ratio of the mean square of the lack of fit (89.9) by the mean square of the pure error (0.0) generates a high value for the F-test that could be misleading.

Contour surfaces (Figure 2 - a, b, c) were built based on the estimated regression model generated, to express the fermentation of monosaccharides. All contour surfaces were built with the fixed variable in the central point (0). According to the regression model and Figure 2a, monosaccharide fermentation is favored by active dry yeast concentration of 0.35 to 0.5 g L^{-1} and reaction time of 25 h. Concentrations of yeast extract around 6.5 g L^{-1} and active dry yeast ranging from 0.37 to 0.5 g L^{-1} have been shown to maximize monosaccharide fermentation (Figure 2b). Based on estimated regression model and contour surface (Figure 2c), concentrations of yeast extract 6.5 g L−1 and reaction time around 27 h favored monosaccharide fermentation. In general, as shown in Fig. 2a and c, reaction time had the highest effect on the monosaccharide fermentation rate compared with the amount of active dry yeast and yeast extract, with maximum fermentation values observed after 22 h.

3.2 Fermentation of monosaccharides at pilot-scale

Results from the experimental design for the optimization of the monosaccharide fermentation at laboratory-scale were used to guide pilot-scale fermentation. According to the predictive model at lab-scale, complete monosaccharide fermentation can be achieved at 19.5 h when 0.37 g L^{-1} of active dry yeast and 6.5 g L^{-1} of yeast extract are used or at 27 h when 0.5 g L^{-1} of active dry yeast and 3.0 g L^{-1} of yeast extract are used. In our first exploratory pilot-scale fermentation trial, we used 0.4 g L^{-1} of active dry yeast, 3.0 g L^{-1} of yeast extract, at 30°C under 13 min of pump recirculation per hour (Figure 3 – low stirring) due to the possible economic advantage of using less yeast extract without sacrificing the fermentation rate. While all glucose was fermented within 15 hours, approximately 5.4 and 3.3 g L^{-1} galactose remained at 19 and 22 hours of fermentation, being equivalent to a monosaccharide fermentation of 70 and 82%, respectively. Taking into account the use of a reduced amount of yeast extract than the one suggested by the model (3.0 vs. 6.5 g L^{-1}), the 70% fermentation yield at 19 hours at pilot-scale is much lower than the 92% predicted by the lab-scale model at 19.5 hours. Differences in fermentation rates at pilot-and lab-scales were not surprising and can be explained by the substantial differences in fermentation volume between laboratory- and pilot-scale fermentations (80 mL vs. 100 L) as well as with the reactor configuration differences (250 mL Erlenmeyer flasks vs. 200 L automated fermentation reactor). Maintaining all other fermentation parameters constant, a high stirring

rate (58 min of stirring/hour with 2 min interval) was attempted to achieve complete monosaccharide fermentation (Figure 3 - high stirring). Indeed, under these conditions, even using less yeast extract (3.0 g L^{-1} instead 6.5 g L^{-1}), complete fermentation of glucose and galactose was observed within 15 hours, a shorter fermentation time than that achieved at laboratory-scale (19.5 hours).

The addition of nutrients for microbial fermentations is associated with higher processing costs (Sikder et al., 2012). Therefore, we evaluated the effect of omitting yeast extract on the fermentation reaction at pilot-scale (Figure 3 - high stirring, no yeast extract). Glucose and galactose fermentation rates were reduced compared with the addition of 3 g L^{-1} of yeast extract. Glucose and galactose fermentation times increased from 10 to 12 hours and from 15 to 27 hours, respectively. Processing costs must be evaluated to determine if yeast extract should be omitted at the expense of a slower fermentation.

3.3 Membrane concentration of fermented colostrum whey permeate

The residual amount of galactose (3.3 g L^{-1}) observed in preliminary experiments at pilot-scale (Figure 3 - low stirring) resulted in approximately 12 g L^{-1} of galactose when the fermented whey permeate was concentrated by a nanofiltration membrane at CF 10. Thus, complete monosaccharide fermentation prior to the membrane concentration step is crucial to obtain high-purity oligosaccharides.

Figure 4 shows the effects of concentration factor on the permeate flux of whey permeate fermented at a high stirring rate and on the recovery yield of oligosaccharides present in bovine colostrum using a 500–700 Da spiral-wound membrane at 20 bar and feed flow of 9 L min^{-1}. Permeate flux of fermented whey permeate from colostrum decreased from 13.83 ± 2.96 to 7.05 ± 1.84 L m^{-2} h^{-1} and the density of the retentate increased from 993.3 kg m^{-3} to 1014.0 kg m^{-3} at CF 10 (Figure 4a). Increased density at CF 10 is a consequence of increased solids concentration in the retentate (oligosaccharides, peptides, and salts) which could in turn contribute to a higher polarization concentration and membrane fouling (de Moura et al., 2011; Contreras et al., 2009). HPAE-PAD analysis of the retentates and permeates from the nanofiltration of fermented whey permeate at CF 10 indicated that approximately 68, 73, and 69% of 3'-SL, 6'-SL, and 6'-SLN, respectively, were retained by the nanofiltration membrane (Figure 4b). On average, approximately 30% of the acidic oligosaccharides permeated the membrane at these conditions. Oligosaccharide concentration in the retentate at CF 10 was 0.78, 0.15, 0.20 g L^{-1} for 3'-SL, 6'-SL, and 6'-SLN, respectively. It is worth noting that the colostrum whey permeate used in this experiment was diafiltered extensively, resulting in a lower concentration of acidic oligosaccharides ranging from 0.15 – 0.27 g L^{-1} compared with a typical concentration of 1 g L^{-1} (Nakamura et al., 2003). No monosaccharides were detected in the final retentate. The oligosaccharide-rich retentate presented a 100% purity in terms of monosaccharides and lactose in the retentate.

In order to minimize the loss of the target oligosaccharides during the concentration step, a higher transmembrane pressure was evaluated at a higher concentration factor (CF 20). Feed flow of 6 L min^{-1} was shown to be as efficient as 9 L min^{-1} in terms of permeate flux (data

not shown), thus it was used in the subsequent nanofiltration experiments due to the reduced energy requirements.

Figure 5 shows the effects of CF on permeate flux and oligosaccharide yields at transmembrane pressure of 35 bar and feed flow of 6 L min^{-1}. Permeate flux decreased from 16.4 ± 0.012 to 4.8 ± 1.12 L m^{-2} h^{-1} when CF increased from 2.0 to 20, corresponding to an increase in density of the retentate from 988.54 to 1032.2 kg m^{-3} (Figure 5a). Minimum increments in permeate flux were observed at 35 bar compared with 20 bar (16.4 ± 0.012 vs. 13.83 ± 2.96 at CF2 and 8.70 ± 1.30 vs. 7.05 ± 1.84 at CF10), likely a consequence of membrane and/or concentration polarization layer compaction at higher transmembrane pressure (Cheryan, 1998). In some cases, higher transmembrane pressure may lead to increased permeate flux due to the reduction of the membrane thickness. However, the effective membrane pore size reduction and compaction of the gel layer may outweigh the marginal increase in permeate flux at higher pressure and lead to an overall higher solute retention (Choi et al., 2005). At CF 20, the oligosaccharide recovery yield in the retentate was 94.3, 93.7 and 95.7% for 3'-SL, 6'-SL, and 6'-SLN, respectively (Figure 5b). The loss of those three major oligosaccharides into the permeate varied from 4.3 to 6.3%. Increased average retention of the target oligosaccharides at 35 bar compared with 20 bar (95 vs. 70%) is likely due to membrane compaction and/or a concentration polarization layer.

The total oligosaccharide concentration in the retentate (5 g L^{-1}) at CF20 was represented by 3.42 g L^{-1} of 3'-SL, 0.67 g L^{-1} of 6'-SL, and 0.97 g L^{-1} of 6'-SLN. Negligible monosaccharide concentration (0.06 g L^{-1}) was detected in the final retentate at CF20. The high retention of oligosaccharides (95%) and the nearly complete absence of monosaccharides in the final retentate, leading to high purity (99%) with respect to the presence of simple sugars in the retentate, are similar to the ones recently reported by Cohen et al. (2016). In the latter study, the use of alkaline pH during the nanofiltration and diafiltration step enabled the recovery of 96% of bovine milk oligosaccharides from colostrum with a 98% degree of purity with respect to simple sugars in the retentate. Considering the fact that both methods were successful in producing a rich-oligosaccharide retentate free of simple sugars for future biological studies, an economic evaluation must be performed to assess the feasibility of those two processing strategies.

3.4 Oligosaccharide profile of the final retentate

To date, dozens of bovine milk structures have been elucidated and identified (Lee et al., 2016; Mariño et al., 2011; Urashima et al., 2013). Accurate quantification depends upon the availability of pure standards corresponding to the actual isomeric structures found in bovine milk, which as of this writing is quite limited. In this study, the three major anionic oligosaccharides were quantified; however, substantial amounts of other sialylated and neutral oligosaccharides were identified in the final retentate. Mass spectrometry with high mass accuracy and resolution sheds light on relative abundances of many more oligosaccharides, in particular those which are present in trace quantities and for which commercial standards do not yet exist.

To understand the diversity of bovine milk oligosaccharides present in the final nanofiltration retentate, duplicate lyophilized samples were injected into the nano-LC Chip

Q-ToF. Oligosaccharides were identified with a targeted workflow using a glycan database developed in-house (Aldredge et al., 2013). Twenty oligosaccharide compounds were identified in the final retentate, some of which were structural isomers (Figure 6). For example, there are two peaks representing sialyllactose (2_0_0_1_0) which differ solely in the glycosidic bond between sialic acid and galactose; one for the isomer 3'-SL and one for the isomer 6'-SL. The majority (84%) of the oligosaccharides were sialylated, with less than 2% being sialylated with N-glycolylneuraminic acid (NeuGc). Approximately 14% of oligosaccharides were of the neutral type, with structures containing hexose and N-acetylhexosamine moieties in various configurations.

Importantly, results from the nano-LC-Chip Q-ToF corroborate the findings of the HPAE-PAD quantification, in that the most abundant oligosaccharides are sialyllactose and sialyllactosamine, with 3'-SL being more abundant than 6'-SL and 6'-SLN (Figure 6). Similar to findings in the literature, the next observed abundant structure was 2_1_0_0_0, which is most likely N-acetylgalactosaminyl-lactose or N-acetylglucosaminyl-lactose and their isomers (Altmann et al., 2015; Tao et al., 2008; Lee et al., 2016). By utilizing both quantitative and qualitative glycoprofiling tools, important processing parameters can be identified and robust food processes can be developed in order to deliver the next generation of high value food ingredients.

4 Conclusions

A novel approach based on the integration of three different processing techniques (enzymatic hydrolysis, yeast fermentation and membrane filtration) has been developed and validated at pilot-scale to produce a purified complex oligosaccharide fraction free of digestible sugars such as lactose, glucose, and galactose. This work shows that bovine colostrum represents a practical source of bioactive milk compounds for use in clinical trials and eventually in therapeutics. Additionally, a complement of sensitive and robust analytical tools has been applied to guide food processing strategies to recover a pool of diverse bioactive components. Fermentation of glucose and galactose released from lactose hydrolysis enabled the complete removal of digestible sugars yielding a material that was concentrated by nanofiltration to produce an oligosaccharide-rich concentrate virtually free from simple sugars (99% purity). The integration of these three processing techniques addresses the major problem associated with isolating oligosaccharides by membrane filtration – namely, concomitant retention of oligosaccharides and simple sugars – the latter of which lack biological specificity towards commensal bacteria. This approach could be applied to other dairy products, including caprine milk, mature bovine milk permeate, and even human milk.

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- A bioprocessing strategy to recover milk oligosaccharides
- Integration of enzymatic reaction, fermentation and membrane filtration
- High purity and high recovery of oligosaccharides for prebiotic evaluation
- Proof-of-concept at pilot-scale

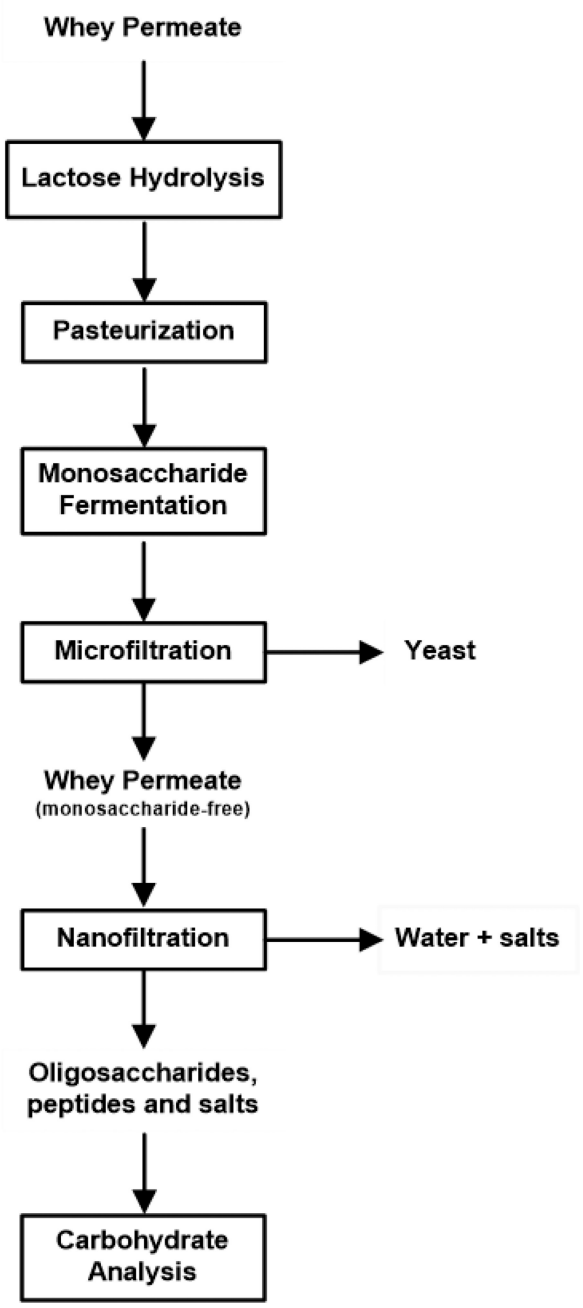

Figure 1.
Process flow diagram for the integrated process to recover oligosaccharides from bovine colostrum at pilot-scale

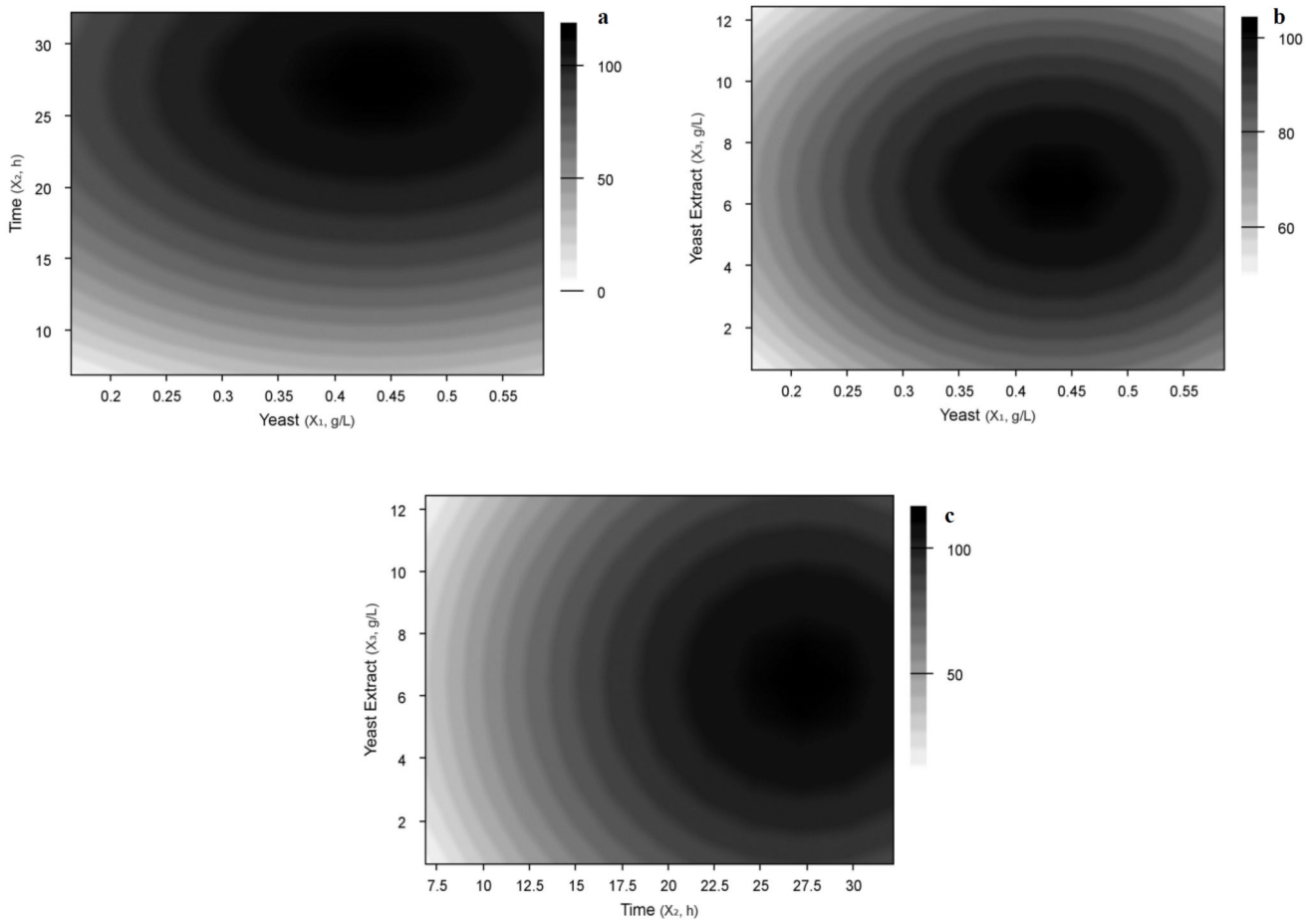

Figure 2.

Effects of operational parameters on monosaccharide fermentation efficiency: reaction time vs. amount of active dry yeast (a); amount of yeast extract vs. amount of active dry yeast (b); amount of active dry yeast vs. reaction time (c).

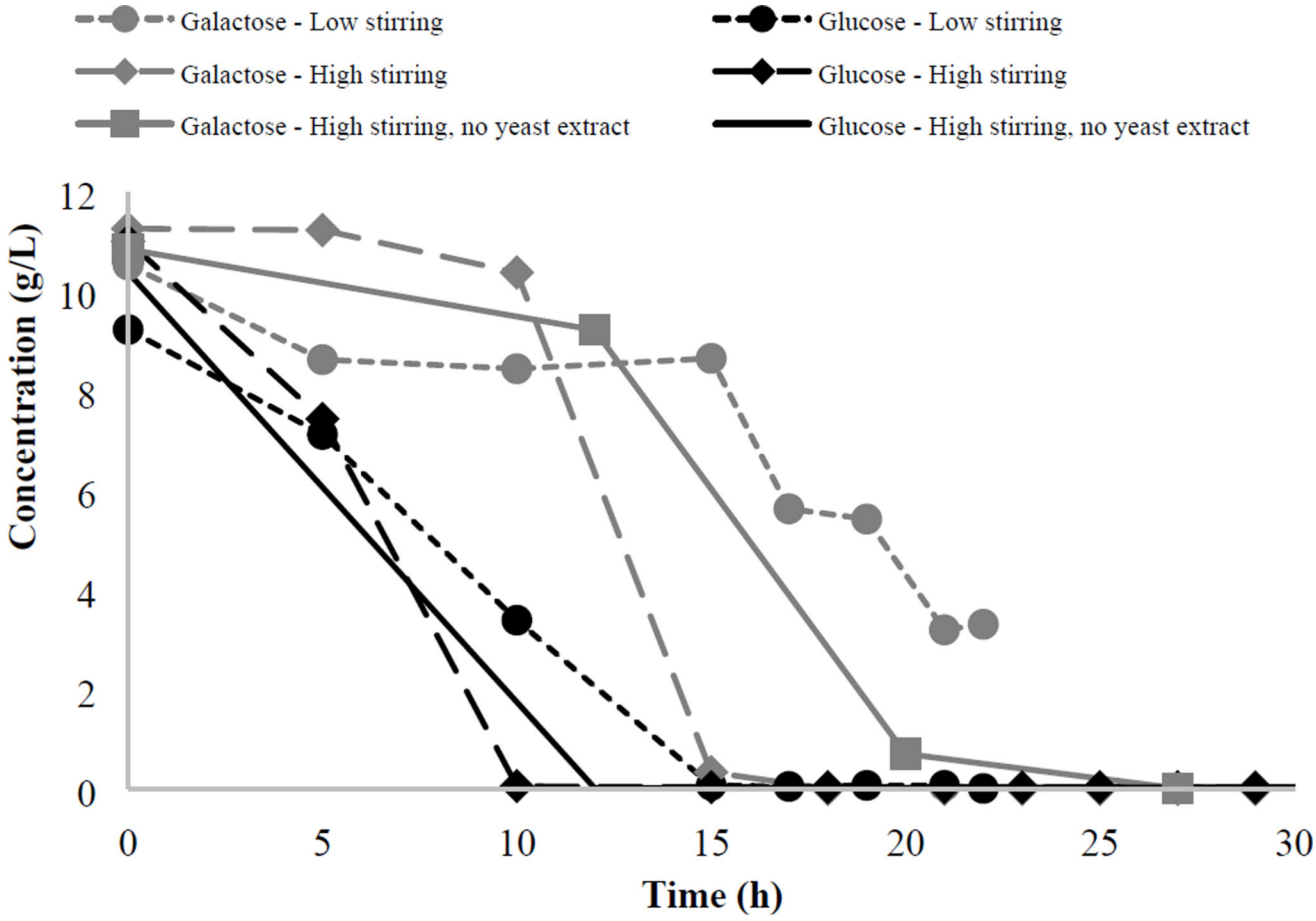

Figure 3.
Effects of stirring configurations and use of yeast extract nutrient supplementation on monosaccharide fermentation rate at pilot-scale

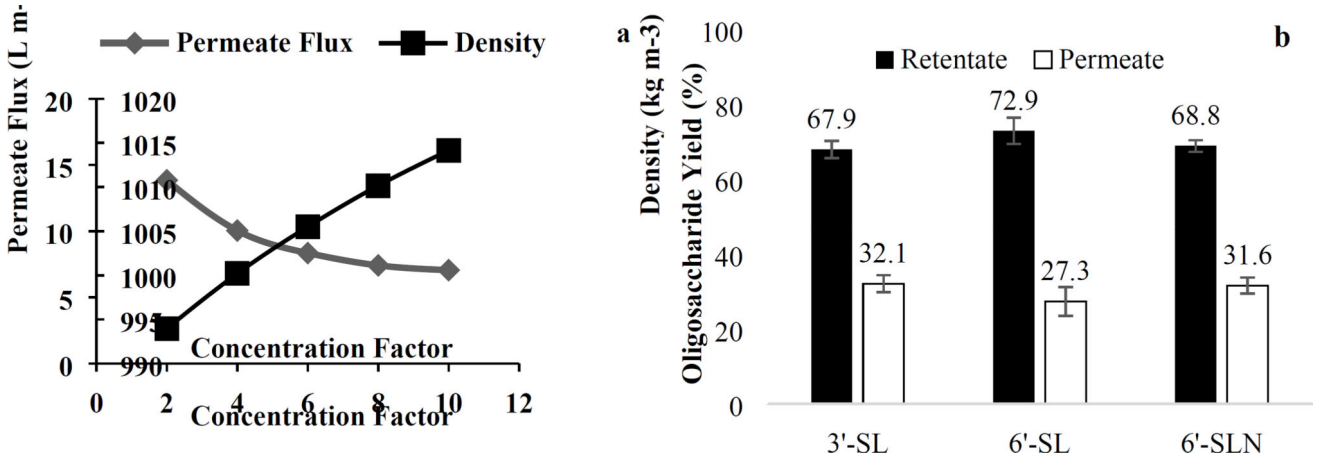

Figure 4.

Effects of concentration factor on permeate flux and density (a) and recovery yield (b) of major oligosaccharides present in whey permeate from colostrum (500–700 Da membrane, 20 bar, 9 L min^{-1}, 50°C).

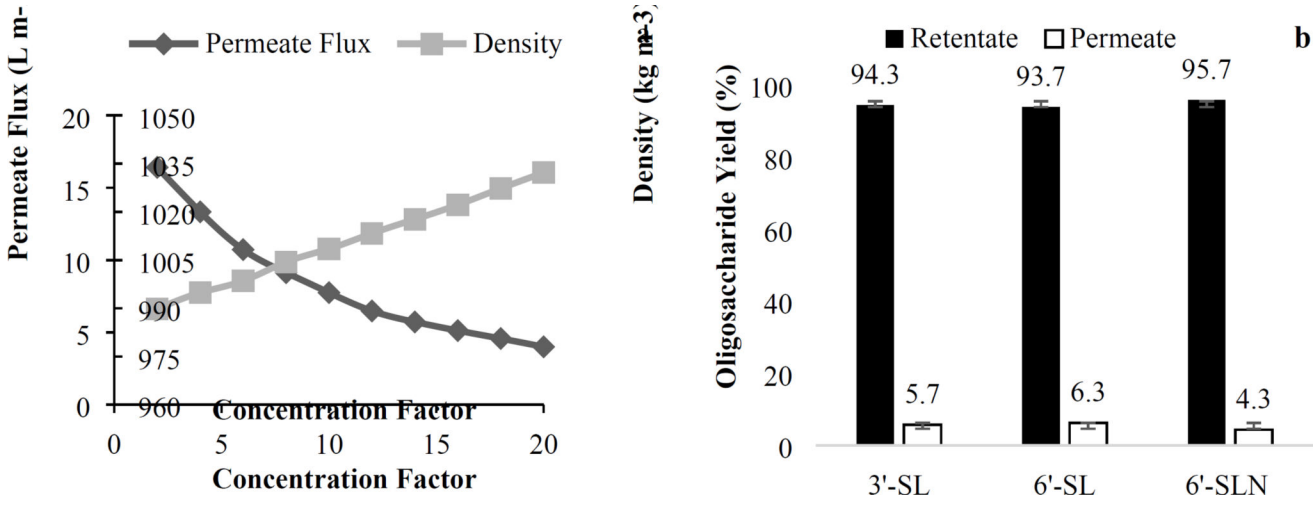

Figure 5.

Effects of concentration factor on permeate flux and density (a) and recovery yield (b) of major oligosaccharides present in whey permeate from colostrum (500–700 Da membrane, 35 bar, 6 L min^{-1}, 50°C)

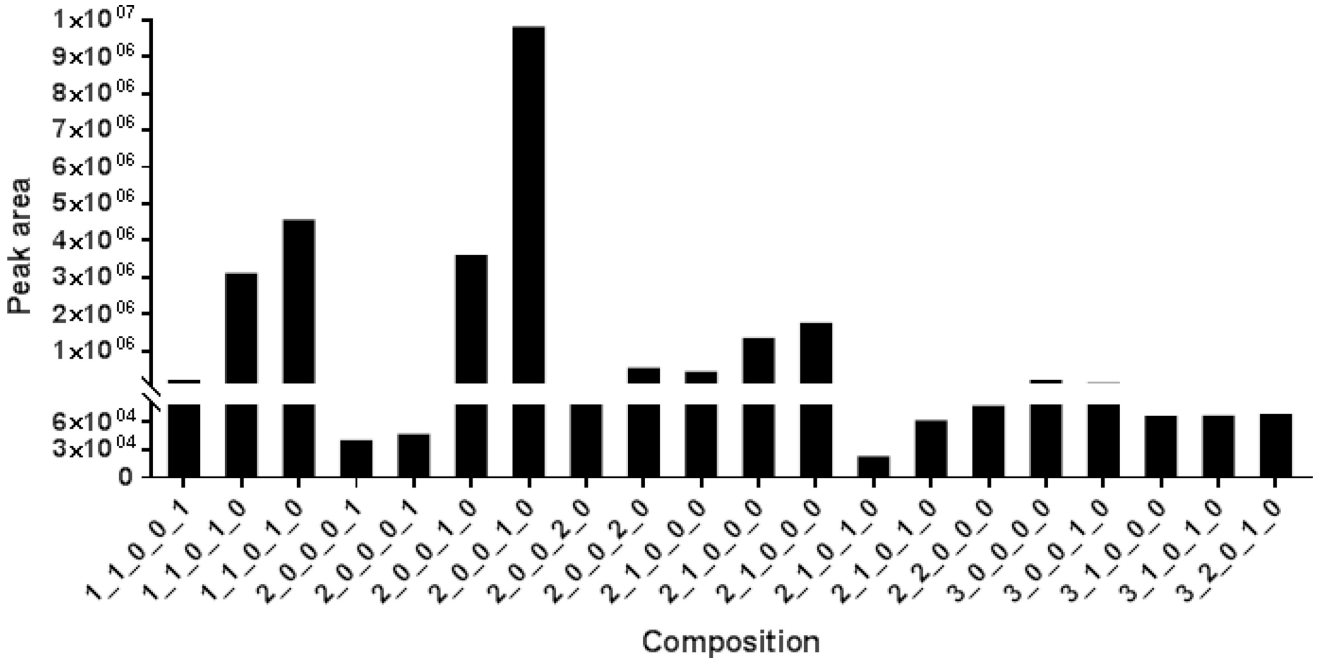

Figure 6.

Peak areas of extracted ion chromatograms from the nano-LC Q-ToF MS analysis of oligosaccharides of retentate sample at a concentration factor of 20. The oligosaccharide composition notation follows the order: Hex_HexNAc_Fuc_NeuAc_NeuGc.

Table 1

Variables and levels evaluated in the experimental design to optimize the fermentation of monosaccharides released from the lactose hydrolysis step

	Levels				
Variables	**−1.68**	**−1**	**0**	**1**	**+1.68**
Active dry yeast (g L^{-1}) − X_1	0.16	0.25	0.37	0.50	0.58
Time (h) − X_2	6.9	12	19.5	27	32.1
Yeast extract (g L^{-1}) − X_3	0.62	3.0	6.5	10	12.38

Complete 2^3 factorial design, with three independent variables in two levels, three repetitions in the central point and six axial points

Table 2

Experimental design for optimizing the fermentation of monosaccharides from bovine colostrum whey permeate by *S. cerevisiae*.

Treatments	Yeast (g L^{-1})	Time (h)	Yeast Extract (g L^{-1})	Fermentation (%)
1	0.25	12.0	3.0	45.66
2	0.50	12.0	3.0	56.30
3	0.25	27.0	3.0	100.00
4	0.50	27.0	3.0	100.00
5	0.25	12.0	10.0	33.35
6	0.50	12.0	10.0	56.39
7	0.25	27.0	10.0	100.00
8	0.50	27.0	10.0	100.00
9	0.16	19.5	6.5	67.82
10	0.58	19.5	6.5	100.00
11	0.37	6.9	6.5	44.41
12	0.37	32.1	6.5	100.00
13	0.37	19.5	0.6	71.84
14	0.37	19.5	12.4	89.39
15	0.37	19.5	6.5	100.00
16	0.37	19.5	6.5	100.00
17	0.37	19.5	6.5	100.00

Table 3

Regression coefficients for the fermentation of monosaccharides from bovine colostrum whey permeate by *S. cerevisiae*.

Factors	Regression Coefficient	Standard Error	Calculated *t*	*p*-value
Mean	100.24	5.37	18.67	0.0000
x1 (L)	6.43	2.52	2.55	0.0381
x1 (Q)	−6.50	2.78	−2.34	0.0516
x2 (L)	22.10	2.52	8.76	0.0001
x2 (Q)	−10.64	2.78	−3.83	0.0064
x3 (L)	1.27	2.52	0.50	0.6309
x3 (Q)	−7.67	2.78	−2.76	0.0280
x1x2	−4.21	3.29	−1.28	0.2420
x1x3	1.55	3.29	0.47	0.6523
x2x3	1.53	3.29	0.46	0.6569

Table 4

Analysis of variance of the estimated model for the fermentation of monosaccharides from hydrolyzed whey permeate from bovine colostrum

Source of variation	Sum of squares	Degrees of freedom	Mean Squares	F-test
Regression	8833.5	5	1766.7	**24.0**[a]
Residual	809.3	11	73.6	
Lack of fit	809.3	9	89.9	**89.9**[b]
Pure error	0	2	0	
Total	9642.8	16		

Values in bold are statistically significant (P<0.05). Coefficient of determination: R^2=0.92; $F_{0.95–5,11}$=3.20; $F_{0.95–9,2}$=19.38