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1 **A sustainable biotechnological process for the efficient synthesis of kojibiose**

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3 Marina Díez-Municio, Antonia Montilla, F. Javier Moreno* and Miguel Herrero

4

5 Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), CEI

6 (UAM+CSIC), C/ Nicolás Cabrera 9, 28049 Madrid, Spain.

7

8 * Corresponding author: Tel.: +34 91 0017948; E-mail address: javier.moreno@csic.es

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10 **ABSTRACT**

11 This work reports the optimization of a cost-effective and scalable process for
12 the enzymatic synthesis of kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucose) from
13 readily available and low-cost substrates such as sucrose and lactose. This
14 biotechnological process is based on the dextransucrase-catalysed initial synthesis of a
15 galactosyl-derivative of kojibiose (4-*O*-β-D-galactopyranosyl-kojibiose) followed by
16 the removal of residual monosaccharides by using a *Saccharomyces cerevisiae* yeast
17 treatment, and a thorough hydrolysis step with *Kluyveromyces lactis* β-galactosidase.
18 Depending on the final purification stage, i.e. extension of the yeast treatment or use of
19 preparative liquid chromatography, the purity of the produced kojibiose ranged from
20 65% to ≥ 99%, respectively. The moderately high-yield achieved (38%, in weight
21 respect to the initial amount of lactose) using this affordable synthesis process could
22 expand the potential applications of kojibiose according to the bioactive properties that
23 have been associated to this disaccharide, so far limited by its low availability.

24

25 ***Keywords:*** kojibiose, dextransucrase, β-galactosidase, yeast, prebiotic
26 properties, anti-viral activities.

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27 **1. Introduction**

28 Kojibiose is a naturally occurring disaccharide comprised of two glucose
29 moieties bound through an α-(1→2) linkage (2-*O*-α-D-glucopyranosyl-α-D-
30 glucopyranose) that can be found in sake and koji (cooked rice and/or soy beans
31 inoculated with a fermentation culture of *Aspergillus oryzae*) extracts,[1] beer,[2] honey,[3,4]
32 and starch hydrolyzate.[5] However, kojibiose is present in those food products at low
33 levels making difficult its isolation from natural sources at high scale.

34 Kojibiose has been reported to be an excellent candidate as a prebiotic ingredient
35 according to its *in vitro* selectivity of microbial fermentation using individual bacteria
36 from *Bifidobacterium*, *Lactobacillus* or *Eubacterium* genera,[6] as well as with cultures of
37 mixed human fecal bacteria.[7] The promising prebiotic potential of kojibiose and
38 derived-oligosaccharides is also supported by the high resistance of α-(1→2) linkages to
39 *in vitro* and *in vivo* gastrointestinal digestion.[8,9] Indeed, new biotechnological and
40 sustainable approaches to the large scale manufacture of 1-2-linked oligosaccharides
41 have been demanded considering that kojibiose is available only in limited amounts.[7]

42 Kojibiose has also the ability to specifically inhibit the α-glucosidase I in
43 different tissues and/or organisms, such as rat liver microsomes,[10] bovine mammary
44 gland,[11] yeast microsomal preparation[12] and mung bean seedlings[13] either on soluble or
45 protein-bound oligosaccharides. Interestingly, glucosidase inhibitors have been shown
46 to display important anti-viral activities[14,15] and, in particular, glycosidase I inhibition
47 compounds, such as kojibose, have been suggested to open up new perspectives for the
48 development of novel drugs, especially of the pseudodisaccharide class, for the
49 treatment of human immunodeficiency virus type 1 (HIV-1) infections.[16] Additionally,
50 due to their glucosidase inhibition activity, these compounds have been disclosed for

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51 limiting digestion of dietary carbohydrates by inhibition of intestinal α-glucosidases
52 thereby providing a regimen for treating diabetes mellitus and obesity.[17,18]

53 The best-known method for preparation of kojibiose is the isolation from a
54 partial acetolyzate of dextran from *Leuconostoc mesenteroides* NRRL B-1299 by using
55 a mixture of acetic anhydride, glacial acetic acid and concentrated sulphuric acid, as
56 well as other chemical reagents such as chloroform or sodium methoxide.[19] Other
57 attempts for the synthesis of kojibiose were based on the partial enzymatic hydrolysis of
58 trisaccharides formed by dextransucrase actions,[20,21] as well as on the use of α-
59 glucosidase, [22] glucoamylase,[23] sucrose phosphorylase[24] and kojibiose phosphorylase[25]
60 using β-D-glucose-1-phosphate or 1,6-anhidro-β-D-glucopyranose as substrates.
61 Nevertheless, all these methods are considered to be tedious, very time-consuming,
62 uneconomical, and are normally characterized by the formation of by-products and low
63 production efficiency.[6] These facts would explain the restricted quantities of kojibiose
64 commercially available and its high cost, which limit its use despite the potential
65 applications described above.

66 In this regard, this work reports the development of a novel biotechnological,
67 cost-effective and environmentally-friendly process for the enzymatic synthesis of
68 kojibiose with relatively high yield and purity from readily available and inexpensive
69 raw materials such as sucrose and lactose. This method could straightforwardly be
70 scaled-up to produce kojibiose at industrial scale, which would allow expanding the
71 potential uses based on its bioactive properties, even allowing the reuse of important
72 food-related by-products, such as cheese whey permeate and beet or cane molasses.

73

74 **2. Experimental**

75 **Chemicals, reagents, standards and enzymes**

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76 All used chemicals and reagents were of analytical grade, purchased from
77 Sigma-Aldrich (St. Louis, MO, USA), VWR (Barcelona, Spain), and Merck
78 (Darmstadt, Germany). Ultra-pure water quality (18.2 MΩcm) with 1–5 ppb total
79 organic carbon (TOC) and <0.001 EU mL^{-1} pyrogen levels was produced in-house using
80 a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica,
81 MA, USA).

82 Carbohydrates (fructose, glucose, galactose, sucrose, leucrose and lactose) were
83 all purchased from Sigma-Aldrich (St. Louis, MO, USA) except kojibiose that was
84 purchased from Carbosynth (Berkshire, United Kingdom), and lactosucrose that was
85 from Wako Pure Chemical Industries (Osaka, Japan).

86 Dextransucrase from *Leuconostoc mesenteroides* B-512F was purchased from
87 CRITT Bio-Industries (Toulouse, France). Specific activity was 0.4 U mg^{-1}, where 1
88 unit is the amount of enzyme required to perform the transfer of 1 μmol of glucose per
89 minute at a working temperature of 30 °C, a sucrose concentration of 100 g L^{-1} at pH
90 5.2 in 20 mM sodium acetate buffer with 0.34 mM of $CaCl_2$. Soluble commercial
91 preparation of β-galactosidase from *Kluyveromyces lactis* (Lactozym Pure 6500 L) was
92 kindly supplied by Novozymes (Bagsvaerd, Denmark).

93

94 **Steps of the process for the synthesis of kojibiose**

95 *Enzymatic synthesis of 4'-galactosyl-kojibiose*

96 The synthesis of the trisaccharide 4'-galactosyl-kojibiose, also denominated 2-α-
97 glucosyl-lactose (O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-α-D-
98 glucopyranose) was carried out by transglucosylation reaction catalyzed by
99 dextransucrase from *L. mesenteroides* B-512F (0.8 U mL^{-1}) at 30 ºC in 20 mM sodium
100 acetate buffer with 0.34 mM $CaCl_2$ (pH 5.2) in the presence of sucrose (donor) and

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101 lactose (acceptor) as described previously by Díez-Municio et al.[26] at two different
102 concentration ratios (25:25 or 10:25, expressed in g/100 mL). The reaction proceeded
103 for 24 h after which the enzyme was inactivated by heating at 100 ºC for 5 min.

104 *Yeast treatment with Saccharomyces cerevisiae*

105 Removal of the monosaccharides (fructose, glucose, galactose) and sucrose
106 present in the carbohydrate mixture was carried out by yeast treatment. Since it is well-
107 known that one of the main affecting factors for removal of carbohydrates by yeast is
108 the initial sugar concentration, this value was fixed at 200 g L^{-1}.[27] Treatment with
109 *Saccharomyces cerevisiae* fresh baker's yeast (Levital, Panibérica de Levadura,
110 Valladolid, Spain) took place at 30 ºC under stirring (1,200 rpm), with the addition of
111 16 g of yeast per 100 g of sugar. When needed, the yeast was removed by centrifugation
112 (5 min at 8,000 rpm) or filtration.

113 *β-galactosidase hydrolysis*

114 The hydrolysis of the remaining lactose and trisaccharide 4'-galactosyl-kojibiose
115 was carried out by adding β-galactosidase enzyme from *K. lactis* (Lactozym Pure 6500
116 L) (65 U mL^{-1}) to the reaction medium without removing the yeast cells. The
117 temperature of the reaction was maintained at 30 ºC. Prior to the addition of the β-
118 galactosidase enzyme, the pH was regulated at 7.3 using potassium hydroxide (5 M),
119 and magnesium chloride (5 mM) was added. The hydrolysis reaction was performed for
120 90 min, after which the enzyme was inactivated by the pH drop caused by the still
121 ongoing yeast treatment.

122 *Purification of kojibiose by preparative liquid chromatography*

123 Kojibiose was isolated and purified by liquid chromatography with refractive
124 index detector (LC-RID) from the reaction mixture obtained after the treatment by β-
125 galactosidase from *K. lactis* on an Agilent Technologies 1260 Infinity LC System

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126 (Boeblingen, Germany) using a Zorbax NH_2 PrepHT preparative column (250 × 21.2
127 mm, 7 µm particle size) (Agilent Technologies, Madrid, Spain). Two mL of reaction
128 mixtures (150 mg of total carbohydrates) were eluted with acetonitrile:water (75:25, *v/v*)
129 as the mobile phase at a flow rate of 21.0 mL min^{-1} for 30 min. The separated kojibiose
130 was collected using an Agilent Technologies 1260 Infinity preparative-scale fraction
131 collector (Boeblingen, Germany), and the fractions were pooled, evaporated in a
132 rotatory evaporator R-200 (Büchi Labortechnik AG, Flawil, Switzerland) below 25 ºC
133 and freeze-dried.
134

135 **Analytical techniques**

136 *Liquid chromatography with refractive index detector (LC-RID).*

137 The progress of the kojibiose synthesis process was monitored by liquid
138 chromatography with refractive index detector (LC-RID) on an Agilent Technologies
139 1220 Infinity LC System – 1260 RID (Boeblingen, Germany). The separation of
140 carbohydrates was carried out with a Kromasil (100-NH_2) column (250 × 4.6 mm, 5 µm
141 particle size) (Akzo Nobel, Brewster, NY, USA) using isocratic elution with
142 acetonitrile:water (75:25, *v/v*) as the mobile phase at a flow rate of 1.0 mL min^{-1} for 40
143 min. Injection volume was 50 µL (1 mg of total carbohydrates). Data acquisition and
144 processing were performed using Agilent ChemStation software (Agilent Technologies,
145 Boeblingen, Germany).

146 Carbohydrates in the reaction mixtures were identified by comparing their
147 retention times with those of standard sugars. Quantitative analysis was performed by
148 the external standard method, using calibration curves in the range 0.1-10 mg mL^{-1} for
149 fructose (quantification of monosaccharides), sucrose, leucrose, lactose, kojibiose and
150 4'-galactosyl-kojibiose. All analyses were carried out in triplicate. Determination

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151 coefficients obtained from these calibration curves, which were linear over the range
152 studied, were high ($R^2 > 0.999$). Reproducibility of the method was estimated on the
153 basis of the intra-day and inter-day precision, calculated as the relative standard
154 deviation (*RSD*) of concentrations of oligosaccharide standards obtained in $n \geq 5$
155 independent measurements, obtaining *RSD* values below 10% in all cases.

156 *Gas chromatography with a flame ionization detector (GC-FID)*

157 The carbohydrate composition of the resulting reaction mixture was determined
158 by GC-FID on an Agilent Technologies 7890A gas chromatograph (Agilent
159 Technologies, Wilmington, DE, USA) equipped with a flame ionization detector, using
160 nitrogen as carrier gas at 1 mL min^{-1}. The trimethylsilyl oxime (TMSO) derivatives
161 were prepared as previously described by Sanz et al.[28] and separated using an fused-
162 silica capillary column (30 m × 0.32 mm i.d. × 0.5 µm film thickness) SPBTM–17,
163 bonded, crosslinked phase (50% diphenyl / 50% dimethylsiloxane) (Supelco,
164 Bellefonte, PA, USA). The oven initial temperature was 200 ºC, increased at a rate of 4
165 ºC min^{-1} to 230 ºC, then at a rate of 2 ºC min^{-1} to 290 ºC and held for 50 min. The
166 injector and detector temperatures were 280 and 290 ºC, respectively. Injections were
167 made in the split mode (1:20). Data acquisition and integration were performed using
168 Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for
169 carbohydrates were calculated from FID peak areas relative to phenyl-β-D-glucoside
170 (internal standard). Mixtures of standard solutions of fructose, galactose, glucose,
171 sucrose, lactose, leucrose, kojibiose and lactosucrose over the expected concentration
172 range were prepared with 0.2 mg of internal standard to calculate the response factor for
173 each sugar.

174 *Gas chromatography with mass spectrometry detection (GC-MS)*

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175 Both synthesized and commercial kojibiose were analyzed by GC-MS on an
176 Agilent Technologies 7890A gas chromatograph coupled to a 5975C MSD quadrupole
177 mass detector (Agilent Technologies, Wilmington, DE, USA) in order to confirm the
178 identification of the purified kojibiose. Sugars separation was performed under the same
179 chromatographic conditions described above, substituting the carrier gas with helium.
180 The mass spectrometry detector was operated in electronic impact mode at 70 eV. Mass
181 spectra were acquired using Agilent ChemStation MSD software (Wilmington, DE,
182 USA).
183
184 **Chemical and microbiological characterization of the synthesized kojibiose**
185 *Chemical analyses of kojibiose*
186 Chemical quality parameters (dry matter, mineral composition, nitrogen and pH)
187 were determined in kojibiose samples.
188 The dry matter (DM) content was gravimetrically determined by drying the
189 kojibiose samples in a conventional oven at 102 ºC until constant weight, according to
190 the AOAC method.[29]
191 Ion composition of the kojibiose samples was determined using an ICP-MS
192 NexION 300XX Perkin Elmer instrument (Perkin Elmer, Waltham, MA, USA). Either a
193 semiquantitative analysis or a quantitative analysis of the elements of interest using the
194 external calibration method and internal standards to correct instrumental drift were
195 carried out.[30] Nitrogen percentage determination was performed on an elemental
196 analyzer LECO CHNS-932 (LECO Corporation, St. Joseph, MI, USA).
197 The pH of kojibiose samples was measured using a pH meter (MP 230, Mettler-
198 Toledo, Barcelona, Spain) at a concentration of 10 mg mL^{-1}.
199 *Microbiological analysis of kojibiose*

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200 In order to evaluate the microbiological quality, samples were analyzed for the
201 presence of yeasts and molds, total and sporulated aerobic microorganisms and
202 enterobacteria. Serial dilutions were performed in triplicate with peptone water (Biocult
203 BV, Roelofarendsveen, The Netherlands). Yeasts and molds were plated on Sabouraud
204 chloramphenicol agar and incubated at 25 ± 1 °C for 5 days. The total and sporulated
205 aerobic bacteria were determined by plating appropriately diluted samples onto plate
206 count agar. The samples were incubated at 30 ± 1 °C for 72 h for total aerobic bacteria
207 and at 37 ± 1 °C for 48 h for sporulated aerobic bacteria after heat treatment of stock
208 dilution at 80 °C for 10 min. For enterobacteria counts, violet red bile dextrose agar was
209 used and incubation was carried out at 30 ± 1 °C for 24 h. All microbial counts were
210 reported as colony forming units per gram (cfu g^{-1}). All culture media were of Difco
211 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).
212
213 **3. Results and Discussion**
214 **Optimization of the process for the efficient synthesis of kojibiose**
215 A general scheme illustrating the process followed for the synthesis of kojibiose
216 is shown in **Figure 1**. Briefly, this procedure, which uses cheap food-grade enzymes
217 and starting substrates as sucrose and lactose, does not generate any toxic substances
218 and is comprised of four steps carried out at constant temperature (30 ºC): i) synthesis
219 of a galactosyl-derivative of kojibiose catalyzed by *Leuconostoc mesenteroides* B-512F
220 dextransucrase; ii) removal of remaining monosaccharides and sucrose by
221 *Saccharomyces cerevisiae* yeast; iii) production of kojibiose by the hydrolytic action of
222 β-galactosidase from *Kluyveromyces lactis*, and; iv) purification stage based on the
223 initial treatment with yeast or on a preparative liquid chromatographic separation. This

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224 process was optimized to maximize kojibiose yield and purity, as well as to reduce
225 operating time as it is explained below.

226 *1st step: Enzymatic synthesis of the trisaccharide 4'-galactosyl-kojibiose*

227 Based on a previous work dealing with the optimization of the enzymatic
228 synthesis of the trisaccharide *O*-β-D-galactopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-
229 (1→2)]-α-D-glucopyranose,[26] the concentration of the initial substrates (sucrose and
230 lactose) was set at 25% *(w/v)* each, whereas the enzyme concentration employed (i.e.,
231 dextransucrase from *L. mesenteroides* B-512F) was 0.8 U mL^{-1} and the reaction time 24
232 h. This trisaccharide was formed by the dextransucrase-catalysed transfer of a glucosyl
233 unit from the hydrolysis of sucrose to lactose acceptor through the formation of an α-
234 (1→2)-glucosyl bond (**Figure 2**). Under these optimized conditions, the final reaction
235 mixture was composed of 31.2% 4'-galactosyl-kojibiose and 38.5% lactose, 21.3%
236 fructose, 5.2% leucrose, 2.8% lactosucrose, 0.9% glucose and 0.1% sucrose as
237 determined by LC-RID (**Table 1**).

238 *2nd step: Yeast treatment with Saccharomyces cerevisiae*

239 Monosaccharides such as glucose, galactose and fructose are potent inhibitors of
240 the hydrolytic action of β-galactosidase.[31] Considering the high levels of fructose
241 present in the reaction mixture, its removal was required prior to the hydrolysis step
242 with the β-galactosidase. Besides, its removal would also help to obtain a more purified
243 kojibiose. *S. cerevisiae* yeast treatments have been already successfully used to remove
244 monosaccharides (mainly glucose and galactose) from galacto-oligosaccharides
245 mixtures.[32-34] Yoon et al.[35] also demonstrated that yeast cells are very efficient for the
246 removal of fructose and sucrose whilst lactose and other oligosaccharides formed by
247 transglycosylation or condensation reactions remained unaffected. Apart from the high
248 efficiency of yeast treatment in the removal of monosaccharides, this procedure can also

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249 be performed directly on synthesis mixtures without the need of significant dilutions (as
250 required by other techniques such as nanofiltration) and is also a low-cost and easily
251 scalable process for industrial uses.[32,36]

252 In order to reduce the treatment time, two different yeast charges were assayed
253 (16 and 32 mg mL^{-1}) and the process was left to stand for 7 h. The carbohydrate
254 composition of the mixture was characterized and quantified by LC-RID (data not
255 shown) and the pH was monitored every 30 min (**Figure 3**). With both yeast charges the
256 pH decreased from 5.2 to 3.7 as sugars were metabolized, and this pH decrease could be
257 caused by the dissolution of CO_2 (produced during glycolysis) resulting in carbonic
258 acid. This treatment was carried out under aeration and vigorous stirring to facilitate the
259 yeast growth and evaporation of ethanol.

260 In the case of the highest yeast charge, a pH plateau was achieved after 4 h of
261 treatment (**Figure 3**). This decrease is in agreement with the efficient decrease in
262 fructose (94.4% of fructose was removed) and the complete removal of lactosucrose,
263 glucose and sucrose observed (**Table 1**). In contrast, leucrose, lactose and 4'-galactosyl-
264 kojibiose were unaffected after the whole yeast treatment. In consequence, a yeast
265 charge of 32 mg mL^{-1} and 4 h were the parameters established for this second stage.

266 *3rd step: Hydrolysis with β-galactosidase from Kluyveromyces lactis*

267 This stage started when the monosaccharides were almost completely removed,
268 although the yeast cells employed were still maintained in the reaction medium. Due to
269 the optimum pH for the hydrolytic action of β-galactosidase from *K. lactis* is in the
270 range 6.5-7.5,[37] after the yeast treatment the pH was increased to 7.3 and three different
271 enzyme concentrations, namely, 6.5, 32.5 and 65 U mL^{-1}, were assayed. As indicated
272 above, the carbohydrate composition of the mixture was quantified by LC-RID and the
273 pH was monitored at different times (data not shown). Under the three enzyme

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274 concentrations studied, the trisaccharide 4'-galactosyl-kojibiose and lactose were
275 efficiently hydrolyzed to release kojibiose and galactose, as well as glucose and
276 galactose, respectively (**Figure 2**). Moreover, no detectable formation of galacto-
277 oligosaccharides derived from the transgalactosylation of lactose was observed. With
278 the highest β-galactosidase concentration assayed, the hydrolysis was faster and after 90
279 min no trace of lactose was detected and the 4'-galactosyl-kojibiose trisaccharide was
280 completely hydrolyzed. Besides, part of the monosaccharides produced during the
281 enzymatic hydrolysis was consumed by the yeast present in the medium producing a pH
282 drop down to 6.0 and, consequently, the enzyme was inactivated. Under these
283 conditions, the reaction mixture after this hydrolysis step was comprised of 46.5%
284 kojibiose, 20.2% glucose, 19.0% galactose, 11.3% leucrose, 2.9% unidentified
285 trisaccharides, and 0.1% fructose (**Table 1**).

286 *4th step: Purification of kojibiose*

287 For the final step, two different strategies were studied as it is shown in **Figure**
288 **1**.

289 i) *Yeast treatment purification*

290 This strategy consists in maintaining the yeast treatment for additional 42 h,
291 thanks to the yeast already present in the reaction medium. This second yeast treatment
292 took longer since, at this point, it was necessary to eliminate both glucose and galactose;
293 during the initial 3 h the yeast mainly metabolized glucose while galactose levels
294 remained constant and, once glucose was removed, galactose assimilation was observed
295 (data not shown) at a slower rate. The main advantage of this step relies on that no
296 additional charge of yeast is required and, consequently, the yeast concentration added
297 during the second step was enough to eliminate the monosaccharide fraction.
298 Nevertheless, other attempts based on the addition of a second charge of yeast during

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299 this step were carried out, although these modifications did not lead to shorten the
300 incubation time (data not shown).

301 The final carbohydrate composition after the incubation with the yeast was as
302 follows: 73.9% kojibiose, 18.4% leucrose and 7.7% unidentified trisaccharides (**Table**
303 **1**). **Figure 4B** displays a GC-FID chromatogram of the final sample and the **Figure 5B**
304 shows the corresponding mass spectrum which allowed the kojibiose identification by
305 comparison with the respective GC retention index and MS data of the commercial
306 standard (**Figures 4A** and **5A**). The high abundance of *m/z* 319 ion (ratio 319/361 > 1)
307 is characteristic of aldohexoses having a 1→2 glycosidic linkage originated by the loss
308 of a *O*-trimethylsilyl (TMS)OH group from the chain C3–C4–C5–C6, as has been
309 previously described.[38,39]

310 Since leucrose was neither metabolized by the yeast treatment nor hydrolyzed by
311 the β-galactosidase and given that its formation proves that fructose can also act as a
312 minor acceptor in the dextransucrase-catalyzed reactions,[40] additional synthesis studies
313 starting with a low concentration of sucrose (10%, *w/v*) and keeping the lactose
314 concentration at 25% *(w/v)* were performed in order to reduce the final content of
315 leucrose. This was attained although substantial levels of di- and trisaccharides derived
316 from the transgalactosylation of lactose were also found and, consequently, the purity of
317 kojibiose could not be increased (**Table 2**). The formation of galacto-oligosaccharides
318 was probably favored because a higher concentration of lactose remained after
319 formation of the 4'-galactosyl-kojibiose, making easier its transgalactosylation.

320 ii) *Chromatographic purification*

321 A second strategy based on the purification by preparative liquid
322 chromatography with refractive index detector (LC-RID) was attempted with the aim of
323 increasing the purity of kojibiose and reducing the total process time. In this case, the

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324 first step was to remove from the medium the remaining yeast either by centrifugation
325 or by filtration. As it is shown in **Figure 6**, kojibiose was well resolved from the rest of
326 carbohydrates present, including disaccharides as leucrose, within only 30 min. This
327 purification step allowed the attainment of kojibiose at the gram scale. Finally, the
328 purity grade of the chromatographically isolated kojibiose was checked by GC-FID
329 (**Figure 4C**), this being comparable to that of the commercial standard which is labeled
330 as min. 99% (**Figure 4A**).
331
332 **Physico-chemical and microbiological characterization of the synthesized kojibiose**
333 Apart from the characterization of the carbohydrate composition, the synthesized
334 kojibiose was also subjected to a physico-chemical and microbiological characterization
335 (**Table 2**). Both the kojibiose purified by the continuous yeast treatment or
336 chromatographically isolated were freeze-dried and white powders were obtained.
337 However, the latter required an evaporation step before freeze-drying to remove the
338 acetonitrile present in the mobile phase. Likewise, the estimated yield of kojibiose
339 synthesized with the process described in this work was 38% (in weight respect to the
340 initial amount of lactose).
341 Additionally, analyses of the dry matter, nitrogen, mineral composition, and pH
342 were carried out for the kojibiose purified with the yeast treatment. Whilst the purity of
343 kojibiose isolated on the preparative LC column could be considered ≥ 99% as it was
344 indicated above, the content of kojibiose purified by the yeast treatment was 65% on dry
345 matter. The second most important compound was leucrose (19%) followed by 8% of
346 yeast metabolites produced when sugars are metabolized (mainly minor amounts of
347 polyalcohols and/or organic acids) (**Table 2**). Lastly, microbiological assays
348 demonstrated that the microbial load (yeast and molds, total and sporulated aerobic

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349 bacteria, enterobacteria) was, in all cases, lower than 3×10^1 cfu g^{-1}, indicating that the
350 kojibiose synthesized by this process is microbiologically safe and could be used as
351 food ingredient, among other applications. Lastly, this procedure, especially when the
352 purification is carried out with yeast treatment, can be considered as an environmental
353 friendly process since the use of toxic solvents is avoided during the whole treatment.
354

355 **4. Conclusions**

356 This work describes a cost-effective and scalable process developed at a
357 moderate temperature, 30 ºC, for the efficient synthesis of kojibiose from abundant and
358 low-cost substrates as sucrose and lactose. This biotechnological process could be an
359 alternative to the chemistry-based procedures used for the production of kojibiose, as
360 well as to be further applied to important agro-industrial residues containing sucrose
361 and lactose, such as beet and cane molasses or cheese whey permeate.[41] The reasonably
362 high-yield and affordable synthesis of such a value-added ingredient, as kojibiose, from
363 food-related by-products provides new opportunities for potential applications of
364 kojibiose considering its limited availability, as well as for the valorization of by-
365 products from the sugar and dairy industries.

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366 ***Acknowledgements***

367 This work has been financed by projects AGL2011-27884 and Consolider Ingenio 2010
368 FUN-C-FOOD CSD2007-00063 both from Ministerio de Ciencia e Innovación
369 (MICINN), ALIBIRD 2009/AGR-1469 from Comunidad de Madrid and project
370 POII10-0178-4685 from Junta de Comunidades de Castilla-La Mancha (JCCM) and
371 European Regional Development Fund (ERDF). M. Díez-Municio is supported by
372 CSIC through JAE-Pre Programme co-funded by European Social Fund (ESF). M.
373 Herrero thanks MICINN for his "Ramón y Cajal" contract. The authors gratefully
374 acknowledge Torsten Paarup of CIAL for the microbiological analysis and Zaida Gil
375 and Pascual Torres of SIdI-UAM for the mineral and nitrogen determination. Authors
376 thank Ramiro Martínez (Novozymes Spain, S.A.) for Lactozym supply.

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377 **REFERENCES**

378 1 K. Matsuda, *Nippon Nogeikagaku Kaishi,* 1959, **33**, 719-723.

379 2 J. S. Hough, D. E. Briggs, R. Stevens and T. W. Young, in *Malting and Brewing*
380 *Science. Volume II Hopped Wort and Beer.* Chapman and Hall Ltd., New York, USA,
381 1982, ch. 22, pp. 776-838.

382 3 T. Watanabe and K. Aso, *Nature,* 1959, **183**, 1740.

383 4 R. Consonni, L. R. Cagliani and C. Cogliati, *J. Agric. Food Chem.*, 2012, **60**, 4526-
384 4534.

385 5 A. Sato and K. Aso, *Nature,* 1957, **180**, 984-985.

386 6 T. Nakada, T. Nishimoto, H. Chaen and S. Fukuda, in *Oligosaccharides in Food and*
387 *Agriculture,* ed. G. Eggleston and G. L. Côté, American Chemical Society, Washington,
388 DC, USA, 2003, vol. 849, pp. 104-117.

389 7 M. L. Sanz, G. R. Gibson and R. A. Rastall, *J. Agric. Food Chem.,* 2005a, **53**, 5192-
390 5199.

391 8 P. Valette, V. Pelenc, Z. Djouzi, C. Andrieux, F. Paul, P. Monsan and O. Szyli, *J.*
392 *Sci. Food Agr.*, 1993, **62**, 121-127.

393 9 M. Remaud-Siméon, R. M. Willemot, P. Sarçabal, G. Potocki de Montalk and P.
394 Monsan, *J. Molec. Catal. B: Enzym.,* 2000, 10, 117-128.

395 10 M. Takeuchi, K. Kamata, M. Yoshida, Y. Kameda and K. Matsui, *J. Biochem.,*
396 1990a, **108**, 42-46.

397 11 K. Shailubhai, M. A. Pratta and I. K. Vijay, *Biochem. J.,* 1987, **247**, 555-562.

398 12 E. Bause, R. Erkens, J. Schweden and L. Jaenicke, *FEBS Lett.,* 1986, **206**, 208-212.

399 13 T. Szumilo, G. P. Kaushal and A. D. Elbein, *Arch. Biochem. Biophys.,* 1986, **247**,
400 261-271.

401 14 A. D. Elbein, *Ann. Rev. Biochem.* 1987, **56**, 497-534.

Green Chemistry Accepted Manuscript

402 15 A. Tan, L. van den Broek, S. van Boeckel, H. Ploegh and J. Bolscher, *J. Biol.*
403 *Chem.,* 1991, **266**, 14504-14510.
404 16 E. Borges de Melo, A. da Silveira Gomes and I. Carvalho, *Tetrahedron,* 2006, **62**,
405 10277-10302.
406 17 M. Takeuchi, N. Takai, N. Asano, Y. Kameda and K. Matsui, *Chem. Pharm. Bull.,*
407 1990b, **38**, 1970-1972.
408 18 *E. Pat.*, 0 830 365 B1, 2002.
409 19 K. Matsuda, H. Watanabe, K. Fujimoto and K. Aso, *Nature*, 1961, **191**, 278.
410 20 K. Aso, K. Shibasaki and M. Nakamura, *Nature,* 1958, **182**, 1303-1304.
411 21 R. W. Bailey, S.A. Barker, E. J. Bourne, P. M. Grant and M. Stacey, *J. Chem. Soc.,*
412 1958, 1895-1902.
413 22 M. Takahashi, T. Shimomura and S. Chiba, *Agric. Biol. Chem.,* 1969, **33**, 1399-
414 1410.
415 23 H. Fujimoto, H. Nishida and K. Ajisaka, *Agric. Biol. Chem.*, 1988, **52**, 1345-1351.
416 24 S. Kitao, S. Yoshida, T. Horiuehi, H. Sekine and I. Kusakabe, *Biosci. Biotech.*
417 *Biochem.*, 1994, **58**, 790-791.
418 25 H. Chaen, T. Nishimoto, T. Nakada, S. Fukuda, M. Kurimoto and Y. Tsujisaka, *J.*
419 *Biosci. Bioeng.,* 2001, **92**, 173-176.
420 26 M. Díez-Municio, A. Montilla, M. L. Jimeno, N. Corzo, A. Olano and F. J. Moreno,
421 *J. Agric. Food Chem.,* 2012, **60**, 1945-1953.
422 27 M. L. Sanz, N. Polemis, V. Morales, N. Corzo, A. Drakoularakou, G. R. Gibson and
423 R. A. Rastall, *J. Agric. Food Chem.,* 2005b, **53**, 2914-2921.
424 28 M. L. Sanz, J. Sanz and I. Martínez-Castro, *J. Chromatogr. A.* 2004, **1059**, 143-148.
425 29 AOAC method 950.01. Official Methods of Analysis of AOAC, ed. K. Helrich,
426 Association of Official Analytical Chemists, Arlington, VA, USA, 1990, vol. 1, pp. 684.

Green Chemistry Accepted Manuscript

427 30 J. Zuluaga, N. Rodríguez, I. Rivas-Ramirez, V. de la Fuente, L. Rufo and R. Amils,
428 *Biol. Trace Elem. Res.* 2011, **144**, 1302-1317.
429 31 D. H. Alpers and J. E. Gerber, *J. Lab. Clin. Med.*, 1971, **78**, 265-274.
430 32 A. Goulas, G. Tzortzis and G.R. Gibson, *Int. Dairy J.,* 2007, **17**, 648-656.
431 33 Z. Li, M. Xiao, L. Lu and Y. Li, *Process Biochem.,* 2008, **43**, 896-899.
432 34 O. Hernández, A. I. Ruiz-Matute, A. Olano, F. J. Moreno and M. L. Sanz, *Int. Dairy*
433 *J.,* 2009, **19**, 531-536.
434 35 S. -H. Yoon, R. Mukerjea and J. F. Robyt, *Carbohydr. Res.,* 2003, **338**, 1127-1132.
435 36 F. J. Moreno, C. Carrero-Carralero, O. Hernández-Hernández, and M. L. Sanz, in
436 *Food Oligosaccharides: Production, Analysis and Bioactivity*, ed. F. J. Moreno and M.
437 L. Sanz, Wiley-Blackwell, Chichester, UK, 2014, ch. 15, pp. 257-283.
438 37 C. Martínez-Villaluenga, A. Cardelle-Cobas, N. Corzo, A. Olano and M. Villamiel,
439 *Food Chem.,* 2008, **107**, 258-264.
440 38 M. L. Sanz, J. Sanz and I. Martínez-Castro, *Chromatographia,* 2002, **56**, 617-622.
441 39 O. Hernández-Hernández, F. Montañés, A. Clemente, F. J. Moreno and M. L. Sanz,
442 *J. Chromatogr. A.,* 2011, **1218**, 7691-7696.
443 40 F. J. Plou, M. T. Martín, A. G. de Segura, M. Alcalde and A. Ballesteros, *Can. J.*
444 *Chem.,* 2002, **80**, 743-752.
445 41 *ES Pat.*, P201331333, 2013.
446

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447 **Figure legends**

448 **Figure 1.** Biotechnological process scheme implemented for the efficient synthesis of
449 kojibiose by transglucosylation of lactose catalyzed by dextransucrase from
450 *Leuconostoc mesenteroides* B-512F, yeast treatment with *Saccharomyces cerevisiae* and
451 hydrolysis with β-galactosidase from *Kluyveromyces lactis* with and without subsequent
452 chromatographic purification.

453 **Figure 2.** Chemical structures of the carbohydrates involved in the developed kojibiose
454 synthesis process.

455 **Figure 3.** Representative time course showing the different pH values achieved during
456 the treatment with *Saccharomyces cerevisiae* at two different yeast charges (16 and 32
457 mg mL^{-1}). The carbohydrate mixture (produced in the dextransucrase-catalysed
458 reaction) used for the yeast treatment had a 200 mg mL^{-1} total sugar concentration.

459 **Figure 4.** Profiles obtained by gas chromatography with a flame ionization detector
460 (GC-FID) of the commercial kojibiose standard (A) and the synthesized kojibiose
461 obtained with yeast (B) or chromatographic (C) purification.
462 Peak identification: 1 and 2 (yeast metabolites), 3 (internal standard), 4 and 5 (leucrose),
463 6 and 7 (kojibiose), 8 (trisaccharides).

464 **Figure 5.** Mass spectra obtained by gas chromatography coupled to mass spectrometry
465 (GC-MS) analysis using the corresponding trimethylsilyl oximes (TMSO) of the
466 commercial kojibiose standard (A) and the synthesized kojibiose obtained with yeast
467 purification (B).

468 **Figure 6.** Preparative liquid chromatography with refractive index detector (LC-RID)
469 profile obtained in the chromatographic purification step.

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471 **Table 1.** Carbohydrate composition (mg mL^{-1}) of the different mixtures determined by
472 LC-RID during the four steps involved in the process for the efficient synthesis of
473 kojibiose. The concentration of the initial substrates (sucrose and lactose) was set at
474 25% *(w/v)* each (500 mg mL^{-1} total sugar concentration).

	1st step	2nd step	3rd step	4th step	
	Dextransucrase Synthesis	**Yeast Treatment**	**β-galactosidase Hydrolysis**	**Yeast Treatment**	**LC-RID Purification**
Fructose	99.55 ± 3.40	5.55 ± 0.05	0.13 ± 0.06	-	-
Glucose	4.25 ± 0.29	-	41.34 ± 4.68	-	-
Galactose	-	-	38.81 ± 0.26	-	-
Sucrose	0.67 ± 0.01	-	-	-	-
Leucrose	24.22 ± 0.64	24.96 ± 0.11	23.18 ± 0.15	24.12 ± 0.29	-
Kojibiose	-	-	95.06 ± 1.84	96.58 ± 2.49	97.42 ± 8.38
Lactose	179.95 ± 5.53	193.09 ± 0.44	-	-	-
Lactosucrose	13.28 ± 0.38	-	-	-	-
4'-galactosyl-kojibiose	145.86 ± 5.61	150.04 ± 1.50	-	-	-
Unidentified trisaccharides	-	-	5.93 ± 0.08	10.05 ± 0.32	-

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477 **Table 2.** Chemical and microbiological characterization of the product with high
478 content in kojibiose obtained by dextransucrase-catalysed reaction, subsequent
479 treatment with *Saccharomyces cerevisiae* yeast and β-galactosidase from
480 *Kluyveromyces lactis*, including a final yeast purification step.

481

	Suc 25% + Lac 25%[†]	Suc 10% + Lac 25%[‡]
Chemical analysis		
Dry matter (DM)	93% on product	93% on product
Kojibiose[§]	65% on DM	52% on DM
Leucrose[§]	19% on DM	8% on DM
Disaccharides[§] (galactosylated derivatives)	-	16% on DM
Trisaccharides[§]	6% on DM	14% on DM
Yeast metabolites[§]	8% on DM	8% on DM
Minerals	2% on DM	2% on DM
Potassium	1.40%	1.40%
Magnesium	0.20%	0.20%
Nitrogen	0.10%	0.10%
Sodium	0.10%	0.10%
Others	0.10%	0.10%
pH[¶]	5.0	5.0
Microbiological analysis		
Yeasts and molds	$< 3 \times 10^1$ cfu g^{-1}	$< 3 \times 10^1$ cfu g^{-1}
Total viable aerobic at 30 °C	$< 3 \times 10^1$ cfu g^{-1}	$< 3 \times 10^1$ cfu g^{-1}
Aerobic sporulated at 30 °C	$< 1 \times 10^1$ cfu g^{-1}	$< 1 \times 10^1$ cfu g^{-1}
Enterobacteriaceae	$< 1 \times 10^1$ cfu g^{-1}	$< 1 \times 10^1$ cfu g^{-1}

482 [†]The concentration of the initial substrates, sucrose (Suc) and lactose (Lac)
483 was set at 25% *(w/v)* each (500 mg mL^{-1} total sugar concentration).

484 [‡]The concentration of the initial sucrose (Suc) was 10% *(w/v)* and lactose
485 (Lac) 25% *(w/v)* (350 mg mL^{-1} total sugar concentration).

486 [§]Determined by GC-FID.

487 [¶]Values obtained by measuring the pH of a solution of 10 mg of product
488 dissolved in 1 ml of ultrapure water.

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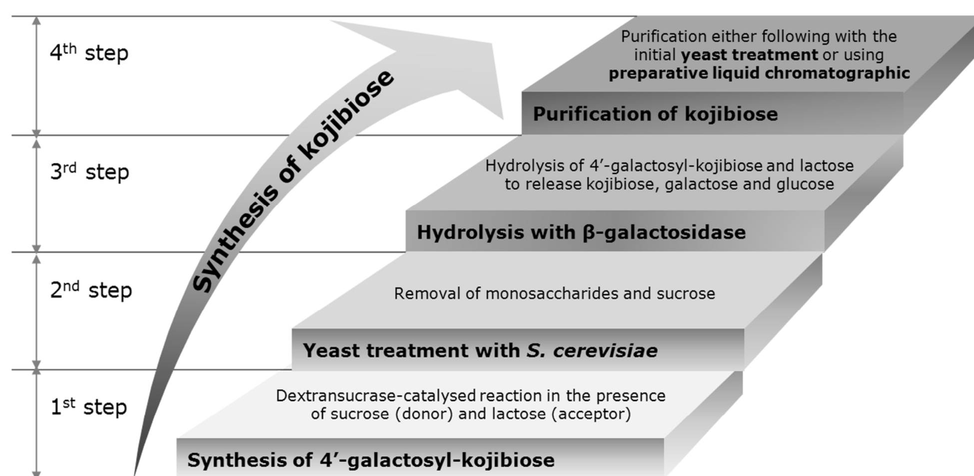

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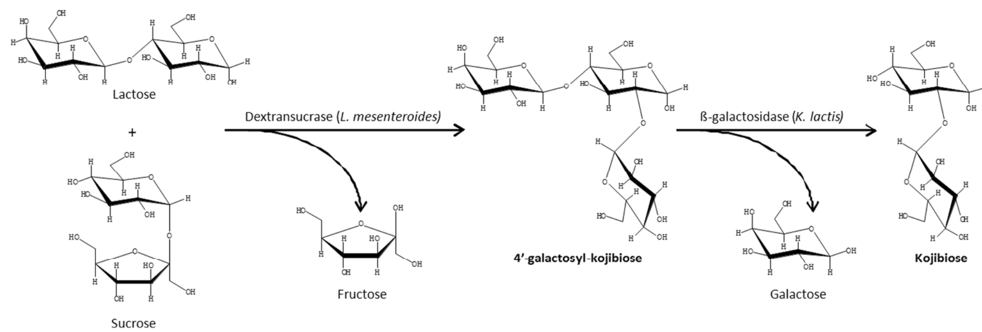

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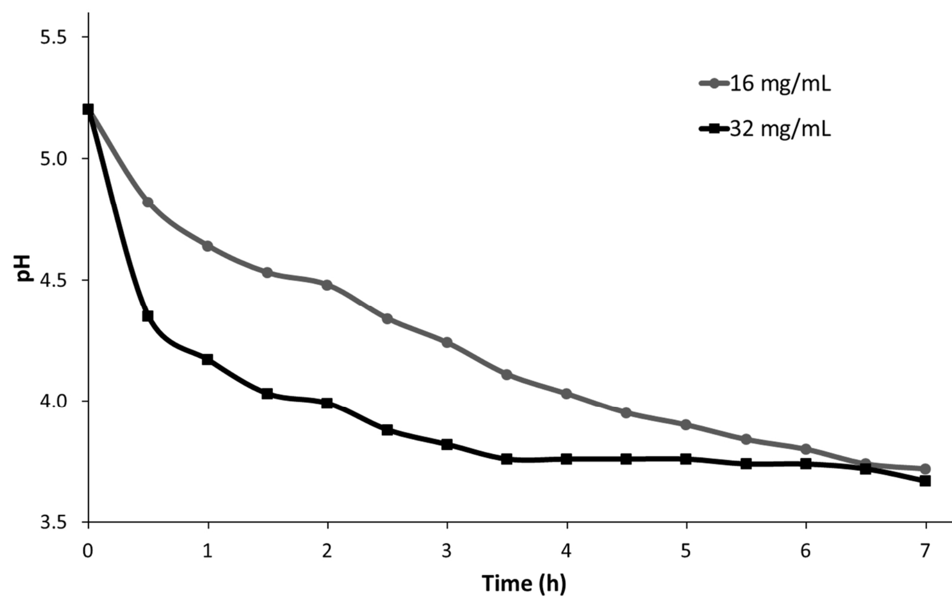

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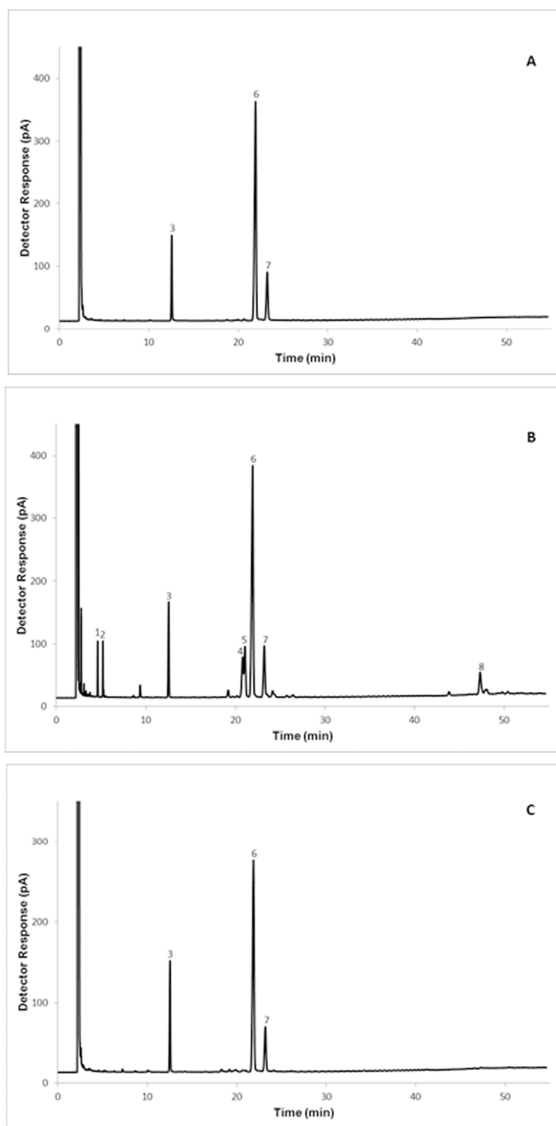

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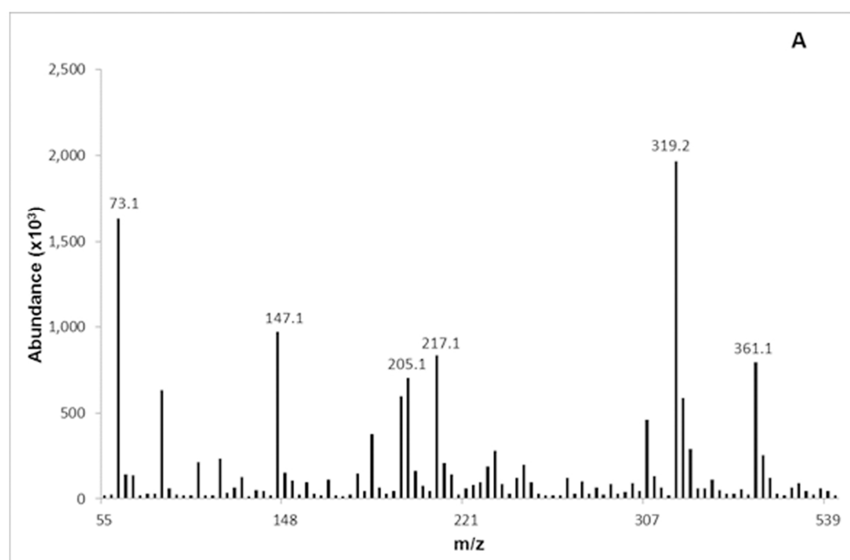

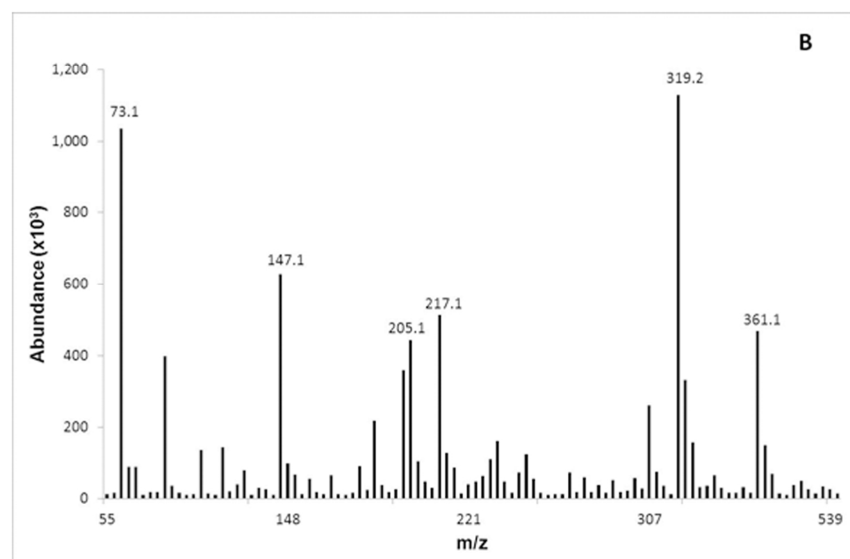

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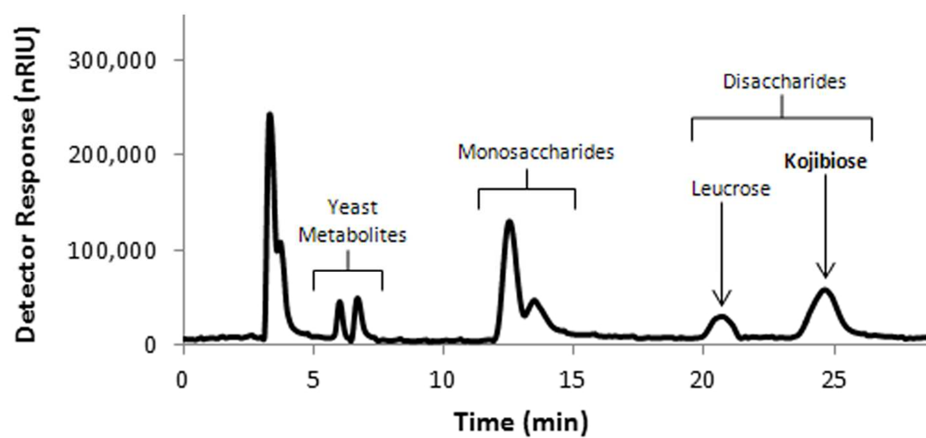

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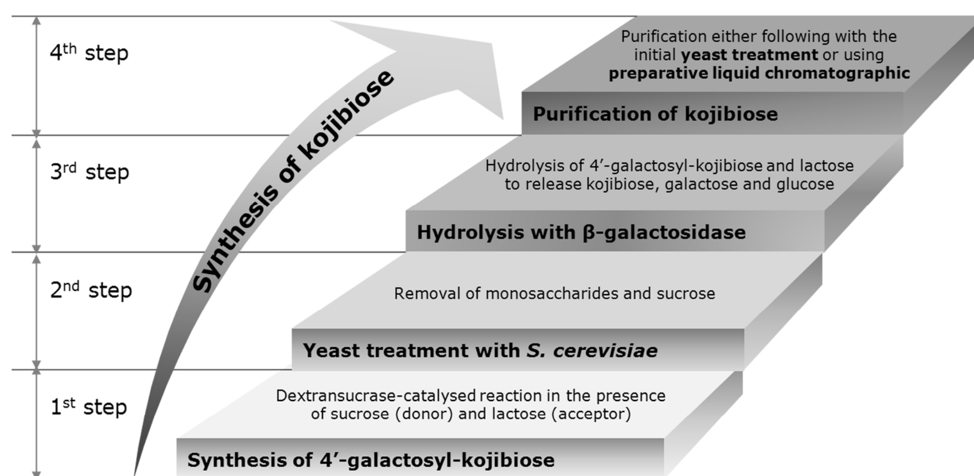

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