J Ind Microbiol Biotechnol (2014) 41:479–488
DOI 10.1007/s10295-014-1400-0

BIOCATALYSIS

SIMB
Society for Industrial Microbiology and Biotechnology

Two promising alkaline β-glucosidases isolated by functional metagenomics from agricultural soil, including one showing high tolerance towards harsh detergents, oxidants and glucose

Sophie Biver · Aurore Stroobants · Daniel Portetelle · Micheline Vandenbol

Received: 12 November 2013 / Accepted: 1 January 2014 / Published online: 12 January 2014

Abstract New β-glucosidase activities were identified by screening metagenomic libraries constructed with DNA isolated from the topsoil of a winter wheat field. Two of the corresponding proteins, displaying an unusual preference for alkaline conditions, were selected for purification by Ni-NTA chromatography. AS-Esc6, a 762-amino-acid enzyme belonging to glycoside hydrolase family 3, proved to be a mesophilic aryl-β-glucosidase with maximal activity around pH 8 and 40 °C. A similar pH optimum was found for AS-Esc10, a 475-amino-acid GH1-family enzyme, but this enzyme remained significantly active across a wider pH range and was also markedly more stable than AS-Esc6 at pH greater than 10. AS-Esc10 was found to degrade cellobiose and diverse aryl glycosides, with an optimal temperature of 60 °C and good stability up to 50 °C. Unlike AS-Esc6, which showed a classically low inhibitory constant for glucose (14 mM), AS-Esc10 showed enhanced activity in the presence of molar concentrations of glucose. AS-Esc10 was highly tolerant to hydrogen peroxide and also to sodium dodecyl sulfate, this being indicative of kinetic stability. This unique combination of properties makes AS-Esc10 a particularly promising candidate whose potential in biotechnological applications is worth exploring further.

Keywords Alkaline enzyme · β-Glucosidase · Detergent · Functional metagenomics · Glucose tolerance

S. Biver (✉) · A. Stroobants · D. Portetelle · M. Vandenbol
Microbiology and Genomics Unit, Gembloux Agro-Bio Tech, University of Liège, Avenue Maréchal Juin 6, 5030 Gembloux, Belgium
e-mail: Sophie.Biver@ulg.ac.be; microbio.gembloux@ulg.ac.be

Introduction

Besides being a major component of plant cell walls, cellulose is found in some algae, bacteria, fungi and invertebrate organisms. This makes it the most abundant organic polymer on earth and a promising source of renewable energy. In nature, this linear polysaccharide composed of glucose units linked by β-1,4-glucosidic bonds is degraded mainly by fungi and bacteria, thanks to the synergic action of endoglucanases (EC 3.2.1.4) and exoglucanases (EC 3.2.1.91). This synergy yields cellobiose, which is then converted to glucose by β-glucosidases (EC 3.2.1.21) [20]. These enzymes are thus crucial for carbon cycling. They are also becoming increasingly attractive to industrialists for diverse biotechnological applications, such as bioethanol production from lignocellulosic biomass, waste management and the improvement of arable soil quality and feed digestibility. Furthermore, cellulases are used in the food and pulp and paper industries [1, 16, 17] and are widely employed in textile finishing, mainly to eliminate superficial microfibrils from cotton fibres and hence to improve colour brightness and reduce pilling propensity (bio-polishing) [33].

β-Glucosidases are key enzymes in most of these applications, as they relieve product inhibition of endo- and exoglucanases by limiting the accumulation of cellobiose, thus increasing the overall rate of cellulolysis [37]. Interestingly, although some β-glucosidases exhibit a strong 'preference' for cello-oligosaccharides and notably cellobiose (true cellobiases, class II), most of them are also able to catalyse hydrolysis of β-glucosidic bonds linking glycoside residues to a wide variety of aglycones (broad substrate specificity enzymes, class III). On the other hand, some are highly specific for aryl glucosides (aryl-β-glucosidases, class I) [36]. This wide substrate-specificity variability, along with the fact that reverse hydrolysis and transglycosylation can

also take place under certain conditions, further extends the range of applications of β-glucosidases [27, 38, 40]. They are particularly helpful in the food industry, e.g. for enhancing the flavour of juices and wines, debittering citrus juices, and degrading cyanogenic glucosides for detoxification of cassava. They are also useful in producing fine chemicals and pharmaceuticals [2].

To fully exploit the potential of such enzymes, a challenge is to discover β-glucosidases with new or improved properties. Particularly sought-after properties include high catalytic efficiency and stability under industrial process conditions and reduced sensitivity to end-product inhibition. A new approach to finding new enzymes is functional metagenomics [5, 35]. In the present study, by applying this approach to agricultural soils, we have isolated several β-glucosidases. We present here the characterization of two of them.

Materials and methods

Sample collection and DNA extraction

Topsoil samples were taken from a well-characterized experimental field located in Gembloux (50° 33′ N, 4° 42′ E), Belgium, at a depth between 15 and 20 cm. The soil is a luvisoil (Food and Agriculture Organization of the United Nations (FAO) classification) composed of a 30-cm-deep silt-loam topsoil (clay, 14–16 %; silt, 75–80 %; sand, 5–6 %) of neutral pH (6.5–7.0) and a slightly acidic (pH 6.2–6.5) silt-loam subsoil (clay, 20–25 %; silt, 70–75 %; sand, 3–6 %, 30–100 cm depth). The field was subjected to conventional tilling with a ploughing depth of 30 cm and was sown with winter wheat on 22 November 2010 and soil samplings (three replicates/date) were performed after 2 and 6 months of culture (on 17 January and 18 May 2011). Soil samples were frozen in liquid nitrogen and stored at −80 °C until DNA extraction. Eight grams of each bulk sample was used to extract metagenomic DNA with the PowerMax™ Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. After extraction, the metagenomic DNA was pooled equally per date.

Construction and screening of metagenomic libraries

Each DNA pool was used to construct one metagenomic library. After partial digestion with *Sau*3AI, DNA was precipitated with 5 % polyethylene glycol 6000 and 0.6 M NaCl to remove small DNA fragments [3] before separation in a 1 % low-melting-point agarose gel. DNA fragments 5–20 kb long, recovered from the agarose with AgarACE enzyme (Promega), were inserted into the *Bam*HI-linearized and dephosphorylated YEp356 vector. The ligation products were then introduced into electrocompetent DH10B *E. coli* cells (Invitrogen). This yielded libraries with an average insert size of ca. 5.5–6 kb. About 45,000 *E. coli* colonies from each library (ca. 250 Mb/library) were screened for β-glucosidase activity by spreading the transformants onto 2 × YT medium (2 × yeast extract and tryptone) (MP Biomedicals) supplemented with 15 g/l agar (Oxoid), 50 μg/ml ampicillin, 5 g/l esculin and 1 g/l ammonium iron(III) citrate (Sigma-Aldrich). The plates were incubated at 37 °C for 2 days and then transferred to room temperature (ca. 22 °C) for 2–3 weeks. Activities were detected by the appearance of a dark halo surrounding positive colonies.

DNA sequencing and sequence analysis

Plasmid inserts were sequenced at GATC Biotech (Germany). Sequence similarity searches were carried out with the NCBI Blast programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein signatures were sought with InterProScan [25]. Signal peptides were predicted with the SignalP 4.1 server [24]. Theoretical isoelectric points and molecular weights were computed with the ProtParam tool of ExPASy (http://www.expasy.org).

First estimation of the β-glucosidase activity of positive clones

The β-glucosidase activity of the positive clones found in the screen was estimated at several pH values and temperatures with *p*-nitrophenyl-β-D-glucopyranoside (pNPG), as previously described [22]. Briefly, 100-μl aliquots of overnight cultures diluted to $OD_{600nm} = 1$ were harvested by centrifugation. The pellets were then mixed with 100 μl of 4 mM pNPG in 100 mM buffer (sodium acetate pH 4–6, sodium phosphate pH 6–8 or Tris–HCl pH 8–10) and incubated for 2–3 h at 20, 40 or 60 °C. The enzymatic reaction was stopped by addition of 100 μl of 1 M Na_2CO_3 before quantification of the released *p*-nitrophenol by measuring the absorbance at 405 nm.

Subcloning the coding sequences of the two β-glucosidases in the pET-30b expression vector

The entire coding sequences of both β-glucosidase genes were amplified with Platinum *Pfx* DNA Polymerase and 0.5 × PCR × Enhancer Solution (Invitrogen). The following pairs of primers were used for AS-Esc6: 5′-gggaattcCATATGACCGACACCCTGAACCCG-3′ and 5′-cgcGGATCCTCATCA**GTGGTGGTGGTGGTGGTG**CGACGCCGCGTTCGCC-3′, and for AS-Esc10: 5′-gggaattcCATATGAACAATGGTTGGAGTCATGAATTG-3′ and 5′-cgcGGATCCTC

ATCA**GTGGTGGTGGTGGTGGTG**GCTGACGCCGA CAGGGTC-3′. The restriction sites (*Nde*I, *Bam*HI) used for subcloning are underlined and the histidine tag sequence appears in bold. PCR products were cloned into the pET-30b(+) vector (Novagen) and the resulting plasmids introduced into *E. coli* Rosetta 2 (DE3) (Novagen) after checking the inserts by DNA sequencing.

Overproduction and purification of recombinant β-glucosidases

To overproduce the β-glucosidases, overnight cultures of transformed *E. coli* Rosetta cells were diluted to OD_{600nm} = 0.005 in 1-l shake flasks containing 200 ml Terrific Broth medium (Difco) and antibiotics (30 μg/ml chloramphenicol and kanamycin). The cultures were grown at 37 °C under shaking at 160 rpm until the OD_{600nm} reached about 0.4. Overproduction was induced by adding 1 μM (AS-Esc10) or 5 μM (AS-Esc6) isopropyl β-D-1-thiogalactopyranoside (IPTG) and then incubating the cultures at 16 °C for 16 h. The cells were harvested by centrifugation and lysed at 37 °C for 30 min in 20 mM Tris–HCl buffer (pH 8) containing 0.3 M NaCl, Triton X-100 (1 ml/l) and lysozyme (5 mg/ml). After sonication on ice (4 × 5 s, 100 W), the lysates were centrifuged at 10,000*g* for 20 min (4 °C) to separate the insoluble from the soluble proteins. The recombinant proteins were purified from the soluble fractions by Ni-NTA affinity chromatography (Qiagen). For this, the protein mixtures were incubated overnight at 4 °C with 500 μl Ni-NTA agarose pre-equilibrated with binding buffer (20 mM Tris–HCl pH 8, 0.5 M NaCl, 1 ml Triton X-100 per litre). The resin was recovered by centrifugation at 800*g* for 5 min and washed twice with binding buffer (flow-through 1) and twice with washing buffer (20 mM Tris–HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole) (flow-through 2) before elution with increasing concentrations of imidazole (100, 250 and 500 mM) in 20 mM Tris–HCl pH 7.5 and 0.5 M NaCl (5 ml eluate/imidazole concentration). The purified proteins were dialysed at 4 °C against 20 mM Tris–HCl pH 7.5 and 0 mM (AS-Esc10) or 300 mM (AS-Esc6) NaCl and analysed by SDS-PAGE. AS-Esc6 was maintained in the presence of NaCl to prevent protein aggregation. As the first eluate obtained for AS-Esc10 still contained many protein contaminants, it was repurified by a similar protocol.

Characterization of the recombinant proteins

β-Glucosidase activities were determined by measuring the amount of *p*-nitrophenol released from pNPG via the absorbance at 405 nm. They were expressed as specific activities (U/mg protein), 1 U being defined as the release of 1 μmol *p*-nitrophenol per minute. Standard assay conditions were as follows: the reaction was performed at 40 °C in 100 mM phosphate buffer pH 8 containing 4 mM (AS-Esc6) or 6 mM (AS-Esc10) pNPG. After pre-incubation of the mixture (390 μl), the reaction was started by adding 10 μl of an appropriate dilution of the enzyme and stopped after 10 min with 400 μl ice-cold 1 M Na_2CO_3. The optimal temperature of each enzyme was estimated by incubating the reaction mixture at various temperatures from 10 to 80 °C. To determine the optimal pH, activities were measured at 40 °C over a pH range of 4–12 in various buffers at 0.1 M (sodium acetate pH 4–6, sodium phosphate pH 6–8, Tris–HCl pH 8–10, glycine–NaOH pH 8–12). For the thermostability studies, the enzymes were pre-incubated at various temperatures (4–60 °C) for 1 h before measurement of the residual activity under standard assay conditions. The effect of pH on enzyme stability was evaluated after a 1-h pre-incubation at 25 °C. To estimate the effects of ions, ethylenediaminetetraacetic acid (EDTA), organic solvents, detergents and H_2O_2, enzyme activities were measured after a 30-min pre-incubation at 30 °C (AS-Esc6) or 40 °C (AS-Esc10) with each additive. Residual activities were then measured after a 20-min incubation with the substrate at the same temperature. Tris–HCl buffer pH 8 was used instead of sodium phosphate to avoid precipitate formation.

Substrate specificity was estimated with several nitrophenyl derivatives (pNPG, *p*-nitrophenyl-α-D-glucopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) and natural substrates (cellobiose, esculin, salicin and arbutin), all purchased from Sigma-Aldrich and tested at 4 mM under the standard assay conditions. For the natural glycosides, the reaction mixtures were boiled for 5 min to inactivate the enzymes before quantification of the glucose released with a glucose oxidase–peroxidase assay kit (Sigma-Aldrich) as recommended by the manufacturer. β-Glucosidase activity was expressed as specific activity (U/mg protein), 1 U corresponding to the production of 1 μmol glucose from salicin, esculin or arbutin or of 2 μmol glucose from cellobiose in 1 min.

The kinetic parameters of AS-Esc6 were estimated by measuring the initial velocities of the reaction at 40 °C at various concentrations of substrate (0.4–6 mM pNPG or 2–40 mM cellobiose) diluted in phosphate buffer pH 8. For AS-Esc10, the reactions were performed in the same buffer but at 50 °C (the kinetic parameters were not evaluated at 60 °C because the enzyme was too unstable at this temperature). Lineweaver–Burk plots were then used to determine the K_m and V_{max}.

To evaluate enzyme sensitivity to end-product inhibition, activities were measured at various concentrations

of glucose (0–2.5 M) and pNPG (0.5–6 mM) in sodium phosphate buffer (pH 8) at 40 °C (AS-Esc6) or 50 °C (AS-Esc10). The inhibitory constant (K_i) of AS-Esc6 for 1 M glucose was deduced from a Dixon plot.

All experiments were performed at least twice in triplicate, and the results of one representative experiment are shown. They are expressed as means ± standard error of the mean. Comparisons with control conditions were performed with Student's *t* test. In all assays, controls without enzyme were included so as to take non-enzymatic hydrolysis of substrates into account.

Nucleotide sequence accession numbers

The nucleotide sequences of the inserts containing the *AS-Esc6* and *AS-Esc10* genes have been deposited in the GenBank database under accession numbers KF660587 and KF660588.

Results

Identification of new β-glucosidase genes by functional metagenomics

To find new β-glucosidase activities, two agricultural-soil metagenomic libraries were screened on 2 × YT agar containing ammonium iron(III) citrate and the aryl-β-glucoside esculin. Both plasmid libraries were constructed in *E. coli* with DNA extracted from the topsoil of a winter wheat field, the difference between them being the sampling date. So far, about 45,000 colonies from each library have been screened, corresponding to a total of ca. 500 Mb, and already four positive candidates have been identified (two per library). To test their temperature and pH preferences, preliminary activity assays were performed on intact *E. coli* cells with pNPG as substrate (data not shown). On the basis of the results obtained, two candidates seeming to act preferably at alkaline pH were selected for further characterization.

The first chosen clone was isolated with library 1 (sampling date 17 January 2011) and contained a 3,396-bp DNA insert very rich in bases G and C (70.3 %). A Blastx search revealed an open reading frame of 2,286 bp coding for a 762-amino-acid protein showing high similarity to various actinomycetales β-glucosidases. This new enzyme (named AS-Esc6) was found to be most similar (83 % similarity, 72 % amino acid identity over 743 aa) to an uncharacterized β-glucosidase of *Rhodococcus rhodochrous* (WP_016694245). An InterProScan analysis revealed signatures typical of proteins belonging to glycoside hydrolase family 3 (GH3), notably the conserved catalytic nucleophile (D247) in the amino-terminal domain of the protein. Its carboxyl-terminal part contains a fibronectin-type-III-like domain (IPR026891) of unknown function, frequently encountered in GH3 enzymes, and the conserved glutamate residue acting as catalytic acid/base (E433). The second protein selected, AS-Esc10, shows several features of family-1 glycoside hydrolases (GH1), with two glutamate residues acting as nucleophilic and acid/base catalysts (G367, G173). This 475-aa protein displays 67 % similarity (52 % amino acid identity over 458 aa) to a β-glucosidase of *Sorangium cellulosum* So ce56 (YP_001618312). Its coding sequence was identified on a 5,573-bp insert (56 % GC) isolated by screening library 2 (18 May 2011). Analysis of the amino acid sequences of these two new enzymes suggested that AS-Esc6 and AS-Esc10 are intracellular proteins (no signal peptide detected). Their calculated p*I* values are 4.64 and 5.79, respectively.

Purification of recombinant β-glucosidases

The coding sequences of these two proteins were cloned in fusion with a carboxy-terminal His-tag in the IPTG-inducible pET-30b(+) expression vector to allow purification by Ni-NTA affinity chromatography. When overproduced in the *E. coli* Rosetta 2 strain, both recombinant proteins were found to be at least partly soluble in the cell extract. This allowed their purification under non-denaturing conditions by standard protocols. SDS-PAGE analysis of the fractions obtained during purification (Fig. 1) revealed molecular weights for AS-Esc6 and AS-Esc10 close to their theoretical ones (80.9 and 54.8 kDa). Although eluates containing each protein at sufficient purity were obtained, for AS-Esc10 it was necessary to repurify the eluate obtained at the lowest imidazole concentration (E1), which still contained many contaminants. After purification, AS-Esc10 was dialysed against 20 mM Tris–HCl (pH 7.5) to remove NaCl and imidazole. In the case of AS-Esc6, only the imidazole was eliminated, because in the absence of NaCl, the formation of macroscopic protein aggregates a few days after dialysis resulted in lower hydrolytic activity than in undialysed fractions.

Biochemical characterization of the purified β-glucosidases

Temperature and pH dependence

To estimate the temperature and pH preferences of the purified enzymes, their activities were measured in the presence of pNPG substrate between 10 and 80 °C and between pH 4 and 12. AS-Esc10 was active across the entire range of temperatures tested (Fig. 2a), with an optimum at 60 °C, whereas AS-Esc6 appeared more mesophilic, with maximal activity at 40 °C and hydrolysis detectable only below 60 °C. In thermostability assays, both β-glucosidases

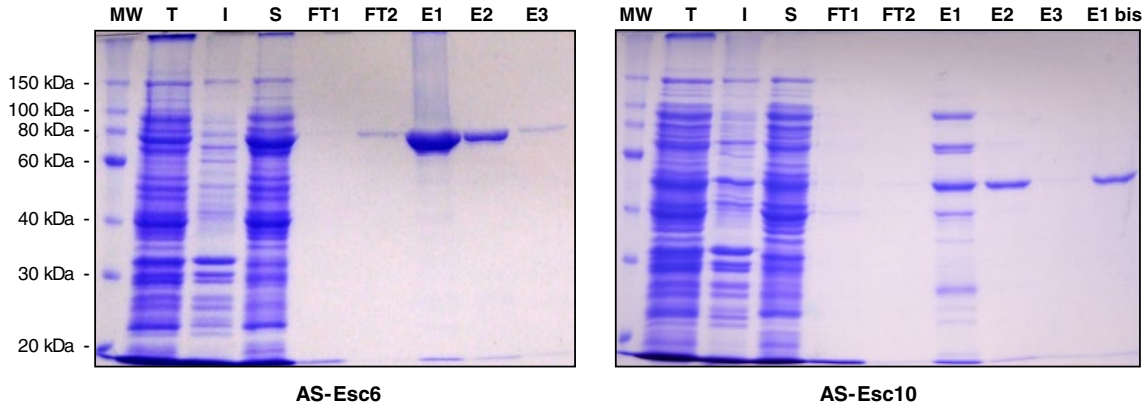

Fig. 1 SDS-PAGE analysis of the recombinant β-glucosidases. Proteins recovered at different purification steps were separated by SDS–10 % polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. *MW* protein molecular weight markers, *T* total extracts of *E. coli* Rosetta cells overproducing AS-Esc6 or AS-Esc10; insoluble (*I*) and soluble (*S*) fractions obtained after cell lysis; *FT1* and *FT2* flow-throughs 1 and 2; *E1–E3* fractions obtained after elution with 100, 250 and 500 mM imidazole; *E1 bis* E1 repurified

proved stable up to temperatures about 10 °C below their respective optima, showing no to very low residual activity after a 1-h incubation at 60 °C. Both enzymes showed a pH optimum near pH 8, but AS-Esc10 proved active over a broader pH range: it displayed more than 75 % maximal activity between pH 5 and 10, whereas AS-Esc6 showed less than 20 % maximal activity at pH 5 and 10 (Fig. 2b). Interestingly, AS-Esc10 was very stable across a wide pH range and notably under alkaline conditions: when incubated for 1 h at 25 °C and pH 12, it retained 68 % of its activity, whereas AS-Esc6 was almost completely inactivated (Fig. 2c).

Substrate specificity and kinetic parameters

The substrate specificities of AS-Esc6 and AS-Esc10 were evaluated by replacing pNPG with various other synthetic aryl glycosides or with natural substrates (Table 1). Although pNPG was clearly the 'preferred' nitrophenyl derivative tested, AS-Esc10 showed a significant hydrolysis rate with *o*-nitrophenyl-β-D-galactopyranoside and AS-Esc6 with *p*-nitrophenyl-β-D-xylopyranoside, as frequently observed with GH1 and GH3 enzymes, respectively [9, 10, 18, 45]. Both enzymes showed lower specific activities with the disaccharide cellobiose and the natural aryl glycosides esculin, salicin and arbutin than with pNPG, with a 'preference' for salicin. Surprisingly, AS-Esc6 showed practically no cellobiose-hydrolysing activity, even at cellobiose concentrations up to 40 mM (data not shown). This suggests that this enzyme is an aryl-β-glucosidase.

To better estimate the efficiency of pNPG and cellobiose degradation by the enzymes, their kinetic parameters were determined at optimal pH (8). This was done at optimal temperature (40 °C) for AS-Esc6 and at 50 °C for AS-Esc10, because of the latter's instability at optimal temperature (60 °C). The highest maximal velocity (V_{max}) was obtained for AS-Esc6, which hydrolysed pNPG at the calculated rate of 46.1 U/mg protein (k_{cat} = 62.1/s) and showed a Michaelis affinity constant (K_m) of 1.14 mM. Lower V_{max} and k_{cat} values (7.9 U/mg and 7.1/s) were found for AS-Esc10, which compensated for this with a higher affinity for pNPG (K_m = 0.20 mM). For cellobiose degradation, the K_m of this enzyme was 16.9 mM (V_{max} = 2.2 U/mg, k_{cat} = 2.0/s), consistent with the general finding that K_m (cellobiose) is greater than K_m (pNPG) [23, 39].

Effects of various additives

The activity of AS-Esc6 and AS-Esc10 was next tested in the presence of several ions and other additives (Table 2). Both enzymes were severely inhibited by 5 mM Zn^{2+} or Cu^{2+} and moderately affected by Mn^{2+}, Mg^{2+}, and Ca^{2+}. Co^{2+} was found to reduce the activity of AS-Esc6 to ca. 10 % and that of AS-Esc10 to ca. 70 % of that measured under control conditions. A metal cofactor thus appeared clearly unnecessary, as deduced also from the absence of inhibition by the chelating agent EDTA, which appeared even to cause slight activation when added at 5 mM.

Addition of 10 % v/v ethanol also somewhat stimulated the activity of AS-Esc6 but not that of AS-Esc10. Both enzymes were significantly inhibited by methanol and dimethyl sufloxide (DMSO), although inhibition was less pronounced for AS-Esc10 in the presence of 10 % DMSO. Activation of β-glucosidases by ethanol is sometimes indicative of transferase activity, with alcohol as nucleophilic acceptor instead of water [14, 39], but it might be attributable to increased stability and/or hydrolytic activity owing to a change in medium polarity.

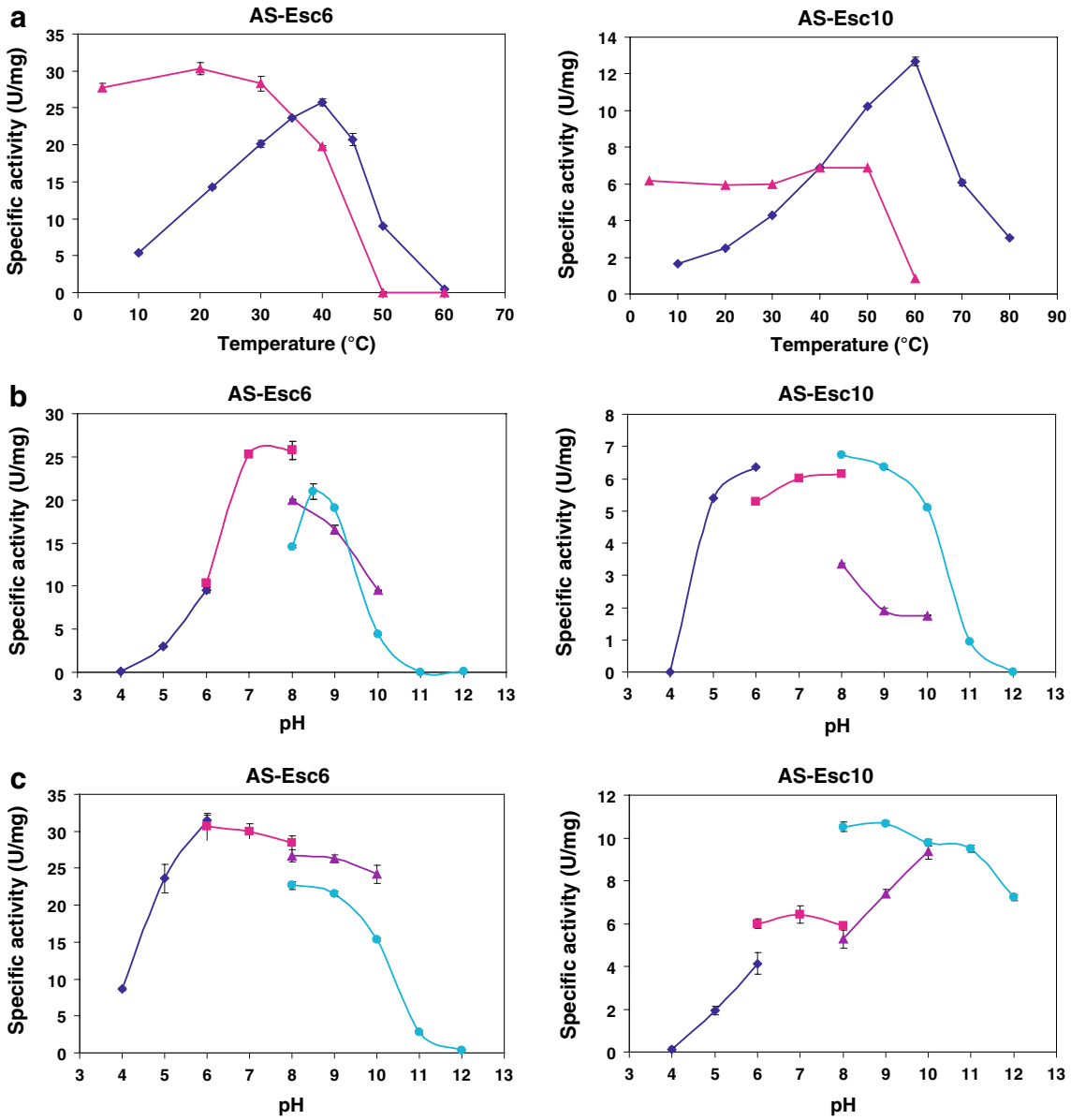

Fig. 2 Effect of pH and temperature on the activity and stability of purified β-glucosidases. **a** Effect of temperature on the activity (*filled diamond*) and stability (*filled triangle*) of AS-Esc6 and AS-Esc10. Thermal stability was estimated by measuring the residual enzyme activity (pNPG, 40 °C, pH 8) after incubation for 1 h at the indicated temperature. **b** pH-dependent activity of β-glucosidases. The ability to hydrolyse pNPG was assayed at 40 °C in different buffers: *filled diamond* 100 mM sodium acetate buffer pH 5–6, *filled square* 100 mM sodium phosphate buffer pH 6–8, *filled triangle* 100 mM Tris–HCl buffer pH 8–10, *filled circle* 100 mM glycine–NaOH buffer pH 8–12. **c** Effect of pH on the stability of AS-Esc6 and AS-Esc10. The enzymes were incubated at 25 °C for 1 h in each buffer before estimation of their residual activity at 40 °C in 100 mM phosphate buffer pH 8

The β-glucosidases were found to be relatively resistant to detergents and notably to 1–2 % v/v Triton X-100, which enhanced the activity of both enzymes. SDS and Tween 80 affected the two enzymes differently. AS-Esc6 was completely inactivated by 0.25 % w/v SDS but strongly stimulated by Tween 80 up to a concentration of at least 2 % v/v (a 140 % activity increase). AS-Esc10, on the other hand, showed a lesser but significant and unexpected activity increase (ca. 30 %) in the presence of 0.25–2 % SDS and a ca. 70 % activity reduction in the presence of the non-ionic detergent Tween 80 (1–2 % v/v).

In addition to being particularly resistant to the harsh detergent SDS, AS-Esc10 was highly tolerant to hydrogen peroxide. In the presence of 1 % w/v H_2O_2 it retained 50 % of its activity, and in the presence of 0.1 % H_2O_2, its activity was actually increased by ca. 70 %. AS-Esc6, in contrast, was inactive at both concentrations tested.

Table 1 Substrate specificity of AS-Esc6 and AS-Esc10 evaluated at 40 °C and pH 8

Substrate (4 mM)	Relative activity (%)	
	AS-Esc6	AS-Esc10
Synthetic aryl glycosides		
p-Nitrophenyl-β-D-glucopyranoside	**100.0 ± 1.2**	**100.0 ± 0.2**
p-Nitrophenyl-α-D-glucopyranoside	1.86 ± 0.01	3.60 ± 0.24
o-Nitrophenyl-β-D-galactopyranoside	ND	36.1 ± 0.1
p-Nitrophenyl-β-D-xylopyranoside	15.1 ± 0.3	1.05 ± 0.12
p-Nitrophenyl-β-D-cellobioside	1.29 ± 0.02	7.81 ± 0.04
p-Nitrophenyl-α-L-arabinofuranoside	0.37 ± 0.00	0.24 ± 0.05
p-Nitrophenyl-*N*-acetyl-β-D-glucosaminide	ND	0.33 ± 0.20
Natural glycosides		
Cellobiose	0.09 ± 0.04	30.8 ± 0.7
Esculin	88.7 ± 5.0	43.9 ± 1.1
Salicin	**100.0 ± 2.8**	**100.0 ± 7.8**
Arbutin	86.3 ± 2.5	33.0 ± 2.2

The specific activities of AS-Esc6 and AS-Esc10 for pNPG were respectively 25.77 and 4.08 U/mg. The specific activities of AS-Esc6 and AS-Esc10 for salicin were respectively 3.20 and 1.32 U/mg

ND not detected

The highest activities of each enzyme are indicated in bold

Table 2 Effect of various additives on the β-glucosidase activity of AS-Esc6 and AS-Esc10

Additive	Concentration	Relative activity (%)	
		As-Esc6	AS-Esc10
No additive (control)		100.0 ± 0.2	100.0 ± 3.4
EDTA	5 mM	115.2 ± 4.7	103.6 ± 0.3
K_2SO_4	5 mM	100.2 ± 0.5	92.9 ± 0.1
KCl	5 mM	93.6 ± 3.5	97.9 ± 0.5
$MnSO_4$	5 mM	63.8 ± 1.7	85.8 ± 3.6
$MnCl_2$	5 mM	65.0 ± 2.7	80.1 ± 2.0
$ZnSO_4$	5 mM	ND	1.11 ± 3.01
$ZnCl_2$	5 mM	ND	0.20 ± 0.20
$CuSO_4$	5 mM	ND	0.16 ± 0.00
$CoCl_2$	5 mM	11.7 ± 1.9	70.9 ± 0.3
$MgCl_2$	5 mM	90.9 ± 1.3	90.9 ± 0.8
$CaCl_2$	5 mM	84.3 ± 2.5	86.4 ± 0.8
Ethanol	10 % (v/v)	116.0 ± 1.5	22.7 ± 0.4
Methanol	10 % (v/v)	40.0 ± 1.0	22.7 ± 0.2
DMSO	10 % (v/v)	15.3 ± 0.4	80.1 ± 9.0
SDS	0.25 % (w/v)	1.55 ± 2.98	125.5 ± 0.8
SDS	1 % (w/v)	ND	117.2 ± 0.7
SDS	2 % (w/v)	ND	129.3 ± 3.7
Triton X-100	1 % (v/v)	167.3 ± 3.6	109.8 ± 0.6
Triton X-100	2 % (v/v)	200.7 ± 5.4	116.1 ± 1.7
Tween 80	1 % (v/v)	187.6 ± 5.4	35.7 ± 0.6
Tween 80	2 % (v/v)	237.6 ± 4.9	33.3 ± 5.5
H_2O_2	0.1 % (w/v)	1.41 ± 0.03	168.7 ± 9.1
H_2O_2	1 % (w/v)	0.74 ± 0.87	46.1 ± 12.0

The enzymes were pre-incubated for 30 min (at 30 °C for AS-Esc6 and 40 °C for AS-Esc10) with each additive before activity measurement with pNPG as substrate. All additives except KCl and K_2SO_4 modified the activity of these β-glucosidases significantly ($p < 0.05$)

ND not detected

Effect of glucose

We next investigated to what extent the activity of the new β-glucosidases is affected by the reaction end-product, D-glucose (Fig. 3). AS-Esc6 showed a typical decrease in pNPG hydrolysis rate with increasing D-glucose concentration (calculated K_i 14 mM). In contrast, the activity of AS-Esc10 was enhanced by D-glucose up to a rather high concentration, above which it gradually returned to normal and then decreased further. In the presence of 6 mM pNPG, a 2.7-fold activity increase was observed for glucose at ca. 500–1,000 mM concentration, with inhibition appearing only at concentrations above 2.5 M. Kinetic analysis performed in the presence of 1 M glucose showed enzyme stimulation to occur despite a reduced affinity for pNPG (8.2 mM), thanks to a 7.4-fold increase in V_{max} (58.1 U/mg).

Discussion

Most β-glucosidases characterized so far show maximal activity in the acidic pH range (pH 4–6.5), retaining only low activity and/or showing instability under mildly alkaline conditions [2, 34] (see also the BRENDA database: http://www.brenda-enzymes.org [31]). Our present screen of two agricultural-soil metagenomic libraries has identified two enzymes acting preferably around pH 8 and remaining relatively stable up to at least pH 10 (and even pH 12 for AS-Esc10). These very uncommon features are accompanied, in the case of AS-Esc10, by high glucose tolerance. This is a very sought-after property, as the high sensitivity of most β-glucosidases to glucose inhibition is a major obstacle limiting their use in many industrial processes [34, 37]. Efforts are thus devoted to finding new enzymes with lower sensitivity to end-product inhibition. Among the β-glucosidases identified to date, some show high K_i values for glucose [11, 26, 28, 29, 43], but very few show stimulation by glucose [23, 39, 42] and none has an alkaline pH optimum. We show here that D-glucose at molar concentrations increases both the K_m and the V_{max} of AS-Esc10, causing enhanced activity at some substrate concentrations. Similar modifications of the kinetic parameters have been highlighted recently for Td2F2 [39], a β-glucosidase isolated from a compost microbial

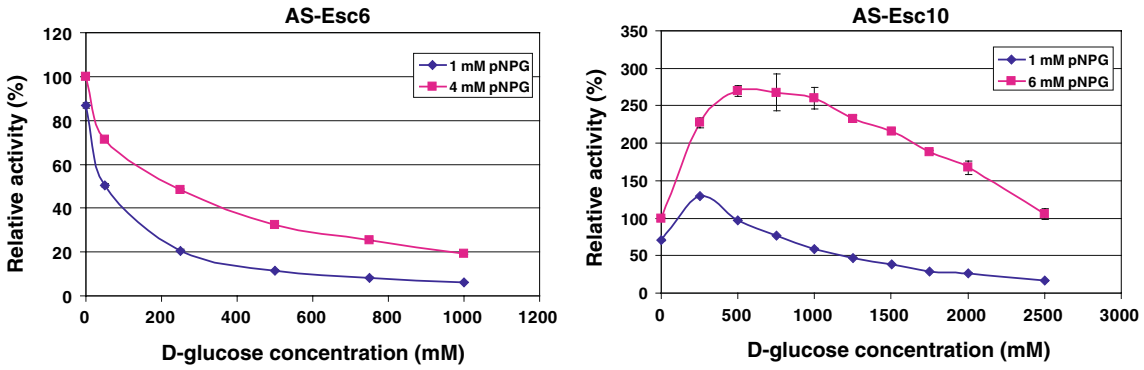

Fig. 3 Effect of D-glucose concentration on the activity of AS-Esc6 and AS-Esc10. β-Glucosidase activities were evaluated in the presence of 1 and 4 mM (AS-Esc6) or 6 mM (AS-Esc10) pNPG and of increasing concentrations of D-glucose diluted in 100 mM phosphate buffer pH 8. The incubation temperature was 40 °C for AS-Esc6 and 50 °C for AS-Esc10

metagenome and displaying high transglycosylation activity. The authors propose that the observed increase in pNPG hydrolysis might be due to high transglycosylation activity leading to production of sophorose and laminaribiose from pNPG and glucose. Further work will be necessary to determine whether the same applies to AS-Esc10.

Also of considerable interest is the resistance of AS-Esc10 to SDS. Strikingly, the enzyme retains maximal activity at detergent concentrations (>10 mM, 0.29 % w/v) that denature most proteins [8], β-glucosidases included [7, 19, 28]. Globular proteins are usually highly vulnerable to SDS because of an equilibrium between their native folded states and the (partially) unfolded conformations that the detergent targets. Some proteins, however, require a high activation energy to reach the transition state between the native and unfolded states, so that they are virtually trapped in the folded state and therefore generally highly resistant to denaturation by SDS [21, 30]. Interestingly, kinetically stable proteins tend to be both SDS resistant and less sensitive to proteolytic cleavage, in addition to having extended half-lives under diverse extreme chemical and physical conditions even if the unfolded state is thermodynamically more favourable [4, 12, 44]. Kinetic stability is thus a clear asset for enzymes of biotechnological interest. The resulting ability of such enzymes to work in combination with surfactants is particularly advantageous, as these chemicals are used in a wide variety of industrial processes and notably in biomass conversion: added surfactants favour cellulose hydrolysis by increasing substrate accessibility for cellulases while decreasing their nonproductive adsorption onto lignin [32]. In this respect SDS appears superior to non-ionic surfactants [6], but its use is currently limited by the low tolerance of the enzymes available. Surfactants also have applications in the textile industry, e.g. in reducing back-staining of indigo dye during bio-stoning of denim garments [46]. Surfactant-stable β-glucosidases could further be helpful in the pulp and paper industry, which uses diverse cellulolytic enzymes and surfactants [15]. AS-Esc10 might thus find many biotechnological applications, the most obvious being its use as a detergent additive in the textile and laundry industries, where it could help alkaline endo- and exoglucanases to remove dirt from cotton garments and improve colour brightness, by degrading their end-product inhibitor cellobiose even in the presence of glucose concentrations that are inhibitory for most other β-glucosidases. Besides the marked resistance of AS-Esc10 to harsh detergents and its high pH optimum, its unusual stability under oxidative conditions should be a major asset in the detergent industry, as oxidants are generally included in detergents as bleaching agents.

Although AS-Esc6 is highly tolerant to gentler detergents such as Tween 80 and Triton X-100 and although these detergents clearly enhance its activity, applications such as those just described are not feasible for this protein. It is too sensitive to SDS and oxidants and is also among the few β-glucosidases characterized so far having practically no detectable cellobiose-hydrolysing activity [13, 41]. Most β-glucosidases are like AS-Esc10 in that they can act on both cello-oligosaccharides and aryl glycosides. Yet enzymes capable of hydrolysing aryl glycosides and/or of catalysing transglycosylations involving them also have various applications in the food, chemical and pharmaceutical industries [2], and AS-Esc6, with its unusually high pH optimum, might extend the already wide range of applications for such enzymes to new ones requiring more alkaline conditions. This is also true of AS-Esc10, although this very promising enzyme can also work efficiently at slightly acidic pH.

Conclusions

Exploring the metagenome of an agricultural soil has thus led us to identify two novel β-glucosidases with very

uncommon properties. Both show a high optimal pH, one of them is among the rare β-glucosidases unable to hydrolyse cellobiose efficiently, and the other displays features that industrialists seek and that make it a promising candidate for use in diverse biotechnological applications: marked resistance to detergents and oxidants combined with high glucose tolerance. To better estimate its potential, this protein will be characterized more extensively, notably as regards its kinetic stability. We will also pursue screening of this metagenomic library, which has so far been particularly fruitful.

Acknowledgments We thank Benjamin Dubois for technical assistance. Sophie Biver is a Postdoctoral Researcher of the Fonds National de la Recherche Scientifique (F.R.S-FNRS).

References

1. Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18(5):355–383
2. Bhatia Y, Mishra S, Bisaria VS (2002) Microbial beta-glucosidases: cloning, properties, and applications. Crit Rev Biotechnol 22(4):375–407. doi:10.1080/07388550290789568
3. Biver S, Vandenbol M (2013) Characterization of three new carboxylic ester hydrolases isolated by functional screening of a forest soil metagenomic library. J Ind Microbiol Biotechnol 40(2):191–200. doi:10.1007/s10295-012-1217-7
4. Cunningham EL, Jaswal SS, Sohl JL, Agard DA (1999) Kinetic stability as a mechanism for protease longevity. Proc Natl Acad Sci USA 96(20):11008–11014
5. Daniel R (2004) The soil metagenome–a rich resource for the discovery of novel natural products. Curr Opin Biotechnol 15(3):199–204. doi:10.1016/j.copbio.2004.04.005
6. Eriksson T, Börjesson J, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb Technol 31(3):353–364
7. Fan H-X, Miao L-L, Liu Y, Liu H-C, Liu Z-P (2011) Gene cloning and characterization of a cold-adapted β-glucosidase belonging to glycosyl hydrolase family 1 from a psychrotolerant bacterium *Micrococcus antarcticus*. Enzyme Microb Technol 49(1):94–99. doi:10.1016/j.enzmictec.2011.03.001
8. Gudiksen KL, Gitlin I, Whitesides GM (2006) Differentiation of proteins based on characteristic patterns of association and denaturation in solutions of SDS. Proc Natl Acad Sci USA 103(21):7968–7972. doi:10.1073/pnas.0602816103
9. Harvey AJ, Hrmova M, De Gori R, Varghese JN, Fincher GB (2000) Comparative modeling of the three-dimensional structures of family 3 glycoside hydrolases. Proteins 41(2):257–269
10. Hill AD, Reilly PJ (2008) Computational analysis of glycoside hydrolase family 1 specificities. Biopolymers 89(11):1021–1031. doi:10.1002/bip.21052
11. Jabbour D, Klippel B, Antranikian G (2012) A novel thermostable and glucose-tolerant β-glucosidase from *Fervidobacterium islandicum*. Appl Microbiol Biotechnol 93(5):1947–1956. doi:10.1007/s00253-011-3406-0
12. Joo H-S, Chang C-S (2005) Oxidant and SDS-stable alkaline protease from a halo-tolerant *Bacillus clausii* I-52: enhanced production and simple purification. J Appl Microbiol 98(2):491–497. doi:10.1111/j.1365-2672.2004.02464.x
13. Kalyani D, Lee K-M, Tiwari MK, Ramachandran P, Kim H, Kim I-W, Jeya M, Lee J-K (2012) Characterization of a recombinant aryl β-glucosidase from *Neosartorya fischeri* NRRL181. Appl Microbiol Biotechnol 94(2):413–423. doi:10.1007/s00253-011-3631-6
14. Karnaouri A, Topakas E, Paschos T, Taouki I, Christakopoulos P (2013) Cloning, expression and characterization of an ethanol tolerant GH3 β-glucosidase from *Myceliophthora thermophila*. PeerJ 1:e46. doi:10.7717/peerj.46
15. Kaya F, Heitmann JA Jr, Joyce TW (1995) Influence of surfactants on the enzymatic hydrolysis of xylan and cellulose. TAPPI J 78(10):150–157
16. Kuhad RC, Gupta R, Singh A (2011) Microbial cellulases and their industrial applications. Enzyme Res 2011:280696. doi:10.4061/2011/280696
17. Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35(5):377–391. doi:10.1007/s10295-008-0327-8
18. Di Lauro B, Rossi M, Moracci M (2006) Characterization of a beta-glycosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*. Extremophiles 10(4):301–310. doi:10.1007/s00792-005-0500-1
19. Li G, Jiang Y, Fan X, Liu Y (2012) Molecular cloning and characterization of a novel β-glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and glucose-tolerance from soil metagenomic library. Bioresour Technol 123:15–22
20. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506–577
21. Manning M, Colón W (2004) Structural basis of protein kinetic stability: resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias toward beta-sheet structure. Biochemistry 43(35):11248–11254. doi:10.1021/bi0491898
22. Matteotti C, Thonart P, Francis F, Haubruge E, Destain J, Brasseur C, Bauwens J, De Pauw E, Portetelle D, Vandenbol M (2011) New glucosidase activities identified by functional screening of a genomic DNA library from the gut microbiota of the termite *Reticulitermes santonensis*. Microbiol Res 166(8):629–642. doi:10.1016/j.micres.2011.01.001
23. Pérez-Pons JA, Rebordosa X, Querol E (1995) Properties of a novel glucose-enhanced β-glucosidase purified from *Streptomyces* sp. (ATCC 11238). Biochim Biophys Acta 2:145–153
24. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8(10):785–786. doi:10.1038/nmeth.1701
25. Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005) InterProScan: protein domains identifier. Nucleic Acids Res 33(Suppl 2):W116–W120. doi:10.1093/nar/gki442
26. Rajasree KP, Mathew GM, Pandey A, Sukumaran RK (2013) Highly glucose tolerant β-glucosidase from *Aspergillus unguis*: NII 08123 for enhanced hydrolysis of biomass. J Ind Microbiol Biotechnol 40(9):967–975. doi:10.1007/s10295-013-1291-5
27. Rather M, Mishra S (2013) β-Glycosidases: an alternative enzyme based method for synthesis of alkyl-glycosides. Sustain Chem Process 1(1):7. doi:10.1186/2043-7129-1-7
28. Riou C, Salmon J-M, Vallier M-J, Gunata Z, Barre P (1998) Purification, characterization, and substrate specificity of a novel highly glucose-tolerant beta-glucosidase from *Aspergillus oryzae*. Appl Environ Microbiol 64(10):3607–3614
29. Saha BC, Bothast RJ (1996) Production, purification, and characterization of a highly glucose-tolerant novel beta-glucosidase from *Candida peltata*. Appl Environ Microbiol 62(9):3165–3170
30. Sanchez-Ruiz JM (2010) Protein kinetic stability. Biophys Chem 148(1–3):1–15. doi:10.1016/j.bpc.2010.02.004
31. Schomburg I, Chang A, Placzek S, Söhngen C, Rother M, Lang M, Munaretto C, Ulas S, Stelzer M, Grote A, Scheer M,

Schomburg D (2013) BRENDA in 2013: integrated reactions, kinetic data, enzyme function data, improved disease classification: new options and contents in BRENDA. Nucleic Acids Res 41(Database issue):D764–D772. doi:10.1093/nar/gks1049
32. Seo D-J, Fujita H, Sakoda A (2011) Effects of a non-ionic surfactant, Tween 20, on adsorption/desorption of saccharification enzymes onto/from lignocelluloses and saccharification rate. Adsorption 17(5):813–822. doi:10.1007/s10450-011-9340-8
33. Shah SR (2013) Chemistry and applications of cellulase in textile wet processing. Res J Eng Sci 2(7):1–5
34. Sørensen A, Lübeck M, Lübeck P, Ahring B (2013) Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomolecules 3(3):612–631. doi:10.3390/biom3030612
35. Steele HL, Jaeger KE, Daniel R, Streit WR (2009) Advances in recovery of novel biocatalysts from metagenomes. J Mol Microbiol Biotechnol 16(1–2):25–37. doi:10.1159/000142892
36. Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B 109(1):1–62
37. Teugjas H, Väljamäe P (2013) Selecting β-glucosidases to support cellulases in cellulose saccharification. Biotechnol Biofuels 6(1):105. doi:10.1186/1754-6834-6-105
38. Tsitsimpikou C, Christakopoulos P, Makropoulou M, Kekos D, Macris BJ, Kolisis FN (1997) Role of methanol on the catalytic behavior of β-glucosidase from *Fusarium oxysporum*. Biotechnol Lett 19(1):31–33. doi:10.1023/A:1018306802099
39. Uchiyama T, Miyazaki K, Yaoi K (2013) Characterization of a novel β-glucosidase from a compost microbial metagenome with strong transglycosylation activity. J Biol Chem 288(25):18325–18334. doi:10.1074/jbc.M113.471342
40. Wang Y, Li J, Xu Y (2011) Characterization of novel β-glucosidases with transglycosylation properties from *Trichosporon asahii*. J Agric Food Chem 59(20):11219–11227. doi:10.1021/jf203693v
41. Watt DK, Ono H, Hayashi K (1998) Agrobacterium tumefaciens beta-glucosidase is also an effective beta-xylosidase, and has a high transglycosylation activity in the presence of alcohols. Biochim Biophys Acta 1385(1):78–88
42. Xu H, Xiong A-S, Zhao W, Tian Y-S, Peng R-H, Chen J-M, Yao Q-H (2011) Characterization of a glucose-, xylose-, sucrose-, and D-galactose-stimulated β-glucosidase from the alkalophilic bacterium *Bacillus halodurans* C-125. Curr Microbiol 62(3):833–839. doi:10.1007/s00284-010-9766-3
43. Yan TR, Lin CL (1997) Purification and characterization of a glucose-tolerant beta-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Biochem 61(6):965–970
44. Zheng F, Huang J, Yin Y, Ding S (2013) A novel neutral xylanase with high SDS resistance from *Volvariella volvacea*: characterization and its synergistic hydrolysis of wheat bran with acetyl xylan esterase. J Ind Microbiol Biotechnol 40(10):1083–1093. doi:10.1007/s10295-013-1312-4
45. Zhou J, Bao L, Chang L, Liu Z, You C, Lu H (2012) Beta-xylosidase activity of a GH3 glucosidase/xylosidase from yak rumen metagenome promotes the enzymatic degradation of hemicellulosic xylans. Lett Appl Microbiol 54(2):79–87. doi:10.1111/j.1472-765X.2011.03175.x
46. Zilz L, Rau M, Budag N, Scharf M, Cavaco-Paulo A, Andreaus J (2013) Nonionic surfactants and dispersants for biopolishing and stonewashing with *Hypocrea jecorina* cellulases. Color Technol 129(1):49–54. doi:10.1111/cote.12003