High-yield purification of commercial lactulose syrup

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ABSTRACT

A simple process for purifying lactulose in commercial lactulose syrups was developed. The objective was to establish a selective enzymatic hydrolysis of lactose and epilactose and further removal of monosaccharides by activated charcoal and water/ethanol solutions. Four commercial β-galactosidases from different microorganisms were tested obtaining significant differences in their activities towards the three disaccharides present in the lactulose syrup. β–galactosidases from *Bacillus circulans* (Biolactasa® NTL*2) and *Bifidobacterium bifidum* (Saphera® 2600 L) showed a reduced enzymatic activity towards lactulose and a high enzymatic activity towards lactose. These two enzymes were chosen to optimize the lactose hydrolysis methodology using Response Surface Methodology (RSM). Once chosen the optimum enzymatic conditions to selectively hydrolyse lactose present in the lactulose syrup, the hydrolysed sample was treated with activated charcoal and water/ethanol solutions to eliminate all monosaccharides. The proposed method offers a product with high purity (> 94%) and high recovery (>80%) of lactulose.

Key words: lactulose, purification, enzymes, activated charcoal, β–galactosidases, prebiotic.

1. Introduction

Lactulose, (4-O-β-D-galactopyranosyl-D-fructose), is a synthetic disaccharide which does not occur naturally and can be synthesized by both chemical and enzymatic methods [1-4]. The beneficial effects of lactulose in the human large intestine have been intensively studied for more than 60 years [3-5]. Lactulose is mainly used in medicine in the form of syrup or as a crystalline product for the symptomatic treatment of constipation and the treatment of portal systemic encephalopathy [6]. Although the use of lactulose as a food ingredient was introduced as far back as the 1950s by Petuely [7], with the growing interest in functional foods, the use of lactulose as prebiotic ingredient is increasing considerably as it offers excellent and scientifically tested functional properties and applications [3].

Lactulose can be chemically obtained by isomerization of lactose in basic media including calcium hydroxide, ammonia, sodium sulphite or tertiary amines [8-11]. These methods afford low yields and are tedious, the main difficulty being the purification of lactulose from the reaction mixture. Complexing agents like borate and aluminate increases the lactulose yield up to 70-80% but its removal from reaction mixtures increases the production costs [12].

The enzymatic synthesis of lactulose has been also produced using β-galactosidases from different sources and fructose as acceptor [13-17], as well as glucose isomerase [18] or cellobiose 2-epimerase [1-2].

Despite the numerous research studies addressing the enzymatic synthesis of lactulose, the industrial production is still mainly done by chemical isomerization [4-18]. Most of the lactulose syrups currently available on the market to be used in the

pharmaceutical and food industries, contain 666 mg/mL lactulose, but also contain a relatively high percentage of other carbohydrates mainly lactose (67 mg/mL) and galactose (67 mg/mL) which increase the calorific value of the product. The presence of these carbohydrates may be problematic for therapies that require the administration of pure lactulose, for example, in patients suffering from diabetes, with galactosaemia or who require a diet without lactose. That is why there is an increasing need of a simple inexpensive lactulose purification process.

It is known that removal of lactose impurity from lactulose is a difficult task whereas monosaccharide impurities can be easily removed from lactulose by treatment with activated charcoal [19]. In a previous study on transglycosylation reactions of lactose and lactulose by β-galactosidase from *Bacillus circulans* [20] we observed different rates of hydrolysis for lactose and lactulose. Based on those results, this study is focused on the search for an efficient and affordable method to purify commercial lactulose syrups, using enzymes, to eliminate lactose and monosaccharides and reduce the presence of other carbohydrates and, thus, to extend the scope of lactulose applications. Here, we present a comparative study between four commercial β–galactosidases from *Kluyveromyces lactis* (Lactozym® Pure 6500 L), *Aspergillus oryzae* (Biolactase® F CONC), *Bacillus circulans* (Biolactasa® NTL*2) and *Bifidobacterium bifidum* (Saphera® 2600 L) which were used as reaction catalysts. In addition, response surface methodology (RSM) has been used to optimize the selective enzymatic hydrolysis of lactose present in commercial lactulose.

2. Experimental

2.1. *Materials and samples*

Eleven commercial lactulose syrups (**Table 1**) were purchased in seven different countries. The lactulose standard used to prepare the calibration curve was purchased from Sigma-Aldrich and had a purity degree greater than 95%.

Four commercial β-galactosidases: Saphera® 2600 L from *Bifidobacterium bifidum*, and Lactozym® Pure 6500 L from *Kluyveromyces lactis*, were purchased from Novozyme (Denmark); Biolactasa® NTL*2 from *Bacillus circulans* and Biolactase® F CONC from *Aspergillus oryzae* were purchased from Biocon (Spain).

Analytical standards of galactose, glucose, fructose, lactose, lactulose, raffinose, and phenyl-β-glucoside were acquired from Sigma-Aldrich (St Louis, MO). Activated charcoal (Darco G60) was purchased from J.T. Baker (Netherlands).

2.2. *Selective enzymatic hydrolysis of lactose present in lactulose syrup*

Lactose present in commercial lactulose syrup (sample number 3) was hydrolysed using β-galactosidases from *Bifidobacterium bifidum (*Saphera® 2600 L), *Kluyveromyces lactis* (Lactozym® Pure 6500 L), *Bacillus circulans* (Biolactasa® NTL*2) and *Aspergillus oryzae* (Biolactase® F CONC). Lactose hydrolysis were conducted at the optimum pH and temperatures previously reported for the enzymatic preparation suppliers, i.e. pH 6.0 and 40ºC for Saphera® 2600 L; pH 6.5 and 50ºC for Lactozym® Pure 6500 L and Biolactasa® NTL*2 and pH 4.5 and 50ºC for Biolactase® F CONC. In order to evaluate the β-galactosidase selectivity, each enzyme (3 U/mL) was incubated with commercial lactulose syrup (10% w/v) at the optimal pH and temperatures for each enzyme. Aliquots

were withdrawn at specific time intervals (0, 15, 30, 60, 90, 120, 180 and 240 min) and immediately immersed in boiling water for 5 min to inactivate the enzyme and then stored at -18 °C for subsequent analysis (*n*=3). Those enzymes with selectivity to hydrolyse lactose but no lactulose were chosen to optimize the lactose hydrolysis methodology using Response Surface Methodology (RSM).

2.3. *Optimization of lactose hydrolysis conditions by RSM*

Lactose hydrolysis optimization, using the commercial lactulose preparation (sample number 3) was performed using a Central Composite Design (CCD) with three independent variables: lactulose concentration X_1 (3.15-25 % w/w), enzymatic dose of two selected enzymes (Saphera® and Biolactasa® NTL*2) X_2 (4.0- 15.0 U/mL) and time X_3 (4.1- 48 h). Three responses (dependent variables) were taken into account to optimise lactulose syrup purification by means of RSM: recovery (% w/w) of lactulose, lactose and epilactose, for each enzyme. A total of 34 points (24 axial, 2 center and 8 factorial) for each selected enzyme were conducted. The obtained data were processed using statistical software package Design-Expert 10 (CAMO-ASA, Norway).

2.4. *Fractionation of carbohydrates in selectively hydrolyzed lactulose syrups by activated charcoal*

In order to remove monosaccharides, the selected hydrolysates of lactulose syrups were purified with activated charcoal following the methodology proposed by Julio-González et al. [19] with some modifications. Briefly, mixture reaction (500 mg) and activated charcoal (6 g) were added to 100 mL of ethanol (1%, v/v). The resulting mixture was stirred for 30 min at 25 °C, and then filtered through Whatman No.1 paper (Whatman International Ltd., Maidstone, UK) under vacuum (this process is named by the

125 desorption step 1). The process was repeated for a second time to ensure removal of total
126 monosaccharides (step 2). The remained lactulose in the washed charcoal was desorbed
127 by adding 100 ml of ethanol (8% v/v) and stirring for 30 min at 25ºC, and then filtered
128 through Whatman No.1 paper under vacuum (step 3); this process was done two more
129 times (steps 4 and 5) to ensure removal of total disaccharides. Desorption of
130 oligosaccharides from the washed activated charcoal was carried out by adding 100 mL
131 of ethanol (50%, v/v) and stirring the mixture during 30 min and subsequent filtration
132 (step 6).
133
134 2.5. *Gas chromatographic analysis of carbohydrates*

135 Mono- and disaccharides were analyzed by gas chromatography-flame ionisation
136 detection (GC-FID) as trimethylsilylated ethers (TMSI) prepared following the method
137 of Montilla et al. [21] with some modifications. The dried mixtures were treated with 100
138 µl of dimethylformamide (DMF) and heating at 70ºC for 30 min. To silylate the
139 carbohydrates 150 µl N-trimethylsilylimidazole were added; the reaction was completed
140 in 30 min at 70 °C. TMSI derivatives of carbohydrates were extracted with 600 µl of
141 hexane and 300 µl of water. Separation of carbohydrates was carried out in an Agilent
142 Technologies gas chromatograph (Mod 7890A) equipped with a flame ionization detector
143 (FID) and a fused silica capillary column SPB-50, bonded, crosslinked phase (50%
144 phenyl-50% methylpolysiloxane; 30 m x 250 µm id., 0.25 µm film thickness)
145 (SUPELCO, USA). The initial oven temperature was 200 ºC increasing to 230 ºC at a rate
146 of 4 ºC/min, and finally increased to 250 ºC at 1 ºC/min. The temperature of injector and
147 detector were at 280°C and 295°C, respectively. Injections were carried out in split mode
148 (1:20) using nitrogen as carrier gas at a flow rate of 0.4 mL/min.

When quantification of trisaccharides was required, analyses were performed by GC-FID of trimethylsilylated oximes (TMSO) derivatives prepared following the method of Brobst and Lott. [22]. Sugar oximes were formed by adding 250 µL of hydroxylamine chloride (2.5%) in pyridine to dried samples and heating the mixture at 70ºC for 30 min. Then, they were silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50 ºC for 30 min. Reaction mixtures were centrifuged at 10,000 rpm for 2 min at room temperature. Separation of carbohydrates was carried out in an Agilent Technologies gas chromatograph (Mod 7890A) equipped with a flame ionization detector (FID) and a fused silica capillary column DB-5HT (5%-phenyl-methylpolysiloxane; 30m x 0.25mm x 0.10µm) (Agilent). The oven temperature was initially set at 150 ºC then increased at 3°C/min to 380 ºC. The injector and detector temperatures were set at 280 and 385 ºC, respectively. Injections were carried out in split mode (1:20) using nitrogen at 1 mL/min as the carrier gas [19].

Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software. To calculate the response factors relative to the internal standard, solutions containing glucose, galactose, lactose, lactulose and raffinose were prepared over the expected concentration range in samples. The identities of carbohydrates were confirmed by comparison with relative retention times (RRT) of standard samples. Response factors were calculated after the duplicate analysis of standard solutions over the expected concentration range in samples. The amount of different carbohydrates was expressed as % carbohydrates recovered and % weight of the total carbohydrates content in the reaction mixtures. All analyses were carried out in duplicate and data were expressed as mean ± standard deviation (SD).

2.6. *Statistical analysis*

173 The comparisons of means using analysis of variance (ANOVA) were made using
174 the statistical software package SPSS (SPSS Inc., IL, USA). The differences were
175 considered significant when $P < 0.05$.

176 **3. Results and Discussion**

177 3.1. *Carbohydrate content in commercial lactulose preparations*

178 **Fig. 1 (a)** shows the chromatographic profile (GC-FID) of a commercial lactulose
179 preparation. As can be observed, beside the main component, lactulose (labelled as peak
180 5), appreciable amounts of monosaccharides (galactose, labelled as peak 2) and
181 disaccharides (lactose as peak 6 and epilactose as peak 4) were also detected. **Table 1**
182 shows the content of carbohydrates present in the different commercial samples analysed.
183 Lactulose content (as indicated in the label) varies widely according to manufacturer
184 although most of the samples (ten of the twelve samples) showed comparable lactulose
185 content ranging from 60 to 67 g/100mL, and only two samples showed lactulose contents
186 of 35 and 59 g/100mL due to the presence of other ingredients such as simethicone or
187 propilenglycol that were declared in the label of samples. Regarding carbohydrate
188 composition, all samples showed similar values except one which showed the presence
189 of small amounts of tagatose, isomer of galactose that could be originated from galactose
190 by isomerization under basic conditions. These results suggest that all commercial
191 lactulose samples were produced with similar manufacturing process. Although industrial
192 production of lactulose is mainly achieved by chemical isomerization of lactose in basic
193 media [8], lactulose can also be obtained by enzymatic transglycosylation when the
194 hydrolysis of lactose by β-galactosidase takes place in presence of fructose [17]. The
195 presence of epilactose in all analyzed samples is indicative of isomerization in basic

media since it is a secondary product formed during chemical isomerization of lactose [23].

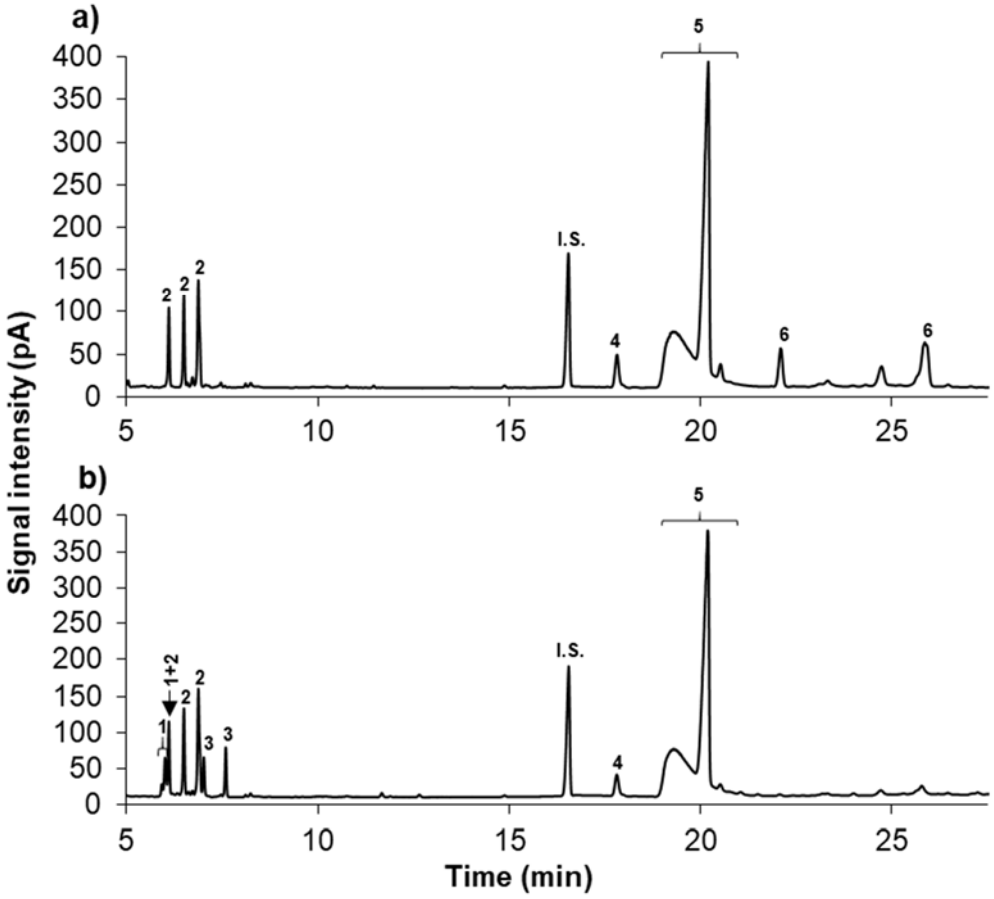

Fig. 1. GC-FID chromatographic profiles of TMSI derivatives of carbohydrates present in a commercial lactulose syrup (sample number 3 in Table 1) before **(a)** and after **(b)** hydrolysis by Saphera® 2600 L, β-galactosidase (11 U/mL) during 4.1 h. 1: fructose; 2: galactose, 3: glucose, 4: epilactose, 5: lactulose, 6: lactose. I.S.: Internal Standard (phenyl-β-glucoside).

Table 1. Content (g/100mL lactulose syrup) of monosaccharides (galactose and tagatose), lactose, lactulose and epilactose found in commercial lactulose preparations.

208

Sample	Country of origin	Carbohydrate content (g/100mL of lactulose syrup)				
		Lactulose	Tagatose	Galactose	Lactose	Epilactose
1	Russia	68.3±5.9	-	6.8±0.2	4.5±0.1	4.2±0.2
2	Germany	66.6±3.8	-	6.6±0.8	4.3±0.1	4.2±0.1
3	Spain	66.3±2.0	-	7.2±0.3	4.7±0.1	3.8±0.1
4	Italy	65.7±1.2	-	6.0±0.1	3.7±0.1	3.7±0.0
5	Russia	65.6±1.5	-	6.2±0.0	4.3±0.4	4.7±1.1
6	Spain	64.6±3.8	0.62±0.0	5.6±0.1	4.5±0.2	2.3±0.0
7	Canada	63.5±0.5	-	6.5±0.0	4.3±0.0	3.6±0.0
8	Spain	63.3±0.0	-	5.5±0.0	3.6±0.0	3.6±0.0
9	Russia	61.7±0.8	-	5.6±0.6	3.5±0.1	3.5±0.2
10	Netherlands	60.2±0.3	-	6.2±0.6	4.7±0.1	4.4±0.1
11	Colombia	58.9±4.5	-	7.4±0.2	4.4±0.1	4.2±0.2
12	Russia	35.2±6.5	-	3.4±0.4	2.3±0.1	2.2±0.3

209

210 Standard deviation (*n*=2)

211

212 *3.2. Selectivity of β-galactosidases to hydrolyze lactose present in commercial lactulose*
213 *syrups.*

214 Since the removal of lactose and epilactose from lactulose is not economically
215 feasible, the previous hydrolysis of both compounds is required. However, since lactulose
216 and epilactose are also substrates for microbial β-galactosidases, the selective hydrolysis
217 of lactose and epilactose in the presence of large amounts of lactulose has to be optimized.

218 In order to choose the right enzyme that better leads to the selective hydrolysis of
219 disaccharides other than lactulose, four different commercial enzyme preparations were
220 tested. **Fig. 2** shows the evolution of content of lactose, lactulose and epilactose during
221 hydrolysis of commercial lactulose syrup (sample 3 in **Table 1**) with the four studied

222 enzymes. According to the results obtained, β−galactosidases from *Bacillus circulans*
223 (Biolactasa® NTL*2) (**Fig. 2 c**) and *Bifidobacterium bifidum* (Saphera® 2600 L) (**Fig. 2**
224 **d**) preferentially hydrolyzed lactose and epilactose over lactulose whereas Lactozym®
225 Pure 6500 L from *Kluyveromyces lactis* and Biolactase® F CONC from *Aspergillus*
226 *oryzae* hydrolyzed lactulose and lactose at similar rates, as can be observed in **Fig. 2 a**
227 and **2 b**. Therefore, these two last enzymes were discarded for the optimization of lactose
228 hydrolysis methodology by RSM.

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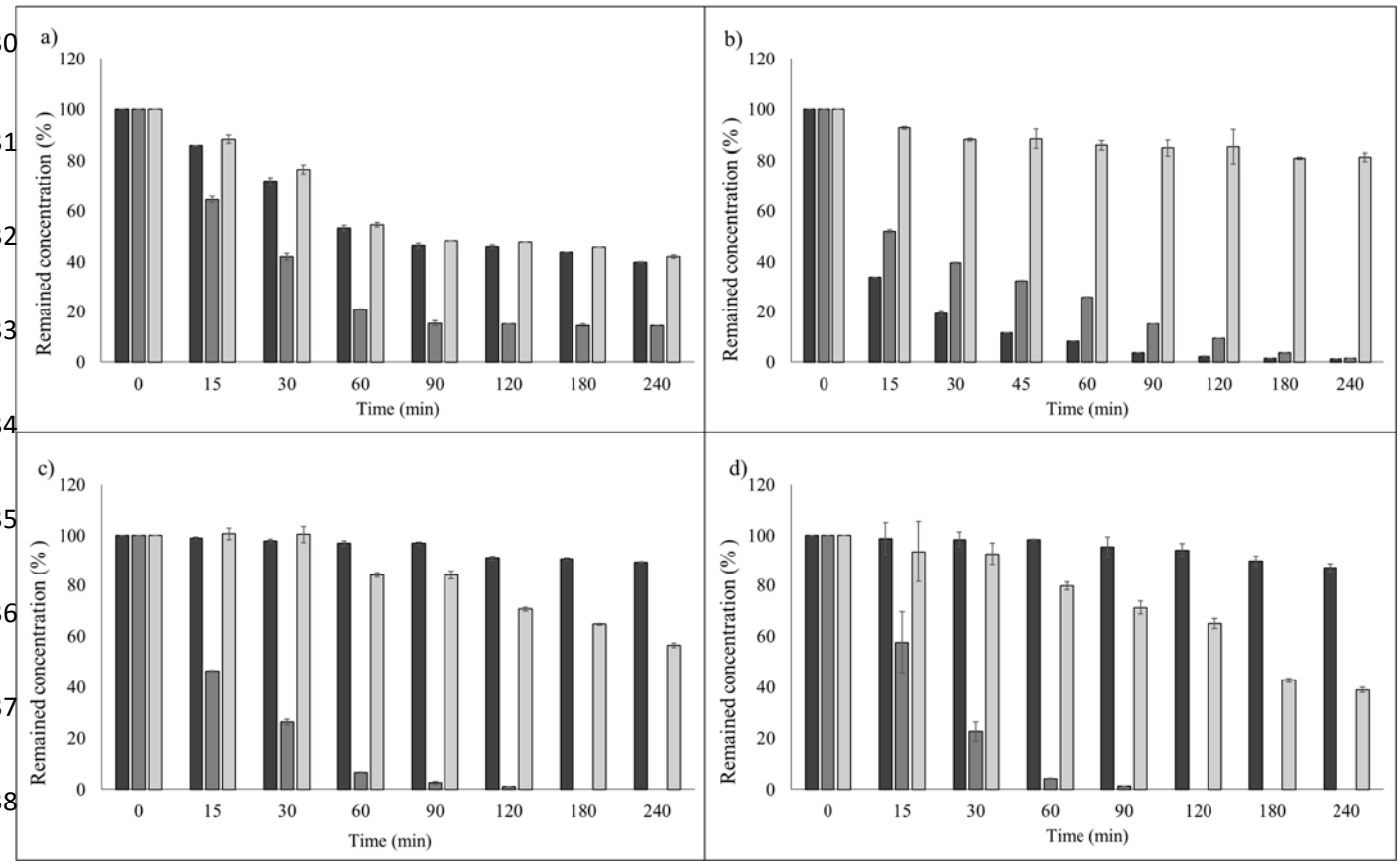

Fig. 2. Remained concentration (%) of lactulose ■, lactose ■, and epilactose □ found during hydrolysis for 240 minutes, of commercial lactulose syrup (10% w/v) (sample number 3 in Table 1) by four commercial β-galactosidases preparations (3 U/mL). **a)** Lactozym® Pure 6500 L *Kluyveromyces lactis* (pH 6.5 and 50 ºC); **b)** Biolactase® F CONC *Aspergillus oryzae* (pH 4.5 and 50 ºC); **c)** Biolactasa® NTL*2 *Bacillus circulans* (pH 6.5 and 50 ºC); **d)** Saphera® 2600 L *Bifidobacterium bifidum* (pH 6 and 40 ºC).

3.3 Optimization of lactose hydrolysis by RSM

The independent variables and experimental responses are shown in **Table 2.** The experimental data was fitted with the CCD model as shown in Equation 1 (Saphera® 2600

L) and Equation 2 (Biolactasa® NTL*2). The analysis of variance ($p \leq 0.05$) was carried out to know the significance of the regression model fit (**Table S1, supplementary material**). In general, the models were significant for all the experimental responses and the two β-galactosidases under study (Saphera® 2600 L and Biolactasa® NTL*2), with the exception of lactose recovery responses for both enzymatic preparations and epilactose recovery responses for Biolactasa® NTL*2 for which there is a significant lack of fit ($p < 0.0001$).

Equation 1 (Saphera® 2600 L)**:**

$$\text{Lactulose recovery (\%)} = 100.8 + 2.5\ X_1 - 7.1\ X_2 - 2.9\ X_3 - 0.02\ X_1^2 + 0.17\ X_2^2 + 0.03\ X_3^2$$

Equation 2 (Biolactasa® NTL*2)

$$\text{Lactulose recovery (\%)} = 106.1 + 2.9\ X_1 - 8.9\ X_2 - 2.5\ X_3 + 0.03\ X_1 * X_2 - 0.03\ X_1^2 + 0.17\ X_2^2 + 0.02\ X_3^2$$

Table 3 shows the optimal operation conditions and the theoretical responses values, calculated by the polynomial equation and the validated model. In order to validate these optimal conditions, confirmation experiments were carried out and the results obtained were compared with the theoretical results (**Table 3**). The aim of the numerical range optimization is to operate in conditions with the higher lactulose recovery where lactose is completely hydrolyzed (Scenario 1 – **Table 3**) or where lactose and epilactose are completely hydrolyzed (Scenario 2 – **Table 3**). The lactulose recovery (%) values were in the prediction interval, which is wider than the confidence interval (95%) since the model includes the sampling bias. Therefore, sample number 1

Table 2. Central Composit Design (CCD) runs. Independent variables and experimental response found during hydrolysis of lactose and epilactose present in commercial lactulose syrup using Saphera® 2600 L or Biolactasa® NTL*2 β-galactosidases.

Run	Lactulose (% w/v) X_1	Enzymatic activity (U/mL) X_2	Time (h) X3	Recovery (%) (Saphera® 2600 L)			Recovery (%) (Biolactasa NTL*2)		
				Lactulose	Lactose	Epilactose	Lactulose	Lactose	Epilactose
1	13.15	15	24.2	30.5	0	0	31.2	0	0
2	3.15	4	4.1	78.1	0	24	87.1	0	37.8
3	13.15	3	24.2	84.8	0	32.3	92.5	3.5	46.5
4	25	9	24.2	70.3	0	21.1	72.9	3.3	31.9
5	13.15	9	0.3	103.3	37.2	88.3	97	55.5	89.2
6	25	9	24.2	67.7	0	20.5	72.7	3.9	30.4
7	13.15	15	24.2	30.5	0	0	32.8	0	0
8	23.1	14	4.1	89.1	0	54.4	90.1	0	63.8
9	3.15	4	44.2	27.6	0	0	24	0	0
10	13.15	9	0.3	96.4	34.3	83	100.3	59.8	97.5
11	13.15	3	24.2	79.6	0	26.4	93.5	3.6	52.9
12	13.15	9	48	37.6	0	0	36.1	0	0
13	13.15	9	48	37.2	0	0	37.5	0	0
14	13.15	9	24.2	54.1	0	5.1	54.9	0	4.6
15	13.15	9	48	29.5	0	0	46.1	0	0
16	13.15	9	0.3	97.8	37	82.3	103.2	54.8	95.6
17	13.15	9	24.2	54.7	0	4.3	52.3	0	5.1
18	13.15	15	24.2	30.5	0	0	30.8	0	0
19	13.15	9	48	30.9	0	0	34.1	0	0
20	25	9	24.2	63.4	0	16.7	66.9	3.4	29.3
21	13.15	15	24.2	28.5	0	0	31.9	0	0
22	1.25	9	24.2	0.8	0	0	0.9	0	0
23	1.25	9	24.2	7.5	0	0	0.4	0	0
24	1.25	9	24.2	6	0	0	1	0	0
25	13.15	3	24.2	75	0	25.6	89.8	2.9	40.1
26	23.1	4	44.2	77.3	0	28.3	79.2	0	44
27	23.1	14	44.2	38.7	0	0	33.5	0	0
28	13.15	9	0.3	99.7	30.9	84.1	98.2	52.6	88.4
29	25	9	24.2	68.9	0	16.9	74.4	3.5	30.8
30	13.15	3	24.2	78.2	0	25.3	87.4	3.3	40
31	3.15	14	4.1	42.4	0	0	55.5	0	0
32	23.1	4	4.1	97	10.6	87.3	95.4	0	81.6
33	3.15	14	44.2	0.2	0	0	0	0	0
34	1.25	9	24.2	2.2	0	0	0	0	0

276

277 **Table 3**. Optimal operation conditions, predicted and experimental response for lactulose
278 recovery, obtained from hydrolysis of commercial lactulose syrup using Saphera® 2600
279 L or Biolactasa® NTL*2 β-galactosidases.

β-Galactosidases	Scenario	Lactulose (% w/v)	Enzymatic activity (U/mL)	Time (h)	Lactulose recovery (%)		Epilactose recovery (%)
					Predicted Mean	Experimental Mean (n=4)	Experimental Mean (n=4)
Saphera® 2600 L	1	21.0	11	4.1	96.9±3.5	95.2±3.3	79.0±2.4
	2	21.8	11.5	38.5	44.33±3.5	48.2±1.4	0.0±0.0
Biolactasa NTL*2	1	21.9	11.4	4.1	90.2±3.5	89.8±2.0	71.3±2.6
	2	23.1	13.4	26.7	51.8±3.5	52.0±1.2	11.3±0.9

280
281
282 **SD**: Standard deviation
283 **Scenario 1**: Maximize initial lactulose content and lactulose recovery; keep in working range enzymatic activity and
284 time; complete hydrolysis of lactose
285 **Scenario 2**: Maximize initial lactulose content and lactulose recovery; keep in working range enzymatic activity and
286 time; complete hydrolysis of lactose and epilactose.
287

288

289 containing 21% of lactulose and hydrolysed during 4.1 h with Saphera® 2600 L β-
290 galactosidase was selected for subsequent activate charcoal treatment to remove
291 monosaccharides. In **Fig. 1b** can be observed a GC-profile of carbohydrates found in this
292 sample, being fructose (peak 1), galactose (peak 2), glucose (peak 3), epilactose (peak 4)
293 and lactulose (peak 5).

294

295 Although enzymatic hydrolysis of high concentrated disaccharide solutions may
296 cause the formation of oligosaccharides via transglycosylation, the time required in this
297 study for complete hydrolysis of lactose was too short to lead to the synthesis of GOS in
298 significant amounts.

299

300 *3.4. Removal of monosaccharides by activated charcoal treatment*

301 Once selected the best enzymatic conditions to selectively hydrolyse lactose
302 present in the lactulose syrup, the hydrolysed sample mixture (above mentioned) was
303 treated with activated charcoal to eliminate all monosaccharides present in these
304 hydrolysates. **Table 4** shows the fractionation of carbohydrates using different
305 water/ethanol solutions. Recovery of purified lactulose was performed in 5 steps applying
306 different water/ethanol solutions (1/99; 8/92; v/v; five steps). The removal of
307 monosaccharides was mainly achieved during the first step using 1% ethanol but a second
308 step under the same conditions was required for their complete removal. Once total
309 monosaccharides were removed high purity lactulose was recovered during the third to
310 fifth steps using 8% ethanol. Through these three steps, 80.3% of total lactulose was
311 recovered with a purity of, at least, 94.2%, the rest being mainly epilactose (3.7%) and
312 other disaccharides (1.2%). The remaining oligosaccharides adsorbed on the activated
313 charcoal were desorbed during the sixth step using 50/50 water/ ethanol solution.
314 Epilactose, present in the purified lactulose in amounts lower than 4%, may contribute to
315 its prebiotic activity since it is considered a potential prebiotic disaccharide [24].
316

317 **4. Conclusions**

318 These results are the first reported in the literature dealing with the purification of
319 commercial lactulose syrup by removing lactose and monosaccharides using a sequential
320 methodology which involves the selective hydrolysis of lactose by commercial β-
321 galactosidases followed by adsorption of carbohydrates on activated charcoal, removal of
322 monosaccharides and recovery of lactulose using different proportions of water/ethanol
323 solutions. Lactulose recovery was of 80.3% with a purity of 94%. The resulting product

can be used in therapies for patients who have diabetes, with galactosaemia or who require a diet without lactose.

Table 4. Stepwise desorption of carbohydrates, from selectively hydrolysed lactulose syrup (Sample hydrolysed with Saphera® 2600 L β-galactosidase, number 1 in table 3) adsorbed onto activated charcoal by applying 200 mL ethanol/water mixtures per g of hydrolysed syrup.

Desorption step	Ethanol/water mixtures	Amount of desorbed carbohydrates (mg/g of hydrolyzed lactulose syrup)*						
		Fructose	Galactose	Glucose	Lactulose	Epilactose	Other disaccharides	Trisaccharides
1	1/99	7.9±0.1	48.5±0.2	15.9±0.1	25.3±1.3	0.5±0.0	0.2±0.0	0.2±0.0
2	1/99	0.2±0.1	6.0±0.3	1.8±0.0	28.9±0.9	0.4±0.0	0.1±0.1	0.1±0.1
3	8/92	0.0±0.0	0.0±0.0	0.0±0.0	338.1±9.5	11.3±0.2	4.3±0.1	2.6±0.1
4	8/92	0.0±0.0	0.0±0.0	0.0±0.0	58.1±3.8	3.7±0.3	0.9±0.1	0.6±0.1
5	8/92	0.0±0.0	0.0±0.0	0.0±0.0	11.4±1.0	1.1±0.3	0.2±0.1	0.2±0.1
6	50/50	0.0±0.0	0.0±0.0	0.0±0.0	5.3±0.6	0.6±0.1	0.3±0.0	0.3±0.0

Standard deviation (n=2)

* Carbohydrate composition (mg/g of hydrolyzed lactulose syrup): Fructose 9.6±0.2; Galactose 66.2±1.8; Glucose 20.8±0.3; Lactulose 507.6±8.0; Epilactose 18.7±0.6; Other disaccharides 7.3±0.3; Trisaccharides 4.8±0.1.

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