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Optimised *N*-acetyl-D-lactosamine synthesis using *Thermus thermophilus* β-galactosidase in bio-solvents

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ABSTRACT

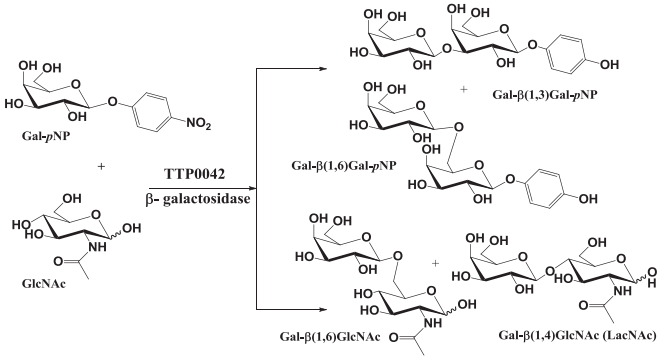
Synthesis of *N*-acetyl-D-lactosamine (Gal-β[1→4]GlcNAc, LacNAc) catalyzed by β-galactosidase from *Thermus thermophilus* (TTP0042) is affected by side reactions that give as result very low yields (about 20%) of LAcNAc when the reaction is performed in buffer. The process is improved (up to 91% of disaccharide yield) when the reaction takes place in the presence of solvents from biomass (bio-solvents) at 2.0 M concentration. Most of the solvents tested increased the LacNAc synthesis and reduced the undesired side reactions. In order to understand the possible effects of these solvents over the enzyme regioselectivity, we developed a conformational study of the enzyme structure in the presence of a selected bio-solvent by circular dichroism and fluorescence. According to this study, we were able to conclude that the presence of bio-solvents in the reaction media modifies the enzyme secondary and tertiary structure and this may be the cause of the regioselectivity changes observed in the transglycosylation reaction.

1. Introduction

Carbohydrates are main molecules related in cell to cell recognition processes, they appear as glycoproteins, glycolipids or proteoglycans anchored in the cell membrane.[1] Due to its strategical situation, oligosaccharides are involved in many biological processes, such as: embryogenesis, neuronal proliferation and apoptosis.[2–4] As the understanding of these biological functions increases, the need for practical synthetic procedures of oligosaccharides in large quantities has become a major issue.

Synthesis of disaccharides and glycoconjugates can be performed by organic chemical methods,[5–7] but these procedures are characterized by several protection and deprotection steps. For that reason the synthesis of these carbohydrates is often achieved by enzymatic methods, using: glycosyltransferases, glycosidases and glycosynthases as biocatalysts.[8–11]

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) catalyses the hydrolysis of the β-galactoside linkages of disaccharides into their monomers but also the transgalactosylation reaction to produce galactooligosaccharides.[12–14] *Thermus thermophilus* β-galactosidase (TTP0042),[15,16] is a thermophilic enzyme, which can synthesize *N*-acetyl-D-lactosamine (LacNAc, Galβ[1→4]GlcNAc) via transglycosylation using *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal) as donor and *N*-acetyl-D-glucosamine (GlcNAc) as acceptor (Scheme 1),[17] but a side reaction is also involved during this synthesis, producing some amounts of self-condensation of the donor (Galβ[1→3]Gal-β-*p*NP and Galβ[1→6]Gal-β-*p*NP).[18]



Scheme 1. Synthesis of *N*-acetyl-D-lactosamine (LacNAc) catalyzed by β-galactosidase TTP0042 from *Thermus thermophilus*.

There has been reported, that many enzymes can maintain their activity in a variety of organic solvents.[19] Recently some β-galactosidases have been tested in presence of environmental friendly solvents made from glycerol,[20–22] solvents from biomass (bio-

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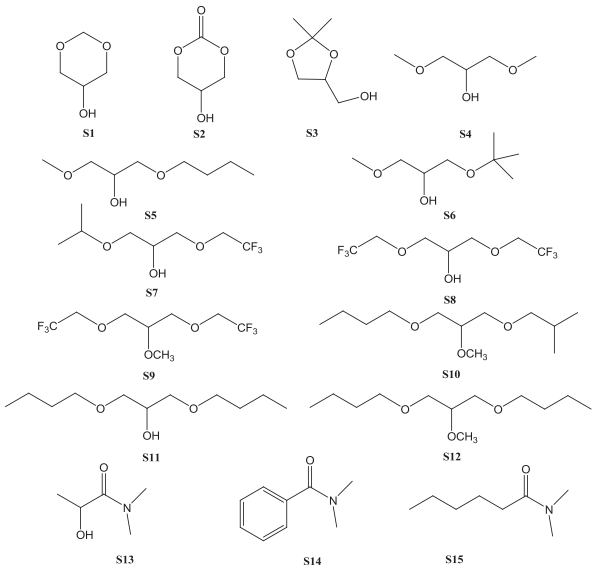
solvents)[21–24] and ionic liquids (ILs).[21,22,25] As a result of these studies, it was found that the use of these solvents decreases unfavourable side reactions and shifts the equilibrium towards the product side, therefore minimising hydrolysis.

In the present work, we have evaluated the effect of different bio-solvents (solvents made from glycerol and from biomass) in the reaction media for transglycosylation using TTP0042 enzyme as a catalyst. To understand the effect of these liquids over the synthetic behaviour of the enzyme, we performed a conformational study by circular dichroism and fluorescence techniques between TTP0042 and selected bio-solvents.

2. Results and discussion

2.1. Effect of bio-solvents over LacNAc synthesis

The effect of three different groups of solvents (Scheme 2) was screened in order to evaluate their effect over TTP0042 β-galactosidase during transglycosylation reaction; 1-glycerol based solvents (cyclic structures, **S1**, **S2** and **S3**); 2-glycerol based solvents (open chain, [**S4**–**S12**]); 3-*N*,*N*-dymethyl amide based solvents (**S13**–**S15**). Main features of these solvents (density, Log P and solubility 2 M) and reaction yields are summarized in Table 1.



Scheme 2. Molecular structure of solvents used in this study.

As can be seen in Table 1, most of the solvents shifted the reaction equilibrium towards synthesis of LacNAc with good yields (>65%) respect the reaction performed in buffer (17%). This result shows an important effect of the solvent in the reaction media and also confirms the sensibility of this enzyme to different solvents. Unfortunately, not all the solvents allow the reactions, and the enzyme seems to be inactive from the beginning of the reaction, these solvents: **S3**, **S7** and **S11** behave like inhibitors or denaturants of the enzyme. Solvents: **S3**, **S5** and **S13** did not complete the reaction and conversion yields were about 55–50% (data not shown). All other solvents present high conversion yields (about 95–100%, data not shown). There is not a clear relationship between solvents structures and the regioselectivity change found in the reactions performed. The three groups of solvents tested display good results with most of their solvents. So, there is a sensibility of the enzyme for the chemical environment, but also, the enzyme seems to be active at very different values of Log*P* and also polarities. As important results, the highest values of LacNAc yields were obtained under biphasic conditions and moderate-low polarities of solvents (**S9**, **S10** and **S11**, Table 1).

2.2. Effect of concentration of solvents in the synthesis of LAcNAc

In order to check the influence of the co-solvent proportion on the enzymatic activity, different concentrations of **S2**, **S4**, **S9**, **S12**, **S13** and **S15** (0.25, 0.5, 1.0, 1.5 and 2 M) were also tested, and the results obtained in these experiments are shown in Fig. 1. Taking into account the solvent effects in the disaccharide synthesis, we selected the following solvents: **S2**, **S4**, **S9**, **S12**, **S13** and **S15**, in order to evaluate their effect in the transglycosylation reactions at different concentrations of each solvent. These solvents are representative of the three kinds of solvents screened and also display different reactions systems, biphasic and monophasic.

Most of the solvents tested, presented their higher effect about 2 M of solvent, there was no difference related to the nature of the system (biphasic and homogeneous systems). The addition of bio-solvent to the mixture of reaction causes a change in the enzyme traditional regioselectivity. Some solvents (**S4**, **S9**, **S12** and **S13**) presented good disaccharide yields when 1.50 M concentrations were used. This effect was previously found for this enzyme by using ionic liquids as co-solvents and was explained as a conformational change in the secondary and tertiary structure of the enzyme due to the presence of co-solvents.[25] In order, to confirm this hypothesis for bio-solvents, we decided to analyse conformational changes in the α-helix from the secondary structure of the

Table 1
Transglycosylation yields (%) catalyzed by TTP0042 in presence of 2 M of solvents and 50 mM of sodium phosphate buffer pH 6.0

Medium	Solvent density (g mL^{-1})	Log *P*	System composition	% Self condensated Galβ(1→3)Gal*p*NP	% Self condensated β(1→6) Galβ(1→6)Gal*p*NP	% Hydrolysis (galactose)	% LacNAc	% Galβ(1→6)GlcNAc
Buffer	1.00	0.00	Monophasic	73	10	0	17	0
S1	1.24	−0.057	Monophasic	33	5	7	55	0
S2	1.41	−0.024	Monophasic	0	6	0	77	17
S3	1.06	0.030	Monophasic	0	0	0	0	0
S4	1.07	−0.60	Monophasic	5	1	4	75	15
S5	0.94	0.14	Monophasic	22	0	0	78	0
S6	0.91	0.27	Monophasic	0	0	0	0	0
S7	1.12	1.14	Biphasic	0	0	0	0	0
S8	1.36	1.42	Biphasic	0	0	3	91	6
S9	1.27	1.71	Biphasic	1	3	1	91	4
S10	0.90	1.93	Biphasic	0	0	4	74	22
S11	0.89	2.07	Biphasic	0	0	0	0	0
S12	0.89	2.48	Biphasic	1	2	0	94	3
S13	1.06	−0.69	Monophasic	7	2	3	80	8
S14	1.15	1.41	Monophasic	35	0	0	65	0
S15	0.91	1.42	Biphasic	0	0	4	68	28

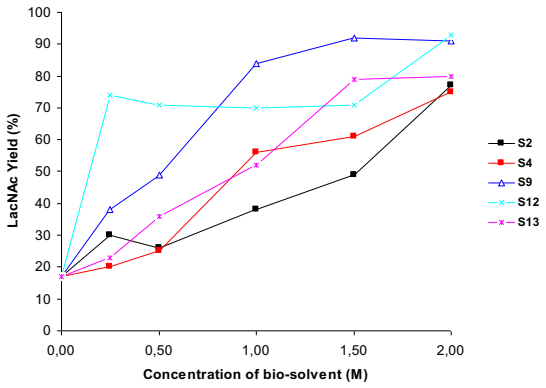


Fig. 1. Synthesis of LacNAc at different solvent concentrations.

protein by circular dichroism[26–28] and in the tertiary structure by fluorescence emission due to tryptophan excitation.[29,30]

2.3. Conformational effects of solvents over enzyme secondary structure

In order to measure the effect of water soluble solvents in the secondary structure of the enzyme, solvents: **S2**, **S4**, **S13** and **S14** were used. These solvents were selected because of their important effect in the enzymatic synthesis of LacNAc and also for their solubility in water at the desired concentration (2.0 M). For this, we analysed their CD spectra at 2.0 M concentrations and their effect in the recording (noise) for the spectra.

According to this result, only **S4** was found able to use it in the CD study for solvent effect on the enzyme secondary structure, because it presented a null observed ellipticity at 2 M concentration, while **S2**, **S13** and **S14** exhibit important changes in the CD spectra and also incremented the noise of the recorded (data not shown). According to this result, we measured the effect of different concentrations of **S4** over the enzyme secondary structure at 60 °C. The final concentrations were: 0.00, 0.25, 0.50, 1.00, 1.50 and 2.00 M. These conditions were selected in order to use the same concentrations and temperature previously developed during the transglycosylation study. As a result of this experiment, we find a modification of the enzyme secondary structure due to the additions of **S4** (Fig. 2).

At the beginning of the procedure (buffer solution), the enzyme displays a typical α-helix spectrum.[31] The CD spectra for the enzyme in buffer solution exhibit a profile with a minimum at 209 nm with a −6700 deg cm^2 dmol^{-1} ellipticity. In contrast, the CD spectra of the enzyme in the presence of solvent **S4** at 2 M shows a significant change in ellipticity (−3600 deg cm^2 dmol^{-1}) at 209 nm.

This result shows an effect of the solvent over the enzyme structure. For this experiment, the enzyme displayed two maximum values of negative absorption at: 217 nm and 209 nm. These CD bands were used as indicators of changes in the secondary structure of the protein. As shown in Fig. 3, the protein decreased ellipticity when the concentration of **S4** is increased. These measurements confirm the solvent effect in the enzyme secondary structure.

This result suggests that the enzyme modifies its native structure during the transglycosylation reaction due to the addition of bio-solvents. This structural modification of the secondary structure of the enzyme could be responsible of the regioselectivity change found in the disaccharide synthesis reactions previously performed.

The interaction between solvent and enzyme could be similar to the found previously when TTP0042 have proven to be sensitive to the addition of ionic liquids, these solvents increase the enzyme regioselectivity and improve the disaccharide yields when they have been employed. This phenomenon also has been explained as modification of the enzyme secondary and tertiary structure by the addition of those solvents that make the enzyme more flexible during synthesis conditions.[32]

2.4. Conformational effects of solvents over enzyme tertiary structure

The effect of the **S4** solvent over the enzyme fluorescence spectrum was investigated. For this experiment, we recorded the emission spectra for the enzyme in buffer solution at 60 °C and then, several additions of **S4** were carried out and the respective fluorescence spectra of them were done.

As a result of this experiment, we noticed that the enzyme was shifting the emission spectra to the blue, from 334 nm as maximum emission wavelength in buffer solution up to 329 nm in 2 M solution of **S4** (Fig. 4). This result reveals that the tertiary structure of the enzyme was modified affecting the tryptophan exposition towards more hydrophobic environment and producing important changes over its native emission spectra in fluorescence. The displacement of the maximum emission wavelength in the fluorescence of this enzyme by using **S4** as co-solvent was confirmed over 1.00 M concentrations (Fig. 5), at lower concentrations no difference was seen. It supposes an important modification of the tertiary structure of the enzyme under our reaction conditions (2 M of S4), which could be related to the change in the enzyme

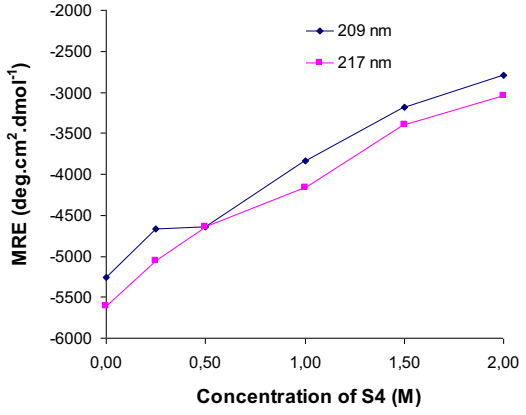


Fig. 2. CD spectra for TTP0042 at different concentrations of **S4**.

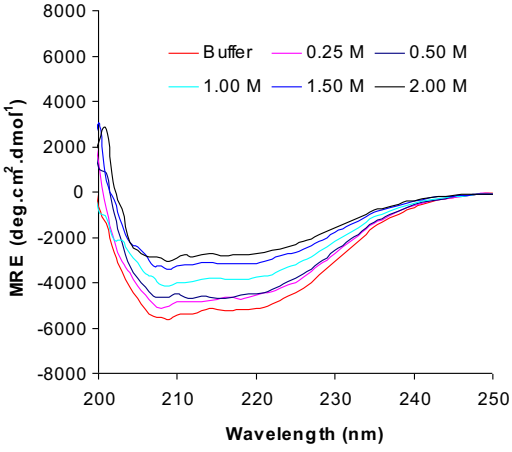


Fig. 3. Effect of **S4** concentration over negative ellipticity bands at 209 nm and 217 nm.

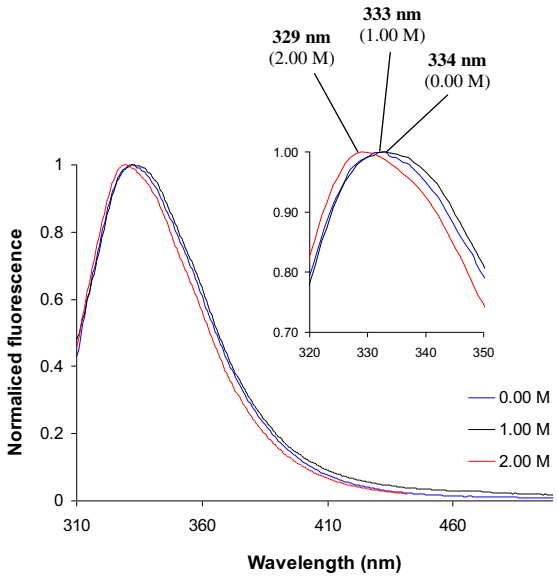


Fig. 4. Fluorescence emission spectra at 65 °C for TTP0042 at different concentrations of **S4**.

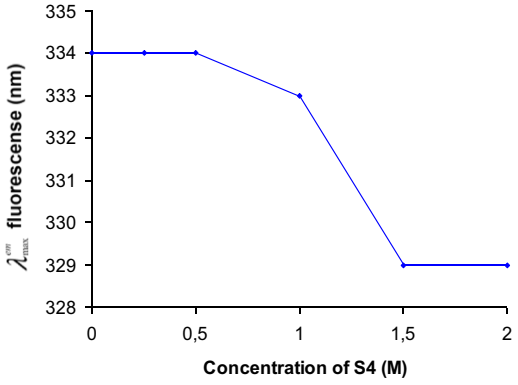


Fig. 5. Maximum emission fluorescence wavelength of TTP0042 at different concentrations of **S4**.

regioselectivity. According to the experimental results, we consider that the enzyme modifies its secondary and tertiary structure during the transglycosylation reactions, due to the conditions involved in these processes. This, might cause a different synthesis of products respect the same reaction when the enzyme reacts in water medium.

3. Conclusions

In this study we have proven that the presence of green solvents from biomass modify the traditional regioselectivity of TTP0042 β-galactosidase towards the synthesis of *N*-acetyl-D-lactosamine. There were several solvents with important yields in the disaccharide synthesis and there was no relation in the nature of the system and the modification of the enzyme regioselectivity, because both: soluble and insoluble solvents were able to modify the enzyme synthetic activity towards the synthesis of LacNAc. The concentration of the solvent is an important factor in order to improve the synthesis of the disaccharide, because at lower concentrations of bio-solvents the synthesis of LacNAc was reduced, the optimal concentration of bio-solvent for these reactions seems to be 2.0 M. The addition of **S4** to the enzyme solution (in buffer) affects the α-helix of the secondary structure of the protein and also affects the tryptophan exposition of the tertiary structure of the enzyme. These conformational changes over secondary and tertiary structure of the enzyme may be the responsible for the modification of the enzyme regioselectivity found in the transglycosylation reactions.

The isolation of the target molecule LacNAc from the reaction mixture proved to be feasible using a simple purification procedure. These results have opened up promising possibilities to follow in trying to make the enzymatic synthesis of regioselective LacNAc industrially feasible. However, further research is clearly required to explore the process implications of using bio-solvents.

4. Experimental section

4.1. General

p-Nitrophenol (*p*NP), *p*NP-β-Gal and GlcNAc were purchased from Sigma Aldrich. Solvents from glycerol were kindly donated by Prof. José I. García, solvents from Bio-mass were a gift from Cognis IP Management GmbH, now part of BASF. All other chemicals were from analytical grade. UV–visible spectra were recorded on a UV-2401 PC Shimadzu. HPLC Jasco using NH2P50-4E amino column (Asahipak, Japan) with acetonitrile/water (80:20) as a mobile phase at a flow of 0.8 mL/min. Chromatograms were recorded using an UV–vis, circular dichroism (CD) and evaporative light scattering (LS) detectors. NMR spectra were performed on Bruker Avance 700 MHz spectrometer.

4.2. Enzyme activity and protein purification

Recombinant TTP0042 enzyme was obtained as describe before.[17,32] Protein concentration was determined by Bradford method,[33] using BSA as standard. Hydrolytic activity was determined by quantification of *p*NP liberated from 5 mM solution of *p*NP-β-Gal in phosphate buffer 50 mM pH 7.0 at 80 °C and 410 nm using continuous method.[17,34] One enzyme unit was defined as the amount of protein that releases 1 μmol of *p*NP-β-Gal per minute under the conditions previously described.

4.3. Disaccharide synthesis

Transglycosylation reactions were carried out in 1.0 mL of solution (2 M solvent)-sodium phosphate buffer (50 mM, pH 6.0), with 51.2 mg (0.17 M) *p*NP-β-Gal (donor) and 188 g (0.85 M) of GlcNAc (acceptor) mixture was preequilibrated to 60 °C. Afterwards, 36 μmol min^{-1} (U) of *T. thermophilus* β-galactosidase were added to the reaction mixture and reaction was incubated during 3 h, then, was stopped (and homogenized in the case of biphasic systems) by addition of methanol. Reaction yields were determined by HPLC-ELSD. The crude mixture was then directly loaded onto a carbon–Celite column eluted with a linear gradient of 0–15% (v/v) of ethanol in water. Solvents were eliminated and disaccharide (LacNAc) was dissolved in D_2O to be characterized by ^1H and ^{13}C NMR spectroscopy. NMR spectra for LacNAc were consistent to previous reports.[17,20,35]

4.4. Effect of ILs concentration on enzyme activity

According to results obtained in transglycosylation, some solvents were selected in order to study their effect in the synthesis of disaccharide, these solvents are: **S2**, **S4**, **S9**, **S12**, **S13** and **S15** (see Scheme 2). Thus, transglycosylations reactions were done as described above, using different concentrations of selected solvents. [0.00, 0.25, 0.50, 1.00, 1.50 and 2.00 M].

4.5. Solvents effects in secondary structure by circular dichroism measurements

In order to understand the effect of solvents in the secondary structure of the protein, we developed a circular dichroism (CD) study, by imitating of the reaction parameters, under these conditions, we try to find a rational explanation for the previous results in transglycosylation.

4.6. General procedures

A Jasco-710 dichrograph, previously calibrated with D-10-camphorsulphonic acid was used to record the far-UV CD spectra of TTP0042. Measurements were performed in 0.1 cm cells at 60 °C (temperature was selected in order to simulate reaction conditions). Five accumulations were acquired for each spectrum. The ellipticity was measured with a 1 nm bandwidth and a 2 s response. For this study, recombinant TTP0042 was used, this is a protein with a molecular weight of 50664.2 Da (higher than native enzyme due to polypeptidic histidine tag attached to the enzyme) and 450 residues. The mean residue ellipticity (MRE) was expressed in deg cm^2 $dmol^{-1}$ and calculated using the following equation:

$$\mathrm{MRE} = \frac{\mathrm{MRW}\cdot\theta_{\mathrm{obs}}}{10\cdot l\cdot c}$$

MRW=molecular weight per residue (112,587)
l=cuvette pathlength
θ_{obs}=observed ellipticity in degrees
l=pathlength in cm
c=concentration in g ml^{-1}

4.7. CD spectra for selected solvents

The following solvents: **S2**, **S4**, **S13** and **S14** were chosen to evaluate their CD spectra and their effect in the recording (noise) for the spectra. These solvents were selected because of their important effect (at 2.0 M) in the enzymatic synthesis of LacNAc.

4.8. Solvents effects over enzyme CD spectra

From the previous procedure, **S4** was chosen as the best solvent to perform a CD study. First a CD spectra for the enzyme solution (1.0 g mL^{-1}), in sodium phosphate buffer 10 mM at pH 6.00, was recorded. Then, several aliquots of **S4** were added to the solution and the mixture was homogenized. Then the CD spectra were recorded. The final concentrations of **S4** used in this study were: 0.00, 0.25, 0.50, 1.00, 1.50 and 2.00 M.

4.9. Solvents effects in tertiary structure by fluorescence study

Fluorescence emission spectra were obtained on a PTI modular spectrometer. 295 nm was set as excitation wavelength because it is highly selective for tryptophan. Emission was acquired from 310 to 500 nm. The excitation and emission bandwidth were adjusted at 2 nm, measurements were done in 0.20 cm pathlength cell at 25 °C with thermoelectric temperature regulator TLC 50. Spectra analysis was done using Felix 32 software. First, the fluorescence spectrum of **S4** was recorded using a 2 M solution of this solvent, then the fluorescence spectrum was done for TTP0042 (1.0 mg mL^{-1}) in sodium phosphate buffer 10 mM at pH 6.00. The emission spectrum of **S4** was negligible with respect to the spectrum of the enzyme in buffer solution. Then, several additions of **S4** were performed in the cuvette in order to evaluate the effect of this solvent in the tryptophan exposition of the enzyme.

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References and notes

1. Hakomori, S. *Glycoconjugate J.* **2004**, *21*, 125–137.
2. Yagi, H.; Yanagisawa, M.; Suzuki, Y.; Nakatani, Y.; Ariga, T.; Kato, K.; Yu, R. K. *J. Biol. Chem.* **2010**, *285*, 37293–37301.
3. Niederer, K. E.; Morrow, D. K.; Gettings, J. L.; Irick, M.; Krawiecki, A.; Brewster, J. L. *Cell. Signalling* **2005**, *17*, 177–186.
4. Yanagisawa, M.; Yu, R. K. *Biochem. Biophys. Res. Commun.* **2009**, *386*, 101–104.
5. Loka, R. S.; Sadek, C. M.; Romaniuk, N. A.; Cairo, C. W. *Bioconjugate Chem.* **2010**, *21*, 1842–1849.
6. Mandal, P. K.; Turnbull, W. B. *Carbohydr. Res.* **2010**, *346*, 2113–2120.
7. Ueki, A.; Takano, Y.; Kobayashi, A.; Nakahara, Y.; Hojo, H. *Tetrahedron* **2010**, *66*, 1742–1759.
8. Bojarová, P.; Krenek, K.; Wetjen, K.; Adamiak, K.; Pelantová, H.; Bezouška, K.; Elling, L.; Kren, V. *Glycobiology* **2009**, *19*, 509–517.
9. Giordano, A.; Andreotti, G.; Tramice, A.; Trincone, A. *Biotechnol. J.* **2006**, *1*, 511–530.
10. Sasaki, A.; Ishimizu, T.; Geyer, R.; Hase, S. *FEBS J.* **2005**, *272*, 1660–1668.
11. Murata, T.; Usui, T. *Biotechnol. Bioprocess Eng.* **2002**, *7*, 263–267.
12. Gosling, A.; Alftrén, J.; Stevens, G. W.; Barber, A. R.; Kentish, S. E.; Gras, S. L. *J. Agric. Food Chem.* **2009**, *57*, 11570–11574.
13. Li, W.; Xiang, X.; Tang, S.; Hu, B.; Tian, L.; Sun, Y.; Ye, H.; Zeng, X. *J. Agric. Food Chem.* **2009**, *57*, 3927–3933.
14. Miyasato, M.; Ajisaka, K. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2086–2090.
15. Dion, M.; Fourage, L.; Hallet, J.-N.; Colas, B. *Glycoconjugate J.* **1999**, *16*, 27–37.
16. Henne, A.; Bruggemann, H.; Raasch, C.; Wiezer, A.; Hartsch, T.; Liesegang, H.; Johann, A.; Lienard, T.; Gohl, O.; Martinez-Arias, R.; Jacobi, C.; Starkuviene, V.; Schlenczeck, S.; Dencker, S.; Huber, R.; Klenk, H.-P.; Kramer, W.; Merkl, R.; Gottschalk, G.; Fritz, H.-J. *Nat. Biotechnol.* **2004**, *22*, 547–553.
17. Sandoval, M.; Ferreras, E.; Pérez-Sánchez, M.; Berenguer, J.; Sinisterra, J. V.; Hernaiz, M. J. *J. Mol. Catal. B: Enzym.* **2012**, *74*, 162–169.
18. Chiffoleau-Giraud, V.; Spangenberg, P.; Dion, M.; Rabiller, C. *Eur. J. Org. Chem.* **1999**, 757–763.
19. Jongejan, J. A. *Organic Synthesis with Enzymes in Non-aqueous Media*; Wiley-VCH GmbH KGaA: 2008, pp 25–46.
20. Pérez-Sánchez, M.; Sandoval, M.; Cortés-Cabrera, A.; García-Marín, H.; Sinisterra, J. V.; García, J. I.; Hernaiz, M. J. *Green Chem.* **2011**, *13*, 2810–2817.
21. Pérez-Sánchez, M.; Sinisterra, J. V.; Henáiz, M. J. *Curr. Org. Chem.* **2010**, *14*, 2366–2383.
22. Hernaiz, M. J.; Alcantara, A. R.; Garcia, J. I.; Sinisterra, J. V. *Chem.—Eur. J.* **2010**, *16*, 9422–9437.
23. Pérez-Sánchez, M.; Cortés-Cabrera, Á.; García-Martín, H.; Sinisterra, J. V.; García, J. I.; Hernaíz, M. J. *Tetrahedron* **2011**, *67*, 7708–7712.
24. Pérez-Sánchez, M.; Sandoval, M.; Hernáiz, M. J. *Tetrahedron* **2012**, *68*, 2141–2145.
25. Sandoval, M.; Cortes, A.; Civera, C.; Trevino, J.; Ferreras, E.; Vaultier, M.; Berenguer, J.; Lozano, P.; Hernaiz, M. J. *RSC Adv.* **2012**, *2*, 6306–6314.
26. Greenfield, N. J. *Nat. Protoc.* **2007**, *1*, 2876–2890.
27. Tello-Solis, S. R.; Jimenez-Guzman, J.; Sarabia-Leos, C.; Gomez-Ruiz, L.; Cruz-Guerrero, A. E.; Rodriguez-Serrano, G. M.; Garcia-Garibay, M. *J. Agric. Food Chem.* **2005**, *53*, 10200–10204.
28. Whitmore, L.; Wallace, B. A. *Biopolymers* **2008**, *89*, 392–400.
29. Michalet, X.; Weiss, S.; Jäger, M. *Chem. Rev.* **2006**, *106*, 1785–1813.
30. Vivian, J. T.; Callis, P. R. *Biophys. J.* **2001**, *80*, 2093–2109.
31. Kelly, S. M.; Jess, T. J.; Price, N. C. *Biochim. Biophys. Acta* **2005**, *1751*, 119–139.
32. Sandoval, M.; Civera, C.; Treviño, J.; Ferreras, E.; Cortés, A.; Vaultier, M.; Berenguer, J.; Lozano, P.; Hernaiz, M. J. *RSC Adv.* **2012**, *2*, 6306–6314.
33. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
34. Fourage, L.; Helbert, M.; Nicolet, P.; Colas, B. *Anal. Biochem.* **1999**, *270*, 184–185.
35. Bridiau, N.; Maugard, T. *Biotechnol. Prog.* **2011**, *27*, 386–394.