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RESEARCH ARTICLE

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Production of high-purity galacto-oligosaccharides by depleting glucose and lactose from galacto-oligosaccharide syrup with yeasts

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Abstract

Galacto-oligosaccharides (GOS) are prebiotic compounds, widely used as ingredients in various food, nutraceutical and pharmaceutical products. Enzymatic synthesis of GOS results in low-purity products that contain high amounts of glucose and lactose beside the valuable GOS. In this study, a systematic approach was used to develop yeast-based fermentation strategies to purify crude GOS. Potentially applicable yeast strains were identified based on an extensive search in literature databases followed by a series of laboratory-scale fermentation tests. Single- and two-step fermentation processes were designed for the removal of glucose alone or together with lactose from crude GOS syrup. Single-step fermentation trials with two strains of previously unreported species, *Cyberlindnera jadinii* NCAIM Y.00499 and *Kluyveromyces nonfermentans* NCAIM Y.01443, resulted in purified products free of both glucose and ethanol from a crude GOS syrup diluted to 15 and 10 w/v%, respectively. Simultaneous removal of glucose and lactose was achieved by *Kluyveromyces marxianus* DMB Km-RK in a single-step fermentation process with a yield of 97.5% and final purity of 100%. A two-step fermentation approach was designed to allow conversion of a glucose-free product into a high-purity GOS by removing glucose with *C. jadinii* Y.00499 in the first step, and lactose by *Kluyveromyces lactis* DMB Kl-RK in the second step, resulting in a final product with a yield of 100% and a final purity of 92.1%. These results indicate that the selected nonconventional yeasts are promising candidates for the removal of non-GOS components from commercial crude GOS products by selective fermentation.

KEYWORDS

Cyberlindnera jadinii (*Candida utilis*), galacto-oligosaccharides (GOS), *Kluyveromyces* spp, purification, selective fermentation

1 | INTRODUCTION

Galacto-oligosaccharides (GOS) are prebiotic polymers used as ingredients in various value-added food, nutraceutical and pharmaceutical products. Several studies have reported beneficial physiological effects associated with their consumption, especially promotion of the growth of probiotic bacteria in the large intestine (Wilson & Whelan, 2017).

GOS are carbohydrates composed of a terminal glucose linked to a chain of galactose units with a degree of polymerization (DP) between 2 and 10 (Gänzle, 2012). At industrial scale, GOS is conventionally manufactured by enzymatic synthesis from lactose as a substrate, using β-galactosidase as a catalyst (Gosling, Stevens, Barber, Kentish, & Gras, 2010; Park & Oh, 2010). The reaction is carried out in batch system under optimized conditions using high substrate concentration in order to shift the reaction from hydrolysis to transgalactosylation (Sangwan, Tomar, Singh, Singh, & Ali, 2011). The enzymatic conversion results in a mixture of carbohydrates consisting of GOS with various DPs, glucose as by-product, considerable amounts of remaining lactose and small amounts of galactose (Coulier et al., 2009). GOS structure depends on the applied β-galactosidase and conditions of the transgalactosylation reaction; oligosaccharides within the DP fractions can differ in glycosidic linkages (Coulier et al., 2009; Torres, do Goncalves, Teixeira, & Rodrigues, 2010). Chemical structures of GOS components highly influence hydrolysis by the human digestive enzymes and their potential prebiotic characteristics. Trimeric or higher GOS constituents were found non-digestible in vitro by human intestinal enzymes, which enables them to exert their prebiotic effects in the large intestine. Disaccharide GOS fractions were heterogeneous from this respect, as some were partially digested under the same conditions (Gosling et al., 2010; Sako, Matsumoto, & Tanaka, 1999). Nevertheless, nonlactose components of crude GOS DP2 fraction are generally considered as disaccharides with prebiotic potential (Guerrero et al., 2014).

After enzymatic synthesis of GOS, a sequence of downstream processing steps including enzyme inactivation, activated carbon treatment, ion exchange and evaporation belong to the production process. The final product is marketed either as a concentrated syrup or in powder form (Kovács et al., 2013).

Due to the limitations of the kinetically controlled enzymatic biotransformation (Torres et al., 2010), commercial products—hereinafter referred to as 'low-purity products'—typically contain only about 50–60 w/w% GOS on total carbohydrate basis. Both the types of glycosidic linkages and the DP distribution of the oligosaccharide fractions may vary to a certain extent depending on the origin of the applied enzyme and the reaction conditions (van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2016). The remaining lactose (generally around 20%–25%), the by-product glucose (approximately 20%), and the galactose (typically less than 1%) are considered as undesired compounds with no or little economic value. Separation of these components from GOS is a challenging task. At industrial scale, purification is done by simulated moving bed (SMB) chromatography, an expensive process regarding both capital expenditures and operating costs. For this reason, there is a continuous interest in developing cost-effective separation methods for removing unwanted monomers and/or dimers from the crude GOS products (Panesar, Kaur, Singh, & Kennedy, 2018; Wisniewski, Pereira, Polakovic, & Rodrigues, 2014).

It should be noted that the carbohydrate composition of high-purity GOS products entering the market is tailored for their intended usage. GOS is one of the key ingredients in infant food formulations. For such an application, removal of lactose from the low-purity products is generally not required, only separation of monosaccharides is aimed at. However, for a number of other applications, especially lactose free and low-calorie products, removal of lactose is also necessary.

In the recent years, several studies have investigated the purification of crude GOS products by selective fermentation using yeasts and lactic acid bacteria, as an alternative process to SMB chromatography. According to the technical and economic analysis performed by Scotta et al. (2016), selective fermentation process may outcompete SMB chromatography if a number of challenges are adequately addressed. The main challenges include reduction (a) in the mass ratio of cells to sugars, (b) in the amount of additional nutrients required for cultivation and (c) in the degree of dilution of the crude GOS syrup prior to fermentation. Besides, the generation of unwanted metabolites, which would require increased efforts and costs for downstream processing, should be minimized.

Removal of monosaccharides and lactose from crude GOS products by yeasts would require the simultaneous utilization of lactose and glucose together with that of galactose by aerobic respiration or ethanolic fermentation. Lactose utilization requires hydrolysis of lactose by the enzyme β-galactosidase (E.C.3.2.1.23) followed by decomposition of glucose and galactose via glycolytic pathway. Simultaneous utilization of lactose, galactose and glucose is influenced by the regulation of β-galactosidase. Many β-galactosidases are inhibited by galactose and—in a much lesser extent—glucose. The inhibition constant Ki for galactose varies between 3 mM and over 100 mM; Ki values determined for glucose inhibition are typically 10-fold higher and the range is from 50 mM to over 1 M (Park & Oh, 2010).

Several mostly *Basidiomycetes* yeast species are able to utilize lactose. The main metabolic route of lactose utilization is the aerobic assimilation; just a few ascomycetous yeast species can ferment it to ethanol (Barnett, Payne, & Yarrow, 1990; Kurtzman, Fell, & Boekhout, 2011a). *Kluyveromyces* species are outstanding not only in lactose assimilation but fermentation, too. All currently accepted *Kluyveromyces* species with the exception of *Kluyveromyces lactis* var. *drosophilarum* and *Kluyveromyces dobzhanskii* are able to assimilate lactose (Kurtzman, Fell, & Boekhout, 2011b). *K. lactis* and *Kluyveromyces marxianus* are the most important species of the *Kluyveromyces* genus from biotechnological point of view. They are able to utilize lactose not only by aerobic respiration but also by anaerobic (ethanolic) fermentation, and both of them are Crabree negative. Two other lactose-utilizing species of the genus, *Kluyveromyces nonfermentans* and *Kluyveromyces wickerhamii*, assimilate but do not ferment lactose. Galactose utilization generally accompanies lactose positivity.

Metabolism of both *K. lactis* and *K. marxianus* is strongly influenced by the aeration. Fermentation starts at lower oxygen content of the medium than in *Saccharomyces cerevisiae*. *K. marxianus* is capable of simultaneous fermentation and respiration, but this balance is strain specific (Lane & Morrissey, 2010). Lane et al. (2011) carried out an extensive study investigating the metabolic diversity of *K. marxianus* and comparing 13 strains for different traits such as tolerance to high osmotic conditions (0.5 M NaCl), moderate heat and cell wall integrity stresses. Very different patterns of sensitivity were found, with some strains showing resistance for multiple stress factors. Lodi and Donnini (2005) demonstrated that transported but unmetabolized lactose has a cytotoxic effect on *K. lactis*, which is the consequence of the osmotic stress. Therefore, lactose as an osmolyte might have a distinguished role among the osmotic stress factors. These data indicate that different pathways and phenotypes could be responsible for the same stress sensing in *Kluyveromyces* and careful selection of suitable, well adapted strains is required for industrial applications.

Cheng et al. (2006) showed that *K. marxianus* ATCC 56497 was able to consume both lactose and monosaccharides when its fermentation was combined with enzymatic synthesis of low-purity GOS. A disadvantage of *K. marxianus* is, however, the close to complete removal of disaccharides that differ from lactose structurally (i.e., possess glycosidic bonds other than the ß(1-6) linkage of lactose). Such nonlactose DP2 compounds are known to exhibit prebiotic effects and are generally classified as valuable GOS fractions (Guerrero et al., 2014). To overcome this problem, *Streptococcus thermophilus* was used by (Giacomelli et al., 2015). Although *S. thermophilus* enables the selective consumption of lactose while preserving other disaccharides, it accumulates galactose. Thus, subsequent removal of galactose requires an additional fermentation step using, for example, *S. cerevisiae*. The major concern against the application of *S. cerevisiae* is, however, generation of high amounts of ethanol as a by-product (Goulas, Tzortzis, & Gibson, 2007; Hernández, Ruiz-Matute, Olano, Moreno, & Sanz, 2009; Li, Xiao, Lu, & Li, 2008). Other attempts, using cells of *K. lactis* (Li et al., 2008; Santibáñez, Guerrero, & Illanes, 2017; Sun et al., 2016) or *Sporobolomyces singularis* (Saravanan et al., 2017) achieved remarkable GOS purity but had to use a very high ratios of cell to substrate concentration in order to reach the desired results.

The aim of the present work was to perform a culture-independent screening of lactose-utilizing species described in the Yeast Monograph (Kurtzman et al., 2011a, 2011b; Kurtzman, Fell, & Boekhout, 2011c) followed by selection of the best performing and safe strains. Applicability of the selected strains was tested in laboratory scale fermentations for producing high-purity GOS from a commercial crude GOS syrup. Single- and two-step fermentation experiments were performed with *Cyberlindnera jadinii*, selected *Dekkera anomala* and *Kluyveromyces* strains to test their potential in selectively depleting glucose and, simultaneously or sequentially, removing lactose from a diluted low-purity GOS product.

2 | MATERIALS AND METHODS

2.1 | Screening of yeast literature database

Culture-independent screening of yeast species potentially applicable for removal of glucose, lactose and galactose from crude GOS syrup was carried out by searching the yeast database published in the Yeast Monograph (Kurtzman et al., 2011a, 2011b, 2011c). A detailed analysis of the species characteristics was performed by considering the following properties: assimilation and fermentation of glucose, lactose and galactose (both +, weak and variable); growth temperature; production of starch and metabolites (acids, carotenoids, etc.); pathogenicity.

2.2 | Culture media used in this work

2.2.1 | Minimal medium

0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4{}^*7H_2O$ and different concentrations of glucose (G) and lactose (L).

2.2.2 | Yeast extract medium

0.5% yeast extract (YE) and different concentrations of glucose (G) and lactose (L).

2.2.3 | GOS fermentation medium

Prepared by the dilution of Vivinal GOS syrup (see specification in Section 3.1) to either 10% or 15% (w/v) and supplemented with the salts of minimal medium (MM) or 0.5% YE.

2.3 | Growth curve analysis in microplate cultures

Minimal medium containing different concentrations of glucose and lactose was inoculated with yeast cells in the concentration of 10^6 cells per ml and 400 µl was pipetted into wells of the microplates. Growth curves were generated by incubating the plates in a microplate reader (Multiscan Ascent, Thermo Electron Corporation) for 24 or 48 h at 30°C and measuring the OD at 595 nm.

2.4 | Osmotic stress tolerance analysis

Growth response to osmotic stress was analysed by spot dilution growth assay. Tenfold dilutions were prepared from yeast stock suspensions (10^7 cells per ml) and 5-µl quantities from a serial dilution were pipetted onto MM plates containing glucose, lactose or Vivinal GOS syrup in different concentrations (2.5, 5.0, 7.5 and 10.0 w/v%)

corresponding to a molarity range of 6–55 mOsmol/L. Colony formation was tested after 48 hours of incubation at 30°C.

2.5 | Laboratory fermentations

A 20- or 100-ml GOS fermentation media in 100- or 500-ml Erlenmeyer flasks, respectively, were inoculated with 5×10^6 cells per ml and incubated by shaking with 220 rpm at 30°C. Samples for growth measurements (OD_{595}) and determination of saccharide composition (by high-performance liquid chromatography [HPLC]) were taken in 24-h intervals. Measurements were carried out in triplicates and repeated once to ensure reproducibility. Results are displayed as averages of the repeats with standard deviations.

2.5.1 | Preparation of primary fermentation medium for two-step GOS purification

GOS mixture obtained from *C. jadinii* NCAIM Y.00499 fermentation of GOS fermentation medium was used as the initial medium for the second fermentation step. To stop the first fermentation, cells were sedimented by centrifugation at 10,000 rmp for 5 min and the obtained supernatants were treated at 80°C for 30 min to inactivate any remaining cells. These media were then supplemented with either 0.4% YE or 0.2× MM, and pH was adjusted to 6.5. Concentrations of the supplementary compounds were determined by decreasing to the lowest possible amounts without effecting metabolic activity of the yeast strains.

2.6 | HPLC analysis

Analysis of GOS carbohydrates (glucose, galactose and DP2–DP6 fractions) and ethanol was carried out by HPLC. Samples were centrifuged at 10,000 rpm for 4 min, then supernatants were treated at 95°C for 10 min and filtered with 0.20-µm PES syringe filters (LAB-EX Ltd. Budapest, Hungary).

RNM Carbohydrate 8% Na+ 300 × 7.8 HPLC column (Phenomenex Inc., Torrance, USA) coupled with a guard column was used at 50°C, 0.2 ml/min with prefiltered (0.2 µm) distilled water as mobile phase. The system consisted of an SCM1000 degasser, a P200 gradient pump, and an AS100 autosampler including a built-in column oven (Thermo Fisher Scientific Inc. Waltham, MA, US). Peaks were detected by Shodex R-101 refractive index detector (Showa Denko Europe GmbH, Munich, Germany) at 50°C. Data collection and peak integration was performed with N2000 Chromatography Data System and N2000 Photographic Data Workstation software package (Science Technology Inc., Hangzhou, China). Peak identification and determination of the concentration of individual carbohydrate fractions was performed as described earlier in (Pázmándi, Maráz, Ladányi, & Kovács, 2018). Analytic grade ethanol was used as a standard to detect and quantify ethanol production.

2.7 | Evaluation of the selective fermentation processes

The performance indicators used to evaluate the selective fermentation processes were adopted from the work of Guerrero et al. (2014).

The initial purity (P_i) of the low-purity GOS product was calculated as follows:

$$P_i[\%] = \frac{C_{DP3-6,i}}{C_{total,i}} * 100, \quad (1)$$

where $C_{DP3-6,i}$ and $C_{total,i}$ are the content of DP3–6 and that of the total amount of carbohydrates (as a sum of glucose, galactose, lactose and nonlactose DP2, and DP3–6 fractions) in g/L concentrations at the initial phase of fermentation.

The final purity (P_f) of the fermented product is calculated as the ratio of DP3–6 fraction ($C_{DP3-6,\ f}$) to the total amount of carbohydrates ($C_{total,f}$) in g/L after fermentation, as follows:

$$P_f[\%] = \frac{C_{DP3-6,f}}{C_{total,f}} * 100, \quad (2)$$

Purification yield (Y) of fermentation processes is defined as follows:

$$Y[\%] = \frac{C_{DP3-6,f}}{C_{DP3-6,i}} * 100, \quad (3)$$

where $C_{DP3-6,i}$ and$C_{DP3-6,f}$ represent the concentration of DP3–6 fractions at the initial and final phase of fermentation, respectively.

2.8 | Data analysis

Creation of figures, hierarchical clustering and visualization by heatmap construction and statistical analysis by principal component analysis (PCA) and was carried out using statistical software RStudio Version 1.2.5042 (RStudio Inc., Boston, MA, USA).

3 | RESULTS AND DISCUSSION

3.1 | Specification of Vivinal GOS syrup

Vivinal GOS syrup (batch number 674796) manufactured by Friesland Campina Domo B. V. (Beilen, The Netherlands) was used as low-purity GOS in the fermentation experiments. An HPLC-refractive index analysis (see Section 2.6) revealed that the product is composed of 40% DP3–DP6 (24% DP3, 12% DP4 and 4% DP5–6), 39% DP2 (as the sum of lactose and nonlactose fractions), 19% glucose and 2% galactose on total carbohydrate basis. The DP profile reported by the manufacturer is in good agreement with the results of this HPLC measurement. Note that according to the manufacturer, the weight percentage of lactose and nonlactose DP2 fractions on total

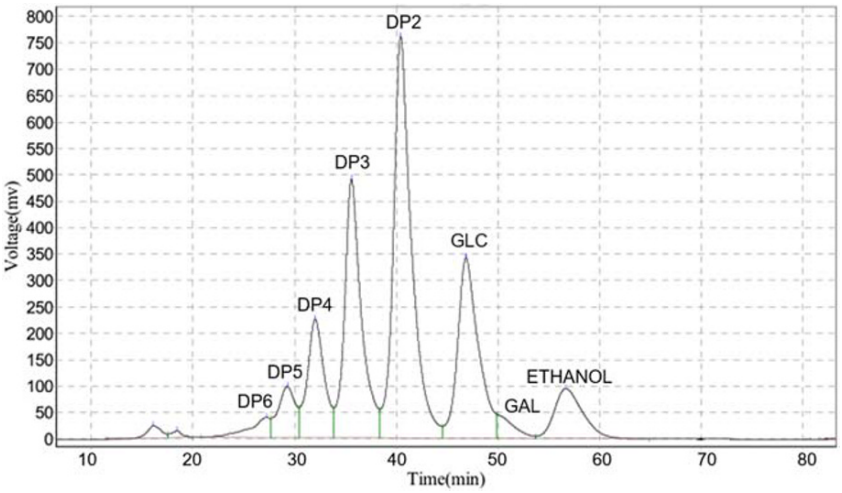

FIGURE 1 High-performance liquid chromatography chromatogram of 10% galacto-oligosaccharides + 0.5% YE fermentation medium spiked with 5 w/v% ethanol. DP, degree of polymerization [Colour figure can be viewed at wileyonlinelibrary.com]

carbohydrate basis are 21% and 18%, respectively. HPLC chromatogram of 10% GOS + YE fermentation medium spiked with ethanol is shown in Figure 1. A detailed structural analysis of the saccharide fractions in Vivinal GOS product can be found in Coulier et al., (2009).

3.2 | Selection of highly active yeast strains for lactose utilization

3.2.1 | Screening for lactose metabolizing species

Culture-independent screening was conducted based on the Yeast Monograph (Kurtzman et al., 2011a, 2011b, 2011c). Distribution of the lactose assimilating species among the ascomycetes and basidiomycetes is shown in Table 1. In total, 330 species representing 60 genera were defined as having either positive, weak or variable lactose assimilation characteristics. This takes up 41.1% and 25.5% of the total genera and species described in the Yeast Monograph, respectively. Lactose-utilizing species are approximately five times more frequent in the basidiomycetes than ascomycetes.

Further selection of the potentially applicable yeast species for GOS purification based on the safety of the species (pathogenicity and growth at 37°C), as well as the production of minor metabolites and starch was carried out in the next step. Following the exclusion of unfit candidates, the number of potentially applicable species was decreased to 143 (29 ascomycetes and 114 basidiomycetes) species, representing 29 genera altogether.

In our study, 75 representative strains of 59 potentially safe species deposited in culture collections were tested for lactose utilization. Based on the results, 34 strains belonging to 25 species proved to be potential lactose utilizing. Growth of the lactose-utilizing strains was compared by spreading the cells on the surface of MM containing 1% glucose (MMG) or lactose (MML). Results of cell growth and clustering of the strains are shown in Figure 2.

The selected 34 lactose-utilizing strains clustered in three main groups. Members of the first group exhibited strong growth on both glucose and lactose, while strains belonging to the second and third groups were stimulated more by glucose than lactose. Species representing the first group included most of the tested *K. lactis* and *K. marxianus* strains together with representatives of the *Candida* and *Cryptococcus* spp., and single strains belonging to *Dekkera anomala* and *Schwanniomyces castellii*. The second group of the strains exhibiting strong growth on glucose but weak on lactose contained majority of the *Candida* spp. along with representatives of *Schwanniomyces*, *Rhodotorula*,

TABLE 1 Distribution of lactose assimilating yeast species among the four main groups of yeasts as revealed by screening of yeast literature databases

	No. of genera			No. of species		
Groups of yeasts	Total	Lactose assimilating	%	Total	Lactose assimilating	%
Ascomycetes, teleomorphic	71	13	18.3	460	40	8.7
Ascomycetes, anamorphic	14	4	28.6	392	54	13.8
Subtotal	85	17	20.0	852	94	11.0
Basidiomycetes, teleomorphic	33	19	57.6	75	36	48.0
Basidiomycetes, anamorphic	28	24	85.7	366	200	54.6
Subtotal	61	43	70.5	441	236	53.5
Total	146	60	41.1	1,293	330	25.5

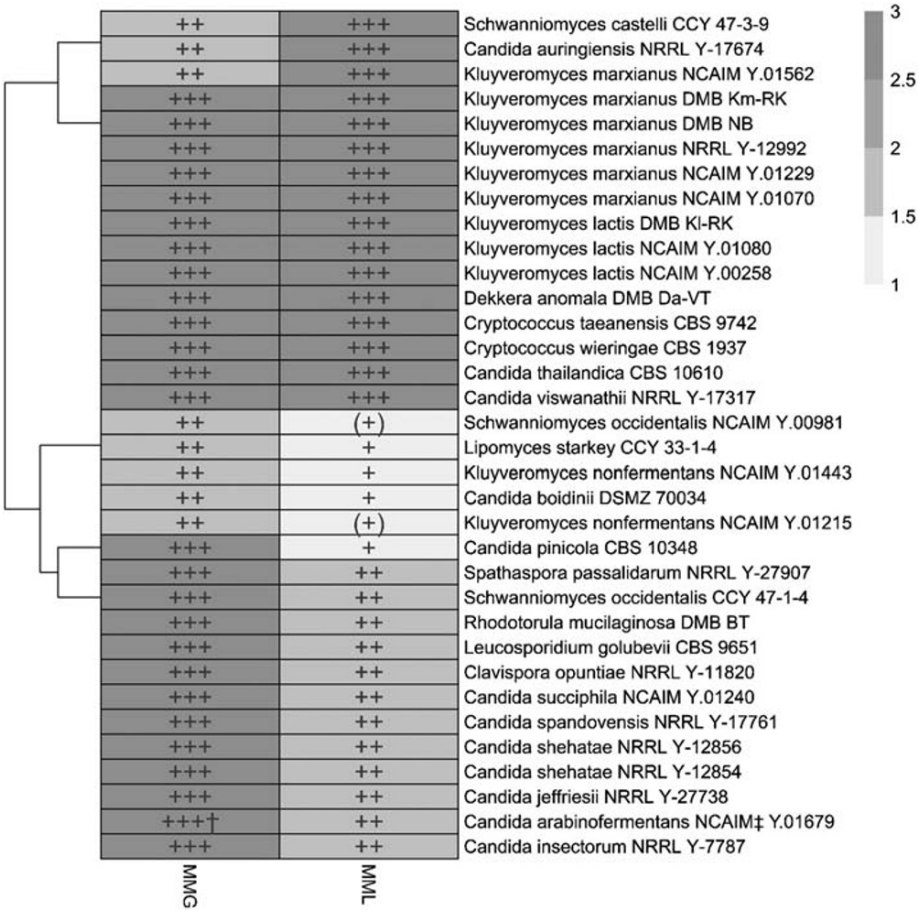

FIGURE 2 Growth of the preselected lactose-utilizing yeast strains on minimal medium (MM) containing 1% glucose (MMG) or lactose (MML) as a carbon source. (+), slight growth; +, moderate growth; ++, good growth; +++, intensive growth. Abbreviations: CBS, Centraalbureau voor Schimmel cultures, Utrecht; CCY, Czechoslovak Culture Collection of Yeasts, Bratislava; DMB, Department of Microbiology and Biotechnology, Szent István University, Budapest; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Szent István University, Budapest; NRRL, ARS Culture Collection, Northern Regional Research Lab, Peoria

Leucosporidium and *Clavispora* species. Strains clustered into the third (smallest) group had limited growth on both glucose and lactose, which included *K. nonfermentans* and some other ascomycetous yeasts.

Growth curves of the preselected 34 strains (listed in Figure 2.) were determined in microplates containing MM with 1% glucose or lactose as a carbon source. Twenty-two strains had poor growth in MML; ΔOD_{595} values after 24 h were negligible and therefore were excluded from further investigations (data not shown). The remaining 12 strains reached ΔOD_{595} between 0.520 and 0.881 in MML after 24-h cultivation, which represented 55%–95% of the ΔOD_{595} values in MMG (Figure 3).

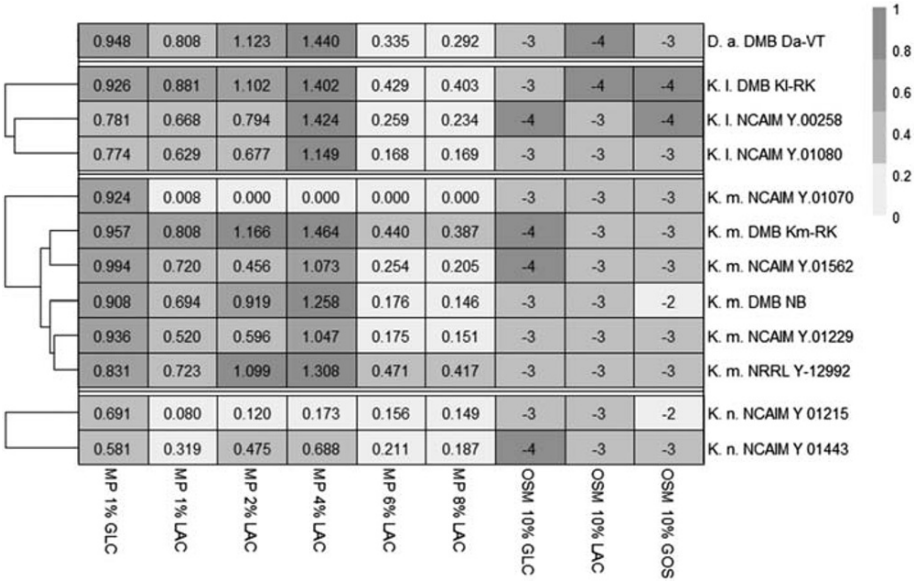

FIGURE 3 Growth of yeast strains in microplate culture (MP) and their osmotic tolerance (OSM) in minimal media (MM) containing various concentrations of glucose (GLC), lactose (LAC) and galacto-oligosaccharides syrup (GOS). In the case of OSM, numbers indicate the last member of the serial dilution where macrocolony formation was observed. In case of the MPs, ΔOD_{595} values belonging to the stationary phase are shown. Abbreviations: K. l.: *Kluyveromyces lactis*; K. m.: *Kluyveromyces marxianus*; K. n: *Kluyveromyces nonfermentans*; D. a.: *Dekkera anomala*

3.2.2 | Influence of the increasing lactose concentration on growth and testing of osmotic sensitivity

Twelve strains with significant growth in MML microcultures belonged to the species *K. lactis*, *K. marxianus*, *K. nonfermentans* and *D. anomala*. In the next step, these strains were investigated for growth in microplates containing elevating lactose concentrations (4%, 6% and 8%) and tested for osmotic sensitivity to glucose, lactose and GOS (2.5%, 5.0%, 7.5% and 10%), respectively. Evaluation of the growth and osmotic tolerance is shown in Figure 3. Elevation of lactose concentration to 2% and 4% resulted in higher ΔOD_{595} values compared with 1% lactose. However, 6% and 8% lactose had an inhibitory effect on growth, as ΔOD_{595} values were only 20%–70% of those in 1% lactose MM. These results confirmed that 2%–3% lactose is optimal for *K. lactis* and *K. marxianus*, and concentrations above 5% decrease the growth rates (Braga, Gomes, & Kalil, 2012; Grubb & Mawson, 1993; Lodi & Donnini, 2005; Manera et al., 2008; Yönten, 2013). Although *K. nonfermentans* is a lactose-positive species and *D. anomala* is variable from this respect (Kurtzman et al., 2011b; Nagahama, Hamamoto, Nakase', & Horikoshil, 1999), their lactose utilization characteristics and growth kinetics on lactose have not been studied yet.

Results of the osmotic sensitivity testing showed that majority of the strains had good osmotic tolerance, as macrocolonies were formed in every member of the decimal dilution series in the presence of up to 7.5% sugars. Growth was observed in the 10^{-3} or 10^{-4} dilutions on MMG and MML, but differences were the most apparent in the presence of 10% GOS. Majority of the strains formed colonies in the10^{-3} dilutions, while some of them only in the 10^{-2} dilution (Figure 3). *D. anomala* (anamorph *Brettanomyces anomalus*) is a stress-tolerant spoilage yeast with exceptional metabolic diversity (Cosentino, Fadda, Deplano, Mulargia, & Palmas, 2001; Steensels et al., 2015). This explains why the strain DMB Da-VT, isolated from a dairy product (Vasdinyei & Deák, 2003), has the potential for efficient lactose utilization and osmotolerance.

Strain selection was evaluated by PCA, including the results of the osmotic tolerance tests in the presence of 10% GOS syrup and growth of the strains in MM containing glucose (1% glc, 24 h) or lactose (1% lac, 24 h and 4% lac, 48 h), as these traits showed the greatest variance among the strains (Figure 4). *K. lactis* DMB KI-RK and *K. marxianus* DMB Km-RK (both originated from cottage cheese) excelled in terms of their ability to increase the cell mass as the concentration of lactose increased up to 4% and were therefore selected for further investigations. Although *K. nonfermentans* NCAIM Y.01443 did not show outstanding results in growth on lactose, its acceptable osmotic tolerance and, more importantly, its inability to produce ethanol makes it a promising candidate for purification of GOS syrup and was therefore included in further experiments. *D. anomala* and numerous *Kluyveromyces* strains showed comparable 'average' results, but *D. anomala* DMB Da-VT was selected for additional experiments as the behaviour of this species has not been studied yet under GOS fermentation conditions.

3.2.3 | Screening for glucose metabolizing species with low or no ethanol production

An additional target of the strain selection was to find robust, Crabtree negative glucose assimilating yeast species—irrespective of their lactose utilization properties—which are weak or zero ethanol producers and could be safely used for depletion of glucose from low-purity GOS.

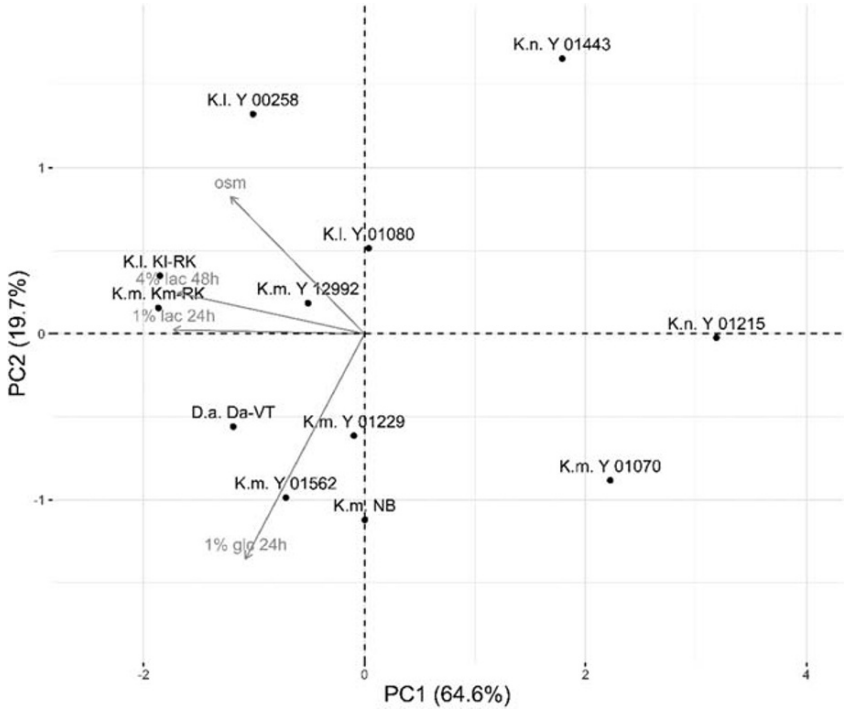

FIGURE 4 Principal component analysis (PCA) generated from the analysis of osmotic tolerance in the presence of 10% galacto-oligosaccharides syrup as well as from the growth analysis in minimal medium containing glucose (1% glc 24 h) or lactose (1% lac 24 h and 4% lac 48 h). Abbreviations: K.l.: *Kluyveromyces lactis*; K.m.: *Kluyveromyces marxianus*; K.n: *Kluyveromyces nonfermentans*; D.a.: *Dekkera anomala*

Yeast strains *Komagatella pastoris* (syn. *Pichia pastoris*) NCAIM Y.01243, *Komagatella pseudopastoris* (syn. *Pichia pseudopastoris*) NCAIM Y.01541, *Schwanniomyces occidentalis* NCAIM Y.00981 and *Cyberlindnera jadinii* (Anamorph: *Candida utilis*) NCAIM Y.00383 and NCAIM Y.00499 were tested for this purpose by cultivation in 20-ml MM culture media containing 5% glucose or 10% GOS syrup in 100-ml Erlenmeyer flasks. Results of the 72-h long fermentation experiments, shown in Table 2, revealed great differences in the growth and glucose assimilation ability of the strains in both fermentation media. *K. pastoris*, *K. pseudopastoris* and *S. occidentalis* strains had poor growth under the applied conditions as cell number increased only by one order of magnitude during 72-h cultivation. Glucose depletion was below 25% irrespective if glucose was present as single sugar component of MM (5%) or together with the other saccharides of the 10% GOS syrup. Final cell concentrations of the *C. jadinii* strains was one order of magnitude higher than that of the previously mentioned strains and the glucose depletion was above 50%. *C. jadinii* NCAIM Y.00499 excelled with glucose consumption close to 100% in both fermentation media; therefore, it was selected for scale-up studies focusing on glucose removal from diluted crude GOS syrup. Ethanol content of the culture media fermented with any of the five strains was negligible. It has to be mentioned that neither of the tested strains decreased the galactose content of the GOS medium and the amount of the DP2–DP6 fractions were not influenced.

3.3 | Fermentation of GOS syrup for the depletion of glucose by *C. jadinii* NCAIM Y.00499

The selected *C. jadinii* NCAIM Y.00499 was inoculated into 200 ml either 10% or 15% GOS syrup supplemented with MM or 0.5% YE. Growth of this strain was similar in all types of GOS media as shown in Table 3.

Carbohydrate profiles obtained throughout the fermentation (Figure 5) revealed that *C. jadinii* Y.00499 depleted glucose in 24 h from both 10% or 15% GOS syrup supplemented with 0.5% YE (Figure 5a,c) or 10% GOS syrup supplemented with 1 × MM (Figure 5b). Glucose utilization was somewhat slower in 15% GOS syrup + MM as complete removal took 48 h (Figure 5d).

TABLE 2 Growth, glucose utilization and ethanol production of yeast strains in 5% glucose + MM and 10% GOS + MM media during 72 h of cultivation

	5% Glucose + MM			10% GOS + MM		
Yeast strains	Cell cc. ($\times 10^8$ cell per ml)	Glucose utilization (%)	Ethanol (g/L)	Cell cc. ($\times 10^8$ cell per ml)	Glucose utilization (%)	Ethanol (g/L)
Komagatella pastoris NCAIM Y.01243	0.48 ± 0.02	17.13 ± 0.73	0.00 ± 0	0.28 ± 0.01	8.73 ± 0.16	0.00 ± 0
Komagatella pseudopastoris NCAIM Y.01541	0.35 ± 0.00	11.35 ± 0.23	0.00 ± 0	0.50 ± 0.01	8.60 ± 0.04	0.00 ± 0
Schwanniomyces occidentalis NCAIM Y.00981	0.57 ± 0.02	24.76 ± 0.10	0.19 ± 0.07	0.49 ± 0.00	17.93 ± 0.30	1.03 ± 0.03
Cyberlindnera jadinii NCAIM Y.00499	2.51 ± 0.11	99.69 ± 3.70	0.00 ± 0	2.66 ± 0.12	95.25 ± 0.69	1.06 ± 0.02
Cyberlindnera jadinii NCAIM Y.00383	1.95 ± 0.04	52.06 ± 1.08	0.00 ± 0	2.07 ± 0.02	73.05 ± 1.01	0.00 ± 0

Abbreviations: GOS, galacto-oligosaccharides; MM, minimal medium.

TABLE 3 Cell concentrations of yeast strains after 72-h fermentation of 10% or 15% GOS syrup supplemented with yeast extract (YE) or minimal salts (MM). Initial cell concentration: 5×10^6 cells per ml

		Cell concentrations (cells per ml $\times 10^8$)			
Fermentation	Yeast strains	10% GOS + YE	10% GOS + MM	15% GOS + YE	15% GOS + MM
Single-step	*Cyberlindnera jadinii* NCAIM Y.00499	3.37 ± 0.10	3.14 ± 0.16	3.50 ± 0.10	3.73 ± 0.13
	Dekkera anomala DMB Da-VT	1.62 ± 0.18	1.13 ± 0.26	–	–
	Kluyveromyces lactis DMB Kl-RK	3.03 ± 0.20	2.17 ± 0.10	–	–
	Kluyveromyces marxianus DMB Km-RK	3.36 ± 0.19	2.47 ± 0.49	–	–
	Kluyveromyces nonfermentans NCAIM Y.01443	1.66 ± 0.03	1.50 ± 0.00	–	–
Two-step	*K. lactis* DMB Kl-RK	2.42 ± 0.02	2.20 ± 0.00	2.16 ± 0.01	2.88 ± 0.05
	K. marxianus DMB Km-RK	1.46 ± 0.06	1.96 ± 0.05	1.81 ± 0.04	2.92 ± 0.07
	K. nonfermentans NCAIM Y.01443	0.65 ± 0.13	0.89 ± 0.06	1.611 ± 0.05	1.29 ± 0.00

Abbreviations: GOS, galacto-oligosaccharides; MM, minimal medium.

Unlike in previous reports on selective fermentation of crude GOS by *S. cerevisiae* (Giacomelli et al., 2015; Goulas et al., 2007; Hernández et al., 2009; Li et al., 2008), ethanol was either not produced by *C. jadinii* Y.00499 in detectable levels (Figure 5b) or it was consumed during prolonged fermentation (Figure 5a,c,d). No cell lysis or degradation of DP2–DP6 compounds was detected; thus, *C. jadinii* Y.00499 has a great potential in manufacturing glucose-free products, with the added benefit of the final product being ethanol free. Here, we report for the first time the efficacy of *C. jadinii* in selectively depleting glucose from a crude GOS syrup without affecting the quantity of GOS components.

C. jadinii can be considered as a safe yeast because it has been approved as GRAS (generally recognized as safe) by the Food and Drug Administration and obtained the QPS (Qualified Presumption of Safe) status for production purposes from the EFSA (European Food Safety Authority) (EFSA Panel on Biological Hazards (BIOHAZ), 2012). Therefore, *C. jadinii* could be used safely for the production of glucose-free GOS; moreover, the yeast biomass can be considered as a valuable by-product and could be valorised as dried fodder yeast and for preparation of bioemulsifiers, cell wall mannans and glucans as well (Buerth, Tielker, & Ernst, 2016; Campos, Stamford, & Sarubbo, 2014).

3.4 | One-step fermentation of GOS syrup for simultaneous removal of glucose and lactose: Application of *D. anomala*, *K. lactis*, *K. marxianus* and *K. nonfermentans* strains

Simultaneous removal of glucose and lactose from diluted GOS syrup in a single-step fermentation was performed with the selected *D. anomala* DMB Da-VT and *Kluyveromyces* strains (*K. lactis* DMB

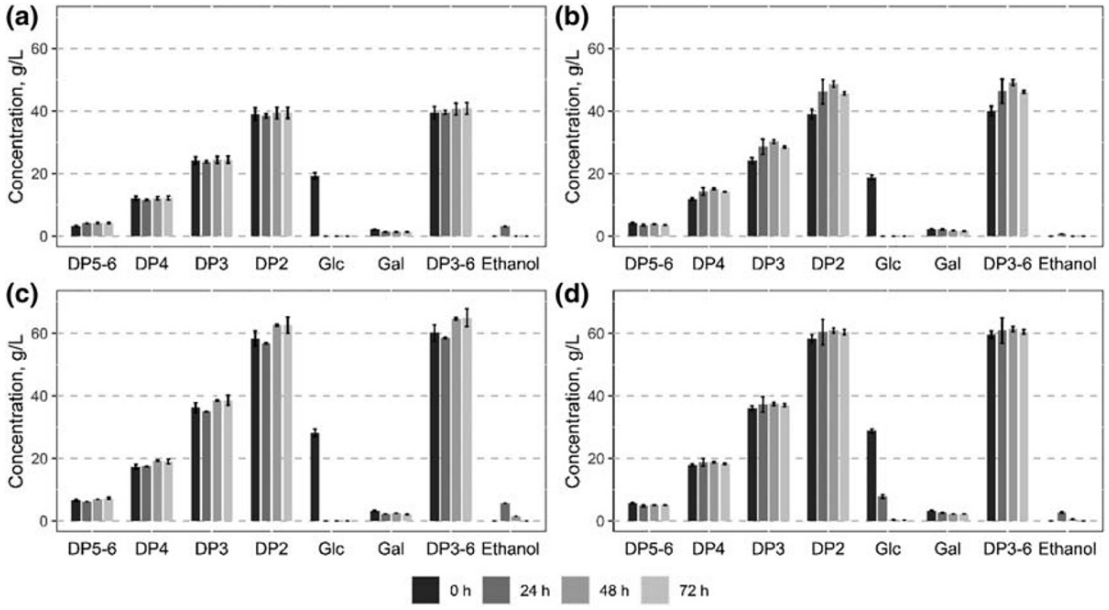

FIGURE 5 Changes in the carbohydrate concentrations during fermentation of diluted galacto-oligosaccharides (GOS) syrup with *Cyberlindnera jadinii* NCAIM Y.00499 (a) 10% GOS syrup + 0.5% yeast extract (YE) (b) 10% GOS syrup + minimal medium (MM) (c) 15% GOS syrup + 0.5% YE and (d) 15% GOS syrup + MM

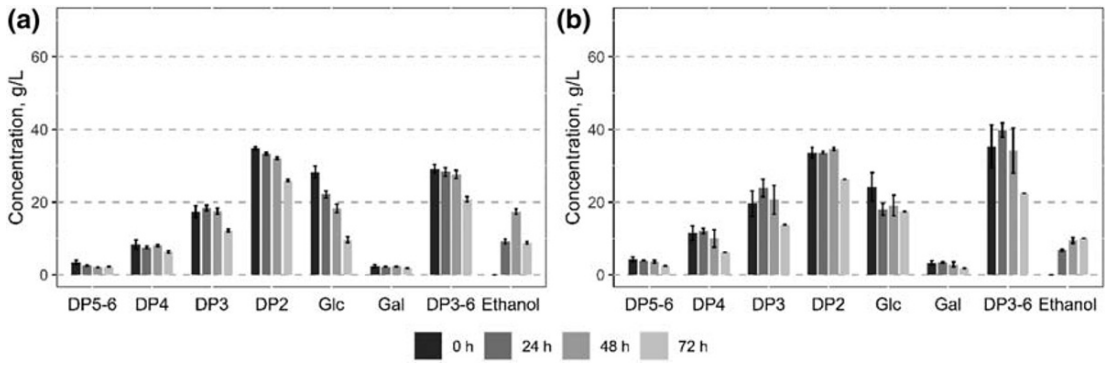

FIGURE 6 Changes in the carbohydrate concentrations during fermentation of diluted GOS syrup with *Dekkera anomala* DMB Da-VT (a) 10% GOS syrup + 0.5% YE (b) 10% GOS syrup + MM

KI-RK, *K. marxianus* DMB Km-RK and *K. nonfermentans* NCAIM Y.01443). Three of these species (*D. anomala*, *K. lactis* and *K. marxianus*) are known to possess lactose fermenting ability, while *K. nonfermentans* can assimilate but not ferment any sugars.

In the fermentation trials, diluted GOS syrup (10%) was complemented with 0.5% YE or MM salts for supporting the metabolic activity of these strains. Final cell concentrations of the cultures, shown in Table 3, indicate that the growth of *K. lactis* DMB KI-RK and *K. marxianus* DMB Km-RK in 10% GOS + YE medium was comparable with that of *C. jadinii*, while complementation of 10% GOS with MM salts resulted slightly decreased cell mass production. *D. anomala* DMB Da-VT and *K. nonfermentans* Y.01443 displayed less vigorous growth than the other three strains in GOS fermentation medium supplemented with either YE or MM salts. Figures 6–9 illustrate consumption of sugars throughout the 72-h fermentation cycles with *D. anomala* DMB Da-VT, *K. lactis* DMB KI-RK, *K. marxianus* DMB Km-RK and *K. nonfermentans* NCAIM Y.01443, respectively.

Depletion of monosaccharides and disaccharides by *D. anomala* DMB Da-VT from 10% GOS medium was slow and very limited (Figure 6). About half of the glucose was consumed for the end of fermentation and decrease of the DP2 fraction was not significant. Galactose content did not change at all, while considerable amount of ethanol was produced. The DP3–DP6 fractions decreased in the last period of fermentation, which was probably the consequence of partial cell lysis and activity of the released β-galactosidase. Cell lysis was monitored with discrimination of the viable and dead cells by microscopic analysis of rhodamine B stained samples taken in the time intervals, which indicated the progress in cell lysis when the ratio of dead cells increased above approximately 50% (data not shown). Cell lysis resulted in releasing of ß-galactosidase into the GOS fermentation medium and, as the consequence, not only DP2 but also the DP3–DP6 fractions were hydrolysed partly as it could be observed in the HPLC chromatograms. In some cases, there was an increase in the glucose without rise in the galactose content, indicating significant hydrolysis of the cell wall glycan. These observations point to the fact that *D. anomala* DMB Da-VT is unfit for purification of GOS as cells easily disintegrate and result in uncontrolled hydrolysis of GOS fractions. This strain was therefore left out from the potential candidates and not included in further experiments.

Results of the HPLC analysis showed that both *K. lactis* and *K. marxianus* strains consumed glucose fast and completely, irrespectively if GOS was complemented with MM or YE (Figures 7 and 8), with *K. marxianus* DMB Km-RK being more vigorous in glucose depletion. Consumption of lactose (included in the DP2 fraction) was negligible by these two strains in GOS + MM medium (Figures 7b and 8b); however, when GOS was complemented with YE (Figures 7a and 8a), majority of the lactose content was removed by *K. lactis* and *K. marxianus*, respectively. In contrast, when GOS was complemented

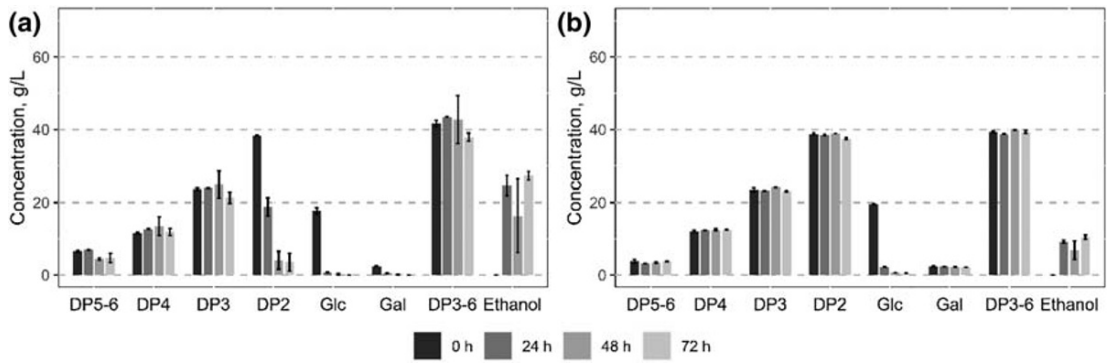

FIGURE 7 Changes in the carbohydrate concentrations during fermentation of diluted GOS syrup with *Kluyveromyces lactis* DMB KI-RK (a) 10% galacto-oligosaccharides (GOS) syrup + 0.5% yeast extract (b) 10% GOS syrup + minimal medium

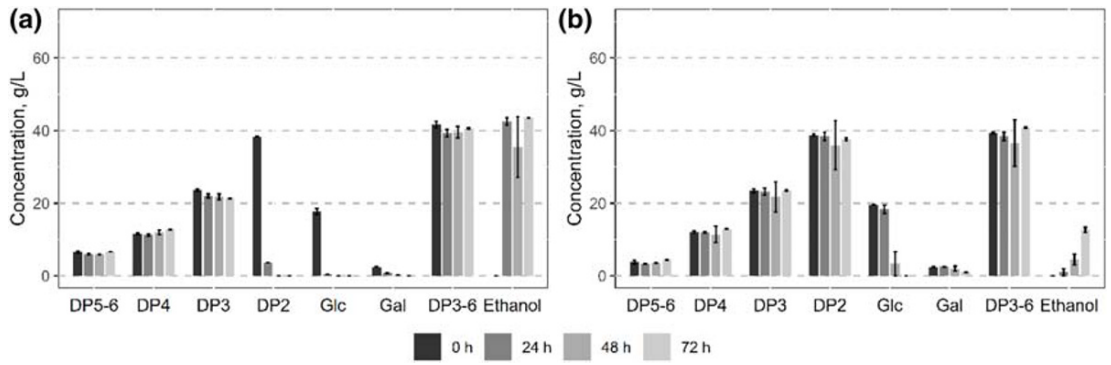

FIGURE 8 Changes in the carbohydrate concentrations during fermentation of diluted GOS syrup with *Kluyveromyces marxianus* DMB Km-RK (a) 10% galacto-oligosaccharides (GOS) syrup + 0.5% yeast extract (b) 10% GOS syrup + minimal medium

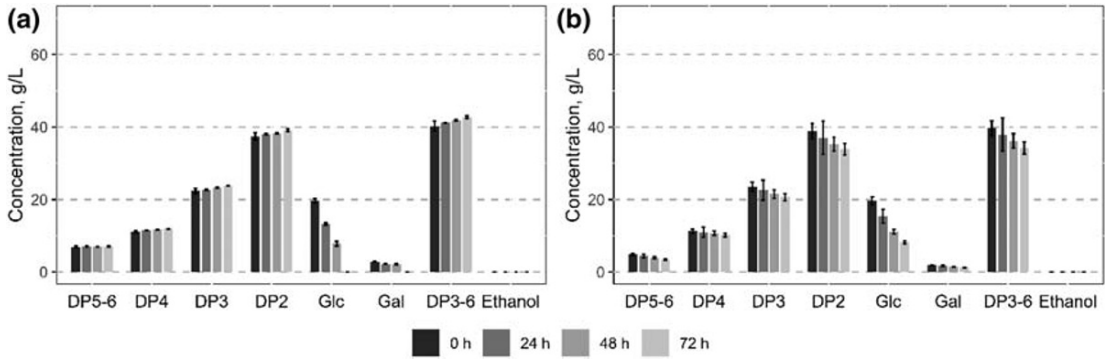

FIGURE 9 Changes in the carbohydrate concentrations during fermentation of diluted GOS syrup with *Kluyveromyces nonfermentans* NCAIM Y.01443 (a) 10% galacto-oligosaccharides (GOS) syrup + 0.5 % yeast extract (b) 10% GOS syrup + minimal medium

with YE (Figures 7a and 8a), lactose depletion was extensive or complete by *K. lactis* and *K. marxianus*, respectively. Considering that non-lactose components comprise about half of the DP2 fraction, it could be concluded that both *K. lactis* DMB Kl-RK and *K. marxianus* DMB Km-RK are able to utilize not only lactose but also the other DP2 components of GOS. Galactose was used up completely by both strains in GOS + YE medium but only partially if diluted GOS syrup was complemented with MM salts. We also note that both strains generated considerable amounts of ethanol (up to 4%) depending on the fermentation conditions. Therefore, although high-purity GOS can be produced via fermentation by *K. lactis* DMB Kl-RK and *K. marxianus* DMB Km-RK, removal of ethanol from the final product is necessary.

Glucose and lactose utilization by *K. nonfermentans* Y.01443 was less vigorous than by the other two *Kluyveromyces* strains (Figure 9). Complete removal of glucose and galactose took 72 h when GOS medium was complemented with YE (Figure 9a), with no detectable change in the other saccharide fractions. In 10% GOS + MM medium, sugar depletion was even less intense (Figure 9b). Only about half of the glucose was consumed without a pronounced change in the concentration of the other saccharide fractions. When using 10% GOS with 0.5% YE, a glucose-, galactose- and ethanol-free product was obtained. A notable advantage of this strain is its inability to produce ethanol, which reduces the complexity of the required downstream processes for product purification. An extensive strain selection or genetic improvement might lead to metabolically more active strains in GOS purification.

Table 4 summarizes the observed yield (Y) and purity (P) values for the single-step fermentation by *Kluyveromyces* strains. It could be concluded that propagation of *K. lactis* DMB Kl-RK and *K. marxianus* DMB Km-RK in GOS fermentation medium supplemented with minimal salts resulted in no loss of the DP3–DP6 fractions (i.e., $Y \simeq 100\%$) but with a moderate P_f levels (approximately 50%) in both cases. A further improvement in final purity was realized for both strains when GOS syrup was supplemented with YE instead of MM salts. Among the two strains, *K. marxianus* DMB Km-RK had better performance, resulting in a yield of 97.5% and a final purity of 100%. These observations are in good agreement with the yields reported in previous studies on *K. lactis* (Li et al., 2008; Santibáñez et al., 2017; Sun et al., 2016) and *K. marxianus* (Cheng et al., 2006; Guerrero et al., 2014). However, initial cell loads (0.0005–0.0007 $g_{cell}/g_{carbohydrate}$) used in this study are considerably lower than in the previous reports by Santibáñez et al. (2017), Sun et al. (2016) and Li et al. (2008) (0.375, 0.08 and 2.5 $g_{cell}/g_{carbohydrate}$, respectively). The use of low initial cell concentrations raises the competitiveness of the selective fermentation as a potential downstream purification process that could replace the SMB chromatography. Only partial purification of 10% GOS + MM was achieved by *K. nonfermentans* Y.01443. However, when 10% GOS medium was complemented with 0.4% YE, the DP3-DP6 fractions were conserved, and the resulted product was free from glucose, galactose and ethanol. Comparison of growth results (Table 3) with data of GOS syrup purification (Table 4) reveals that complementation of GOS fermentation medium with YE instead of MM salts is advantageous regarding both growth and purification (Y and P) performance of the *Kluyveromyces* strains.

3.5 | Two-step fermentation of GOS syrup: Removal of glucose in the first step by *C. jadinii* and lactose in the second step by *Kluyveromyces* strains

As we demonstrated in Section 3.3, *C. jadinii* Y.00499 effectively removes glucose from the GOS syrup while preserving the DP2–DP6 fractions. However, it lacks the ability to utilize lactose and galactose. In order to produce a lactose-, galactose- and glucose-free GOS formulation, implementation of a two-step fermentation process was investigated. In the first step, *C. jadinii* was used to deplete glucose from the diluted GOS syrup. The resulted product was then refermented with *Kluyveromyces* strains in the second step to remove lactose and galactose.

In our approach, as the first fermentation step, culture media containing either 10% or 15% GOS enriched with 0.5% YE were fermented for 48 h by *C. jadinii* Y.00499 for glucose removal. Then, the separated fermentation media were applied in the second fermentation step, as described in Section 2.5.1. The aim of the secondary fermentation was further depletion of lactose and galactose from partially purified GOS by the application of *Kluyveromyces* strains already tested in single-step fermentations. It was necessary to supply these strains with inorganic or organic macroelements and

TABLE 4 Performance of *Kluyveromyces* strains in terms of yield (*Y*) and purity (*P*) in single-step and two-step fermentation trials

Fermentation	Culture medium	Strains	Y^a (%)	P_i^b (%)	P_f^c (%)
Single-step	10% GOS syrup + 1 × MM	*Kluyveromyces lactis* KI-RK	100.0 ± 0.42	39.3 ± 0.29	49.57 ± 0.21
		***Kluyveromyces marxianus* Km-RK**	**100.0** ± 1.00	**39.3 ± 0.29**	**51.44 ± 0.51**
		Kluyveromyces nonfermentans Y.01443	86.37 ± 0.41	39.6 ± 0.00	44.16 ± 0.21
	10% GOS syrup + 0.5% YE	*K. lactis KL-RK*	90.94 ± 0.86	41.6 ± 0.88	91.49 ± 0.87
		***K. marxianus* Km-RK**	**97.43** ± 2.51	**41.6 ± 0.88**	**100.0 ± 2.58**
		K. nonfermentans Y.01443	100.0 ± 3.24	39.7 ± 0.11	52.18 ± 1.46
Two-step	10% GOS syrup + 0.5% YE fermented with *Cyberlindnera jadinii* Y.00499 + 0.2 × MM	***K. lactis* KI-RK**	**64.60 ± 0.10**	**49.2 ± 0.15**	**78.12 ± 0.12**
		K. marxianus Km-RK	100.0 ± 2.73	49.2 ± 0.15	55.68 ± 1.52
		K. nonfermentans Y.01443	89.19 ± 1.96	49.2 ± 0.15	50.80 ± 1.12
	10% GOS syrup + 0.5% YE fermented with *Cyberlindnera jadinii* Y.00499 + 0.4% YE	***K. lactis* KI-RK**	**100.0 ± 0.43**	**49.2 ± 0.15**	**92.07 ± 0.40**
		K. marxianus Km-RK	100.0 ± 0.39	49.2 ± 0.15	61.79 ± 0.24
		K. nonfermentans Y.01443	95.76 ± 2.76	49.2 ± 0.15	53.66 ± 1.55
	15% GOS syrup + 0.5% YE fermented with *Cyberlindnera jadinii* Y.00499 + 0.2 × MM	***K. lactis* KI-RK**	**99.10 ± 0.06**	**44.3 ± 0.04**	**66.47 ± 0.04**
		K. marxianus Km-RK	97.47 ± 2.83	44.3 ± 0.04	48.94 ± 1.42
		K. nonfermentans Y.01443	97.26 ± 2.37	44.3 ± 0.04	46.29 ± 1.13
	15% GOS syrup + 0.5% YE fermented with *Cyberlindnera jadinii* Y.00499 + 0.4% YE	***K. lactis* KI-RK**	**93.16 ± 4.38**	**44.3 ± 0.04**	**65.45 ± 3.08**
		K. marxianus Km-RK	96.91 ± 0.99	44.3 ± 0.04	54.77 ± 0.56
		K. nonfermentans Y.01443	99.75 ± 4.22	44.3 ± 0.04	46.25 ± 1.96

Note. Best results are indicated by bold lettering.
Abbreviation: GOS, galacto-oligosaccharides.
[a]Yield.
[b]Initial purity.
[c]Final purity.

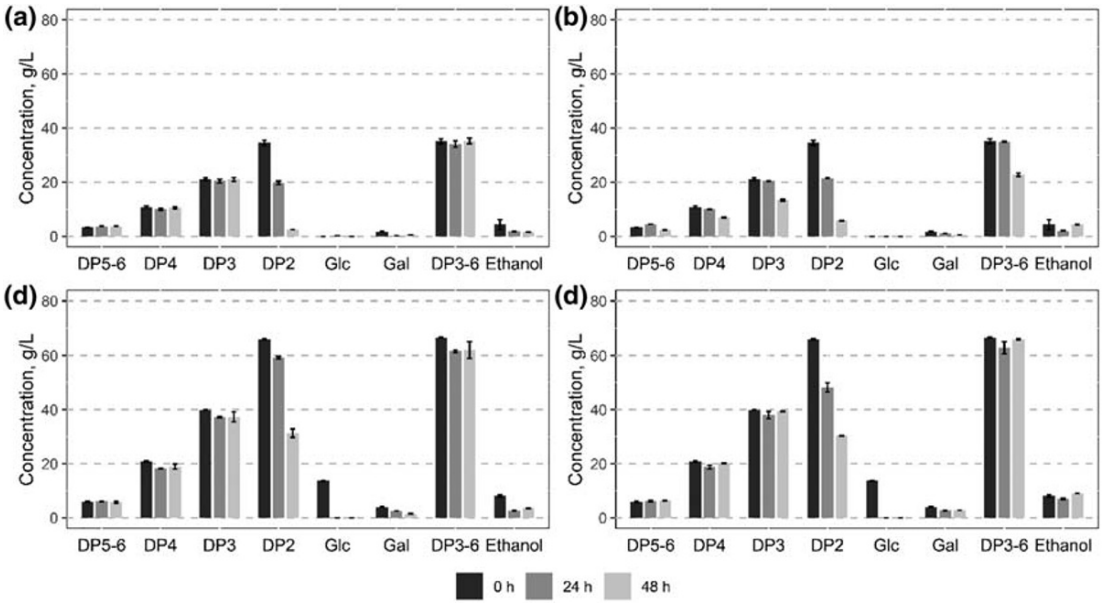

FIGURE 10 Changes in the carbohydrate concentrations during secondary fermentation with *Kluyveromyces lactis* DMB KI-RK. Primary fermentation of 10% (a,b) and 15% (c,d) galacto-oligosaccharides (GOS) syrup + 0.5% yeast extract (YE) was performed with *Cyberlindnera jadinii* NCAIM Y.00499. (a) Primary fermentation medium complemented with 0.4% YE; (b) primary fermentation medium complemented with 0.2×minimal medium (MM); (c) primary fermentation medium complemented with 0.4% YE; (d) primary fermentation medium complemented with 0.2 × MM

microelements, therefore primary fermentation medium were complemented with 0.2 × MM or 0.4% YE. Results are shown in Figures 10–12.

The initial fermentation media, 10% GOS + YE and 15% GOS + YE, obtained after 48 h of fermentation by *C. jadinii* Y.00499, contained only small or no detectable amounts of glucose. Amounts of galactose and DP2–DP6 fractions in the fermentation media were close to the quantity in the original GOS + YE media. Due to the shorter (48 h) fermentation activity of *C. jadinii*, small amounts of ethanol (approximately 0.2%) were also present in the initial fermentation media.

K. lactis DMB KI-RK managed to remove DP2 fraction from 10% GOS + YE (Figure 10a), while fermentation of 10% GOS + MM was

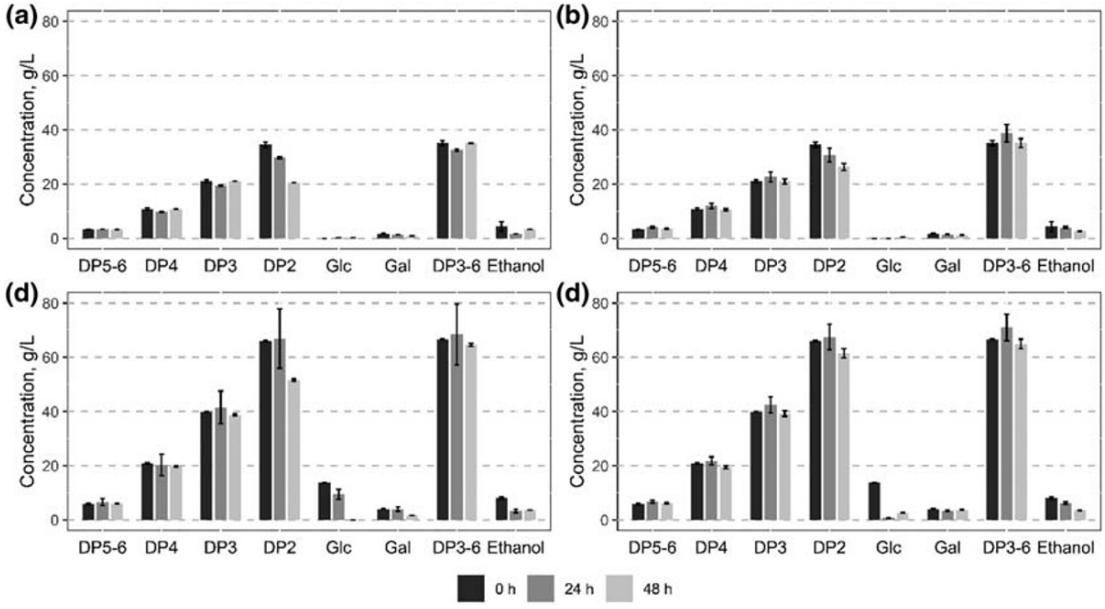

FIGURE 11 Changes in the carbohydrate concentrations during secondary fermentation with *Kluyveromyces marxianus* DMB Km-RK. Primary fermentation of 10% (a,b) and 15% (c,d) galacto-oligosaccharides (GOS) syrup + 0.5% yeast extract (YE) was performed with *Cyberlindnera jadinii* NCAIM Y.00499. (a) Primary fermentation medium complemented with 0.4% YE; (b) primary fermentation medium complemented with 0.2 × minimal medium (MM); (c) primary fermentation medium complemented with 0.4% YE; (d) primary fermentation medium complemented with 0.2 × MM

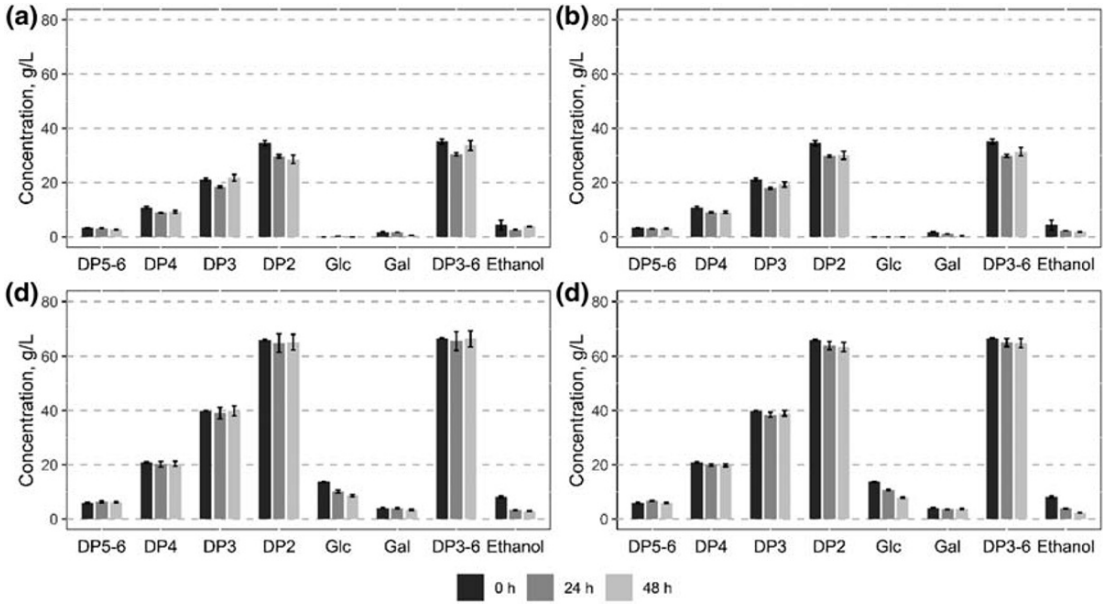

FIGURE 12 Changes of the carbohydrate concentrations during secondary fermentation with *Kluyveromyces nonfermentans* NCAIM Y.01443. Primary fermentation of 10% galacto-oligosaccharides (GOS) syrup+ 0.5% yeast extract (YE) was performed with *Cyberlindnera jadinii* NCAIM Y.00499 (a) primary fermentation medium complemented with 0.4% YE (b) primary fermentation medium complemented with 0.2 × minimal medium (MM) (c) primary fermentation medium complemented with 0.4% YE (d) primary fermentation medium complemented with 0.2 × MM

incomplete, and a drop in ΣDP3–6 level was observed after 48 h (Figure 10b). Purification of 15% GOS was less effective, as only about half of the initial lactose was removed with both types of GOS supplementation (Figure 10c,d).

K. marxianus DMB Km-RK was less active compared with *K. lactis* DMB Kl-RK in all types of media. Only about a third of DP2 was removed from 10% GOS media (Figure 11a,b), and although glucose was removed from 15% GOS media completely, decrease in the concentration of DP2 compounds was limited (Figure 11b,d).

K. nonfermentans Y.01443 was the least vigorous out of the three strains (Figure 12), as in 10% GOS media DP2 fraction was only slightly depleted, regardless of the supplementation (Figure 12a,b). Sugar contents of 15% GOS media remained relatively unchanged, only a small portion of glucose was utilized by this strain and ethanol produced in the first fermentation was consumed only partially (Figure 12c,d).

It is important to note that although concentrations of the DP3–DP6 fractions did not change considerably during 48 h fermentation with the applied *Kluyveromyces* strains, prolonged fermentation time caused the decrease in their concentrations. This was probably the consequence of partial cell lysis and activity of the released β-galactosidase, which was more pronounced in case of complementation with 0.4% YE than 0.2 MM.

The overall performance of the two-step fermentation processes, expressed in terms of yield and purity, is reported in Table 4. Considering the secondary fermentation of all GOS media, it could be concluded that *K. lactis* DMB Kl-RK was most active in the consumption of glucose, galactose and DP2 compounds, followed by *K. marxianus* DMB Km-RK, while *K. nonfermentans* Y.01443 showed the weakest purification characteristics. The best result (Y = 100% and P_f = 92%) was obtained by *K. lactis* DMB Kl-RK in 10% GOS + YE.

The purification performance of the strains (Table 4) is related to their growing potential (Table 3) in different GOS media, indicating that depletion of the unwanted components from a crude GOS syrup is likely associated with complex, growth-dependent metabolic processes rather than bioconversion.

4 | CONCLUSION

In this work, we aimed to investigate the opportunity of applying novel nonconventional lactose-utilizing yeast species for the production of high-purity GOS. For this purpose, screening of a yeast literature database was conducted, followed by the application of culture-dependent techniques, which allowed the selection of candidates for tolerating the harsh fermentation conditions of properly diluted crude GOS syrup media. Not surprisingly, *K. lactis* and *K. marxianus* strains were in the first line, although their wide metabolic diversity and different patterns of sensitivity for stress factors (González-Siso et al., 2000; Lane & Morrissey, 2010) necessitated the selection of the most robust strains. Interestingly, *K. lactis* and *K. marxianus* as well as a further selected *D. anomala* strain all originated from dairy products, which indicates strong metabolic adaptation potential of these yeast species.

Following strain selection, fermentation experiments using diluted low-purity GOS were conducted. Based on our results, we propose three GOS-purification strategies, the first being the selective removal of glucose with either the respiro-fermentative *C. jadinii* Y.00499 or the nonfermentative *K. nonfermentans* Y.01443. *C. jadinii* Y.00499 is a robust strain with high glucose metabolic activity, which consumed the low amount of ethanol produced after glucose is depleted. *K. nonfermentans* Y.01443 requires longer fermentation time and lower substrate concentration for removal of glucose from GOS medium than *C. jadinii* Y.00499, but the produced glucose-free GOS product is obviously free from ethanol. Both applications offer a great advantage as glucose- and ethanol-free GOS is of great value as it can directly be used for infant formula production. The second approach proposed here is the production of high-purity GOS in one step, obtained by fermenting 10% GOS + YE with *K. marxianus* DMB Km-RK. This process results in virtually no loss of GOS fractions (97.5% Y) and100% P_f. We note that similarly to the previous reports with *Kluyveromyces* strains, ethanol is present in the final product, which requires its removal by downstream processes. Finally, a two-step fermentation strategy was worked out on 10% GOS + YE, combining glucose removal by *C. jadinii* NCAIM Y.0499 with lactose and galactose depletion by *K. lactis* DMB Kl-RK, thus achieving 100% Y and 92.1% P_f of the product.

Our experiments on single-step processes show that lactose and glucose are utilized simultaneously by the studied strains; however, depletion of glucose being always faster than that of the lactose. Although the batch processing time of the two-step approach is longer than that of a single-step fermentation, the removal rate of lactose is faster in the second step of the two-step strategy. This is likely associated with the absence of glucose in the secondary step, where no competition between substrates or possible glucose repression can occur.

The main advantage of the two-step process is its flexibility, as either glucose-free or high-purity (glucose- and lactose-free) GOS formulations can be produced, which can justify the observed high fermentation times in comparison with the single-step fermentation process.

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