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# 1 **Improving the environmental compatibility of enzymatic** 2 **synthesis of sugar-based surfactants using green reaction** 3 **media**

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18

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20 The authors declare no competing financial interest.

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25 **ABSTRACT**

26 The enzymatic synthesis of sugar-based surfactants is often performed in non-conventional  
27 media that do not meet longer satisfy the current environmental acceptability, especially  
28 biodegradability and cytotoxicity. In this work, we propose an innovative sustainable route by  
29 replacing the current reference organic solvent, 2-methyl-2-butanol (2M2B), by 2-  
30 methyltetrahydrofuran (MeTHF), an agrosolvent and 2-methyltetrahydrofuran-3-one  
31 (MeTHF-3-one), a food-grade ingredient used as solvent. These two neoteric solvents were  
32 thus evaluated as reaction media *via* lipase-catalyzed esterification of glucose by lauric acid  
33 and revealed a novel matter of interest. The regioselectivity of the reaction was mainly  
34 directed toward the primary alcohol of glucose maintaining the end-product obtained in  
35 2M2B: D-glucose-6-O-laurate. The PLS-Surface Response Design evidenced enzymatic  
36 performances in ester production of 48% in MeTHF and 79% in MeTHF-3-one. The latter  
37 solvent resulted not only in better yields compared to 2M2B, but also in an increased  
38 enzymatic stability allowing better reuse of the catalyst. Demonstrated to be readily  
39 biodegradable according to OECD standards for the first time, MeTHF-3-one was  
40 substantiated as a green medium for efficient, selective and sustainable enzymatic synthesis of  
41 sugar esters.

42

43 **KEYWORDS**

44 readily biodegradable solvent, 2-methyltetrahydrofuran, 2-methyltetrahydrofuran-3-one,  
45 lipase, glucose laurate, PLS surface response design

## 46 INTRODUCTION

47 The development of innovative and environmentally friendly strategies for biomass  
48 transformation into market products falls within the scope of bioeconomy. Glycolipids  
49 represent a significant part of the global market from biorefinery as biocompatible and bio-  
50 based molecules of interest exhibiting for some of them surfactant properties [1]. These non-  
51 ionic biosurfactants offer numerous applications as detergents and wetting agents in the  
52 industry [2]. Moreover, they can exhibit antimicrobial, antiviral or antitumoral properties [3]  
53 which make them suitable candidates for cosmetic, pharmaceutical, therapeutic formulations  
54 and biocontrol for crop protection [4]. Sugar fatty acid esters can be obtained by the chemical  
55 esterification route which often does not meet environmental safety criteria in regard of eco-  
56 cytotoxic organic solvents and hazardous chemical reagents use [5]. Sugar fatty acid esters  
57 can also be produced microbiologically with some drawbacks such as strains selection  
58 regulation or numerous purification steps [6]. As an alternative, enzymatic esterification  
59 reactions have been widely studied with lipases (EC 3.1.1.3) as biocatalysts due to the  
60 selectivity, the efficiency and the more sustainable reaction conditions in comparison to  
61 chemical synthesis since the pioneered works of Klivanov of lipase-catalyzed sugar-esters  
62 synthesis [7]. Enzymatic route offers regioselectivity, catalyst reusability, substrates  
63 versatility while decreasing by-products [8]. However, solubility of both sugar and acyl donor  
64 in the same medium still remains a constraint for the enzymatic catalysis limiting hydroxyl  
65 groups reactivity [9]. In addition, rational solvent selection must be performed keeping in  
66 mind the need to maintain enzymatic activity and eco-cytotoxicity/biodegradability  
67 considerations [10–12]. Enzymatic synthesis of sugar esters has long been performed in  
68 organic solvents such as dimethyl sulfoxide, dimethyl formamide and pyridine, in which both  
69 sugars and fatty acids are soluble [13]. However, these organic solvents do not answer the  
70 current environmental requirements and are incompatible with food, cosmetic and medical

71 applications. For these reasons, enzymatic synthesis of sugar esters is classically implemented  
72 in 2-methyl-2-butanol (2M2B). Ways of improvement include the use of room-temperature  
73 ionic liquids, DMSO or more recently deep eutectic solvents [14–16] but still raise toxicity  
74 questioning to hamper the development of carbohydrate biotransformation [17]. In this  
75 sustainability context, we propose an integrated alternative focusing on the replacement of  
76 these reaction media for enzymatic synthesis of sugar fatty acid esters from renewable  
77 bioresources. Coming from lignocellulosic biorefinery, glucose was selected as sugar  
78 substrate. The choice of the fatty acid from oil biorefinery, was based on the promising  
79 surfactant properties after grafting of lauryl chain on sugar moiety [18]. Moreover, by using a  
80 free fatty acid as substrate instead of classical reactive vinyl (or ethyl/methyl) acyl donor, the  
81 production of volatile acetaldehyde or primary alcohols as side-products is thought to be  
82 overcome. Novozym435®, the commercial immobilized on acrylic resins lipase from  
83 *Candida antarctica*, is the most widely used enzyme for industrial applications and  
84 specifically for esters synthesis [19]. We selected this biocatalyst regarding its specificity  
85 towards the unbranched long chain fatty acids [20] and reusability. This present work aims to  
86 investigate the feasibility of lipase-catalyzed esterification of glucose by lauric acid in 2-  
87 methyltetrahydrofuran (MeTHF), an agro-solvent known for its interesting properties in terms  
88 of safety, health, biodegradability, and environmental score [21,22] and 2-  
89 methyltetrahydrofuran-3-one (MeTHF-3-one) a food-grade ingredient that is used in food  
90 industries to mimic flavors [23]. Optimal conditions were studied *via* a PLS-surface response  
91 design. Reaction performances and regioselectivity were compared to those obtained in the  
92 classically 2M2B used here as a reference. Finally, the stability of the lipase preparation in  
93 these two alternative solvents was evaluated as well as the structural integrity of the  
94 immobilization carrier. Biodegradability and eco-cytotoxicity considerations of these two  
95 alternative solvents are also reported to support the discussion.

## 96 **EXPERIMENTAL SECTION**

97

### 98 **Chemicals and enzyme**

99 Novozym435<sup>®</sup> (*Candida antarctica* lipase (CaLB) immobilized on acrylic resin  
100 formed by poly(methyl-methacrylate) cross-linked with divinylbenzene, EC 3.1.1.3,  
101 5000 U/g), molecular sieves (4 Å) and anhydrous D-(+)-glucose (99%) were purchased  
102 from Sigma-Aldrich (USA). Lauric acid (98%) was supplied by Alfa Aesar (Thermo  
103 Fisher Scientific, Heysham). 2M2B, >99% and MeTHF, >99% were purchased from  
104 Sigma-Aldrich (USA). MeTHF-3-one, >97% was supplied by Alfa Aesar (Thermo  
105 Fisher Scientific, Heysham). HPLC grade trifluoroacetic acid (99%) was purchased  
106 from Sigma-Aldrich (Germany), HPLC grade acetonitrile from Merck (Darmstadt),  
107 and petroleum ether from Fisher Scientific (UK). Dimethyl sulfoxide d6 (99.80%) and  
108 Dimethyl sulfoxide (>99%) were obtained from VWR.

109

### 110 **Biodegradability test**

111 The biodegradability of the three organic compounds: 2M2B, MeTHF and MeTHF-3-  
112 one was evaluated by Eurofins Ecotoxicologie France SAS (Maxéville, France)  
113 according to the OECD 301 F guideline. The three organic compounds tested met the  
114 validity criteria required for this test. The typical procedure consisted in determining  
115 the biodegradability of these organic compounds by microorganisms in an aquatic  
116 environment using a static test system. The tested mixture contained an inorganic  
117 medium, the organic compound as the only nominal source of carbon and energy with  
118 a theoretical oxygen demand of 100 mg.L<sup>-1</sup>, and a mixed inoculation from an urban  
119 wastewater treatment plant (amount of suspended matter inferior to 30 mg.L<sup>-1</sup> in the  
120 final mixture). O<sub>2</sub> generated during microbial degradation was trapped and measured

121 by the OXYTOP system. The O<sub>2</sub> released was compared to the theoretical amount  
122 released and expressed as a percentage of degradation. The test standardly lasted 28  
123 days, but could be extended if the biodegradation curve did not reach a plateau by day  
124 28.

125

### 126 **Fish Embryo Acute Toxicity Test**

127 The acute or lethal toxicity of MeTHF and MeTHF-3-one on the embryonic stages of  
128 *Danio rerio* was evaluated by SGS SA (Rouen, France) according to OECD 236  
129 guideline. The 96h LC<sub>50</sub> corresponds to the concentration (mg.L<sup>-1</sup>) of the tested  
130 solvents leading to 50% mortality of *Danio rerio* eggs in 96 h. The two solvents tested  
131 have a respective 96h LC<sub>50</sub> greater than 500 mg.L<sup>-1</sup>. The safety data sheets for 2M2B  
132 report a 48h LC<sub>50</sub> of 2430 mg.L<sup>-1</sup> evaluated on embryonic stages of *Leuciscus idus*  
133 *melanotus*.

134

### 135 **Sugar solubility**

136 Determination of the solubility of glucose in 2M2B, MeTHF and MeTHF-3-one was  
137 performed by gravimetric analyses. 60 mg of glucose was introduced into 1 mL of each  
138 solvent. The saturated dispersion was stirred for 24h at 50°C. After centrifugation  
139 (13,000 g, 15 min, MiniSpin®, Eppendorf Austria GmbH), the supernatants were  
140 collected and evaporated under vacuum. The resulting amount of glucose was weighed  
141 (using a precision analytical balance, Sartorius Praxium 224-1s, Germany) and used for  
142 concentration determination. All measurements were performed in triplicate and the  
143 solubility values were expressed as mean values with a standard deviation (±) in g.L<sup>-1</sup>.

144

145

## 146 **Enzymatic esterification**

147 Enzymatic synthesis of glucose monoester was performed by esterification of glucose  
148 with lauric acid in 2M2B, 2-MeTHF or MeTHF-3-one catalyzed by Novozym435<sup>®</sup>.

149 Glucose concentration was fixed at 0.17 M all along the study. Acyl donor and enzyme  
150 loadings were described in the “Experimental design” section.

151 All reagents were stored under vacuum in a desiccator with saturated salt solutions  
152 (MgCl<sub>2</sub>) for at least 24h. Substrates, enzyme and solvents water activity ( $a_w$ ) was  
153 measured and controlled before use ( $0.30 \pm 0.05$ ). Molecular sieves of 4 Å were  
154 activated at 300°C for 24h then added to the reaction medium (100 g.L<sup>-1</sup>) according to  
155 literature data [14,24,25]. For the experimental design, reactions were performed in 1.5  
156 mL Eppendorf tubes on a ThermoMixer C (Eppendorf, Hamburg, Germany) vortexed  
157 at 1000 rpm. For the other experiments, reactions were performed at a selected  
158 temperature at 350 rpm in 20 mL glass tubes with a Carousel 12 Plus Reaction  
159 Station<sup>™</sup> (Radleys, United Kingdom) coupled to a Haake K35 thermoregulated bath  
160 (Thermo Fisher Scientific, Heysham) at 10°C to avoid evaporation. At the end of the  
161 reaction, the immobilized enzyme was removed by filtration. Control experiments  
162 were performed (one at 0°C, one without lauric acid, one without enzyme, and one  
163 with thermally inactivated enzyme). No product was detected in these control  
164 experiments.

165

## 166 **Quantitative Analysis by HPLC**

167 Glucose esters were quantified by HPLC (LC 20 AD, Shimadzu, Marne-la-Vallée,  
168 France) equipped with a UV detector at 214 nm and a light-scattering low temperature  
169 evaporative detector (ELSD-LT II, Shimadzu). The reaction medium was diluted in  
170 dimethyl sulfoxide (DMSO) to both stop the reaction and solubilize all the



171 components. Samples were filtered through a 0.2  $\mu\text{m}$  syringe filter and then injected  
172 for analysis. Separation was performed on a C18 amide Alltima HP (5 $\mu$  150mm x 2.1  
173 mm) chromatography column (Grace Davison Discovery Sciences) with an Alltima HP  
174 Guard (7.5 x 2.1 mm), maintained at 30 °C. ELSD parameters were fixed at 40°C,  
175 sensitivity gain of 8 and He pressure of 3.8 bars. The elution method consisted of a  
176 gradient at a flow rate of 0.2 mL.min<sup>-1</sup>: phase A (water/acetonitrile/TFA 30:70:0.3) and  
177 phase B (acetonitrile/TFA 100:0.3). The gradient consisted of a decrease from 100% to  
178 70% of phase A for 15 min. Then for 10 min the proportion of phase A was maintained  
179 at 100%. The results were recorded and processed by a chromatogram processing  
180 software (LC solution, Shimadzu). For analytical extraction and purification, the  
181 reaction media were filtered. The permeate was placed under vacuum to evaporate the  
182 solvent. The resulting product contained residues of lauric acid and glucose ester. It  
183 was washed 3 times in petroleum ether, then centrifuged for 20 min at 13,500 rpm. The  
184 lauric acid was solubilized in petroleum ether and the pellet contained the purified GE.  
185 The purified product was then characterized by LC-MS-MS and NMR analyses. The  
186 concentration of glucose ester was measured based on standard calibration with the  
187 purified product, from 1 to 5 g.L<sup>-1</sup>. All HPLC analyses were performed in triplicate.  
188 The yield of glucose ester was determined following the equation 1:

$$189 \text{ Glucose ester yield (\%)} = \frac{\text{glucose ester produced at the end of the reaction (M)}}{\text{initial glucose concentration (M)}} \times 100 \quad (\text{Eq.1})$$

190

### 191 **Liquid Chromatography-Mass Spectrometry (LC-MS-MS)**

192 Separations were performed using an ACQUITY UPLC H-Class system coupled on-  
193 line with an ACQUITY PDA detector and a Synapt G2-Si QTOF high resolution  
194 hybrid mass spectrometer (Waters, Manchester, UK), equipped with an electrospray  
195 ionization (ESI) source and operated in the resolution mode (Resolution FWHM:

25,000). The reaction medium was diluted in dimethyl sulfoxide (DMSO). The samples were filtered through a 0.2  $\mu\text{m}$  syringe filter and then 5  $\mu\text{L}$  were injected. Products were separated on a C18 amide Alltima HP (5 $\mu$  150mm x 2.1 mm) chromatography column (Grace Davison Discovery Sciences) with an Alltima HP Guard column (7.5 x 2.1 mm), maintained at 30  $^{\circ}\text{C}$ . The elution method was the same as that used for the HPLC experiments. Acquisitions were performed in the continuum mode. The scan range was 50-2000 at 0.2 s/scan. Data acquisition and processing were performed using MassLynx software (V4.1, Waters).

204

### 205 **Nuclear magnetic resonance analyses (NMR)**

206 The purified glucose ester was analyzed by NMR after solubilization in dimethyl  
207 sulfoxide- $\text{d}_6$  (1.0% w/v) using a combination of 1D and 2D NMR techniques. The  
208 experiments were performed on a spectrometer (Bruker Avance III 600 MHz  
209 spectrometer) equipped with a 5 mm probe operating at 150.9128 MHz ( $^{13}\text{C}$  channel)  
210 and 600.1700 MHz ( $^1\text{H}$  channel) at 298 K. The  $^1\text{H}$ ,  $^{13}\text{C}$  decoupled from  $^1\text{H}$ ,  $^1\text{H}$ - $^{13}\text{C}$   
211 HSQC,  $^1\text{H}$ - $^{13}\text{C}$  HMBC and  $^1\text{H}$ - $^1\text{H}$  COSY diffusion NMR experiments were performed  
212 under standard conditions. NMR spectra were processed using Bruker's Topspin 3.6.2  
213 windows processing software.

214 The  $^1\text{H}$  and  $^{13}\text{C}$  assignments for the glucose monoester, i.e.  $\alpha/\beta$ -D-glucose-6-O-laurate,  
215 were as follows:  $^1\text{H}$  NMR (600 MHz, DMSO- $\text{d}_6$ ),  $\delta$  4.897 (d,  $J$  = 4.8 Hz, 1H, H1),  
216 3.132 (m, 1H, H2), 3.430 (t,  $J$  = 9.3 Hz, H3), 3.036 (t,  $J$  = 9.7 Hz, 1H, H4), 3.763 (td,  $J$   
217 = 8.4 Hz, 2H, H5), 4.258 (dd,  $J$  = 11.9 Hz, 2.4 Hz, 1H, H6a), 3.991 (dd,  $J$  = 11.7 Hz,  
218 6.1 Hz 1H, H6b), 2.275 (td, 3.5 Hz, 2H, H8), 1.482 (m, 2H, H9), 1.246 (16H, lauric  
219 acid chain), 0.861 (t,  $J$  = 6.9 Hz, 3H, H18) and  $^{13}\text{C}$  NMR (150 MHz, DMSO- $\text{d}_6$ ),  $\delta$   
220 92.838 (C1), 72.659 (C2), 73.359 (C3), 71.079 (C4), 69.836 (C5), 64.448 (C6),

221 175.108 (C7), 34.607 (C8), 32.1204, 29.462, 29.387, 29.229, 29.187, 29.029, 28.917,  
222 25.282, 22.795 (C lauric acid chain), 14.506 (C18).  
223 The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra can be found in SI (Figure S1). Furthermore, two  
224 chemical shifts for the H-6 indicated that as observed with the LC-MS-MS  
225 experiments, the esterification was performed on the  $\alpha$  and  $\beta$  conformations of the D-  
226 glucose [26]. The structural elucidation of the product was in agreement with literature  
227 [25,27]. In order to demonstrate the ester bond formed, HMBC experiments were  
228 performed (Figure 1). Protons on the C6 of the glucose ring interacted with the  
229 carbonyl carbon indicating the formation of an ester bond. This resulted in the  
230 chemical shift of the C8 to the higher field [25]. Ultimately, the glucose ester produced  
231 was identified as  $\alpha/\beta$ -D-glucose-6-O-laurate.

232

### 233 **Experimental design**

234 Optimization of factors affecting the enzymatic synthesis of glucose fatty acid ester  
235 (GE) was performed following an experimental design plan. Experiments were  
236 designed by Statgraphics Centurion XVIII<sup>®</sup> software (FranceSTAT, Neuilly sur Seine,  
237 France) and MODDE 12<sup>®</sup> software (Umetrics, Umea, Sweeden). Four factors (i.e. X1:  
238 reaction temperature, X2: lauric acid/glucose molar ratio, X3: reaction duration and  
239 X4: enzyme concentration) were selected and the measured response was the yield of  
240 glucose ester production. The levels of the factors were chosen by considering several  
241 limitations. Regarding temperature, Novozym435<sup>®</sup> exhibits significant thermal  
242 stability, specially under high temperatures, even up to 100 °C [28]. However, in the  
243 literature, this reaction is often performed between 30 and 60°C [14,24,25,29]. It has  
244 been shown that better yields of monoester were achieved at 40°C by minimizing the  
245 impact of the subsequent esterification reaction leading to a by-product diester [29].

246 Among the three chosen solvents, the boiling point of MeTHF is the lowest (81°C).  
 247 Based on this knowledge and preliminary single-factor experiments (*i.e.* to obtain the  
 248 optimum for one parameter at a time while keeping others constant), the upper level for  
 249 temperature was 75°C and the lower level was set at 25°C, the room temperature.  
 250 The upper and lower levels for lauric acid/glucose molar ratio, enzyme concentration  
 251 and experiment duration were first selected according to literature data [14,24,25,29–  
 252 31], then confirmed by preliminary single-factor experiments. For lauric acid/glucose  
 253 molar ratio and enzyme concentration, the lower levels were 2:1 and 12.5 g.L<sup>-1</sup>,  
 254 respectively, and the upper levels were 6:1 and 37.5 g.L<sup>-1</sup>, respectively. For the  
 255 duration of the experiments, the lower level was 24 h and the upper level was 72 h, to  
 256 avoid the formation of diester as a byproduct [14,25,29–32]. The levels of the factors  
 257 are shown in Table 1. After defining the factors and response of interest, an appropriate  
 258 design type was selected. The design consisted of 2<sup>4</sup> factorial design and 2 central  
 259 points. The experimental design matrix was generated and presented in Table 2. All  
 260 experiments were performed in random order. Once the glucose ester yield data was  
 261 collected, it was checked for outliers and model quality, after which it could be used  
 262 for response surface optimization. This method uses a polynomial model including  
 263 linear terms and all two factor interactions. The experimental design was subjected to  
 264 goodness of fit routine using a first- and second-order equation by MODDE 12<sup>®</sup>  
 265 software, as shown in Equation 2 and Equation 3:

$$266 \quad Y(GE) = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i,j=1}^4 \beta_{ij} X_i X_j \quad (\text{Eq. 2})$$

$$267 \quad Y(GE) = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i,j=1}^4 \beta_{ij} X_i X_j \quad (\text{Eq. 3})$$

268 Where  $Y(GE)$  is the response variable for glucose ester yield,  $X_i$  are the independent  
 269 factor variables,  $\beta_0, \beta_i, \beta_{ii}$  and  $\beta_{ij}$  are the constant, linear, quadratic and two factor  
 270 interaction coefficients, respectively. Each coefficient in the second-order equation was

271 calculated and the possible interaction effects of the factors were obtained. The method  
272 used to fit the models was PLS supported by NIPALS algorithm [33]. A Variable  
273 Importance to the Projection (VIP) was applied as part of the PLS calculations. The  
274 VIP is based on the weighted sum of square of the weights of the model factors,  
275 allowing hierarchical ordering of the independent variables [33,34].  
276 The significance of the models was checked by Analysis of Variance (ANOVA). The  
277 goodness of fit was expressed by the coefficient of determination  $R^2$  and  $R^2_{adj}$ . Model  
278 significance was determined by F-test ( $p < 0.05$ ). Optimization (best  $Y(GE)$ ) was  
279 determined by the PLS method. VIP charts and surface response plots were drawn  
280 using MODDE 12<sup>®</sup> software.

281

### 282 **Enzyme stability in the solvents**

283 Enzyme stability was determined by measuring glucose ester yields after prior  
284 incubation of the enzyme preparation in 2M2B, MeTHF and MeTHF-3-one. The  
285 enzyme was incubated in the solvents for 3, 6, 9, 12 or 15 days at 75°C. After  
286 centrifugation (10,000 rpm, MiniSpin<sup>®</sup>, Eppendorf Austria GmbH) the solvent  
287 (supernatant) was removed and the enzyme preparation was dried with a SpeedVac  
288 Concentrator (SPD121P, Thermo Savant, Waltham, MA, USA) for 45 min. Finally, the  
289 enzyme preparation was stored under vacuum (at a controlled  $a_w$  of  $0.37 \pm 0.01$ ) until  
290 use. Then, the glucose esterification reactions were performed under the optimal  
291 conditions determined by the experimental design (at 75°C for 72 h, with a lauric  
292 acid/glucose molar ratio of 6:1 and an enzyme concentration of  $12.5 \text{ g.L}^{-1}$ ). The  
293 reaction medium was analyzed by HPLC to determine the yield of ester production, as  
294 described previously.

295

296 **Scanning electron microscopy**

297 After incubation in the three distinct organic solvents (2M2B, MeTHF and MeTHF-3-  
298 one) for up to 15 days at 75°C, the morphological properties of the Novozym435®  
299 carriers were observed with a high-resolution environmental scanning electron  
300 microscope (ESEM, FEI Quanta 200 FEG, OR, USA). A small amount of each sample  
301 was placed on double-sided carbon tape adhered to aluminum SEM stubs. The  
302 specimens were examined and the secondary electron images were acquired under low-  
303 vacuum mode at a pressure of 0.5 Torr using a pressure limiting apertures (PLA).

304

305 **RESULTS AND DISCUSSION**

306

307 The potential of an agro-solvent and a food grade ingredient used as solvents was  
308 investigated to challenge the 2M2B classically used for enzymatic synthesis of  
309 glycolipids. The rational selection was based on physicochemical and environmental  
310 considerations: biodegradability and cytotoxicity. Indeed, MeTHF constitutes a bio-  
311 based solvent derived from biomass and MeTHF-3-one listed as generally recognized  
312 as safe (GRAS). The scheme of lipase-catalyzed esterification of glucose, main  
313 platform molecule from lignocellulosic biorefinery, with lauric acid leading to one  
314 product with interesting surfactant properties [18] is presented in Figure 2. The  
315 challenge was to develop a system in which the substrate and the acyl donor would be  
316 available for the reaction despite opposite polarity while directing and maintaining the  
317 enzymatic activity towards ester synthesis.

318

319

320

## 321 **Environmental considerations and substrate availability**

322 Biodegradability and ecotoxicity of the three solvents were first compared according to  
323 OECD guideline. Concerning the fish embryo acute toxicity test, MeTHF and MeTHF-  
324 3-one both exhibited a respective 96 h LC<sub>50</sub> superior to 500 mg.L<sup>-1</sup>. Likewise, the  
325 Material Safety Data Sheets of 2M2B reported a 48h LC<sub>50</sub> of 2430 mg.L<sup>-1</sup>. These data,  
326 evaluated on embryonic stages of soft water fishes according to the OECD 236  
327 guideline, suggested a similar ecotoxicity between these three organic compounds.  
328 Then, the biodegradability kinetics for each solvent were presented in Figure 3A. The  
329 biodegradability rates after 28 days are provided in SI (Table S1). MeTHF and 2M2B  
330 exhibited similar biodegradation kinetic profiles with 5 and 6 % of biodegradability  
331 level after 28 days, respectively. In opposite, the biodegradation kinetic of MeTHF-3-  
332 one showed a biodegradation level of 69 % at 28 days within a 10-day window in the  
333 28-day period. This behavior allowed MeTHF-3-one to be classified as “readily  
334 biodegradable” in agreement with the OECD standards [35]. Based on these  
335 environmental considerations, we therefore confirmed the suitability of selecting the  
336 MeTHF and MeTHF-3-one in this study to improve the sustainability of glycolipids  
337 synthesis by enzymatic route.

338 All enzymatic reaction systems implemented in this study included reactants, enzyme  
339 and organic solvents with controlled initial water activity ( $a_w$ ) for maintaining a  
340 sufficient hydration state of lipase for enzyme stability while favoring the synthesis  
341 activity [36]. Among the other numerous factors influencing performances of  
342 enzymatic esterification of glucose in organic solvent, the availability of the polar  
343 substrate and the acyl donor in the solvent constitute a key factor [7,9,25]. Lauric acid  
344 was soluble in all the three solvents with a solubility value much higher than 500 g.L<sup>-1</sup>  
345 confirming the full availability of the acyl donor in the reaction systems. The solubility

346 values of glucose were also determined and presented in Figure 3B as a function of the  
347 log P of each solvent where P constitutes the solvent partition coefficient between 1-  
348 octanol and water. As log P of MeTHF-3-one was not available in literature, ACD/log  
349 P calculator procedure [37] was used for establishing a relative comparison between  
350 the three solvents. The use of MeTHF-3-one with a log P of -0.65 led to a glucose  
351 solubility of 5.05 g.L<sup>-1</sup> *versus* 1.15 and 0.85 g.L<sup>-1</sup> in 2M2B (log P = 1.04) or MeTHF  
352 (log P = 0.85), respectively. Although the solubility of glucose in a solvent cannot be  
353 exclusively related to log P, the results are consistent with an increase of solubility  
354 with a decrease of log P. The selected alternative solvents meet thus the sustainability  
355 criteria while ensuring practical availability of both substrates, totally for acyl donors  
356 and partially for the sugar as well as in the reference organic solvent.

357

### 358 **Feasibility of glucose ester synthesis in the neoteric solvents**

359 Lipase-catalyzed esterification in organic solvent has been widely reported in the  
360 literature since 1990's [38–41]. 2M2B has often been presented as a reference solvent  
361 for this type of reaction. It seems to receive a renewed interest in recent studies despite  
362 physicochemical properties far away from durability criteria [42,43]. Based on  
363 literature data, we firstly set the following experimental conditions as a basis for  
364 comparison with 2M2B: equimolar ratio of lauric acid/glucose, 100 g.L<sup>-1</sup> of molecular  
365 sieve, 25 g.L<sup>-1</sup> of Novozym435<sup>®</sup>, 50 °C, 350 rpm. After 72 h, the enzymatic reactions  
366 were stopped and the identification of the products was performed by LC-MS. After  
367 each reaction, NMR analysis of the recycled solvents was done. These results are  
368 presented in SI (see SI, Figure S2).

369 Regardless of the solvent, LC-MS-MS analyses revealed at least the presence of three  
370 distinct compounds after 72 h of reaction. In all cases, glucose laurate mono-ester



371 ( $C_{18}H_{34}O_7$ ) is evidenced by  $R_T = 2.88 - 2.98$  min,  $[M + Na]^+$   $m/z$  385.220 and  
372  $[MM + Na]^+$   $m/z$  785.445 with the presence of both anomers [27] (Figure S3). The two  
373 other signals were identified as unreacted substrates: the residual glucose ( $R_T = 1.89$   
374 min,  $[M + H]^+$   $m/z$  181.073,  $[M + Na]^+$   $m/z$  203.055) and the residual lauric acid ( $R_T =$   
375 7.41 min,  $[M - H]^-$   $m/z = 199.171$ ). Total and Reconstructed Ion Currents (TIC and RIC  
376 respectively,  $m/z$  385.220) of the reaction media in 2M2B are presented in SI (Figure  
377 S4). The sodium adduct led to two fragments ions at  $m/z$  345.220 and 327.217, issued  
378 respectively from subsequent losses of NaOH and  $H_2O$ . Furthermore, the daughter ion  
379 at  $m/z$  327.217 was consecutively fragmented by MS-MS, leading to a pyran fragment  
380 ion at  $m/z$  127.041 ( $C_6H_7O_3$ ) due to the loss of a lauric acid molecule ( $C_{12}H_{24}O_2$ ). The  
381 MS-MS spectrum of the ion at  $m/z$  327.217 is presented in SI (Figure S5) and a  
382 proposed fragmentation pattern of glucose laurate mono-ester sodium adduct is  
383 presented in SI (Figure S6). These data demonstrated the glucose laurate monoester  
384 formation in the three distinct solvents. In 2M2B and MeTHF, a minor compound can  
385 be detected ( $R_T = 14.21$  min,  $[M + Na]^+$   $m/z$  567.407,  $[M + NH_4]^+$   $m/z$  562.446,  $[MM +$   
386  $Na]^+$   $m/z$  1111.821) suggesting the formation of traces of glucose dilaurate. Similar  
387 results were recently observed by Martinez-Garcia et al. (2021) reporting up to 4% of  
388 diester synthesized in 2M2B [43]. In MeTHF-3-one, the selectivity of the reaction  
389 seems exclusively orientated toward monoester synthesis. Indeed, only traces of diester  
390 product were detected. Total ion current (TIC) of the reaction of glucose with lauric  
391 acid in MeTHF-3-one showed however two additional impurity at 5.08 and 5.32 min  
392 (Figure S7). Mass spectra (molecular mass region) of these side products are presented  
393 in SI (Figure S8). These products are hardly detectable on NMR spectra. The structural  
394 characterization of these compounds as well as a proposed mechanism of formation in  
395 the reaction system have been described in SI (Figure S9). Briefly, these two

396 compounds were obtained from lipase-catalyzed esterification of a derivative resulting  
397 from the ring opening of MeTHF-3-one, in the presence of lauric acid (Table S2 for  
398 details on the control experiments performed). Finally, the monoester compound was  
399 extracted and purified from the three distinct reaction media and then analyzed by  
400 NMR for structural elucidation (Figure 1). Complementary NMR data was presented in  
401 SI (Figure S1). NMR results confirmed the obtention of D-glucose-6-O-laurate in large  
402 amounts whatever the three distinct organic solvents used, showing also that the  
403 anomeric carbon of glucose remained not esterified. MeTHF and MeTHF-3-one were  
404 thus demonstrated for the first time as suitable alternative to 2M2B for lipase-catalyzed  
405 glucose esterification while preserving the regioselectivity *i.e.* the glucose-6-  
406 monolaurate, the product of interest. We confirmed also that log P would be useful for  
407 predicting glucose solubility but failed to predict enzyme catalysis feasibility in our  
408 reaction systems as suggested in literature [44].

409

#### 410 **Reaction optimization through model fitting and statistical analysis**

411 The effects of temperature, lauric acid/glucose molar ratio, reaction duration and  
412 enzyme concentration were investigated and optimized in 2M2B, MeTHF and  
413 MeTHF-3-one using a design of experiments using PLS methodology to ensure a  
414 robust comparison. Table 2 shows the experimental design matrix of the three factors  
415 and glucose ester yields in 2M2B, MeTHF and MeTHF-3-one generated by PLS  
416 statistical tool. The results were fitted using Equation 2 and Equation 3. The factor  
417 coefficients are presented in Table 3. Analysis of variance (ANOVA) was performed  
418 and VIP charts were used to obtain the linear, quadratic and interaction effects on the  
419 response. The models F-values were between 33.496 and 41.445 and the p-values of  
420 “Prob>F” were always < 0.0001 (Table 3), which means that the models were

421 considered highly significant at the 95% confidence level (p-value of “Prob>F” < 0.05  
422 and F-value > 4). In other words, the models adequately predicted the glucose ester  
423 yields from the 4 factors. Furthermore, the coefficients of correlation ( $R^2$ ) ranged from  
424 0.96 to 0.97. In general, quadratic models are considered as models with high degrees  
425 of correlation when their  $R^2$  values are higher than 0.90 [34,45,46]. The  $R^2_{Adj}$  values  
426 (0.93 to 0.94) were another proof of the significance of the models. The precision of  
427 the models and their reproducibility were assessed using the RSD% (Table 3), whose  
428 values went from 2.93 to 4.69% (less than 10%).

429 The effect of the factors on the glucose ester yield  $Y(GE)$  was studied using VIP charts  
430 (Figure 4 – D, E, F). For all solvents, the factors with the higher significant effects  
431 were temperature and lauric acid/glucose molar ratio (Figure 4 – D, E, F). However,  
432 for 2M2B and MeTHF-3-one, the quadratic terms did not have a significant influence  
433 over the reaction yields, whereas they all did for MeTHF at the 95% confidence level  
434 (Figure 4E). For all solvents, the interaction of temperature and lauric acid/glucose  
435 molar ratio was statistically significant according to the model, with a p-value < 0.05.  
436 The other interactions were not significant. Finally, the success of the predictions of  
437 the models were evaluated by comparing the experimental values of  $Y(GE)$  and the  
438 ones predicted by the model (Figure 4 - A, B, C). The experimental values were  
439 closely regrouped around the line of best fit, which means that the model was highly  
440 efficient to predict the experimental values. All statistical analyses proved the model  
441 was reliable and efficient. The predicted higher glucose ester yields were 38.1% in  
442 2M2B, 35.3% in MeTHF and 56.7% in MeTHF-3-one (Figure 5). The obtained  
443 experimental glucose ester yields for these optimal conditions were in perfect  
444 agreement with those predicted ( $39.1 \pm 2.0\%$  for 2M2B,  $37.0 \pm 11.7\%$  for MeTHF and  
445  $57.4 \pm 2.4\%$  for MeTHF-3-one). One can note that the lowest enzyme concentration

446 was the optimal one as already reported [47–50]. This may be due to the precipitation  
447 of produced ester in the enzyme microenvironment preventing the access of the  
448 substrate to the active site at higher enzyme concentration [51–53]. In the three  
449 solvents, the highest substrate molar ratio was the optimal one likely to an equilibrium  
450 shift toward synthesis with higher content of the fatty acid [53]. Most certainly thanks  
451 to a decrease in viscosity, increasing the encounters between enzymes and substrates  
452 [53], the highest tested temperature of 75°C provide the best yields afforded by  
453 Novozym435<sup>®</sup> stability up to 80 to 100 ° C [28,54]. The optimal conditions for  
454 obtaining the higher glucose ester yields were the same in all solvents, namely 75 °C  
455 lauric acid/glucose molar ratio of 6:1, during 72 h with an enzyme concentration of  
456 12.5 g.L<sup>-1</sup> and will be used for the following experiments. The highest glucose ester  
457 yields were similar in 2M2B and MeTHF, but significantly higher in MeTHF-3-one.  
458 The excellent yields obtained in MeTHF-3-one seemed to be due to the solubility of  
459 glucose which was 5 fold higher than in the other two solvents (5.05 ± 0.35 g.L<sup>-1</sup> in  
460 MeTHF-3-one *versus* 1.15 ± 0.07 g.L<sup>-1</sup> and 0.85 ± 0.21 g.L<sup>-1</sup> in 2M2B and MeTHF,  
461 respectively). Based on these results, MeTHF can be proposed as a biobased  
462 alternative to 2M2B preserving the performances. However, MeTHF-3-one, with its  
463 food grade label, was demonstrated as a better substitute to 2M2B by significantly  
464 improving the reaction efficiency in mild conditions.

465

#### 466 **Reaction kinetics**

467 The reaction kinetics in the three solvents were then compared (Figure 6A). The  
468 kinetics in MeTHF and 2M2B were very similar and reached a plateau at 48h while the  
469 plateau in MeTHF-3-one was not reached at 72h. Initial rates (Figure 6B,  $V_i$ ) in 2M2B  
470 or MeTHF were 16.3 ± 2.9 mmol.h<sup>-1</sup> and 15.8 ± 0.5 mmol.h<sup>-1</sup>, respectively and

471 significantly higher in MeTHF-3-one ( $26.0 \pm 2.8 \text{ mmol.h}^{-1}$ ). The esterification yield at  
472 72 h reached  $79.4 \pm 0.4 \%$  for MeTHF-3-one *versus*  $47.6 \pm 0.8 \%$  and  $48.3 \pm 0.8 \%$  for  
473 2M2B and MeTHF, respectively. These differences in initial rate and yield can be  
474 correlated to the greater solubility of glucose in MeTHF-3-one. Under our  
475 experimental conditions, MeTHF-3-one provided promising alternative with respect to  
476 enzymatic performances (eg. 1.7-fold increase) and regioselectivity towards mono-  
477 esterification on the primary hydroxyl of glucose. Moreover, the yields obtained in  
478 MeTHF-3-one were competitive compared to the rates obtained previously and more  
479 recently in 2M2B [41,43].

480

#### 481 **Enzyme stability**

482 Among the many advantages of immobilized enzymes, recycling and reuse of the  
483 biocatalyst enhances both the sustainability and the economic viability of the strategy  
484 [52,55–59]. In this context, enzyme stability can be studied to assess the impact of  
485 incubation in an organic solvent on the structural integrity of enzyme carriers and  
486 activity. Such an approach provides valuable information on the possible reusability of  
487 biocatalysts [56]. Stability studies on Novozym435<sup>®</sup> in 2M2B have already shown that  
488 the biocatalyst could be reused several times [56,60,61]. To our knowledge, no data on  
489 this enzymatic preparation in MeTHF or MeTHF-3-one have been reported in the  
490 literature. Novozym435<sup>®</sup> was first incubated in the three distinct solvents at 75°C and  
491 then used for esterification under the optimal conditions. The glucose laurate yields  
492 reached after varying incubation times of the enzyme in each solvent are shown in  
493 Figure 7. Likewise, SEM observations of the surface morphology of fresh  
494 Novozym435<sup>®</sup> *versus* Novozym435<sup>®</sup> after incubation in the solvents for 3 and 15 days  
495 (75°C) are presented in Figure 7. Whatever the solvents, an average of 63 % of enzyme

496 activity was preserved after 3 days of incubation. The results also show that 15-days of  
497 incubation did not lead to a total deactivation of the enzyme preparation. However, the  
498 decrease in activity throughout the days of incubation was greater in MeTHF (from  
499  $48.3 \pm 0.8$  % to  $3.6 \pm 0.1$  after 15 days of incubation) than in 2M2B (from  $47.6 \pm 0.8$  to  
500  $10.5 \pm 0.6$  % after 15 days of incubation) or MeTHF-3-one (from  $79.4 \pm 0.4$  to  $16.9 \pm$   
501  $0.1$  % after 15 days of incubation). The loss of activity after incubation in organic  
502 solvent could be due to the interaction of solvents with the surface of enzyme carriers,  
503 as previously reported in the literature [62]. It may be emphasized that the enzymatic  
504 performance in MeTHF-3-one remained superior to that obtained in the reference  
505 organic solvent 2M2B regardless of the prior incubation duration. The stability of the  
506 enzyme preparation would therefore be improved in MeTHF-3-one compared to 2M2B  
507 or MeTHF. Regarding the morphological and textural properties of the enzyme  
508 carriers, no change was observed between the fresh enzyme preparation and those  
509 observed after 3 days of incubation regardless of the solvent (Figure 7). Indeed,  
510 carriers kept a spherical shape with a smooth surface before and after 3 days of  
511 incubation in each solvent. After 15 days of incubation, SEM observation of the  
512 enzyme carriers showed that MeTHF did not induce drastic structural changes (Figure  
513 7). In opposite, prolonged incubation in 2M2B resulted in structural changes illustrated  
514 by the appearance of irregularities on the surface of the enzyme carriers. In the case of  
515 MeTHF-3-one, 15-days incubation resulted in an obvious change in the integrity of the  
516 carrier surface (Figure 7). This irregular deposit could be attributed to the adsorption of  
517 MeTHF-3-one onto the surface of the enzyme carrier. This therefore provides  
518 additional strong arguments for the substitution of 2M2B by MeTHF-3-one. The  
519 obvious adsorption of MeTHF-3-one on the surface of the enzyme carriers would

520 minimize the deleterious effect on the lipase microenvironment and activity unlike  
521 other organic solvents used for this type of reaction [63].  
522

## 523 CONCLUSIONS

524 The substitution of 2M2B by the agro-solvent MeTHF or the food-grade ingredient  
525 MeTHF-3-one used as solvent allowed the development of a novel sustainable route  
526 for lipase-catalyzed synthesis of glucose laurate. These two neoteric solvents did not  
527 affect the regioselectivity classically observed in 2M2B toward the primary alcohol of  
528 glucose. The complete statistical assessment confirmed that the established model was  
529 adequate and accurately predicted the glucose ester yields within the range of reaction  
530 conditions. Enzymatic performances achieved in MeTHF were similar to those  
531 obtained in 2M2B while a significant improvement was evidenced in MeTHF-3-one.  
532 This latter, demonstrated as readily biodegradable, also increased the enzymatic  
533 stability of Novozym435® leading to consider its reusability. These results open a  
534 promising field in the design of sustainable enzymatic syntheses for biosurfactants  
535 production from renewable resources.

536

## 537 Supporting Information

538 Additional experimental details, results, figures and tables associated with (i) solvents  
539 environmental data, (ii) LC-MS-MS and NMR analyses of reaction media and purified end-  
540 product (PDF).

541

## 542 Abbreviations

543 2-methyl-2-butanol (2M2B), 2-methyltetrahydrofuran (MeTHF), 2-methyltetrahydrofuran-3-  
544 one (MeTHF-3-one), generally recognized as safe (GRAS).

545

546

547



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- 752

753 **FIGURE LEGENDS**

754

755 **Figure 1.** Heteronuclear multiple bond correlation (HMBC) of the purified glucose ester. The  
756 horizontal and vertical axis indicate  $^1\text{H}$  proton and  $^{13}\text{C}$  chemical shift (ppm) respectively. The  
757 key interactions are represented on the molecule

758

759 **Figure 2.** Reaction scheme of lipase-catalyzed synthesis of glucose laurate monoester  
760 (temperature between 25 and 75°C).

761

762 **Figure 3.** (A) Results from biodegradation test expressed in % for 2M2B (black crosses),  
763 MeTHF (white squares) and MeTHF-3-one (black circles) for 28 days according to the OECD  
764 301 F guideline. (B) glucose solubility ( $\text{g}\cdot\text{L}^{-1}$ ) at 50 °C in the three distinct organic solvents:  
765 2M2B (black cross), MeTHF (white square) and MeTHF-3-one (black circle) in function of  
766 their log P calculated by ACD/Chemsketch.

767

768 **Figure 4.** Correlation graphs between the predicted and observed experimental glucose  
769 ester yield values in (A) 2M2B, (B) MeTHF, (C) MeTHF-3-one and effect of the  
770 factors by Variable Importance to Projection (VIP) charts on glucose ester yields: (D)  
771 in 2M2B, (E) in MeTHF and (F) in MeTHF-3-one. Any factor beyond the significance  
772 level of 0.8 (dotted lines) is statistically important ( $p < 0.05$ )

773

774 **Figure 5.** Response surface plot of glucose ester yield in (A) 2M2B, (B) MeTHF and  
775 (C) MeTHF-3-one as a function of temperature (25 to 75°C) and lauric acid/glucose  
776 molar ratio (2 to 6) with enzyme and duration set in agreement with optimized factors

777 (12.5 g.L<sup>-1</sup> and 72h, respectively). The glucose ester yield (%) was determined by  
778 HPLC according to Eq. 1.

779

780 **Figure 6.** (A) Conversion yield (%) of glucose and lauric acid into glucose monoester after  
781 incubation at 75°C, for a molar ratio lauric acid/glucose of 6:1, an enzyme concentration of  
782 12.5 g.L<sup>-1</sup>. ■, ● and ▲ represent the glucose ester yields in 2M2B, MeTHF-3-one and  
783 MeTHF, respectively. (B) Initial rates (mmol.h<sup>-1</sup>) of the reaction in 2M2B, MeTHF-3-one and  
784 MeTHF. The glucose ester yield (%) was determined by HPLC according to Eq. 1.

785

786 **Figure 7.** Glucose laurate yield after enzyme incubation in the three distinct solvents  
787 (the reactions were carried out at the optimal conditions (*i.e.* for 72h at 75°C with an  
788 enzyme concentration of 12.5 g.L<sup>-1</sup> and a lauric acid/glucose molar ratio of 6:1)) and  
789 corresponding SEM micrographs of Novozym 435 before incubation, after incubation  
790 for 3 days and 15 days in 2M2B (A), MeTHF (B) and MeTHF-3-one (C). The glucose  
791 ester yield (%) was determined by HPLC according to equation 1.

792

### 793 **TABLE LEGENDS**

794

795 **Table 1.** Experimental range of levels and coding of factors used to evaluate the glucose  
796 monoester yields.

797

798 **Table 2.** Experimental design matrix of the three factors and glucose ester yields in 2M2B,  
799 MeTHF and MeTHF-3-one.

800

801 **Table 3.** Regression coefficients and ANOVA of quadratic model.

802 **TABLES**

803 **Table 1.**

Factors	Coding	Range of levels		
		Low	Middle	High
Temperature (°C)	X1	25	50	75
Lauric acid/glucose molar ratio	X2	2	4	6
Reaction duration (h)	X3	24	48	72
Enzyme concentration	X4	12.5	25	37.5

804

805

806 **Table 2.**

Temperature (°C)	Lauric acid/glucose molar ratio	Reaction duration (h)	Enzyme concentration (g/L)	Glucose ester yield <sup>a</sup> (%) in 2M2B	Glucose ester yield <sup>a</sup> (%) in MeTHF	Glucose ester yield <sup>a</sup> (%) in MeTHF-3-one
X1	X2	X3	X4			
25	2	24	12.5	1.0 ± 0.2 <sup>b</sup>	0.9 ± 0.1	2.4 ± 0.1
50	4	48	25	9.8 ± 1.5 <sup>c</sup>	2.9 ± 0.2 <sup>c</sup>	15.2 ± 8.0 <sup>c</sup>
25	2	72	12.5	2.8 ± 0.1	0.5 ± 0.4	3.3 ± 0.4
75	6	72	37.5	34.0 ± 4.6	31.0 ± 3.3	52.9 ± 11.6
50	4	48	25	7.7 ± 1.0 <sup>c</sup>	3.7 ± 0.1 <sup>c</sup>	11.9 ± 2.2 <sup>c</sup>
25	6	24	37.5	1.5 ± 0.4	1.9 ± 0.4	5.0 ± 0.3
25	6	72	12.5	5.5 ± 0.9	2.7 ± 0.2	6.0 ± 0.3
75	2	24	12.5	10. ± 4.5	9.8 ± 4.4	21.2 ± 0.2
75	2	72	12.5	22.9 ± 4.7	12.0 ± 2.5	28.6 ± 2.6
75	2	24	37.5	18.9 ± 1.4	8.8 ± 0.0	19.0 ± 2.8
75	6	24	37.5	25.7 ± 0.9	25.6 ± 4.3	28.4 ± 2.9
75	6	72	12.5	39.1 ± 2.0	37.0 ± 11.7	57.4 ± 2.4
75	2	72	37.5	17.0 ± 4.4	13.8 ± 1.4	25.8 ± 3.5
25	2	24	37.5	1.0 ± 0.1	0.7 ± 0.0	2.2 ± 0.1
75	6	24	12.5	25.2 ± 2.4	33.7 ± 8.3	41.4 ± 8.1
25	6	24	12.5	1.9 ± 0.5	2.3 ± 0.5	4.4 ± 0.8
25	6	72	37.5	3.8 ± 0.3	1.3 ± 0.4	5.5 ± 1.6
25	2	72	37.5	2.1 ± 0.5	0.7 ± 0.2	3.0 ± 1.1

807 <sup>a</sup> Eq. 1808 <sup>b</sup> Mean ± SD of duplicate809 <sup>c</sup> Central point

810

811 **Table 3.**

812

Coefficients	For glucose ester yield in 2M2B	For glucose ester yield in MeTHF	For glucose ester yield in MeTHF-3-one
Constant $\beta_0$	7.088	22.738	20.475
Linear			
$\beta_1$	-0.121	-0.335	-0.135
$\beta_2$	-3.744	-5.200	-5.453
$\beta_3$	-0.161	-0.408	-0.518
$\beta_4$	-0.077	-0.506	-0.285
Quadratic			
$\beta_{11}$	0.002	0.003	0.002
$\beta_{22}$	0.280	0.499	0.349
$\beta_{33}$	0.002	0.003	0.002
$\beta_{44}$	0.007	0.013	0.009
Interaction			
$\beta_{12}$	0.062	0.094	0.094
$\beta_{13}$	0.003	0.002	0.005
$\beta_{14}$	0.000	-0.002	-0.004
$\beta_{23}$	0.018	0.002	0.035
$\beta_{24}$	-0.023	-0.036	-0.030
$\beta_{34}$	-0.005	0.001	0.001
Model F-value	41.445	40.938	33.496
p-value Prob >F	< 0.0001	< 0.0001	< 0.0001
R <sup>2</sup>	0.97	0.97	0.96
R <sup>2</sup> <sub>adjusted</sub>	0.94	0.94	0.93
RSD (%)	2.93	2.98	4.69

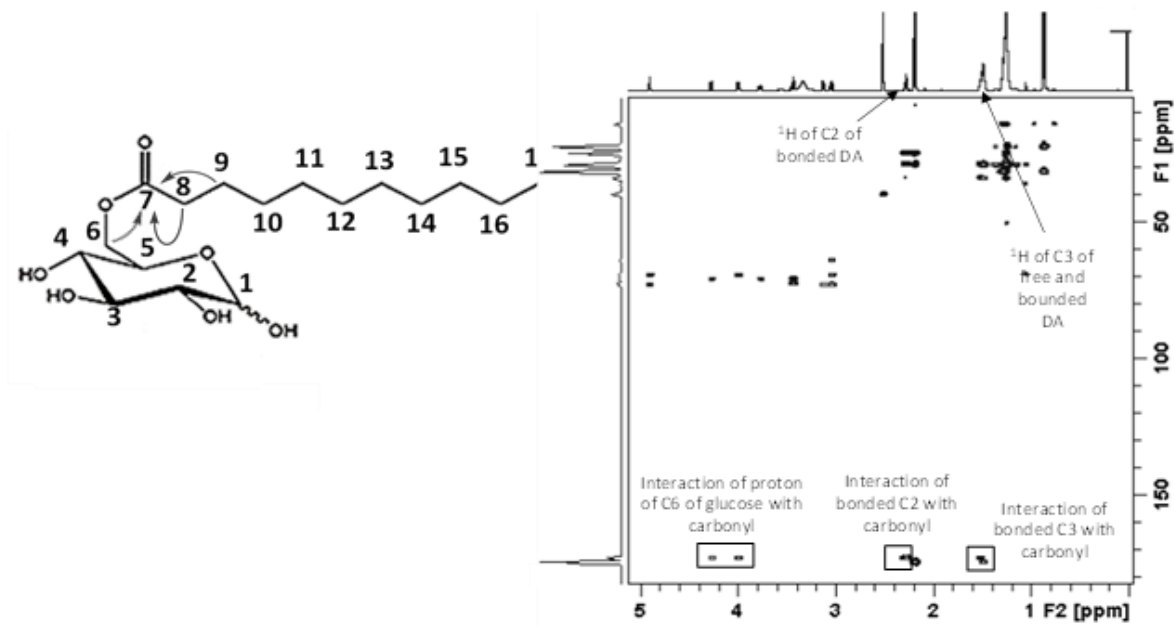
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816 FIGURES

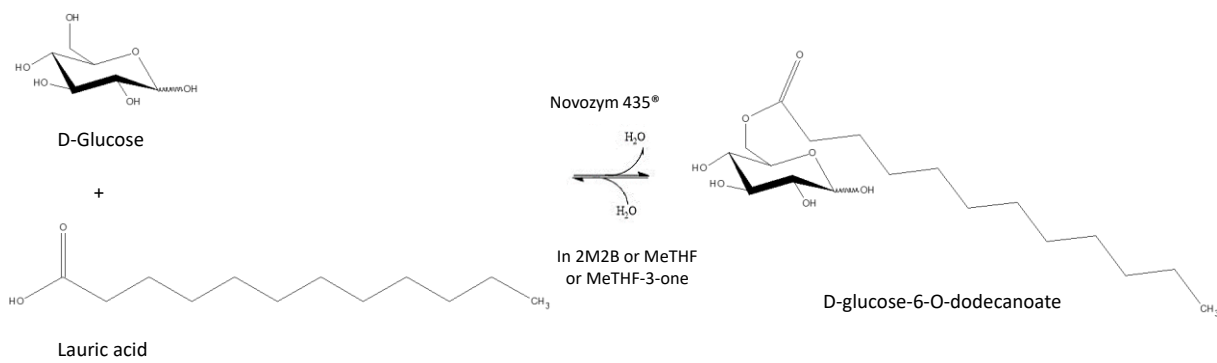
817 Figure 1.



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820 **Figure 2.**



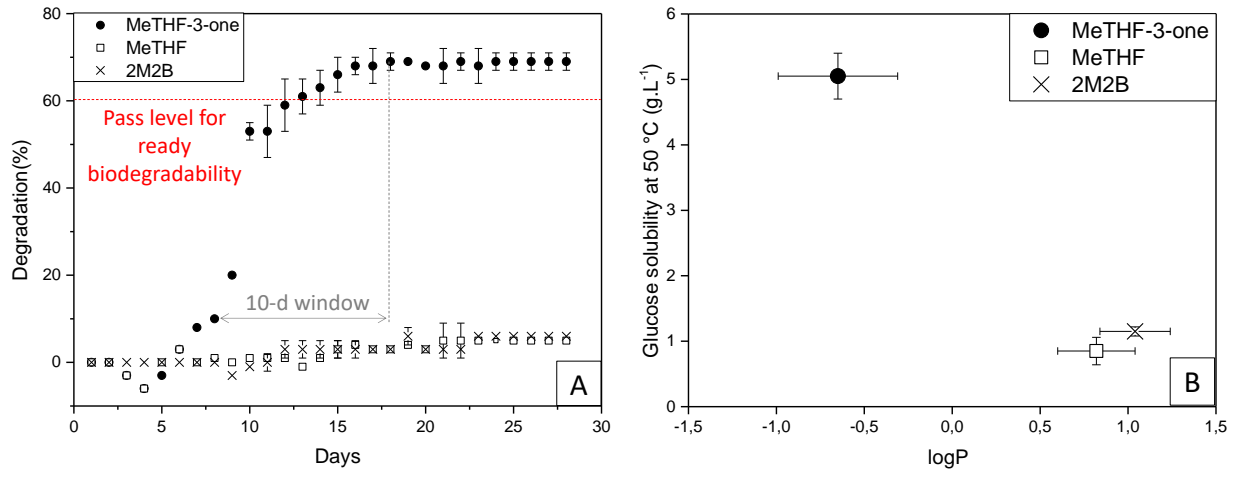
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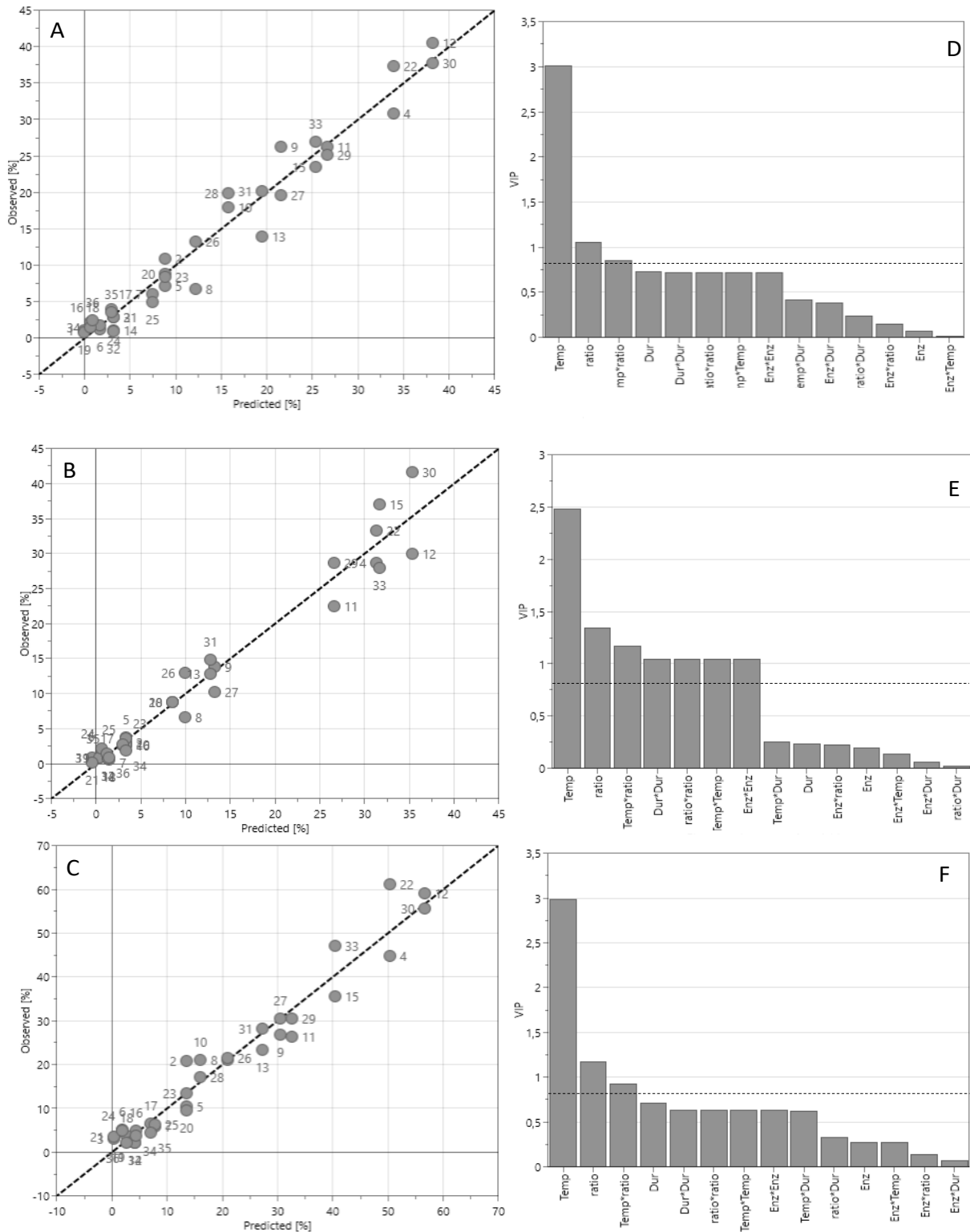
823 **Figure 3.**

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825

826 **Figure 4.**

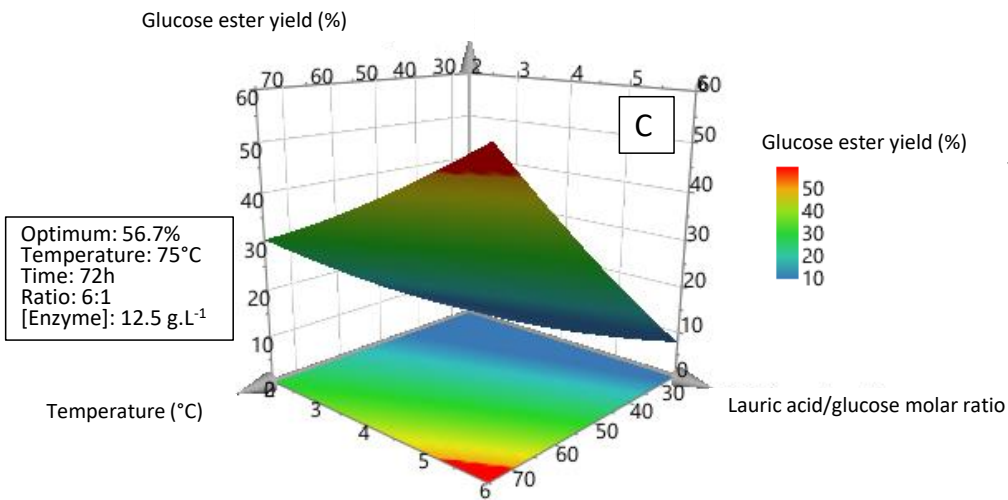
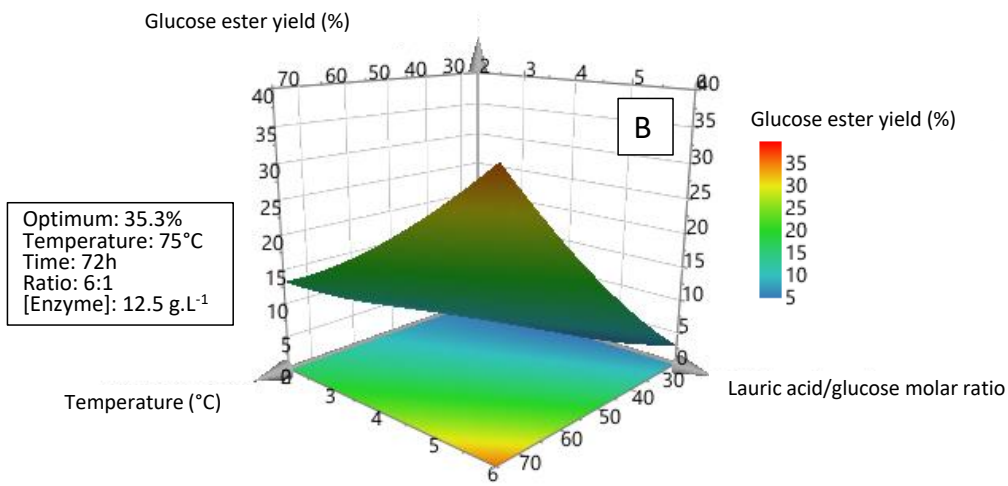
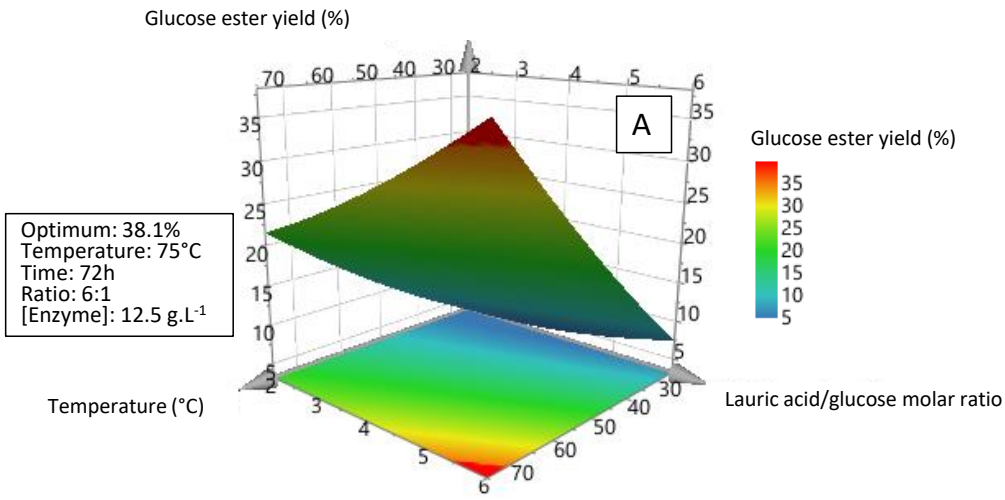


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829 **Figure 5** (in color)

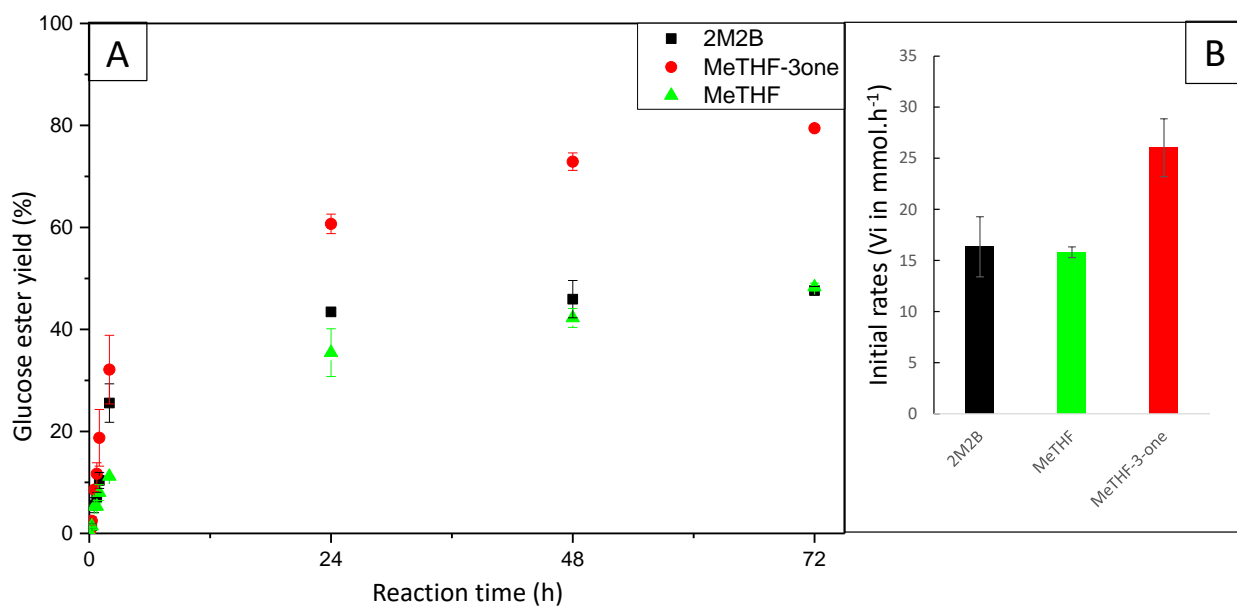
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832 **Figure 6** (in color).

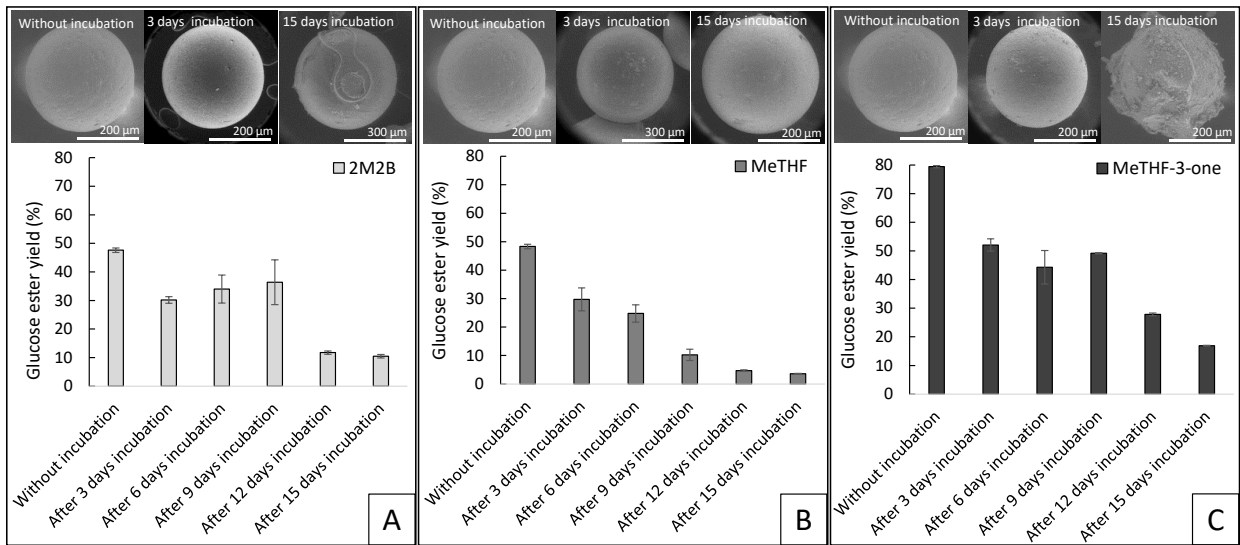
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835 **Figure 7.**

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837