ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Galactooligosaccharides Production by Transgalactosylation of Lactose using Yeast β-Galactosidases Isolated from Milk Kefir Grains

Camargo Vânia Eliza[1], Ishimoto Caroline Kie[1], Araújo Ticiana Vasques[1]*, Nurmberguer Jussimara Monteiro[2], Ayub Marco Antonio Zachia[3] and Esposito Elisa[1]

[1]Departamento de Ciência e Tecnologia, Universidade Federal de São Paulo, São José dos Campos Brazil

[2]Associação Fundo de Incentivo a Pesquisa, AFIP, Departamento de Pesquisa e Desenvolvimento de Medicina Diagnostica, São Paulo, Brazil

[3]Instituto de Ciência e Tecnologia dos Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

***Corresponding Author:** Araújo Ticiana Vasques, Departamento de Ciência e Tecnologia, Universidade Federal de São Paulo, São José dos Campos Brazil.

Received: May 14, 2021; **Published:** May 24, 2022

DOI: 10.31080/ecmi.2022.18.01229

Abstract

Kefir grains from two different origins were cultivated in different media containing lactose as a carbon source. The isolates were grown in liquid medium with 20% lactose to access their ability to produce β-galactosidase, which was measured by permeabilizing cells and measuring the enzymatic activity. The microorganisms showing the highest enzymatic activities were submitted to a high lactose concentration solution in order to induce the transgalactosylation reaction and form galactooligosaccharides (GOS) molecules. A total of twenty-six isolates were obtained, of which twenty produced β-galactosidase activity and seven strains were further used for GOS production. The isolate SP - J, which showed the highest enzymatic activity, was identified by the MALDI-TOF/MS technique as being the yeast Kluyveromyces marxianus (99.99 % confidence). Incubations of this yeast in 200 mg/mL of lactose solutions at pH 6.5 and 40 °C produced the highest concentration of GOS (39.34 mg/ml) using a small amount of β-galactosidase (0.51 U/mL). Kefir grains proved to be a source of efficient microorganisms, in addition to low cost to produce β-galactosidase by lactose transgalactosylation of the prebiotic GOS.

Keywords: *Galactooligosaccharides; B-Galactosidase; Kefir Grains; Kluyveromyces Marxianus; Lactose; Transgalactosylation*

Introduction

Galactooligosaccharides (GOS) are one of the most important non-digestible oligosaccharides demonstrating prebiotic, bifidogenic properties, modulate the immune system and regulate cholesterol. GOS are important not only because they can be obtained from by-products of the dairy industry, such as residual lactose from cheese whey, but also because they possess properties that could be used as a substitute for human milk oligosaccharides (HMOS) (ABURTO et al., 2016; *et al*., 2019) [1,2].

GOS is predominantly produced by the transgalactosylation reaction of lactose, which are catalyzed by β-galactosidases. In this reaction, a galactose molecule provenient from the hidrolization of lactose is transferred to a nucleophilic molecule other than water, which may be any sugar present in the reaction medium. In this way, the molecules achieve a higher degree of polymerization (DP) and form different types of GOS (JENAB et al., 2017;YAÑEZ-ÑECO et al., 2017) [3,4].

The galactooligosaccharides are formed by 2 to 8 units of monosaccharides and are composed by molecules of galactose and a terminal glucose. However, transgalactosylated disaccharides formed by two galactose units are also considered GOS (MARTINS et al., 2019) [5].

GOS molecules are considered non-digestible prebiotics because they have a β configuration and are resistant to hydrolysis in the digestive tract of hosts, whereas most of human digestive enzymes present specificity for α configurations. In this way, GOS are selectively metabolized in the colon by beneficial microorganisms that colonize the intestinal tract, such as bifidobacteria and lactobacilli (PERDIJK et al., 2019;VERA et al., 2016) [6,7].

By metabolizing GOS, these microorganisms release by-products such as short chain fatty acids (SCFA) among others, which cause a change in the environment, making it slightly more acidic and thereby altering the composition of the gastrointestinal microbiota (AZCARATE-PERIL et al., 2017) [8].

As for the β-galactosidases enzymes, they can be extracted from vegetables and filamentous fungi, the latter being thermostable, extracellular, and generally have a pH optimum between 2.5 - 5.4, the main industrial enzyme being the one isolated from *Aspergillus sp*. While most yeasts produce the β-galactosidase intracellularly, they have a pH optimum between 6.0 - 7.0 and due to their high hydrolytic action, it is widely used in the industry to reduce lactose in dairy products. The *Kluyveromyces sp.* species are widely used, being *Kluyveromyces lactis* the most employed, since it is always found in dairy products (HUSAIN, 2010;SELVARAJAN; MOHANASRINIVASAN, 2015) [9,10].

An enzymatic reaction for GOS production, with the enzyme β-galactosidase, can be performed are immobilized enzymes, free enzymes and whole microbial cells are used (HUSAIN, 2010;JENAB et al., 2017;PANESAR et al., 2018) [3,9,11].

For industrial production, however, most of the commercially available enzymes are produced by yeast and, mostly intracellular, have a high extraction cost, low stability and an expensive *downstream* (GOBINATH; PRAPULLA, 2015) [12].

Kefir is a carbonated fermented milk beverage with a slight acidic taste and small amounts of ethanol in its composition. It was first reported in the Caucasus Mountain region in Russia and the Balkans in Eastern Europe (PRADO; BLANDÓN; VANDENBERGHE, 2015) [13]. Kefir differs from other dairy fermented products because its fermentation is made by kefir grains, which are an aggregation of yeasts, lactic-acid and acetic-acid bacteria in a symbiotic community (ROSA et al., 2017) [14]. The microbial composition according to the "Codex Alimentairus Standard for fermented milks #243" (WORLD HEALTH ORGANIZATION, 2018) [15] is generally composed of 65 to 80% of *Lactobacillus* and *Lactococcus* and the remaining microorganisms are yeasts, such as *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Saccharomyces fragilis*, among others.

The microbial composition of kefir grains is very complex and is known to vary from region to region, however there are characteristic groups such as lactic acid bacteria, acetic bacteria, yeasts, and fungi. The cultivation environment, preservation and storage conditions are determinant for microbial diversity (ROSA et al., 2017) [14]. In Brazil, kefir grains are seldom commercialized, as cultivation conditions vary between regions with artisanal production and manufacture. (LEITE et al., 2013;ZANIRATI et al., 2015) [16,17].

In view of these considerations, the objectives of this study were to isolate and identify yeasts with industrial characteristics and at a low cost for the production of β-galactosidase by lactose transgalactosylation of the prebiotic GOS; derived from two types of kefir grains grown in two different regions of Brazil.

Materials and Methods

Kefir grains

The kefir grains evaluated in this study were donated by postgraduate student of the laboratory Eduardo Ferreira Martins and there was no information available on the exact location where the grains came from, except for that they originated from two regions of Brazil (São Paulo and Santa Catarina). These kefir grains were chosen because they were morphologically different and had a faster fermentation kinectics (aproximately 12 hours). The grains were placed in glass bottles and kept on the following substrates: raw cow milk,

pasteurized cow milk, 40% cheese whey solution, and 40% lactose solution. These four treatments aimed to make available different sources of lactose to the micro-organisms so that a greater variety of microorganisms would be isolated. A total of 100 ml of each medium was fermented for 2 days at 27°C; subsequently, an additional 100 ml of each medium was added to its corresponding glass bottle and fermented for another 2 days.

Microorganisms isolation

A total of 5 g of Kefir grains were removed from the substrates mentioned above, resuspended in 10 mL of sterile saline solution (0.9% sodium chloride), macerated in a graduated tube, and homogenized in a vortex stirrer for 2 min. The medium used for the isolation of strains was sterilized for 15 min at 121°C and 1 atm; it was composed of 17 g/L yeast extract, 9 g/L ammonium sulfate ($(NH_4)_2SO_4$), 5 g/L monopotassium phosphate (KH_2PO_4), 0.4 g/L magnesium sulfate ($MgSO_4$), 30 g/L lactose, and 20 g/L agar in 0.1 M sodium phosphate buffer pH 6.5 (SRIVASTAVA; MISHRA; CHAND, 2015) [18]. The medium was then poured into Petri dishes; after solidification, the macerated kefir grains suspension spread on the plates and was incubated at 28°C for 48 h. After growth, single colonies of morphologically different microorganisms were again striated in Petri dishes containing the medium above by using the streaking technique; they were then incubated at 28°C for 48 h.

Microorganism cultivation and β - galactosidase production

The isolated single-yeast-colonies were then inoculated into 10 mL of liquid medium described by SRIVASTAVA; MISHRA; CHAND, (2015) [18] and incubated in an orbital shaking incubator for 24h at 12 x g and 28°C (Hettich, Rotina 420R, Germany). Then, 40 mL of new medium were added to the tubes, which were incubated again (28°C, 12 x g) for 72 h.

Cell permeabilization

Cell samples were recovered by centrifugation (6,452 x g, 10 min); the cell pellet was subjected to permeabilization using 50% (volume fraction) of ethanol and sodium phosphate buffer (pH 6.5) and was incubated in an orbital shaking incubator for 15 min at 25°C. Cells were then separated from the solvent by centrifugation (6,452 x g for 10 min), washed twice with cold 0.1 M sodium phosphate buffer (pH 6.5), and stored in the same buffer solution at 4°C for later use (SRIVASTAVA; MISHRA; CHAND, 2015;YAÑEZ-ÑECO et al., 2017) [4,18].

β-galactosidase activity assay

The enzymatic activity of β-galactosidase was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate of the reaction (YAÑEZ-ÑECO et al., 2017) [4]. A 50 µL sample of permeabilized cell suspension was mixed with 2 mL of 15 mM ONPG in sodium phosphate buffer and incubated at 37°C for 5 min. The enzymatic reaction was stopped by adding 500 µL of 10% sodium carbonate solution. The o-nitrophenol (ONP) released in the reaction was measured by spectrophotometry at 420 nm (MANERA et al., 2010;MARTARELLO et al., 2019) [19,20]. One unit (U) of β-galactosidase activity was defined as the quantity of enzyme required for the hydrolysis of 1 µmol of ONPG per minute under the conditions of the experiment (MANERA et al., 2010) [19].

Galactooligosaccharides (GOS) production

The reaction for GOS synthesis was carried out in 15 mL graduated tubes containing 4 mL of lactose (20%, mass fraction) in sodium phosphate buffer and permeabilized cells, which corresponded to 0.51 U/mL of β-galactosidase enzyme. The reactions were carried out in an orbital shaking incubator at 40°C for 6 h. At 1 h intervals, 100 µL samples were collected and placed in a dry bath at 100°C for 5 min to stop the reaction (CAREVIĆ et al., 2018;SRIVASTAVA; MISHRA; CHAND, 2015) [18,21]. The glucose concentration was then measured by using an enzymatic kit for glucose determination (Lab test, MG, Brazil) in a spectrophotometer at 505 nm (DUTRA ROSOLEN et al.,

2015) [22]. After the incubation period, the reaction mixture was heated at 100°C for 15 min for enzyme deactivation (SANGWAN et al., 2011) [23]. The GOS production was monitored by determining the glucose concentration and the reaction was stopped when the glucose concentration stabilized or began to decay. The samples were frozen for later analysis.

GOS identification and quantification

The identification of the GOS was carried out by paper chromatography, and 20% glucose, 20% lactose, and 40% commercial GOS (Kenkou Ichiba – Japan) solution were used as standards. Whatman #1 filter paper and 0.05 µL of the supernatant of each sample were used. The mobile phase was composed of butanol: pyridine: water (6: 4: 3, volume fractions) and the spray reagent was composed of a mixture of 4 mL of aniline in 100 mL of acetone, 4 g of diphenylamine in 100 mL of acetone, and 16 mL of phosphoric acid in 4 mL ultrapure water. After being left to dry at room temperature, the paper was incubated at 100°C for 5 min (EYLAR; JEANLOZ, 1962;TOBA et al., 1981) [24,25].

Carbohydrate concentrations of glucose, lactose, galactose, and GOS were determined using HPLC (Shimadzu, Japan) equipped with an Aminex HPX-87C column (300 mm × 7.8 mm × 9 µm) and a refractive index detector (RID). The carbohydrates were quantified using six-point analytical curves of lactose, glucose, and galactose. The HPLC condition was in isocratic separation mode using ultrapure and sonicated water as the mobile phase, temperature of 85°C, pressure of 34- 43 kgf, flow rate of 0.6 mL/min, run time of 14 min, and volume of 20 µL of injected sample (DUARTE et al., 2017) [26].

Microorganism identification

The microorganisms were identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) technology using Vitek MS system and Myla software database version 3.2 (BioMerieux, Marcy-I'Etoile, France). The standard yeast identification protocol was performed by the Vitek MS direct from culture plates, according to the Vitek MS workflow user Manual for Clinical Use (BioMerieux, 2011). For each isolate, a 1µL plastic loop was used to pick up a small amount of colony from the agar plate, which was transferred into a single well of a disposable barcode-labeled target slide (Vitek MS-DS; BioMerieux). The isolates were tested in quadruplicate and after the sample was dried, 0.5 µL of formic acid and 1 µL of the Vitek MS-CHCA matrix solution (α-cyano-4 hydroxy-cinnamic acid solution) were applied to each isolate spot. The calibration of the mass spectrometer for every acquisition group was performed using LyfoCults plus Escherichia coli ATCC®8739™ (BioMerieux). The identification on the Vitek MS was based on a mass spectral fingerprint generated and identified automatically by Myla software database version 3.2.

Statistical analysis

Statistical analysis of the data was performed using Graphpad Prism 7 software (version 7.00, GraphPad Software, Inc. San Diego, CA, USA) and analyzes of variance (ANOVA). The media were compared using Bonferroni test at 5%.

Results and Discussion

Microorganisms from kefir grains

Kefir grains are composed of different species of microorganisms, such as lactic acid bacteria, acetic acid bacteria, yeasts, and other fungi (BENGOA et al., 2019) [27]. The interaction between these microorganisms has been described as a symbiosis occurring as follows: homofermentative and heterofermentative lactic acid bacteria, lactose-assimilating, and non-lactose-assimilating yeasts (FAZIO et al., 2020;PRADO; BLANDÓN; VANDENBERGHE, 2015) [13,28].

Because the objective of this research was to isolate β-galactosidase-producing yeasts that could be used for GOS production, the study

focus was the lactose-assimilating yeasts, such as *Kluyveromyces marxianus* var. *marxianus*, *fragilis*, and *lactis, Candida kefir* and *Candida pseudotropicalis* (FAZIO et al., 2020;NEJATI; JUNNE; NEUBAUER, 2020) [28,29]. Table 1 presents the obtained isolates.

Kefir Grains	Microorganisms isolated from Kefir grains originated from	
Growth Medium	**Sao paulo**	**Santa Catarina**
Raw cow milk	4 (J, K, L, M)	3 (B1, B2, B3)
Pasteurized cow milk	3 (D, E, F)	4 (F1, F2, F3, F3a)
40% lactose solution	3 (A, B, C)	3 (C1, C1a, C2)
40% cheese whey solution	3 (G, H, I)	3 (D1, D1a, D2)

Table 1: *Isolates present in the different studied kefir grains and media where they grew.*

Several studies have shown that kefir microbial composition varies according to the region of origin, the substrate used for the fermentation, and the methods for maintaining the culture. For this reason, kefir grains coming from the donation of different regions and presenting different morphologies were fermented on different substrates to try to isolate a larger variety of microorganisms (ROSA et al., 2017) [14].

Screening for β-galactosidase-producing yeasts

The isolated microorganisms were cultivated in liquid media and cells were permeabilized in order to verify β-galactosidase production. The results of São Paulo kefir grains are shown in figure 1 and those of Santa Catarina kefir grains are presented in figure 2.

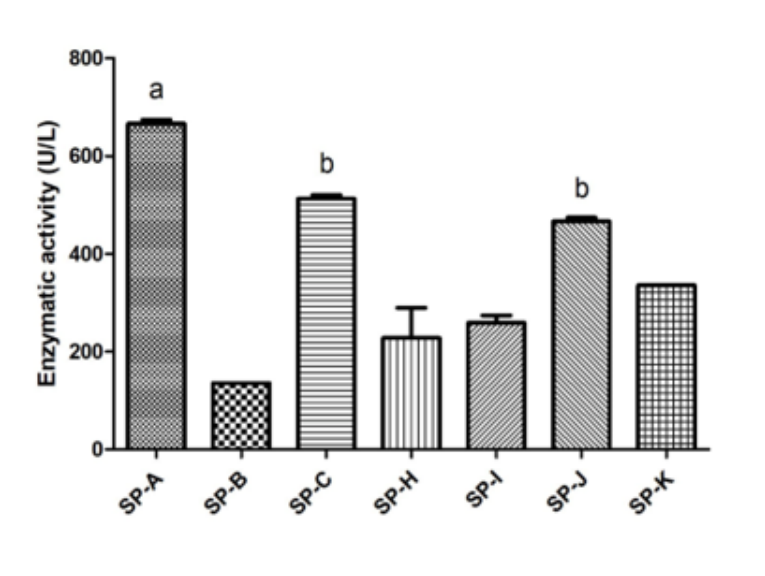

Figure 1: *β-galactosidase enzymatic activities of isolated kefir microorganisms from São Paulo region cultivated in submerged media, as described by (SRIVASTAVA; MISHRA; CHAND, 2015).*

ab Means followed by different lowercase letters refer to the differences between isolates using the Bonferroni test (p <0.05).

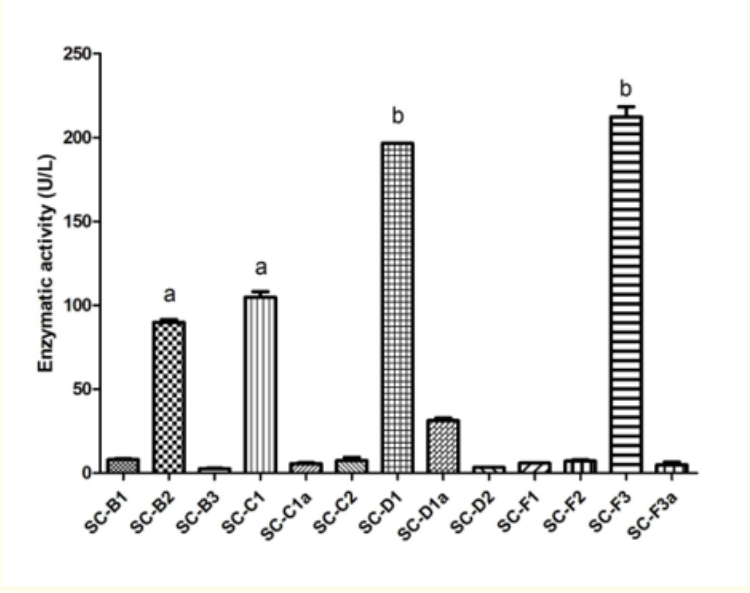

Figure 2: *β-galactosidase enzymatic activities of isolated kefir microorganisms from Santa Catarina region cultivated in submerged media, as described by (SRIVASTAVA; MISHRA; CHAND, 2015).*

ab Means followed by different lowercase letters refer to the differences between isolates using the Bonferroni test ($p < 0.05$).

Fig 1 β-galactosidase enzymatic activities of isolated kefir microorganisms from São Paulo region cultivated in submerged media, as described by (SRIVASTAVA; MISHRA; CHAND, 2015) [18].

Fig 2 β-galactosidase enzymatic activities of isolated kefir microorganisms from Santa Catarina region cultivated in submerged media, as described by (SRIVASTAVA; MISHRA; CHAND, 2015) [18].

The production of β-galactosidase by yeasts is a intracellular process and, in these cases, downstream processes involve cell disruptions, which causes an increase in purification costs as well as low yields and decreased activity (GOBINATH; PRAPULLA, 2015) [12]. For this reason, it was necessary to use the whole cell permeabilization method.

Various studies have used this approach and obtained higher GOS production compared to that of soluble β-galactosidases (PANESAR et al., 2018;SRIVASTAVA; MISHRA; CHAND, 2015) [11,18] and demonstrated higher stability of the enzyme when permeabilized whole cell was used (SUN et al., 2016) '[30]. Different methods of cell permeabilization can be used, such as physical or chemical processes, and the chemical processes is related to the presence of solvent or detergent residues in treated cells, which can contaminate the final food products (VIANA et al., 2018) [31]. The liposoluble nature of ethanol and the permeability of cell membranes can be highly increased, which facilitates the accessibility of the substrate to β-galactosidase and the release of products. Ethanol is considered a non-toxic compound because this solvent is present in many fermented dairy products used for human consumption. Easily biodegradable and hydrosoluble in nature, this reagent has many favorable properties for its utilization (SUN et al., 2016) [30]. Thus, many studies have used this solvent for bioprocesses, which allows the access to the intracellular enzyme in a simple and safe manner (SRIVASTAVA; MISHRA; CHAND, 2015;VIANA et al., 2018) [18,31].

Thus, β-galactosidase enzymatic activities was accessed for all isolates, as shown in figures 1 and 2.

It can be observed that some of the microorganisms isolated from São Paulo kefir (Table 1) did not present any enzymatic activity (SP-D, SP-E, SP-F, SP-G, SP-L, SP-M). This may be due to the fact that these microorganisms are non-lactose-assimilating yeasts (PRADO; BLANDÓN; VANDENBERGHE, 2015) [13].

Among the seven isolates that presented activity (Figure 1), the SP-A isolate activity (666.20 ± 10.88 U/L) was significantly higher ($p < 0.05$) than the other ones, followed by SP-C (512.30 ± 10.88 U/L) and SP-J (466.12 ± 10.88 U/L). The other isolates presented considerable values and enzymatic activities higher than 100 U/L; however, their values were not high enough to be considered for subsequent use in the GOS production or statistical analysis.

Among the isolates of the SC kefir grains, the SC-F3 isolate (212.33 ± 8.36 U/L) and the SC-D1 isolate (196.50 ± 0.07 U/L) were not significantly different; however, both presented a β-galactosidase enzymatic activity significantly higher than that of SC-C1 (104.83 ± 4.72 U/L) and SC-B2 (89.83 ± 2.35 U/L) ($p < 0.05$). It is important to note that only four isolates presented activity above 80 $U.L^{-1}$; the other nine isolates showed little enzymatic activity and therefore were not statistically analyzed (Figure 2). Thus, the four isolates with the highest enzymatic activities were used for GOS production.

Production, identification and quantification of GOS

Paper chromatography

GOS production was carried out using the three most promising isolates (A, C, and J) of the São Paulo kefir, which had the highest enzymatic activity, and the four isolates (B2, C1, D1 and F3) of Santa Catarina kefir that demonstrated higher enzymatic activity. In addition, the GOS production was performed using a commercial enzyme from *Kluyveromyces lactis* (Sigma-Aldrich) for later comparison. GOS production was monitored by measuring the glucose concentration of the samples collected at each hour of cultivation, and the reaction was stopped when the glucose concentration stabilized or began to decay.

After production, the samples were analyzed by paper chromatography (Figure 3) using lactose, glucose, and commercial GOS standards to confirm whether GOS had been produced.

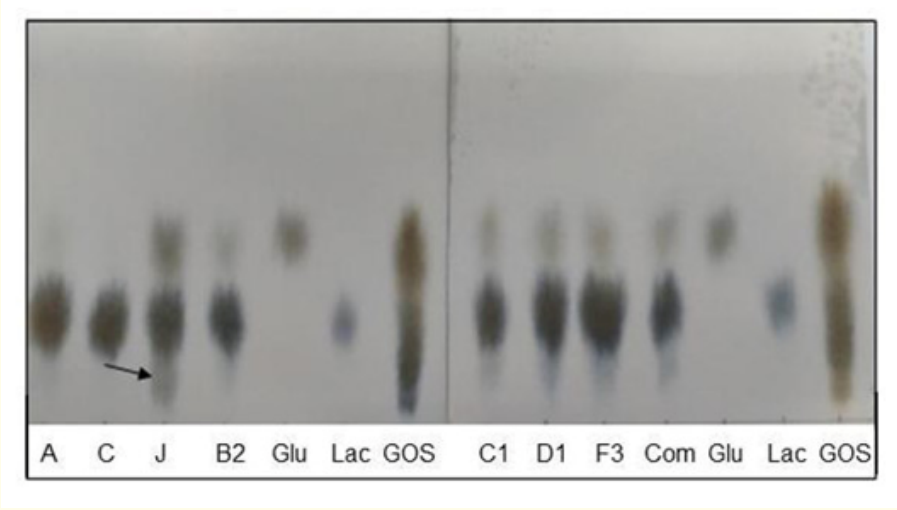

Figure 3: *Paper-chromatography showing the GOS production by strains A, C, J, B2, C1, D1, F3, and the commercial enzyme (Com). Glucose (Glu), lactose (Lac), and commercial GOS standards were also loaded for comparison.*

The spots corresponding to lowest solubility (Rf) are of great molecular weight, correspond to oligosaccharides, and can be attributed to GOS by comparing it to the GOS standard (SANTOS; SIMIQUELI; PASTORE, 2009) [32].

Commercial GOS preparations are not pure; they contain 55% GOS and the remaining is composed of monosaccharides, mainly glucose, galactose, and disaccharide lactose. The smeared spot of GOS indicates that the GOS mixture in the commercial sample used in this work is formed by polymers ranging in size from trisaccharides to hexasaccharides and contains 2 to 5 units of galactose (CAO et al., 2017) [33]. It can be observed that GOS was produced by all isolates; this was more clearly observed for isolates J, D1, F1, and for the commercial enzyme.

High performance liquid chromatography (HPLC)

GOS quantification of the samples from isolates A, C, J, B2, C1, D1, and commercial enzyme were analyzed by HPLC. The chromatograms presented in Figure 4 represent only samples C1, D1, J, the commercial GOS, and the commercial enzyme, which had significant amount of GOS.

The reaction for conversion of lactose to GOS produces molecules with different degrees of polymerization (DP), which range in size from disaccharides to pentasaccharides (MARTINS et al., 2019;NATH et al., 2016) [5,34]. In order to determine the GOS degree of polymerization produced by the tested isolates, we used calibration curves previously made using sugars formed from sucrose (Glu-Fru) by a reaction using α-galactosidases as catalyst. These sugars were raffinose, which is a trisaccharide (Gal-Gli-Fru) that has the same retention time as the highest peak of the commercial GOS (Figure 4e), and stachyose, which is a tetrasaccharide (Gal-Gal-Gli-Fru) that has the same retention time as the lowest GOS peak (Figure 4e) (LEBLANC et al., 2004) [35].

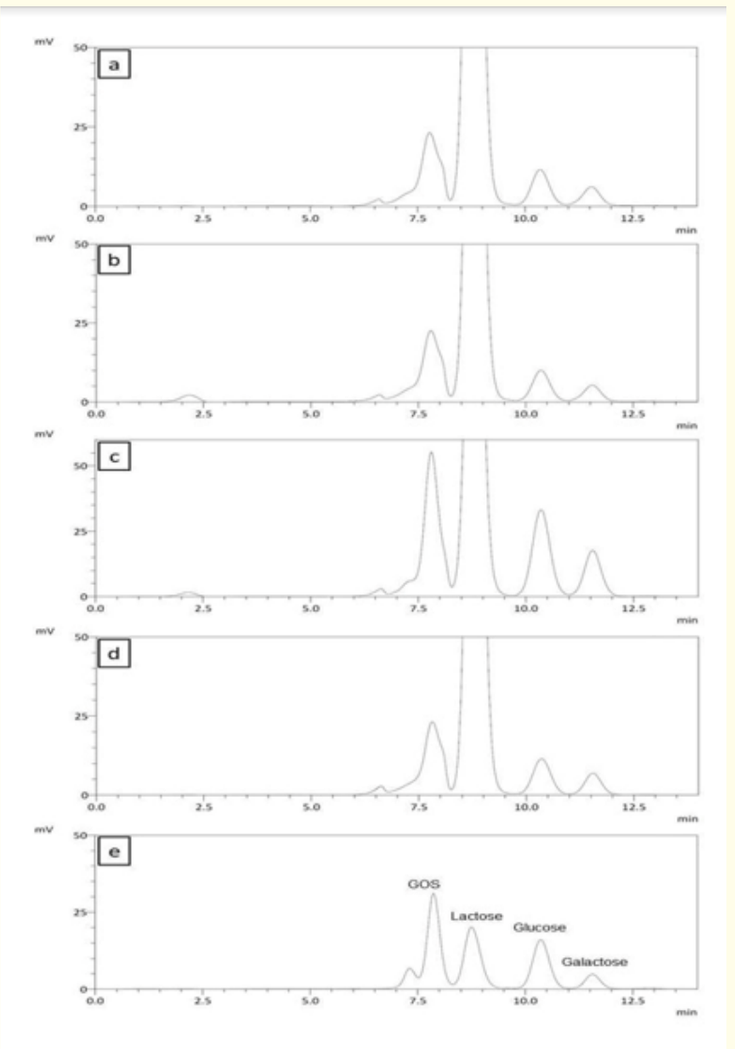

Figure 4: *Chromatograms of GOS quantification by HPLC: (a) Isolate C1; (b) Isolate D1; (c) Isolate J; (d) commercial enzyme; (e) commercial GOS. These data were obtained and compared with calibration curves previously made for each type of sugar.*

By comparison, we can assume that all the isolates produced GOS in the form of trisaccharides (highest amount), tetrasaccharides, and pentasaccharides as can be observed in the smaller peaks to the left of the trisaccharides peak (Figure 4).

After the analysis of the chromatograms, the calibration curve with the commercial GOS (Fig. 4e) (concentration of 5 g/L) was used to obtain an approximate quantification of total GOS produced as well as the production yield by each isolate (Table 2).

Isolates	GOS (mg/ml)	GOS yield (%)
A	8.353 ± 1.489	4.177 ± 0.745
C	5.158 ± 0.525	2.579 ± 0.263
J	39.340 ± 3.945	19.670 ± 1.973
B2	11.020 ± 0.000	5.509 ± 0.000
C1	25.040 ± 1.725	12.520 ± 0.862
D1	25.080 ± 2.435	12.540 ± 1.218
F3	12.570 ± 0.000	6.283 ± 0.000
Com (1)	21.540 ± 1.775	10.770 ± 0.887

Table 2: *Quantification of galactooligosaccharides (GOS) production obtained in the cultures of isolates and the GOS yield. (1) Commercial enzyme β-galactosidases from K. lactis (Sigma).*

The highest yields of GOS were obtained for isolate J (19.67%). By comparing the yield of GOS obtained by LISBOA et al., (2012) [36] which was equivalent to 41.9% (400 mg/mL of lactose and 167.5 mg/mL of GOS) using 5U/mL of commercial enzyme of *Kluyveromyces lactis* (Lactozym®), and the yield of isolate J, one can observe that the latter is almost half of the former. However, the conditions used in our study were 200 mg/mL of lactose and 0.51 U/mL. This results in a GOS/enzyme ratio of 33.5 mg/U for the work by LISBOA., *et al.* [36] and a ratio of 77.14 mg/U for the J isolate, since 0.51 U/ml of enzyme was used and an amount of GOS equivalent to 39.34 mg/mL was obtained.

By using enzymes from different isolates but under the same conditions, the different GOS yield values points to the existence of different affinity between transgalactosylation and hydrolysis reactions performed by β-galactosidases using different sources of microorganisms [37,38]. Thus, the SP-J isolate presented the highest affinity.

Microorganism identification

Two strains were identified as *Torulaspora delbrueckii* (C1 and D1) and one strain was identified as *Kluyveromyces marxianus* (J) by MALDI-TOF/MS analyses. *Torulaspora delbrueckii* is known as *Saccharomyces delbrueckii* or *Rosei Saccharomyces* (an anamorph called *Candida colliculosa*), and *Kluyveromyces marxianus* is the sexual stage of *Candida kefyr* [39,40]. They were all identified with a high confidence level (99.9%) by Vitek MS system. Isolates A, C, B2, F3 were not analyzed because they could no longer grow in the culture medium used during the experiment.

MALDI-TOF / MS has been widely used in clinical microbiology laboratories because it is a rapid technique that requires only a small portion of microorganisms from a primary colony of a Petri dish. This is a breakthrough compared to the commonly used molecular techniques, which are time consuming and sometimes imprecise [41].

Several yeasts have been isolated from the different kefir grains originated from different regions, including *Torulaspora delbrueckii* and especially *Kluyveromyces marxianus* [27,42]. This demonstrates that, in addition to being used to identify pathogenic microorganisms, this technique can be widely used to identify microorganisms in the food industry.

Conclusion

This study showed that kefir grains are a good source of β-galactosidase enzyme-producing microorganisms. Seven of the twenty-six isolates studied here were selected because of their β-galactosidase yield, which ranged from 89.83 to 673.85 U/L. Among them, two isolates presented yield values similar to that of the commercial enzyme, and one isolate, identified as *Kluyveromyces marxianus* (SP-J), presented an excellent GOS yield and a high GOS/enzyme ratio, which demonstrates that it is a β-galactosidases strain of great interest in the transgalactosylation of GOS.

The results of the present study reveal that the search for new strains in kefir grains in addition to having a low cost is of great value for obtaining organisms with industrial characteristics and that the synthesis of GOS using permeabilized cells can be advantageous in the industrial processes and allow an increased yield.

Acknowledgements

We thank Eduardo Ferreira Martins for providing the grains Kefir. This project was funded by Brazilian funding agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESP (Fundação de Amparo e Pesquisa do Estado de São Paulo) project process number: 14/15423-7.

Conflict of Interest

I wish to confirm that there are no known conflicts of interest associated with this publication and that significant financial support for this work was acknowledged. I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of them.

Sources of Support

This project was funded by Brazilian funding agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESP (Fundação de Amparo e Pesquisa do Estado de São Paulo) project process number: 14/15423-7.

Bibliography

1. Aburto Carla., *et al*. "Simultaneous Synthesis and Purification (SSP) of Galacto-Oligosaccharides in Batch Operation". *LWT - Food Science and Technology* 72 (2016): 81-89.

2. Oh Nam Su., *et al*. "Enhanced Production of Galactooligosaccharides Enriched Skim Milk and Applied to Potentially Synbiotic Fermented Milk with Lactobacillus Rhamnosus 4B15". *Food Science of Animal Resources* 39.5 (2019): 725-741.

3. Jenab Ehsan., *et al*. "Enzymatic conversion of lactose into galacto-oligosaccharides: The effect of process parameters, kinetics, foam architecture, and product characterization". *Journal of Food Engineering* 222 (2017): 63-72.

4. Yañez-Ñeco Claudia., *et al*. "Galactooligosaccharide Production from Pantoea Anthophila Strains Isolated from 'Tejuino', a Mexican Traditional Fermented Beverage". *Catalysts* 7.8 (2017): 242.

5. Martins Gonçalo N., *et al*. "Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis". *Frontiers in Nutrition* (2019): 1-24.

6. Perdijk Olaf., *et al*. "Corrigendum: Sialyllactose and Galactooligosaccharides Promote Epithelial Barrier Functioning and Distinctly Modulate Microbiota Composition and Short Chain Fatty Acid Production In Vitro". *Frontiers in Immunology* (2019): 762.

7. Vera Carlos., *et al*. "Synthesis and Purification of Galacto-Oligosaccharides: State of the Art". *World Journal of Microbiology and Biotechnology* 32.12 (2016).

8. Azcarate-Peril M Andrea., *et al*. "Impact of Short-Chain Galactooligosaccharides on the Gut Microbiome of Lactose-Intolerant Individuals". *Proceedings of the National Academy of Sciences of the United States of America* 114.3 (2017): E367-E375.

9. Husain Qayyum. "β Galactosidases and Their Potential Applications: A Review". *Critical Reviews in Biotechnology* 30.1 (2010): 41-62.

10. Selvarajan E and V Mohanasrinivasan. "Kinetic Studies on Exploring Lactose Hydrolysis Potential of β-Galactosidase Extracted from Lactobacillus Plantarum HF571129". *Journal of Food Science and Technology* 52.10 (2015): 6206-6217.

11. Panesar Parmjit S., *et al*. "Biocatalytic Strategies in the Production of Galacto-Oligosaccharides and Its Global Status". *International Journal of Biological Macromolecules* 111 (2018): 667-679.

12. Gobinath Duraiswamy and Siddalingaiya Gurudutt Prapulla. "Transgalactosylating β-Galactosidase from Probiotic Lactobacillus Plantarum MCC2156: Production and Permeabilization for Use as Whole Cell Biocatalyst". *Journal of Food Science and Technology* 52.9 (2015): 6003-6009.

13. Prado Maria R., *et al*. "Milk Kefir: Composition, Microbial Cultures, Biological Activities, and Related Products". *Frontiers in Microbiology* (2015): 1-10.

14. Rosa, Damiana D., *et al*. "Milk Kefir: Nutritional, Microbiological and Health Benefits". *Nutrition Research Reviews* 30.1 (2017): 82-96.

15. WORLD HEALTH ORGANIZATION. "Standarts for Fermented Milks". Codex Alimentarius 3 edition (2018): 12.

16. Leite Analy Machado de Oliveira., *et al*. "Microbiological, Technological and Therapeutic Properties of Kefir: A Natural Probiotic Beverage". *Brazilian Journal of Microbiology* 44.2 (2013): 341-349.

17. Zanirati Debora Ferreira., *et al*. "Selection of Lactic Acid Bacteria from Brazilian Kefir Grains for Potential Use as Starter or Probiotic Cultures". *Anaerobe* 32 (2015): 70-76.

18. Srivastava Anita., *et al*. "Transgalactosylation of Lactose for Synthesis of Galacto-Oligosaccharides Using Kluyveromyces Marxianus NCIM 3551". *New Biotechnology* 32.4 (2015): 412-418.

19. Manera Ana Paula., *et al*. "Galacto-Oligosaccharides Production Using Permeabilized Cells of Kluyveromyces Marxianus". *International Journal of Food Engineering* 6.6 (2010): 1-13.

20. Martarello Raquel Dall'Agnol., *et al*. "Optimization and Partial Purification of Beta-Galactosidase Production by Aspergillus Niger Isolated from Brazilian Soils Using Soybean Residue". *AMB Express* 9.1 (2019): 1-13.

21. Carević Milica., *et al*. "Evaluation of β-Galactosidase from Lactobacillus Acidophilus as Biocatalyst for Galacto-Oligosaccharides Synthesis: Product Structural Characterization and Enzyme Immobilization". *Journal of Bioscience and Bioengineering* 126.6 (2018): 697-704.

22. Dutra Rosolen Michele., *et al*. "Lactose Hydrolysis in Milk and Dairy Whey Using Microbial β-Galactosidases". *Enzyme Research* 2015 (2015): 1-7.

23. Sangwan Vikas., *et al*. "Galactooligosaccharides: Novel Components of Designer Foods". *Journal of Food Science* 76.4 (2011): 103-111.

24. Eylar EH and RW Jeanloz. "Oligosaccharides from the a, Acid of Human Plasma". *The Journal of Biological Chemistry* 237.3 (1962): 622-628.

25. Toba Takahiro., *et al*. "β-Galactosidases of Lactic Acid Bacteria: Characterization by Oligosaccharides Formed During Hydrolysis of Lactose". *Journal of Dairy Science* 64.2 (1981): 185-192.

26. Duarte Lovaine Silva., *et al*. "A New Bioprocess for the Production of Prebiotic Lactosucrose by an Immobilized β-Galactosidase". *Process Biochemistry* 55 (2017): 96-103.

27. Bengoa AA., *et al*. "Kefir Micro-Organisms: Their Role in Grain Assembly and Health Properties of Fermented Milk". *Journal of Applied Microbiology* 126.3 (2019): 686-700.

28. Fazio Alessia., *et al*. "Effect of Addition of Pectins from Jujubes (Ziziphus Jujuba Mill.) on Vitamin C Production during Heterolactic Fermentation". *Molecules* 25.11 (2020): 1-15.

29. Nejati Fatemeh., *et al*. "A Big World in Small Grain: A Review of Natural Milk Kefir Starters". *Microorganisms* 8.2 (2020): 1-10.

30. Sun Huaisheng., *et al*. "Recyclable Strategy for the Production of High-Purity Galacto-Oligosaccharides by Kluyveromyces Lactis". *Journal of Agricultural and Food Chemistry* 64.28 (2016): 5679-5685.

31. Viana Caroline Dos Santos., *et al*. "Determination of Cell Permeabilization and Beta-Galactosidase Extraction from Aspergillus Oryzae CCT 0977 Grown in Cheese Whey". *International Journal of Chemical Engineering* 2018 (2018): 1-6.

32. Santos R., *et al*. "Galactooligosaccharide Production by Scopolariopis Sp". *Ciencia y Tecnología De Alimentos* 29.3 (2009): 682-689.

33. Cao Lichuang., *et al*. "Improving the Secretion Yield of the β-Galactosidase Bgal1-3 in Pichia Pastoris for Use as a Potential Catalyst in the Production of Prebiotic-Enriched Milk". *Journal of Agricultural and Food Chemistry* 65.49 (2017): 10757-10766.

34. Nath Arijit., *et al*. "Synthesis of Lactose-Derived Nutraceuticals from Dairy Waste Whey-a Review". 9 (2016).

35. LeBlanc Jean Guy., *et al*. "Reduction of Non-Digestible Oligosaccharides in Soymilk: Application of Engineered Lactic Acid Bacteria That Produce Alpha-Galactosidase". *Genetics and Molecular Research: GMR* 3.3 (2004): 432-440.

36. Lisboa Cristiane Reinaldo., *et al*. "Synthesis of Galactooligosaccharides from Lactose Using Commercial β-Galactosidase from Kluyveromyces Lactis". *Brazilian Journal of Food Technology* 15.1 (2012): 30-40.

37. Frenzel Monika., *et al*. "Comparison of the Galacto-Oligosaccharide Forming Activity of Different β-Galactosidases". *LWT - Food Science and Technology* 60.2 (2015): 1068-1071.

38. Kittibunchakul Suwapat., *et al*. "Structural Comparison of Different Galacto-Oligosaccharide Mixtures Formed by β-Galactosidases from Lactic Acid Bacteria and Bifidobacteria". *Journal of Agricultural and Food Chemistry* 68.15 (2020): 4437-4446.

39. Mainville I., *et al*. "Accurate Identification of Yeasts in Kefir by Polyphasic Analysis". *EC Microbiology* 12.2 (2017): 104-113.

40. Ramírez Manuel and Rocío Velázquez. "The Yeast Torulaspora Delbrueckii: An Interesting but Difficult-to-Use Tool for Winemaking". *Fermentation* 4.4 (2018): 1-15.

41. Ge Mao-Cheng., *et al*. "Routine Identification of Microorganisms by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry: Success Rate, Economic Analysis, and Clinical Outcome". *Journal of Microbiology, Immunology and Infection* 50.5 (2017): 662-668.

42. Tan Kei Xian., *et al*. "Prospects of Kefiran as a Food-Derived Biopolymer for Agri-Food and Biomedical Applications". *RSC Advances* 10.42 (2020): 25339-25351.

Volume 18 Issue 6 June 2022