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

Lundemo, Pontus; Nordberg Karlsson, Eva; Adlercreutz, Patrick

Published in:
Journal of Molecular Catalysis B: Enzymatic

DOI:
10.1016/j.molcatb.2014.06.009

2014

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

Author: Pontus Lundemo Eva Nordberg Karlsson Patrick Adlercreutz

PII: S1381-1177(14)00188-X
DOI: http://dx.doi.org/doi:10.1016/j.molcatb.2014.06.009
Reference: MOLCAB 2980

To appear in: *Journal of Molecular Catalysis B: Enzymatic*

Received date: 6-2-2014
Revised date: 27-5-2014
Accepted date: 21-6-2014

Please cite this article as: P. Lundemo, E.N. Karlsson, P. Adlercreutz, Preparation of two glycoside hydrolases for use in micro-aqueous media, *Journal of Molecular Catalysis B: Enzymatic* (2014), http://dx.doi.org/10.1016/j.molcatb.2014.06.009

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

Pontus Lundemo[a], Eva Nordberg Karlsson[a], Patrick Adlercreutz[a]

[a]Lund University, Dept of Chemistry, Biotechnology, P.O. Box 124, SE-221 00 Lund, Sweden

Corresponding author:

Pontus Lundemo

Phone: +46462220858

Email: pontus.lundemo@biotek.lu.se

Eva Nordberg Karlsson

Email: eva.nordberg_karlsson@biotek.lu.se

Patrick Adlercreutz

Email: patrick.adlercreutz@biotek.lu.se

Abstract

Enzymatic synthesis of alkyl glycosides using glycoside hydrolases is well studied, but has yet to reach industrial scale, primarily due to limited yields. Reduced water content should increase yields by limiting the unwanted hydrolytic side reaction. However, previous studies have shown that a reduction in water content surprisingly favors hydrolysis over transglycosylation. In addition, glycoside hydrolases normally require a high degree of hydration to function efficiently. This study compares six enzyme preparation methods to improve resilience and activity of two glycoside hydrolases from *Thermotoga neapolitana* (*Tn*Bgl3B and *Tn*Bgl1A) in micro-aqueous hexanol. Indeed, when adsorbed onto Accurel MP-1000 both enzymes increasingly favored transglycosylation over hydrolysis at low hydration, in contrast to freeze-dried or untreated enzyme. Additionally, they displayed 17-70x higher reaction rates compared to freeze-dried enzyme at low water activity, while displaying comparable or lower activity for fully hydrated systems. These results provide valuable information for use of enzymes under micro-aqueous conditions and build towards utilizing the full synthetic potential of glycoside hydrolases.

Keywords: Transglycosylation; Hydrolase; Immobilization; Organic solvent; Alkyl glycoside

Abbreviations: a_w, water activity; r_s, transglycosylation rate; r_h, hydrolysis rate; HG, hexyl-*β*-D-glucoside; *p*NPG, *p*-nitrophenol-*β*-D-glucopyranoside; *p*NP, *p*-nitrophenol; AOT, dioctyl sodium sulfosuccinate.

1. Introduction

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

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

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

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

In this paper, we attempt to increase the synthetic usefulness of two *β*-glycosidases by improving their selectivity and activity in micro-aqueous media. Six enzyme preparation methods are compared for synthesis of hexyl-*β*-D-glucoside (HG) from *p*-nitrophenol-*β*-D-glucopyranoside (*p*NPG) in hexanol. As model enzyme the *β*-glucosidase with the highest reported r_s/r_h, from *Thermotoga neapolitana* (*Tn*Bgl3B), is used. It belongs to the glycoside hydrolase family 3 and has been reported to have very low activity in micro-aqueous media, when no enzyme preparation method was used [16]. In contrast, *β*-glycosidase from *P. furiosus*, belonging to glycoside hydrolase family 1, has been shown to be most active in absence of a separate aqueous phase [7]. To avoid bias from this potential discrepancy between the two glycoside hydrolase families, *Thermotoga neapolitana* enzymes from both families are studied in parallel. For lipases, up to 400-fold activation has been demonstrated by selecting a proper enzyme preparation method [17], but to the best of our knowledge, no such attempt has previously been made for *β*-glycosidases.

2. Materials and Methods

2.1. Material.

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

2.2. Mutagenesis

The genes encoding *Tn*Bgl1A and *Tn*Bgl3B were previously cloned into PET22b(+) (Novagen, Madison, WI, USA) [16, 18]. Mutagenesis for construction of the N220F mutant was performed in a previous study, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the sequence with GenBank accession number AF039487 as the template and the primer 5'-GGAAAGATAGGGATTGTTTTCTTCAACGGATACTTCGAACCTGC-3' [10]. The resulting plasmid was transformed into *E. coli* Nova Blue cells for storage and into *E. coli* BL21 (Novagen) for expression. The complete gene was sequenced by GATC Biotech AG (Konstanz, Germany) to confirm the mutations.

2.3. Expression and purification.

The enzymes were synthesized in 0.5 L cultivations of *E. coli* BL21 (Novagen) in Erlenmeyer flasks at 37 °C, pH 7 in Luria-Bertania (LB) media containing 100 µg/ml Ampicillin, inoculated with 1 % over night precultures. After reaching an OD_{620} of 0.6 *Tn*Bgl1A and *Tn*Bgl3B gene expression was induced by addition of 0.5 ml 100 mM isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) and production was continued for 20 h. Cells were harvested by centrifugation for 10 min (4 °C, 5500 x g), re-suspended in binding buffer (20 mM imidazole, 20 mM Tris-HCl, 0.75 M NaCl, pH 7.5) and lysed by sonication 6 x 3 min at 60 % amplitude and a cycle of 0.5 using a 14 mm titanium probe (UP400 S, Dr. Hielscher). Heat treatment (70 °C, 30 min) and centrifugation (30 min, 4 °C, 15000 x g) was used to remove most of the native *E.coli* proteins before purification by immobilized metal affinity chromatography using an ÄKTA prime system (Amersham Biosciences, Uppsala, Sweden). The protein slurry was applied to a Histrap FF crude column (GE Healthcare) pretreated with 0.1 M Copper (II) sulphate. Bound proteins were eluted using elution buffer (250 mM imidazole, 20 mM Tris-HCl, 0.75 M NaCl, pH 7.5).

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

2.4. Lyophilisation.

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

2.5. Surfactant modification.

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

2.6. Factorial immobilization test

The influence of buffer strength, pH and incubation time for adsorption and covalent immobilization of *Tn*Bgl1A and *Tn*Bgl3B on the supports listed in sections 2.7 and 2.8 was tested using a 2^3 factorial design. The software Minitab® (Release 14.1) was used to evaluate the data. Three factors were studied (buffer strength 0.05, 0.15 and 0.25 mM; buffer pH 4, 5.5 and 7 and incubation time 1, 7 and 24 h). Two replicates and 4 central points was used giving a total number of 20 runs per enzyme.

2.7. Adsorption.

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

2.8. Covalent immobilization.

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

2.9. Protein determination.

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

2.10. Water activity.

Substrate solutions (34 mM *p*NPG in hexanol) were incubated over saturated salt solutions to defined water activities. The salts used for equilibration were KCH_3CO_2 (a_w = 0.23), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), KCl (a_w = 0.84) and K_2SO_4 (a_w = 0.97).

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

2.11. Transferase reaction.

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

2.12. HPLC analysis.

Transferase reactions were monitored using RP-HPLC (LaChrom; pump L-7100, interface L-7000, autosampler L-7250 with a 20 ml injection loop, UV-detector L7400, Hitachi Ltd. Tokyo, Japan) equipped with an evaporative light scattering detector (Alltech 500 ELSD, Alltech Associates Inc., Deer-field, USA) with evaporator temperature 94 °C, a nebulizer gas flow of 2.5 standard liters per minute and a Kromasil 100 5C18 column (4.6 µm * 250 mm, Kromasil, EkaChemicals AB, Separation Products, Bohus, Sweden). A gradient was applied from 50 % to 70 % methanol in 0.1 % acetic acid in MQ H_2O over 5 minutes and kept at 70 % for one minute before returning to initial conditions for re-equilibration. A constant flow rate of 1.0 ml/min was used. *p*NPG elutes after 3.5 min and is followed at 405 nm as well as with ELSD. HG and *p*NP both have a retention time of 7.5 min, but HG does not absorb at 405 nm and *p*NP is too volatile

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

3. Results and Discussion

3.1. Enzyme preparation.

Two *β*-glycosidases from *Thermotoga neapolitana* (*Tn*Bgl1A and *Tn*Bgl3B) were modified by six different enzyme preparation methods; adsorption onto Accurel MP1000 (porous polypropylen) or Amberlite IRA-400 (ion-exchange resin), covalent linking to Eupergit C250L, freeze-drying, surfactant encapsulation using AOT as well as application of a gentle drying method on adsorption onto MP1000. Before formulating the enzyme preparations, protein purity was established to above 95 % using SDS-PAGE (Figure S1). For the methods involving adsorption or binding to a support material (Eupergit C250L, Amberlite IRA-400 and Accurel MP1000), parameters that can affect the immobilization yield (buffer strength, buffer pH and incubation time) was studied using a 3 factor 2 level factorial design. The estimated effects and coefficients for each parameter are presented in Supplementary Table S1. Buffer pH had the highest influence on immobilization yield, and a low pH (pH 4) was preferred for all preparations. The effect was, unsurprisingly, especially prominent for immobilization on the ion-exchange resin Amberlite IRA-400. However, for use in organic solvents, it is advisable to generate the enzyme preparations under the pH optimum for the enzyme. This is because the protonation state of the enzyme is conserved when transferred into an organic medium [23]. For incubation time and buffer strength, the lower values (1 h incubation or 0.05 mM buffer strength) 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 *Tn*Bgl1A and *Tn*Bgl3B 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 *β*-
205 glycosidases as the other two. For *Tn*Bgl1A, 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 *p*NPG 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, *p*NPG is converted into *p*NP 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 *p*NP 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 *Tn*Bgl1A and *Tn*Bgl3B
226 display exponential increase of reaction rates with increasing a_w. This correlates well with
227 previous characterizations of β-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 nmol min^{-1} g^{-1} and 1.65 nmol min^{-1} g^{-1} for *Tn*Bgl1A and *Tn*Bgl3B
230 respectively at $a_w \approx 0.7$), while freeze-dried enzymes are amongst the least suited for low
231 hydration (0.049 nmol min^{-1} g^{-1} and 0.023 nmol min^{-1} g^{-1} for *Tn*Bgl1A and *Tn*Bgl3B respectively
232 at $a_w \approx 0.7$). However, the opposite relation applies at high a_w for *Tn*Bgl1A, 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 *Tn*Bgl3B as for *Tn*Bgl1A, although the freeze-dried preparation only reached an
238 activity equal to the MP1000 adsorbed *Tn*Bgl3B at water saturation. The results emphasize the
239 importance of choosing enzyme treatment based on intended hydration condition. For example,
240 *Tn*Bgl1A 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

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

3.3. Increased ratio of transglycosylation/hydrolysis at low hydration.

Previous reports of transglycosylation catalyzed by a wide range of *β*-glycosidases have shown reduced selectivity (r_s/r_h) at low a_w [9, 11, 12]. As seen in Figure 4, this unwanted and counter-intuitive trend was observed for untreated, freeze-dried and covalently bound enzyme preparations of both *Tn*Bgl1A and *Tn*Bgl3B. Nevertheless, for *Tn*Bgl3B adsorbed onto Accurel MP1000 the r_s/r_h ratio was maintained at low hydration. Furthermore, *Tn*Bgl1A on Accurel MP1000 even displayed increased selectivity at low water activity. Consequently, we have successfully showed that reduced hydration can be used as means to improve alkyl glycoside yields from transglycosylation catalyzed by *Tn*Bgl1A, when immobilized on Accurel MP1000. We believe the, previously observed, reduced selectivity at low hydration for *β*-glycosidases has been due to detrimental protein-protein interactions when the enzymes are transferred to an organic solvent. Gentle immobilization onto a suitable support can reduce these interactions without introducing new harmful alterations to the enzyme. Why the effects of immobilization are less prominent for *Tn*Bgl3B is, however, not clear.

3.4. Proper enzyme preparation in combination with *Tn*Bgl1A mutation N220F.

In a previous study, we found that the single mutation N220F of *Tn*Bgl1A increased r_s/r_h 7-fold (up to 1.38 ± 0.20) for HG synthesis in a biphasic water:hexanol system (15:85) without enzyme preparation [10]. We can now show that immobilizing onto Accurel MP1000 enables further increase in the r_s/r_h up to 3.16 ± 0.06 at a_w = 0.85, as demonstrated in Figure 5. However, this increase in specificity comes at the cost of a severely reduced reaction rate (2.4 ± 0.3 μmol min^{-1} mg^{-1} at a_w = 0.85 compared to 87.8 ± 3.2 μmol min^{-1} mg^{-1} with 15 % water).

4. Conclusions

Previous studies on *β*-glycosidase catalyzed transglycosylation in micro-aqueous media have, counter-intuitively, shown increased ratio of undesired hydrolysis at low water activity. This paper demonstrates, for the first time, that reduced water content can be a viable method of reducing the hydrolytic side reaction, provided the enzyme is properly prepared for use in micro-aqueous media. We show that deposition onto Accurel MP-1000 is a suitable preparation method, especially when dried using the propanol washing method described by Moore et al [21]. However, the reaction rates at low hydration still require significant enhancement to reach an enzymatic method competitive to the classical chemical routes used for synthesis of alkyl glycosides today. Feasible routes to get there include screening for *β*-glycosidases with desirable properties in micro-aqueous media or alternatively protein engineering of promising candidates such as *Tn*Bgl3B. Nevertheless, deposition on MP1000 provides one step towards unlocking the full synthetic potential of *β*-glycosidases.

Acknowledgements

This work was supported by the Swedish Research Council (VR) and the EU FP7 program AMYLOMICS.

References

[1] S. Matsumura, K. Imai, S. Yoshikawa, K. Kawada, T. Uchibor, J Am Oil Chem Soc, 67 (1990) 996-1001.
[2] B.A.L. Elias Meezan, B.A.L. Dennis J. Pillion, in, US 4868289 (Sep, 1989) Magnusson et al. 36/4..1; US 5182258 (Jan, 1993) Chiou 514/3; US 5369095 (Nov, 1994) Kee et al. 514/24, US, 1997.
[3] M. le Maire, P. Champeil, J.V. Moller, Biochim Biophys Acta, 1508 (2000) 86-111.
[4] W. von Rybinski, K. Hill, Angew Chem Int Ed, 37 (1998) 1328-1345.

[5] B.M. de Roode, M.C. Franssen, A. van der Padt, R.M. Boom, Biotechnol progress, 19 (2003) 1391-1402.
[6] F. van Rantwijk, M. Woudenberg-van Oosterom, R.A. Sheldon, J Mol Catal B: Enzym, 6 (1999) 511-532.
[7] T. Hansson, P. Adlercreutz, Biotechnol Bioeng, 75 (2001) 656-665.
[8] F.M. Kone, M. Le Bechec, J.P. Sine, M. Dion, C. Tellier, Prot Eng Des Sel, 22 (2009) 37-44.
[9] I. Mladenoska, C.E. Grey, E. Winkelhausen, S. Kuzmanova, P. Adlercreutz, Biocat Biotrans, 25 (2007) 382-385.
[10] P. Lundemo, P. Adlercreutz, E.N. Karlsson, Appl Environ Microb, 79 (2013) 3400-3405.
[11] T. Hansson, M. Andersson, E. Wehtje, P. Adlercreutz, Enzyme Microbial Technol, 29 (2001) 527-534.
[12] I. Mladenoska, E. Winkelhausen, S. Kuzmanova, Food Technol Biotech, 46 (2008) 311-316.
[13] A.M. Klibanov, Trends Biotechnol, 15 (1997) 97-101.
[14] G. Ljunger, P. Adlercreutz, B. Mattiasson, Enzyme Microb Tech, 16 (1994) 751-755.
[15] A.E. Petersson, P. Adlercreutz, B. Mattiasson, Biotechnol Bioeng, 97 (2007) 235-241.
[16] P. Turner, D. Svensson, P. Adlercreutz, E.N. Karlsson, J Biotechnol, 130 (2007) 67-74.
[17] M. Persson, I. Mladenoska, E. Wehtje, P. Adlercreutz, Enzyme Microb Tech, 31 (2002) 833-841.
[18] C. Turner, P. Turner, G. Jacobson, K. Almgren, M. Waldeback, P. Sjoberg, E.N. Karlsson, K.E. Markides, Green Chem, 8 (2006) 949-959.
[19] U.K. Laemmli, Nature, 227 (1970) 680-&.
[20] A. Blinkovsky, in, Novo Nordisk Biotech, Inc., USA . 1998, pp. 5 pp., Cont.-in-part of U.S. 5,693,516.
[21] B. Moore, J. Partridge, P. Halling, Very High Activity Biocatalysts for Low-Water Systems, in: E. Vulfson, P. Halling, H. Holland (Eds.) Enzymes in Nonaqueous Solvents, Humana Press, 2001, pp. 97-104.
[22] M.M. Bradford, Anal Biochem, 72 (1976) 248-254.
[23] A. Zaks, A.M. Klibanov, Proc Natl Acad Sci USA, 82 (1985) 3192-3196.
[24] D.G. Rees, P.J. Halling, Enzyme Microb Tech, 28 (2001) 282-292.

327

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 ± 1σ from duplicate reactions.

	*Tn*Bgl1A			*Tn*Bgl3B		
Support	Protein yield *%*	Load *mg/g*	Rel. activity *%*	Protein yield *%*	Load *mg/g*	Rel. activity *%*
Eupergit C250L[a]	93 ± 3	1.3 ± 0.0		98 ± 5	1.7 ± 0.2	
Eupergit C250L[b]	42 ± 3	0.6 ± 0.1	1.7	64 ± 11	1.1 ± 0.3	3.2
Accurel MP1000[a]	98 ± 1	1.8 ± 0.0		96 ± 1	1.7 ± 0.1	
Accurel MP1000[b]	94 ± 1	1.4 ± 0.5	12.4	59 ± 3	1.0 ± 0.1	48.4
Amberlite IRA-400[a]	29 ± 1	0.5 ± 0.0		22 ± 2	0.4 ± 0.1	
Amberlite IRA-400[b]	4 ± 3	0.1 ± 0.4	2.9	1 ± 4	0.0 ± 0.1	2.5

[a] Best results obtained in any of the tested conditions
[b] Immobilized under conditions for use in organic media , incubated 24h in 0.1M citrate phosphate buffer pH 5.6.

328
329

329

TABLE II: Specific initial formation of hexyl-β-D-glucopyranoside (μmol min^{-1} mg^{-1}) at each approximated water activity (a_w). Data is presented as average ± 1σ from duplicate reactions.

	*Tn*Bgl1A				*Tn*Bgl3B			
a_w	**0.7**	**0.8**	**0.9**	**1.0**	**0.7**	**0.8**	**0.9**	**1.0**
Untreated	0.24 ± 0.05	0.79 ± 0.04	4.75 ± 0.90	14.73 ± 0.21	0.49 ± 0.05	0.90 ± 0.17	8.49 ± 0.87	25.45 ± 1.76
Freeze-dried	0.05 ± 0.05	0.33 ± 0.19	4.53 ± 1.35	6.78 ± 2.35	0.02 ± 0.03	0.04 ± 0.02	0.91 ± 0.64	9.65 ± 0.72
AOT	0.10 ± 0.06	0.40 ± 0.00	0.42 ± 0.05	1.84 ± 0.21	0.15 ± 0.08	0.38 ± 0.00	1.14 ± 1.18	6.15 ± 0.00
Eupergit C250L	0.00 ± 0.01	0.02 ± 0.01	0.11 ± 0.03	0.25 ± 0.08	0.01 ± 0.01	0.03 ± 0.03	0.11 ± 0.20	0.61 ± 0.23
Accurel MP1000								
Vacuum dried	0.16 ± 0.02	0.38 ± 0.06	0.84 ± 0.09	1.34 ± 0.22	0.41 ± 0.03	0.92 ± 0.14	2.60 ± 0.46	13.95 ± 2.60
Propanol dried	0.83 ± 0.15	1.03 ± 0.15	2.03 ± 0.16	4.52 ± 0.30	1.65 ± 0.37	1.93 ± 0.13	3.69 ± 1.09	9.38 ± 0.13
Amberlite IRA-400	0.00 ± 0.00	0.21 ± 0.19	0.45 ± 0.23	1.08 ± 0.90	0.03 ± 0.11	0.05 ± 0.05	0.16 ± 0.00	0.98 ± 0.44

330
331

- Two β-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.

*Graphical Abstract (for review)

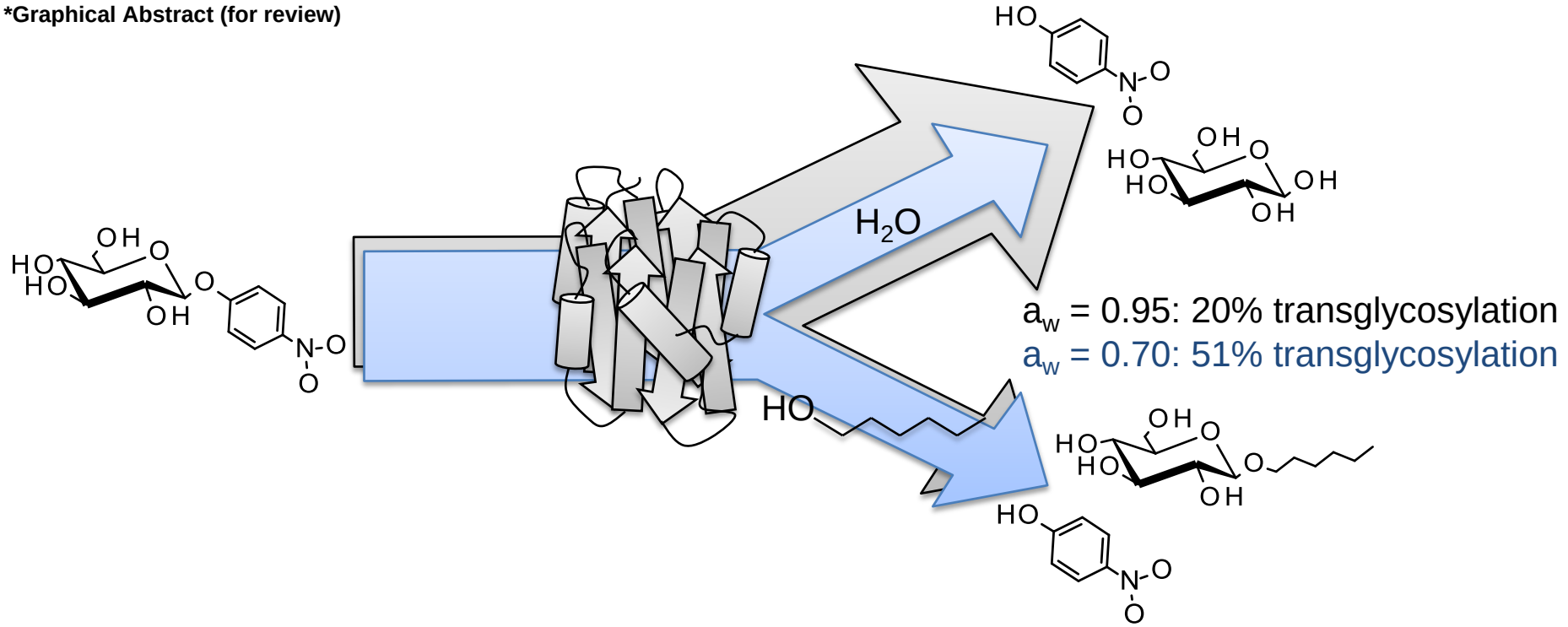

Figure 1, Schematic representation of the enzymatic conversion of *p*-nitrophenyl-*β*-D-glucoside to hexyl-*β*-glucoside at the rate r_s and to glucose at the rate r_h catalyzed by a retaining *β*-glucosidase.

Figure 2, Initial transglycosylation activity of *Tn*Bgl1A (left) and *Tn*Bgl3B (right) plotted versus experimentally determined water activities (a_w). The enzyme was freeze-dried (◊), deposited on Accurel MP1000; vacuum dried (■) or propanol dried (♦), covalently linked to Eupergit C250L (Δ) or added as aqueous solution (○). Error bars represent 1σ, based on triplicate measurements.

Figure 3, Initial transglycosylation activity of *Tn*Bgl1A (left) and *Tn*Bgl3B (right) normalized against untreated enzyme at each approximate water activity (a_w). The compared preparation methods are freeze-drying (◊), deposition on Accurel MP1000; vacuum dried (■) or propanol dried (♦), and covalent linking to Eupergit C250L (Δ).

Figure 4, Selectivity for transglycosylation (r_s/r_h) of *Tn*Bgl1A (left) and *Tn*Bgl3B (right) as a function of water activity (a_w). The enzyme was freeze-dried (◊), deposited on Accurel MP1000; vacuum dried (■) or propanol dried (♦), and covalently linked to Eupergit C250L (Δ). Error bars represent 1σ, based on triplicate measurements.

Figure 5, Initial transglycosylation activity (left) and selectivity for transglycosylation (r_s/r_h) for mutant N220F of *Tn*Bgl1A as a function of water activity (a_w). The enzyme was deposited on Accurel MP1000 and dried by propanol wash. Error bars represent 1σ, based on triplicate measurements.

Figure 1