Biochemistry and Biophysics Reports 7 (2016) 52–55

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

A mutant β-glucosidase increases the rate of the cellulose enzymatic hydrolysis

Fábio K. Tamaki [1,2], Éverton M. Araujo [1], Roberto Rozenberg [1], Sandro R. Marana *

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, São Paulo, SP 05513–970, Brazil

ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form
25 April 2016
Accepted 17 May 2016
Available online 18 May 2016

Keywords:
Cellulose Hydrolysis
β-Glucosidase
Spodoptera frugiperda

ABSTRACT

The enzymatic hydrolysis of cellulose and lignocellulosic materials is marked by a rate decrease along the reaction time. Cellobiohydrolase slow dissociation from the substrate and its inhibition by the cellobiose produced are relevant factors associated to the rate decrease. In that sense, addition of β-glucosidases to the enzyme cocktails employed in cellulose enzymatic hydrolysis not only produces glucose as final product but also reduces the cellobiohydrolase inhibition by cellobiose. The digestive β-glucosidase GH1 from the fall armyworm *Spodoptera frugiperda*, hereafter called Sfβgly, containing the mutation L428V showed an increased k_{cat} for cellobiose hydrolysis. In comparison to assays conducted with the wild-type Sfβgly and cellobiohydrolase TrCel7A, the presence of the mutant L428V increased in 5 fold the initial rate of crystalline cellulose hydrolysis and reduced to one quarter the time needed to TrCel7A produce the maximum glucose yield. As our results show that mutant L428V complement the action of TrCel7A, the introduction of the equivalent replacement in β-glucosidases is a promising strategy to reduce costs in the enzymatic hydrolysis of lignocellulosic materials.

1. Introduction

The enzymatic hydrolysis of cellulose and lignocellulosic materials is marked by a rate decrease along the reaction time. This is observed for reactions conducted with isolated cellobiohydrolases, such as TrCel7A, and with combinations of endoglucanases and cellobiohydrolases. Experiments and kinetic models have suggested that the slow dissociation of TrCel7A from crystalline cellulose and its inhibition by the produced cellobiose are relevant factors to the rate decrease [1–3].

The processive action of TrCel7A starts when an isolated cellulose chain is detached from the crystalline surface and introduced into its tunnel-shaped active site, which contains 10 subsites for glucosyl units binding. The cellobiose formed after glycosidic bond hydrolysis is released from the active site, freeing subsites +1 and +2. Their subsequent filling propels TrCel7A along the cellulose chain [4]. Nevertheless, the energetically favorable interactions between substrate and subsites +1 and +2 also favor the cellobiose interaction. Thus, the increase of product concentration allows it to diffuse back into +1 and +2 subsites, blocking cellulose sliding and inhibiting TrCel7A processive activity. Indeed cellobiose inhibits TrCel7A with K_i ranging from 20 μM to 1.6 mM, and the energy involved in cellobiose binding corresponds to about 12 kcal/mol [4–6].

Therefore, the addition of β-glucosidases to enzyme cocktails designed to cellulose hydrolysis is not only planned to produce glucose as final product, but also aims at reducing the cellobiose inhibition upon TrCel7A. Due to this relevant role, efforts have been conducted to develop mutant β-glucosidases exhibiting suitable properties for the cellulose hydrolysis process, as improved thermal stability [7–9], increased catalytic activity [10–12] and reduced inhibition by glucose [13]. In this sense, in this manuscript we characterized the participation of a new mutant GH1 β-glucosidase on the enzymatic hydrolysis of crystalline cellulose, which increases the rate of glucose production and decreases the TrCel7A inhibition by cellobiose.

2. Materials and methods

2.1. Random mutagenesis and library screening

A sample of the expression vector pCAL coding for the wild-type Sfβgly was submitted to random mutagenesis using the GeneMorph II kit (Agilent Technologies; Santa Clara, US) following

Abbreviations: Sfβgly, β-glucosidase GH1 from the fall armyworm *Spodoptera frugiperda*; TrCel7A, cellobiohydrolase I from *T. reesei*; NPβfuc, p-nitrophenyl β-fucoside; NPβgal, *p*-nitrophenyl β-galactoside; NPβglc, *p*-nitrophenyl β-glucoside

* Corresponding author.
E-mail address: srmarana@iq.usp.br (S.R. Marana).
[1] These authors contributed equally to this manuscript.
[2] Current address: The Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK.

http://dx.doi.org/10.1016/j.bbrep.2016.05.014

the manufacturer's instructions. The random Sfβgly mutants library was used to transform *E. coli* NovaBlue(DE3) (Novagen, Darmstadt, Germany). Isolated colonies (960) were screened following the procedures previously described [14]. Sfβgly mutants were selected based on the activity upon 4 mM p-nitrophenyl β-glucoside (NPβglc) and 14 mM cellobiose. These substrates were prepared in 100 mM sodium citrate - sodium phosphate buffer pH 6.0.

2.2. Production and purification of the mutant and wild-type Sfβgly

Expression vectors pET46 coding for the wild-type and mutant Sfβgly were separately introduced in *E. coli* BL21(DE3) (Merck Millipore; Darmstad, Germany) by heat shock following manufacturer's procedures. Isolated colonies were used recombinantly express mutant and wild-type Sfβgly following the procedures previously described [15]. Recombinant proteins were also purified based on previously described methods [15]. The purity of the Sfβgly samples were checked by using SDS-PAGE [16]. Protein concentration was determined by absorbance at 280 nm in 6 M guanidinium hydrochloride prepared in 20 mM sodium phosphate buffer pH 6.0. Theoretical extinction coefficients ($\varepsilon_{280\ nm}$) were calculated based on the proteins primary sequences using the ProtParam server.

2.3. Determination of β-glucosidase kinetic parameters

The k_{cat} and K_m of the purified wild-type and mutant Sfβgly were determined by measuring the hydrolysis initial rates (v_0) of at least 10 different concentrations of cellobiose and p-nitrophenyl-β-d-glycopyranosides (Sigma, St. Louis, MO, USA) prepared in 100 mM sodium citrate – sodium phosphate pH 6.0. Experiments were performed at 30 °C in a final volume of 100 μL. Concentration of the wild-type Sfβgly was 0.18 μM, whereas L428V mutant was 0.46 μM. Glucose production from cellobiose was detected using the Glicose PP kit (Gold Analisa Diagnóstica, Belo Horizonte, Brazil). Since each Sfβgly catalytic cycle hydrolyzes cellobiose into two glucoses, this assay determination overestimate in 2-fold the Sfβgly activity (measured by the product production). Thus, the cellobiose hydrolysis rate was calculated from the glucose production rate divided by 2. Release of *p*-nitrophenolate was detected following the absorbance at 420 nm after addition of 250 mM sodium carbonate – sodium bicarbonate pH 11.0. The v_0 and [S] data were fitted in the Michaelis-Menten equation using the Enzfitter software (Elsevier-Biosoft, Cambridge, UK).

2.4. Determination of TrCel7A activity

Cellobiohydrolase TrCel7A (0.1 mg; Megazyme) was used to hydrolyze crystalline cellulose Avicel PH101 5 mg/mL suspension in sodium acetate buffer 50 mM pH 5.2. Cellulose hydrolysis assays were performed in 1.55 mL under constant shaking (200 rpm) in an orbital shaker at 37 °C. The enzymatic reaction was interrupted by boiling for 5 min Then reaction mixtures were centrifuged (10,000 rpm for 5 min at 4 °C) to sediment the remaining substrate. The supernatant was employed for glucose detection using the Glicose PP kit and also to determination of the total reducing carbohydrate using the dinitrosalicylic acid method [17]. TrCel7A activity was also detected in the presence of wild-type or mutant Sfβgly (0.11 nmol) following the same procedures. For calculation of the TrCel7A activity (cellobiose rate production), the amount of glucose detected in the supernatant (G) was initially converted in the corresponding absorbance in the dinitrosalicylic acid method, which was subtracted from the absorbance detected in the total reducing carbohydrate measurement. This allows the correct estimation of reducing ends only from cellobiose, but not from glucose. From the remaining value, if any, the amount of remaining cellobiose in the supernatant was calculated (RC) using a standard curve previously prepared. Then, the total cellobiose (TC) produced by TrCel7A was calculated by adding the remaining cellobiose (RC), if any, and the amount of glucose (G) divided by two (see above).

3. Results and discussion

A mutant of the GH1 β-glycosidase from the armyworm *Spodoptera frugiperda* (Sfβgly; AF052729) containing the single mutation L428V was selected from a library of random mutants. This mutant was picked based on its higher activity upon both cellobiose and NPβglc relative to the wild-type Sfβgly in library screening conditions. Mutant Sfβgly L428V, hereafter called L428V, was further produced as recombinant protein in BL21(DE3) bacteria and purified (Fig. S1).

Enzyme kinetic parameters were determined for L428V, whose turnover constants (k_{cat}) are 1.8–3 times higher than wild-type Sfβgly activity upon cellobiose and synthetic substrates, respectively (Fig. 1; Table 1). In addition, K_m tends to decrease to half for NPβglc and *p*-nitrophenylβ-fucoside (NPβfuc), whereas it was doubled for cellobiose. Hence, the specificity, represented by the ratio k_{cat}/K_m, was clearly increased for synthetic substrates, which accords to the increased activity observed in the library screening. Mutation L428V is placed far from the active site (around 11 Å; Supplementary material Fig. S2) in an α-helix near the protein surface, so the mechanism by which this mutation affects the enzyme activity remains to be elucidated. Nevertheless it has been shown that mutational effects traveling through interaction pathways reach the active site of β-glucosidases [14,15,18].

Regardless the mechanism involved in the mutational effect on the kinetic parameters, the increased k_{cat} for cellobiose (Table 1) indicates that under substrate saturation L428V hydrolyzes cellobiose twice faster than the wild-type Sfβgly. Hence, because of this enhanced cellobiasic activity, L428V is an interesting candidate to complement and reduce the inhibiton of TrCel7A activity by cellobiose during the enzymatic hydrolysis of cellulose.

In order to test this hypothesis, activity assays of isolated TrCel7A upon crystalline cellulose (Avicel PH101) were performed in presence of wild-type Sfβgly and L428V. Cellobiose production in the absence of β-glucosidases is low and reaches a plateau after about 5 h (Fig. 2A). In agreement, the production of glucose directly by TrCel7A is barely detectable (Fig. 2B). Conversely, in the presence of β-glucosidases only glucose was detected, showing that they promptly hydrolyze the produced cellobiose. In this situation, the rate of cellobiose production by TrCel7A had to be calculated based on the amount of glucose produced as described in Materials and Methods. Briefly, glucose was specifically detected using a method based on glucose oxidase, then compared to the total reducing carbohydrate detected using dinitrosalycilic acid method to assure that no cellobiose remained. Thus, confirmed that only glucose was present, the concentration of cellobiose produced by TrCel7A was calculated based on the ratio of 1 cellobiose to 2 glucoses.

The rate of cellobiose production by TrCel7A in the first 4 h is approximately the same (25 μM/h) in both the presence and absence of the wild-type Sfβgly. However, after this period the rate of cellobiose production is constant only when wild-type Sfβgly is added. After 15 h, even in the presence of wild-type Sfβgly, there is a clear decrease in the cellobiose production rate (Fig. 2A). This observed rate decrease is not due to TrCel7A inactivation, as enzymes pre-incubated for 24 h without substrate, but under the same experimental conditions, remained fully active (Fig. S3).

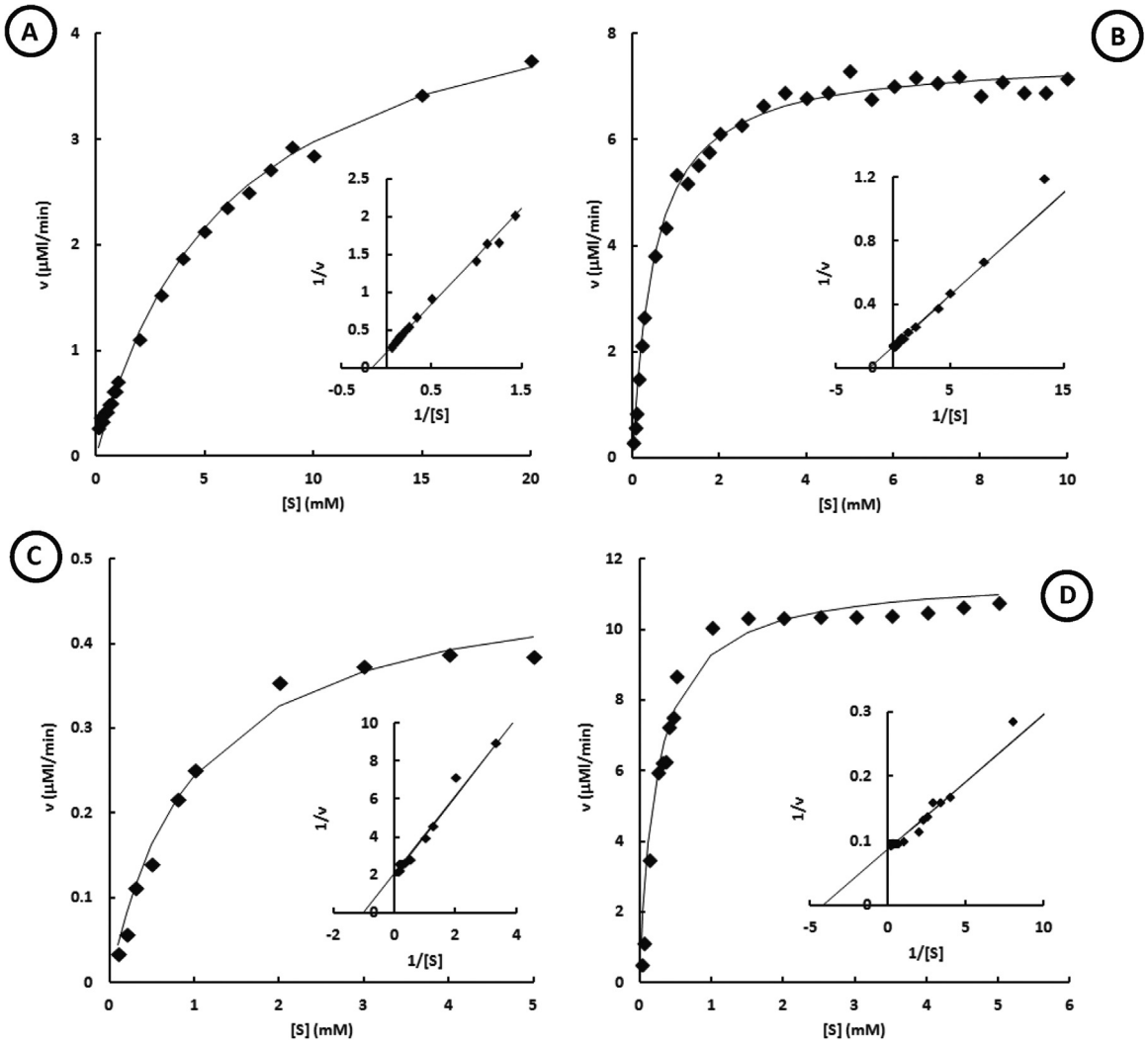

Fig. 1. – Effect of the substrate concentration on the initial rate of hydrolysis catalyzed by L428V. (**A**), cellobiose; (**B**), *p*-nitrophenyl β-glucoside; (**C**), *p*-nitrophenyl β-galactoside; (**D**), *p*-nitrophenyl β-fucoside. Continuous lines were calculated based on the best fitting of the experimental data (♦) in the Michaelis-Menten equation. Inserts are Lineweaver-Burk plots.

Table 1
Enzyme kinetic parameters for wild-type and mutant Sfβgly.

		Enzyme	
Substrate		**Wild-type**	**Mutant L428V**
cellobiose	K_m (mM)	3.2 ± 0.5	6.1 ± 0.4
	k_{cat} (min^{-1})	5.7 ± 0.3	10.2 ± 0.3
	k_{cat}/K_m (min^{-1} mM^{-1})	1.8 ± 0.3	1.7 ± 0.1
NPβglc	K_m (mM)	1.7 ± 0.1	0.49 ± 0.02
	k_{cat} (min^{-1})	5.1 ± 0.1	16.1 ± 0.1
	k_{cat}/K_m (min^{-1} mM^{-1})	3.0 ± 0.2	32 ± 1
NPβgal	K_m (mM)	0.9 ± 0.1	1.0 ± 0.1
	k_{cat} (min^{-1})	0.5 ± 0.3	1.05 ± 0.03
	k_{cat}/K_m (min^{-1} mM^{-1})	0.6 ± 0.3	1.0 ± 0.1
NPβfuc	K_m (mM)	0.48 ± 0.02	0.24 ± 0.02
	k_{cat} (min^{-1})	9.2 ± 0.1	24.5 ± 0.4
	k_{cat}/K_m (min^{-1} mM^{-1})	19.2 ± 0.8	102 ± 8

The *k*cat and Km of the purified wild-type and mutant Sfβgly were determined by measuring the hydrolysis initial rates (v_0) of at least 10 different concentrations of cellobiose and p-nitrophenyl β-D-glycopyranosides prepared in 100 mM sodium citrate – sodium phosphate buffer pH 6.0. Experiments were performed at 30 °C. The v_0 and [S] data were fitted in the Michaelis-Menten equation using the Enzfitter software (Elsevier-Biosoft, Cambridge, UK).

Nonetheless, as a result of this kinetics, the amount of cellobiose produced using the same initial concentrations of TrCel7A and cellulose is about 3.5 times higher in the presence of the wild-type β-glucosidase (Fig. 2A). Interestingly, the addition of L428V increases about five times the rate of cellobiose production (120 µM/h) during initial times (upto 4 h) (Fig. 2A). Hence, although the final amount of cellobiose produced in the presence of the wild-type Sfβgly and L428V is similar (580 µM), TrCel7A activity in the presence of L428V is higher than using the wild-type enzyme.

Considering the first 4 h of cellulose hydrolysis (Fig. 2A), the increment in cellobiose hydrolysis rate due to the addition of the more active β-glucosidase L428V suggests that TrCel7A activity had been inhibited by cellobiose in the absence of β-glucosidases or even in the presence of the wild-type Sfβgly. In turn, the high cellobiase activity of L428V reduces even more the inhibitory pressure upon TrCel7A by hydrolyzing cellobiose into glucose (Fig. 2B), as evidenced for the higher rate of glucose production, which increases the activity of TrCel7A and consumes the cellulose faster (around 4 h; Fig. 2). The decrease in TrCel7A activity and convergence to the same final level of cellobiose produced (580 µM) in the presence of either L428V (after 4 h) or wild-type Sfβgly (after 15 h) suggest that the cellulose chains are not available for TrCel7A to initiate new processive cycles. To check this possibility, the cellulose remaining after a 24 h hydrolysis assay

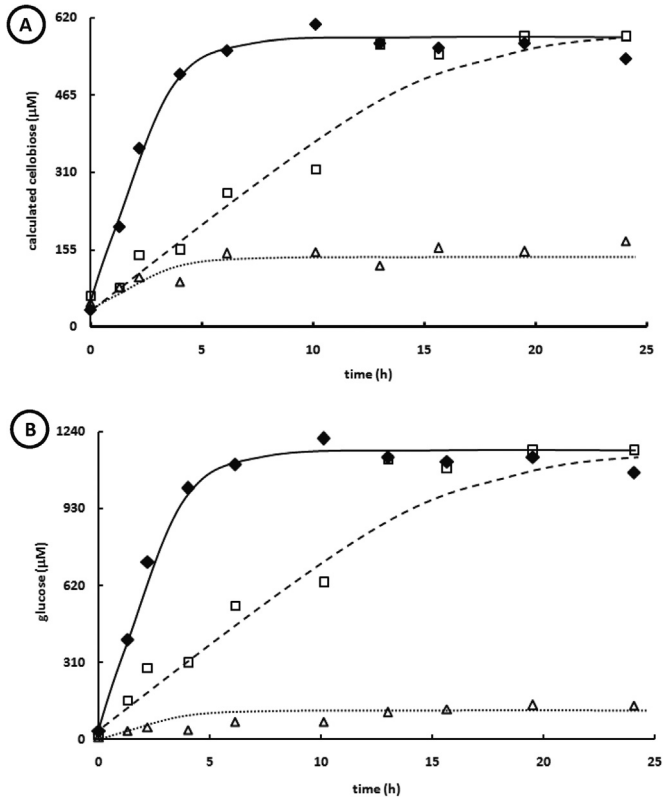

Fig. 2. – Crystalline cellulose (5 mg/mL Avicel PH101) hydrolysis catalyzed by TrCel7A (0.1 mg). (**A**), Calculated production of cellobiose. (**B**), Production of glucose. Assay performed in the absence of β-glucosidases (△). Assays in the presence of 0.11 nmols of the wild-type Sfβgly (□) and L428V (♦). Due the presence of β-glucosidases (□ and ♦), cellobiose produced by TrCel7A was converted into glucose, thus the amount of cellobiose produced (**B**) was calculated based on the glucose concentration (**A**).

was recovered, thoroughly washed and used again as substrate for fresh TrCel7A. Indeed, barely any product is formed when using the remaining cellulose as substrate (Fig. S4). This result supports that substrate sites where TrCel7A initiates processive hydrolysis were not available, as previously proposed [1]. Moreover, it reinforces that using more active βglycosidases speeds up the cellulose enzymatic hydrolysis, reducing the time required to reach its end.

In conclusion, the utilization of the mutant L428V presenting higher cellobiase activity to complement the action of TrCel7A upon crystalline cellulose reduced to a quarter the reaction time to obtain the maximum glucose yield, showing that the introduction of this mutation in β-glucosidases is a promising strategy to reduce costs in the enzymatic hydrolysis of lignocellulosic materials.

Authors' contributions

Planned experiments: S. R. M.; Performed experiments: F. K. T., E. M. A. and R. R.; Analyzed data: F. K. T., E. M. A., R. R. and S. R. M.; Writing, reviewing and editing: F. K. T., E. M. A., R. R. and S. R. M.; Contributed with resources and funding acquisition: S. R. M.

Acknowledgments

This project was supported by Fapesp (Fundação de Amparo à Pesquisa do Estado de São Paulo; Grants 08/55914–9, 14/21900–2 and 14/19439–5), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), "Instituto Nacional de Ciências e Tecnologia para o Bioetanol (Grants: 08/57908–1 and 574002/2008–1) and CAPES. S. R. M is a CNPq research fellow and a staff member of the "Departamento de Bioquímica – IQUSP". F. K. T and R. R were post-docs supported by Fapesp. F. K. T. is currently a "Science Without Borders" Postdoctoral fellowship. E. M. A. was an undergraduate student supported by CNPq.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.05.014.

References

[1] J. Jalak, P. Väljamäe, Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis, Biotechnol. Bioeng. 106 (2010) 871–883.
[2] K. Igarashi, T. Uchihashi, A. Koivula, et al., Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface, Science 333 (2011) 1279–1282.
[3] S.R. Marana, Structural and mechanistic fundamentals for designing of cellulases, Comput. Struct. Biotech. J. 2 (2012) e201209006.
[4] B.C. Knott, M.F. Crowley, M.E. Himmel, et al., Carbohydrate–protein interactions that drive processive polysaccharide translocation in enzymes revealed from a computational study of cellobiohydrolase processivity, J. Am. Chem. Soc. 136 (2014) 8810–8819.
[5] S. Kuusk, M. Sorlie, P. Valjamae, The predominant molecular state of a bound enzyme determines the strength and type of product inhibition in the hydrolysis of recalcitrant polysaccharides by processive enzymes, J. Biol. Chem. 290 (2015) 11678–11691.
[6] M. Gruno, P. Väljamäe, G. Petterson, et al., Inhibition of the *Trichodermareesei* cellulase by cellobiose is strongly dependent on the nature of the substrate, Biotech. Bioeng. 86 (2004) 503–511.
[7] M.J. Arrizubieta, J. Polaina, Increased thermal resistance and modification of the catalytic properties of a β-glucosidase by random mutagenesis and *in vitro* recombination, J. Biol. Chem. 275 (2000) 28843–28848.
[8] G. González-Blasco, J. Sanz-Aparicio, B. González, et al., Directed evolution of β-glucosidase a from *Paenibacillus polymyxa* to thermal resistance, J. Biol. Chem. 275 (2000) 13708–13712.
[9] X.-Q. Pei, Z.-L. Yi, C.-G. Tang, et al., Three amino acid changes contribute markedly to the thermostability of β-glucosidase BglC from *Thermobifida fusca*, Bioresour. Technol. 102 (2011) 3337–3342.
[10] J.H.G. Lebink, T. Kaper, P. Bron, et al., Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* β-glucosidase CelB by directed evolution, Biochemistry 39 (2000) 3656–3665.
[11] J.K. McCarthy, A. Uzelac, D.F. Davis, et al., Improved catalytic efficiency and active site modification of 1,4-β-D-glucan glucohydrolase a from *Thermotoga neapolitana* by directed evolution, J. Biol. Chem. 279 (2004) 11495–11502.
[12] E. Hardiman, M. Gibbs, R. Reeves, et al., Directed evolution of a thermophilic *β*-glucosidase for cellulosic bioethanol production, Appl. Biochem. Biotechnol. 161 (2010) 301–312.
[13] L. Cao, Z. Wang, G. Ren, et al., Engineering a novel glucose tolerant β-glucosidase as supplementation to enhance the hydrolysis of sugarcane bagasse at high glucose concentration, Biotechnol. Biofuels 8 (2015) 202.
[14] L.M.F. Mendonça, S.R. Marana, Single mutations outside the active site affect the substrate specificity in a β-glycosidase, Biochim. Biophys. Acta Proteins Proteom. 1814 (2011) 1616–1623.
[15] F.K. Tamaki, L.C. Textor, I. Polikarpov, et al., Sets of covariant residues modulate the activity and thermal stability of GH1 β-glucosidases, PLoS One 9 (2014) e96627.
[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[17] G. Noelting, P. Bernfeld, Sur les enzymes amylolytiques. III La b-amylase dosage d'activité et controlê de l'absence d'amylase, Helv. Chim. Acta 31 (1948) 286–290.
[18] W. Chuenchor, S. Pengthaisong, R. Robinson, et al., Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation, J. Mol. Biol. 377 (2008) 1200–1215.