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1 Preparation of two glycoside hydrolases for use in micro-aqueous media

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15  
16 **Abstract**  
17 Enzymatic synthesis of alkyl glycosides using glycoside hydrolases is well studied, but has yet to  
18 reach industrial scale, primarily due to limited yields. Reduced water content should increase  
19 yields by limiting the unwanted hydrolytic side reaction. However, previous studies have shown  
20 that a reduction in water content surprisingly favors hydrolysis over transglycosylation. In  
21 addition, glycoside hydrolases normally require a high degree of hydration to function  
22 efficiently. This study compares six enzyme preparation methods to improve resilience and  
23 activity of two glycoside hydrolases from *Thermotoga neapolitana* (*TnBgl3B* and *TnBgl1A*) in  
24 micro-aqueous hexanol. Indeed, when adsorbed onto Accurel MP-1000 both enzymes  
25 increasingly favored transglycosylation over hydrolysis at low hydration, in contrast to freeze-  
26 dried or untreated enzyme. Additionally, they displayed 17-70x higher reaction rates compared  
27 to freeze-dried enzyme at low water activity, while displaying comparable or lower activity for  
28 fully hydrated systems. These results provide valuable information for use of enzymes under  
29 micro-aqueous conditions and build towards utilizing the full synthetic potential of glycoside  
30 hydrolases.

31  
32 **Keywords:** Transglycosylation; Hydrolase; Immobilization; Organic solvent; Alkyl glycoside

33  
34 **Abbreviations:**  $a_w$ , water activity;  $r_s$ , transglycosylation rate;  $r_h$ , hydrolysis rate; HG, hexyl- $\beta$ -D-  
35 glucoside; *p*NPG, *p*-nitrophenol- $\beta$ -D-glucopyranoside; *p*NP, *p*-nitrophenol; AOT, dioctyl sodium  
36 sulfosuccinate.

37  
38

## 39 1. Introduction

40 Alkyl glycosides are a group of attractive surfactants. They exhibit antimicrobial activity,  
41 biodegradability and low toxicity [1], and find their use in cosmetics, biochemistry and  
42 pharmaceutical industry [2-4]. Currently, they are produced using conventional chemistry, which  
43 lead to a mixture of anomers [4], and require complicated separation techniques for purification.  
44 Enzymatic synthesis using glycoside hydrolases ( $\beta$ -glycosidases) is an attractive alternative, as it  
45 provides an anomerically pure product and reduced waste, thereby constituting a more  
46 environmentally sustainable option [5]. There are two possible enzymatic strategies: the  
47 thermodynamically controlled reverse hydrolysis or the kinetically controlled transglycosylation  
48 reaction [6].

49 Currently, the alkyl glycoside yields from enzymatic synthesis are too low for an  
50 economically feasible industrial process. This issue is intimately linked to the presence of water.  
51 In reverse hydrolysis the amount of water directly influences the equilibrium yield, while  
52 enzyme properties are a significant factor for transglycosylation. The catalytic mechanism  
53 involves a glycosyl-enzyme intermediate, which can be deglycosylated either by water or by  
54 alcohol, yielding hydrolysis or alkyl glycoside respectively. Therefore, the yield is determined by  
55 the acceptor specificity of the enzyme, often quantified as the ratio of transferase over hydrolase  
56 activity ( $r_s/r_h$ ). Several previous studies have been aimed at increasing alkyl glycoside yield by  
57 improving  $r_s/r_h$  through protein engineering [7-10].

58 Another way to impair the hydrolytic side reaction, and increase the alkyl glycoside  
59 yield, is to reduce the water content in the reaction media. However, previous reports of  
60 transglycosylation, catalyzed by a wide range of  $\beta$ -glycosidases has, counter-intuitively, shown  
61 reduced selectivity ( $r_s/r_h$ ) at low  $a_w$  [9, 11, 12]. In addition, most enzymes are not well suited for

62 anhydrous conditions [13].  $\beta$ -glycosidases in particular have been reported to require a water  
63 activity ( $a_w$ ) as high as 0.6 [11, 14], in contrast e.g. lipases such as CALB, which has been shown  
64 to retain activity at  $a_w$  as low as 0.02 [15].

65 In this paper, we attempt to increase the synthetic usefulness of two  $\beta$ -glycosidases by  
66 improving their selectivity and activity in micro-aqueous media. Six enzyme preparation  
67 methods are compared for synthesis of hexyl- $\beta$ -D-glucoside (HG) from *p*-nitrophenol- $\beta$ -D-  
68 glucopyranoside (*p*NPG) in hexanol. As model enzyme the  $\beta$ -glucosidase with the highest  
69 reported  $r_s/r_h$ , from *Thermotoga neapolitana* (*TnBgl3B*), is used. It belongs to the glycoside  
70 hydrolase family 3 and has been reported to have very low activity in micro-aqueous media,  
71 when no enzyme preparation method was used [16]. In contrast,  $\beta$ -glycosidase from *P. furiosus*,  
72 belonging to glycoside hydrolase family 1, has been shown to be most active in absence of a  
73 separate aqueous phase [7]. To avoid bias from this potential discrepancy between the two  
74 glycoside hydrolase families, *Thermotoga neapolitana* enzymes from both families are studied in  
75 parallel. For lipases, up to 400-fold activation has been demonstrated by selecting a proper  
76 enzyme preparation method [17], but to the best of our knowledge, no such attempt has  
77 previously been made for  $\beta$ -glycosidases.

78

## 79 **2. Materials and Methods**

### 80 **2.1. Material.**

81 Hexyl- $\beta$ -D-glucoside (HG), *p*-nitrophenol (*p*NP) and *p*-nitrophenol- $\beta$ -D-glucoside (*p*NPG) were  
82 obtained from Sigma-Aldrich (St Louis, Missouri, USA) and all other chemicals from VWR  
83 International (Stockholm, Sweden).

### 84 **2.2. Mutagenesis**

85 The genes encoding *TnBgl1A* and *TnBgl3B* were previously cloned into PET22b(+) (Novagen,  
86 Madison, WI, USA) [16, 18]. Mutagenesis for construction of the N220F mutant was performed  
87 in a previous study, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA),  
88 with the sequence with GenBank accession number AF039487 as the template and the primer 5'-  
89 GGAAAGATAGGGATTGTTTTCTTCAACGGATACTTCGAACCTGC-3' [10]. The resulting  
90 plasmid was transformed into *E. coli* Nova Blue cells for storage and into *E. coli* BL21  
91 (Novagen) for expression. The complete gene was sequenced by GATC Biotech AG (Konstanz,  
92 Germany) to confirm the mutations.

93

### 94 **2.3. Expression and purification.**

95 The enzymes were synthesized in 0.5 L cultivations of *E. coli* BL21 (Novagen) in Erlenmeyer  
96 flasks at 37 °C, pH 7 in Luria-Bertania (LB) media containing 100 µg/ml Ampicillin, inoculated  
97 with 1 % over night precultures. After reaching an OD<sub>620</sub> of 0.6 *TnBgl1A* and *TnBgl3B* gene  
98 expression was induced by addition of 0.5 ml 100 mM isopropyl-β-D-1-thiogalactopyranoside  
99 (IPTG) and production was continued for 20 h. Cells were harvested by centrifugation for 10  
100 min (4 °C, 5500 x g), re-suspended in binding buffer (20 mM imidazole, 20 mM Tris-HCl, 0.75  
101 M NaCl, pH 7.5) and lysed by sonication 6 x 3 min at 60 % amplitude and a cycle of 0.5 using a  
102 14 mm titanium probe (UP400 S, Dr. Hielscher). Heat treatment (70 °C, 30 min) and  
103 centrifugation (30 min, 4 °C, 15000 x g) was used to remove most of the native *E.coli* proteins  
104 before purification by immobilized metal affinity chromatography using an ÄKTA prime system  
105 (Amersham Biosciences, Uppsala, Sweden). The protein slurry was applied to a Histrap FF  
106 crude column (GE Healthcare) pretreated with 0.1 M Copper (II) sulphate. Bound proteins were  
107 eluted using elution buffer (250 mM imidazole, 20 mM Tris-HCl, 0.75 M NaCl, pH 7.5).

108 Fractions containing protein were pooled and dialyzed against 50 mM citrate phosphate buffer,  
109 pH 5.6, over night using a 3500 Da molecular weight cut-off dialysis membrane (Spectrum  
110 laboratories, Rancho Dominguez, CA, USA) and stored at -20 °C until use. Purity of the  
111 expressed proteins was estimated using SDS-PAGE according to Laemmli [19].

#### 112 **2.4. Lyophilisation.**

113 The glycosidases were diluted up to 1 ml in 0.1 M citrate phosphate buffer, pH 5.6 to roughly  
114 0.25-0.30 mg/ml and centrifuged to remove insoluble residues. The supernatants were  
115 immediately frozen at -80 °C and then freeze-dried for 18 h.

#### 116 **2.5. Surfactant modification.**

117 A reverse micellar system was created according to a previously described method [20]. 150 µl  
118 suspensions of 0.6-0.85 mg/ml glycosidase in 0.1 M citrate phosphate buffer, pH 5.6 was added  
119 to 5 ml 100 mM dioctyl sodium sulfosuccinate (AOT) in 2,2,4-trimethylpentane and shaken  
120 vigorously. The trimethylpentane was removed by rotary evaporation and the residue was further  
121 dried in a vacuum desiccator.

#### 122 **2.6. Factorial immobilization test**

123 The influence of buffer strength, pH and incubation time for adsorption and covalent  
124 immobilization of *TnBgl1A* and *TnBgl3B* on the supports listed in sections 2.7 and 2.8 was  
125 tested using a 2<sup>3</sup> factorial design. The software Minitab<sup>®</sup> (Release 14.1) was used to evaluate the  
126 data. Three factors were studied (buffer strength 0.05, 0.15 and 0.25 mM; buffer pH 4, 5.5 and 7  
127 and incubation time 1, 7 and 24 h). Two replicates and 4 central points was used giving a total  
128 number of 20 runs per enzyme.

#### 129 **2.7. Adsorption.**



130 Both glycosidases were immobilized by adsorption to a hydrophobic support (Accurel MP-1000)  
131 and an anion-exchange resin (IRA-400). 7 ml 0.09-0.11 mg/ml enzyme in 0.1 mM citrate  
132 phosphate buffer, pH 5.6 was added to 400 mg Accurel MP-1000, which was pre-wetted with 3  
133 ml ethanol / g support, and to 400 mg IRA-400, which was pre-washed with 0.1 mM citrate  
134 phosphate buffer, pH 5.6. The enzyme and support was incubated on a nutating mixer overnight  
135 and thereafter filtered and washed with buffer. Finally, the preparations were dried in a vacuum  
136 desiccator. For the MP-1000 support, a milder drying technique previously described by Moore  
137 et al. was also evaluated [21]. After removing the aqueous enzyme solution, the support was  
138 washed three times with n-propanol, the same volume as the original aqueous solution, set to the  
139 desired water activity. This was followed by two washes with the same volume of hexanol, set to  
140 the desired water activity. The hexanol was removed immediately prior to addition of substrate.

#### 141 **2.8. Covalent immobilization.**

142 400 mg epoxy-activated matrix, Eupergit<sup>®</sup> C250L, was washed with 0.1 mM citrate phosphate  
143 buffer, pH 5.6. 7 ml 0.09-0.11 mg/ml enzyme in 0.1 mM citrate phosphate buffer, pH 5.6 was  
144 added, incubated on a nutating mixer overnight and thereafter filtered, washed with buffer and  
145 dried in a vacuum desiccator.

#### 146 **2.9. Protein determination.**

147 Total protein concentration was estimated at 595 nm by the Bradford method [22] using bovine  
148 serum albumin as standard.

#### 149 **2.10. Water activity.**

150 Substrate solutions (34 mM *p*NPG in hexanol) were incubated over saturated salt solutions to  
151 defined water activities. The salts used for equilibration were  $\text{KCH}_3\text{CO}_2$  ( $a_w = 0.23$ ),  $\text{MgCl}_2$  ( $a_w$   
152 = 0.33),  $\text{Mg}(\text{NO}_3)_2$  ( $a_w = 0.53$ ),  $\text{NaCl}$  ( $a_w = 0.75$ ),  $\text{KCl}$  ( $a_w = 0.84$ ) and  $\text{K}_2\text{SO}_4$  ( $a_w = 0.97$ ).

153 Triplicate samples from each equilibrated hexanol sample were injected on a 899 Karl Fischer  
154 coulometer (Metrohm, Herisau, Switzerland). The obtained relation between water activity and  
155 water amount was used to estimate water activity in the transferase reactions. The procedure was  
156 repeated using n-propanol, to allow setting the water activity for drying the MP-1000, as  
157 described above.

#### 158 **2.11. Transferase reaction.**

159 Support corresponding to 21  $\mu\text{g}$  of immobilized enzyme, based on the Bradford assay, was  
160 mixed with 2 ml 34 mM *p*NPG set to desired water activity, based on the Karl-Fisher calibration  
161 curve, and incubated in a ThermoMixer (HLC Biotech, Bovenden, Germany) set to 70 °C, 700  
162 rpm. 150  $\mu\text{l}$  samples were taken at 0, 2, 4, 8, 24, 48, 72 h and diluted with 150  $\mu\text{l}$  2 mM NaOH  
163 and 300  $\mu\text{l}$  methanol before HPLC analysis. 40  $\mu\text{l}$  was withdrawn to check actual water activity  
164 after 1 h reaction by Karl Fischer coulometry.

#### 165 **2.12. HPLC analysis.**

166 Transferase reactions were monitored using RP-HPLC (LaChrom; pump L-7100, interface L-  
167 7000, autosampler L-7250 with a 20 ml injection loop, UV-detector L7400, Hitachi Ltd. Tokyo,  
168 Japan) equipped with an evaporative light scattering detector (Alltech 500 ELSD, Alltech  
169 Associates Inc., Deer-field, USA) with evaporator temperature 94 °C, a nebulizer gas flow of 2.5  
170 standard liters per minute and a Kromasil 100 5C18 column (4.6  $\mu\text{m}$  \* 250 mm, Kromasil,  
171 EkaChemicals AB, Separation Products, Bohus, Sweden). A gradient was applied from 50 % to  
172 70 % methanol in 0.1 % acetic acid in MQ H<sub>2</sub>O over 5 minutes and kept at 70 % for one minute  
173 before returning to initial conditions for re-equilibration. A constant flow rate of 1.0 ml/min was  
174 used. *p*NPG elutes after 3.5 min and is followed at 405 nm as well as with ELSD. HG and *p*NP  
175 both have a retention time of 7.5 min, but HG does not absorb at 405 nm and *p*NP is too volatile

176 to be detected by ELSD. Concentrations were determined by use of 8 point external standard  
177 curves.

178

### 179 **3. Results and Discussion**

#### 180 **3.1. Enzyme preparation.**

181 Two  $\beta$ -glycosidases from *Thermotoga neapolitana* (*TnBgl1A* and *TnBgl3B*) were modified by  
182 six different enzyme preparation methods; adsorption onto Accurel MP1000 (porous  
183 polypropylen) or Amberlite IRA-400 (ion-exchange resin), covalent linking to Eupergit C250L,  
184 freeze-drying, surfactant encapsulation using AOT as well as application of a gentle drying  
185 method on adsorption onto MP1000. Before formulating the enzyme preparations, protein purity  
186 was established to above 95 % using SDS-PAGE (Figure S1). For the methods involving  
187 adsorption or binding to a support material (Eupergit C250L, Amberlite IRA-400 and Accurel  
188 MP1000), parameters that can affect the immobilization yield (buffer strength, buffer pH and  
189 incubation time) was studied using a 3 factor 2 level factorial design. The estimated effects and  
190 coefficients for each parameter are presented in Supplementary Table S1. Buffer pH had the  
191 highest influence on immobilization yield, and a low pH (pH 4) was preferred for all  
192 preparations. The effect was, unsurprisingly, especially prominent for immobilization on the ion-  
193 exchange resin Amberlite IRA-400. However, for use in organic solvents, it is advisable to  
194 generate the enzyme preparations under the pH optimum for the enzyme. This is because the  
195 protonation state of the enzyme is conserved when transferred into an organic medium [23]. For  
196 incubation time and buffer strength, the lower values (1 h incubation or 0.05 mM buffer strength)  
197 were detrimental to the immobilization, while less difference was seen between the high values

198 (15 h incubation and 0.25 mM buffer strength) and the center point (8 h incubation and 0.15 mM  
199 buffer strength).

200 Table I shows the immobilization yield of *TnBgl1A* and *TnBgl3B* onto Eupergit C250L,  
201 Amberlite IRA-400 and Accurel MP1000, based on protein measurements of the initial enzyme  
202 slurry and the filtrate after preparation. Both data at the best immobilization conditions from the  
203 factorial design and at the conditions selected for use in the transferase reaction are presented. It  
204 is clear from the table, that Amberlite IRA-400 is not as well suited for immobilization of  $\beta$ -  
205 glycosidases as the other two. For *TnBgl1A*, Accurel MP1000 maintain high immobilization  
206 yield at pH optimum for the enzyme (pH 5.6). For the remaining two methods, freeze-drying and  
207 AOT modification, 100% protein yields are assumed. Table I also shows the relative specific  
208 total activity (hydrolysis and transglycosylation) of *pNPG* for each enzyme preparation,  
209 compared to untreated enzyme in water-saturated hexanol. Amongst the three preparations, both  
210 enzymes retain the most activity in water-saturated hexanol when adsorbed onto Acurrel  
211 MP1000. However, at high hydration, untreated enzyme still has higher specific activity, which  
212 is not the case at lower hydration.

### 213 **3.2. Improved catalytic** 214 **activity at low hydration.**

215 To determine which enzyme preparation method is most suited for low water activities,  
216 transglycosylation reactions were followed at four different hydration states for each enzyme  
217 preparation. In the transglycosylation reaction monitored, *pNPG* is converted into *pNP* and either  
218 glucose or HG, visualized in Figure 1. Although all of these components were detected using  
219 HPLC, formation of HG was used for comparing the different preparation methods. This is  
220 primarily since *pNP* binds to Amberlite IRA-400 (data not shown), and can therefore not be used

221 to follow all reactions. Table II shows the initial formation rates of HG at the various  $a_w$  levels  
222 based on the amount of water added to the substrate mixture before adding the enzyme  
223 preparations. The enzyme preparations influenced the water activity to different extent, and in  
224 Figures 2 and 3 the reaction rates are plotted versus the experimentally determined water  
225 activities. The first figure, plotted on a logarithmic scale, shows that both *TnBgl1A* and *TnBgl3B*  
226 display exponential increase of reaction rates with increasing  $a_w$ . This correlates well with  
227 previous characterizations of  $\beta$ -glycosidases in micro-aqueous media [7, 14]. Moreover, Table II  
228 demonstrates that enzyme adsorbed to Accurel MP1000 and dried using 1-propanol, retains the  
229 most activity at low  $a_w$  ( $0.83 \text{ nmol min}^{-1} \text{ g}^{-1}$  and  $1.65 \text{ nmol min}^{-1} \text{ g}^{-1}$  for *TnBgl1A* and *TnBgl3B*  
230 respectively at  $a_w \approx 0.7$ ), while freeze-dried enzymes are amongst the least suited for low  
231 hydration ( $0.049 \text{ nmol min}^{-1} \text{ g}^{-1}$  and  $0.023 \text{ nmol min}^{-1} \text{ g}^{-1}$  for *TnBgl1A* and *TnBgl3B* respectively  
232 at  $a_w \approx 0.7$ ). However, the opposite relation applies at high  $a_w$  for *TnBgl1A*, where the freeze-  
233 dried preparation outcompetes the MP1000 adsorbed preparation, as illustrated in Figure 3. The  
234 figure shows the specific initial formation rates, normalized against the activity of untreated  
235 enzyme for each approximate  $a_w$ , and plotted against the experimentally determined water  
236 activities for the untreated enzyme used for normalization. As can be seen in the figure, the trend  
237 is the same for *TnBgl3B* as for *TnBgl1A*, although the freeze-dried preparation only reached an  
238 activity equal to the MP1000 adsorbed *TnBgl3B* at water saturation. The results emphasize the  
239 importance of choosing enzyme treatment based on intended hydration condition. For example,  
240 *TnBgl1A* adsorbed to Accurel MP1000 had 17x higher transglycosylation activity than freeze-  
241 dried enzyme at the lowest studied  $a_w$ , but only 0.45x the rate at  $a_w = 0.9$ . The reason for the  
242 higher enzyme activity when adsorbed on Accurel MP1000 is likely due to an improved  
243 dispersion of the catalyst and thereby an increased surface accessibility [24]. The additional

244 increase in activity when using the propanol drying procedure could be from avoiding the  
245 removal of essential water molecules from the protein [21].

### 246 **3.3. Increased ratio of transglycosylation/hydrolysis at low hydration.**

247 Previous reports of transglycosylation catalyzed by a wide range of  $\beta$ -glycosidases have shown  
248 reduced selectivity ( $r_s/r_h$ ) at low  $a_w$  [9, 11, 12]. As seen in Figure 4, this unwanted and counter-  
249 intuitive trend was observed for untreated, freeze-dried and covalently bound enzyme  
250 preparations of both *TnBgl1A* and *TnBgl3B*. Nevertheless, for *TnBgl3B* adsorbed onto Accurel  
251 MP1000 the  $r_s/r_h$  ratio was maintained at low hydration. Furthermore, *TnBgl1A* on Accurel  
252 MP1000 even displayed increased selectivity at low water activity. Consequently, we have  
253 successfully showed that reduced hydration can be used as means to improve alkyl glycoside  
254 yields from transglycosylation catalyzed by *TnBgl1A*, when immobilized on Accurel MP1000.  
255 We believe the, previously observed, reduced selectivity at low hydration for  $\beta$ -glycosidases has  
256 been due to detrimental protein-protein interactions when the enzymes are transferred to an  
257 organic solvent. Gentle immobilization onto a suitable support can reduce these interactions  
258 without introducing new harmful alterations to the enzyme. Why the effects of immobilization  
259 are less prominent for *TnBgl3B* is, however, not clear.

### 260 **3.4. Proper enzyme preparation in combination with *TnBgl1A* mutation N220F.**

261 In a previous study, we found that the single mutation N220F of *TnBgl1A* increased  $r_s/r_h$  7-fold  
262 (up to  $1.38 \pm 0.20$ ) for HG synthesis in a biphasic water:hexanol system (15:85) without enzyme  
263 preparation [10]. We can now show that immobilizing onto Accurel MP1000 enables further  
264 increase in the  $r_s/r_h$  up to  $3.16 \pm 0.06$  at  $a_w = 0.85$ , as demonstrated in Figure 5. However, this  
265 increase in specificity comes at the cost of a severely reduced reaction rate ( $2.4 \pm 0.3 \mu\text{mol min}^{-1}$   
266  $\text{mg}^{-1}$  at  $a_w = 0.85$  compared to  $87.8 \pm 3.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$  with 15 % water).

#### 267 4. Conclusions

268 Previous studies on  $\beta$ -glycosidase catalyzed transglycosylation in micro-aqueous media have,  
269 counter-intuitively, shown increased ratio of undesired hydrolysis at low water activity. This  
270 paper demonstrates, for the first time, that reduced water content can be a viable method of  
271 reducing the hydrolytic side reaction, provided the enzyme is properly prepared for use in micro-  
272 aqueous media. We show that deposition onto Accurel MP-1000 is a suitable preparation  
273 method, especially when dried using the propanol washing method described by Moore et al  
274 [21]. However, the reaction rates at low hydration still require significant enhancement to reach  
275 an enzymatic method competitive to the classical chemical routes used for synthesis of alkyl  
276 glycosides today. Feasible routes to get there include screening for  $\beta$ -glycosidases with desirable  
277 properties in micro-aqueous media or alternatively protein engineering of promising candidates  
278 such as *TnBgl3B*. Nevertheless, deposition on MP1000 provides one step towards unlocking the  
279 full synthetic potential of  $\beta$ -glycosidases.

280

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284

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**TABLE I:** Immobilization yield, enzyme load based on protein measurements in non-bound fraction as well as total activity relative to untreated enzyme for transglycosylation and hydrolysis of *p*NPG in water saturated hexanol. Data is presented as average  $\pm 1\sigma$  from duplicate reactions.

Support	<i>TnBgl1A</i>			<i>TnBgl3B</i>		
	Protein yield %	Load mg/g	Rel. activity %	Protein yield %	Load mg/g	Rel. activity %
Eupergit C250L <sup>a</sup>	93 $\pm$ 3	1.3 $\pm$ 0.0		98 $\pm$ 5	1.7 $\pm$ 0.2	
Eupergit C250L <sup>b</sup>	42 $\pm$ 3	0.6 $\pm$ 0.1	1.7	64 $\pm$ 11	1.1 $\pm$ 0.3	3.2
Accurel MP1000 <sup>a</sup>	98 $\pm$ 1	1.8 $\pm$ 0.0		96 $\pm$ 1	1.7 $\pm$ 0.1	
Accurel MP1000 <sup>b</sup>	94 $\pm$ 1	1.4 $\pm$ 0.5	12.4	59 $\pm$ 3	1.0 $\pm$ 0.1	48.4
Amberlite IRA-400 <sup>a</sup>	29 $\pm$ 1	0.5 $\pm$ 0.0		22 $\pm$ 2	0.4 $\pm$ 0.1	
Amberlite IRA-400 <sup>b</sup>	4 $\pm$ 3	0.1 $\pm$ 0.4	2.9	1 $\pm$ 4	0.0 $\pm$ 0.1	2.5

<sup>a</sup> Best results obtained in any of the tested conditions

<sup>b</sup> Immobilized under conditions for use in organic media , incubated 24h in 0.1M citrate phosphate buffer pH 5.6.

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**TABLE II:** Specific initial formation of hexyl- $\beta$ -D-glucopyranoside ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) at each approximated water activity ( $a_w$ ). Data is presented as average  $\pm 1\sigma$  from duplicate reactions.

$a_w$	<i>TnBgl1A</i>				<i>TnBgl3B</i>			
	<b>0.7</b>	<b>0.8</b>	<b>0.9</b>	<b>1.0</b>	<b>0.7</b>	<b>0.8</b>	<b>0.9</b>	<b>1.0</b>
Untreated	0.24 $\pm$	0.79 $\pm$	4.75 $\pm$	14.73 $\pm$	0.49 $\pm$	0.90 $\pm$	8.49 $\pm$	25.45 $\pm$
	0.05	0.04	0.90	0.21	0.05	0.17	0.87	1.76
Freeze-dried	0.05 $\pm$	0.33 $\pm$	4.53 $\pm$	6.78 $\pm$	0.02 $\pm$	0.04 $\pm$	0.91 $\pm$	9.65 $\pm$
	0.05	0.19	1.35	2.35	0.03	0.02	0.64	0.72
AOT	0.10 $\pm$	0.40 $\pm$	0.42 $\pm$	1.84 $\pm$	0.15 $\pm$	0.38 $\pm$	1.14 $\pm$	6.15 $\pm$
	0.06	0.00	0.05	0.21	0.08	0.00	1.18	0.00
Eupergit C250L	0.00 $\pm$	0.02 $\pm$	0.11 $\pm$	0.25 $\pm$	0.01 $\pm$	0.03 $\pm$	0.11 $\pm$	0.61 $\pm$
	0.01	0.01	0.03	0.08	0.01	0.03	0.20	0.23
Accurel MP1000	0.16 $\pm$	0.38 $\pm$	0.84 $\pm$	1.34 $\pm$	0.41 $\pm$	0.92 $\pm$	2.60 $\pm$	13.95 $\pm$
	0.02	0.06	0.09	0.22	0.03	0.14	0.46	2.60
Vacuum dried	0.83 $\pm$	1.03 $\pm$	2.03 $\pm$	4.52 $\pm$	1.65 $\pm$	1.93 $\pm$	3.69 $\pm$	9.38 $\pm$
	0.15	0.15	0.16	0.30	0.37	0.13	1.09	0.13
Propanol dried	0.00 $\pm$	0.21 $\pm$	0.45 $\pm$	1.08 $\pm$	0.03 $\pm$	0.05 $\pm$	0.16 $\pm$	0.98 $\pm$
	0.00	0.19	0.23	0.90	0.11	0.05	0.00	0.44

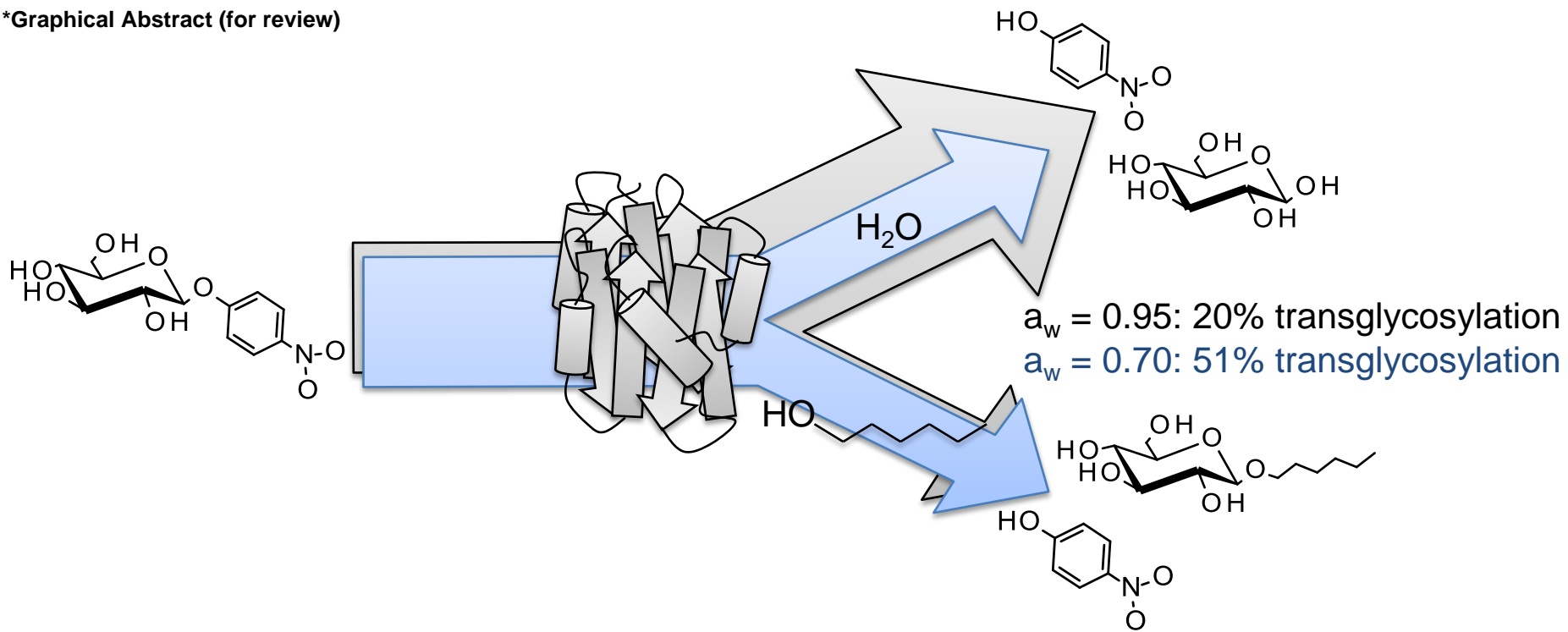
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- Two  $\beta$ -glucosidases were immobilized using six enzyme preparation methods.
  - Adsorbed onto polypropylene, both enzymes favor transglycosylation at low hydration.
  - At low water content, activity is better retained with a proper enzyme preparation.

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**\*Graphical Abstract (for review)**



1 **Figure 1**, Schematic representation of the enzymatic conversion of *p*-nitrophenyl- $\beta$ -D-glucoside  
2 to hexyl- $\beta$ -glucoside at the rate  $r_s$  and to glucose at the rate  $r_h$  catalyzed by a retaining  
3  $\beta$ -glucosidase.

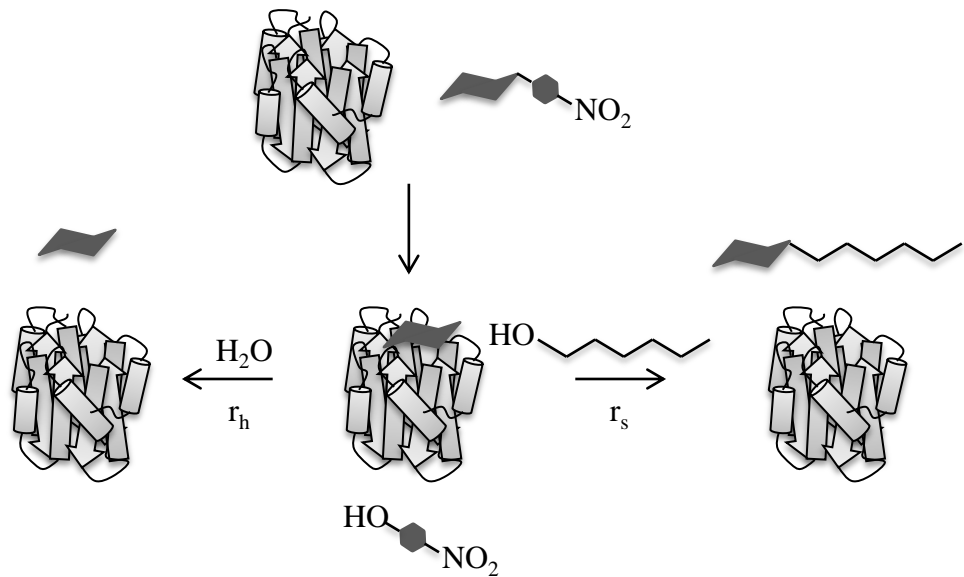
4  
5 **Figure 2**, Initial transglycosylation activity of *TnBgl1A* (left) and *TnBgl3B* (right) plotted versus  
6 experimentally determined water activities ( $a_w$ ). The enzyme was freeze-dried ( $\diamond$ ), deposited on  
7 Accurel MP1000; vacuum dried ( $\blacksquare$ ) or propanol dried ( $\blacklozenge$ ), covalently linked to Eupergit C250L  
8 ( $\Delta$ ) or added as aqueous solution ( $\circ$ ). Error bars represent  $1\sigma$ , based on triplicate measurements.

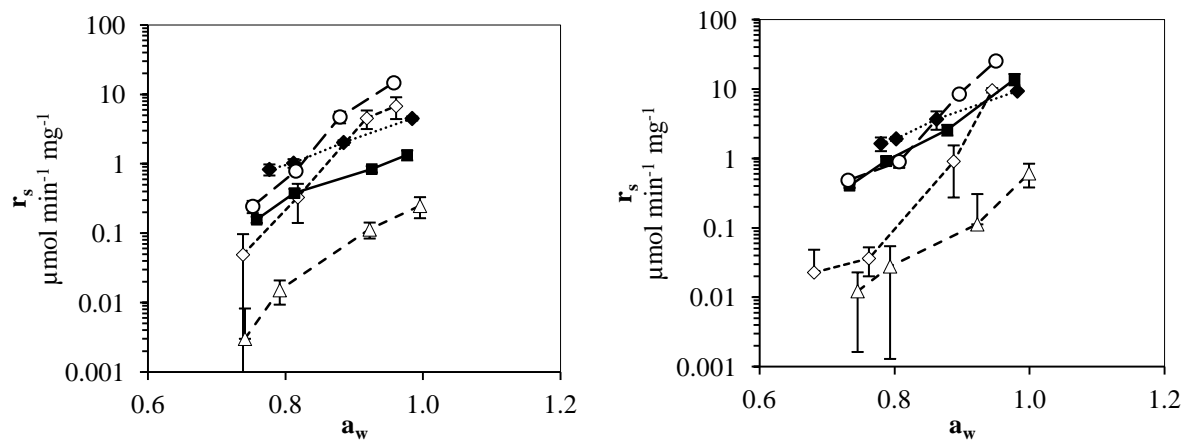
9  
10 **Figure 3**, Initial transglycosylation activity of *TnBgl1A* (left) and *TnBgl3B* (right) normalized  
11 against untreated enzyme at each approximate water activity ( $a_w$ ). The compared preparation  
12 methods are freeze-drying ( $\diamond$ ), deposition on Accurel MP1000; vacuum dried ( $\blacksquare$ ) or propanol  
13 dried ( $\blacklozenge$ ), and covalent linking to Eupergit C250L ( $\Delta$ ).

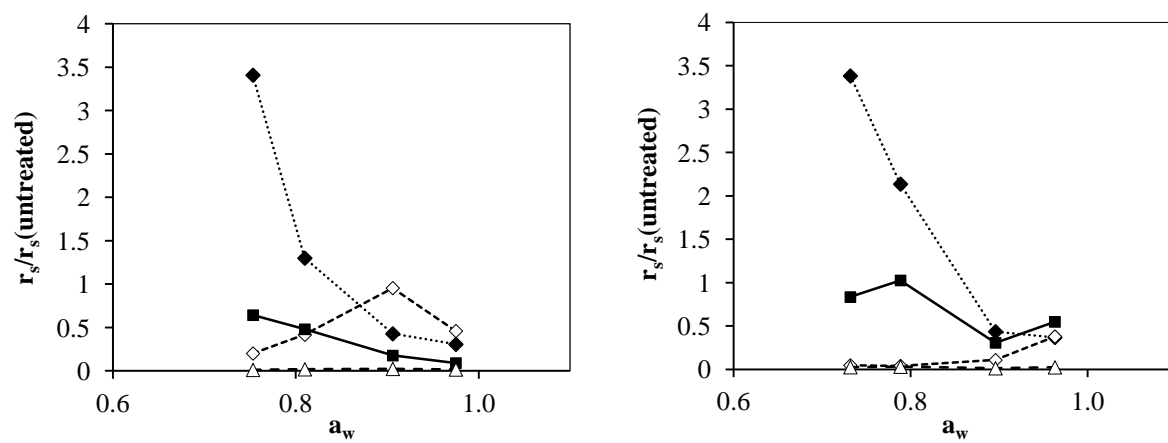
14  
15 **Figure 4**, Selectivity for transglycosylation ( $r_s/r_h$ ) of *TnBgl1A* (left) and *TnBgl3B* (right) as a  
16 function of water activity ( $a_w$ ). The enzyme was freeze-dried ( $\diamond$ ), deposited on Accurel MP1000;  
17 vacuum dried ( $\blacksquare$ ) or propanol dried ( $\blacklozenge$ ), and covalently linked to Eupergit C250L ( $\Delta$ ). Error bars  
18 represent  $1\sigma$ , based on triplicate measurements.

19  
20 **Figure 5**, Initial transglycosylation activity (left) and selectivity for transglycosylation ( $r_s/r_h$ ) for  
21 mutant N220F of *TnBgl1A* as a function of water activity ( $a_w$ ). The enzyme was deposited on  
22 Accurel MP1000 and dried by propanol wash. Error bars represent  $1\sigma$ , based on triplicate  
23 measurements.

Figure 1

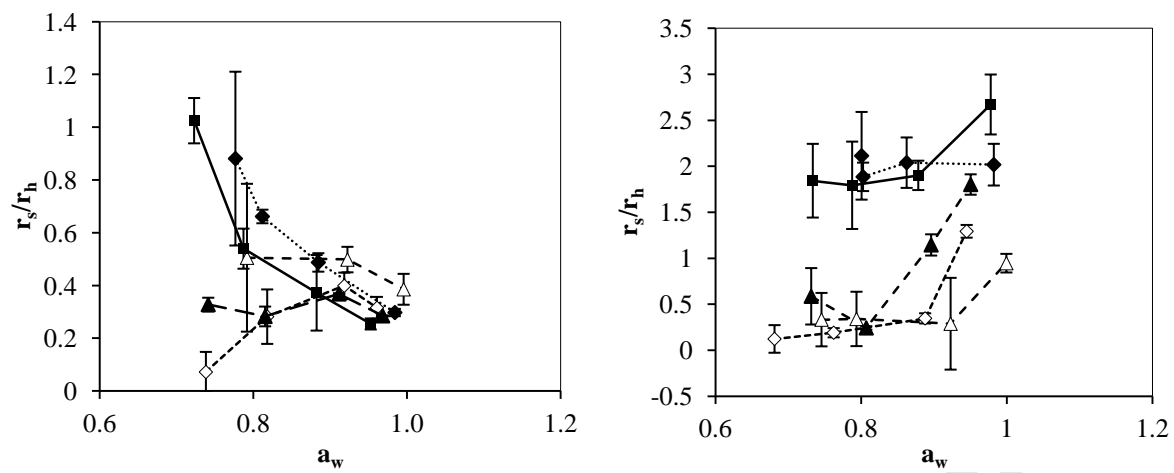




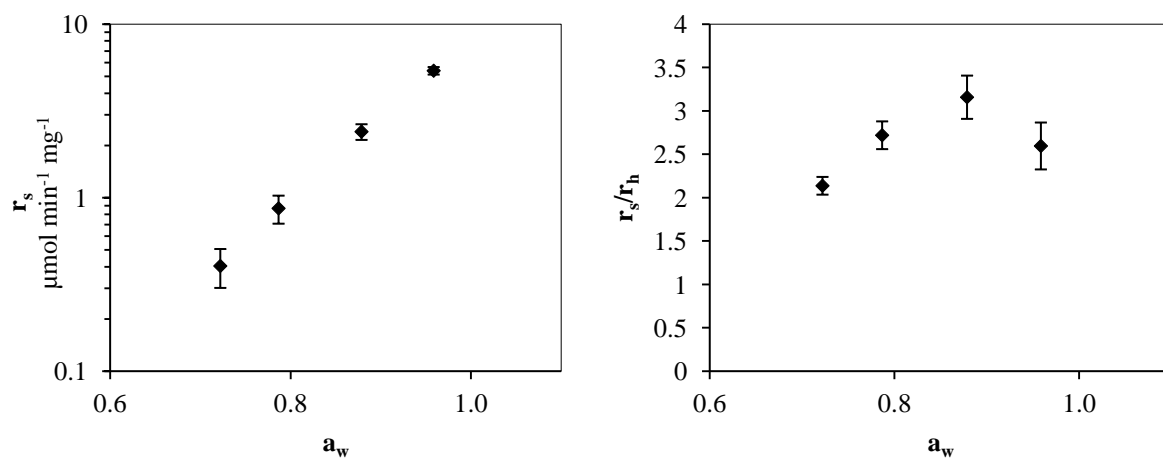


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