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Biochemical characterization of a novel thermostable β-glucosidase from *Dictyoglomus turgidum*

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ABSTRACT

Dtur_0462 gene from the hypertermophilic bacterium *Dictyoglomus turgidum*, encoding a β-glucosidase, was synthetically produced and expressed in *Escherichia coli* BL21(DE3)-RIL. *Dtur*βGlu was purified to homogeneity by affinity chromatography and its homotetrameric structure was determined by gel filtration. The monomer is composed by 418 amino acidic residues and showed high sequence similarity with Glycoside Hydrolases (GHs) belonging to GH1 family. The maximum activity of *Dtur*βGlu was observed at 80 °C and at pH 5.4. *Dtur*βGlu was stable in the range of pH 5–8 and retained 70% of its activity after 2 h of incubation at 70 °C. Metal ions and chemical reagents differently influenced the β-glucosidase activity; furthermore, *Dtur*βGlu displays a good ethanol and glucose tolerance (K_i 750 mM). The enzyme is active on *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu) (K_m 0.84 mM) and *p*-nitrophenyl-β-D-galactopyranoside (*p*NPGal) (K_m 1.36 mM) and shows a broad substrate specificity towards natural compounds as salicin, cellobiose and genistin. The ability to hydrolyze different substrates, the activation in the presence of surfactants, the good thermal resistance, and finally the high glucose and ethanol tolerance make this enzyme a good candidate for industrial applications.

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1. Introduction

β-glucosidases (EC 3.2.1.21) are a heterogeneous group of enzymes that catalyze the hydrolysis of β-D-glycosidic bonds in cellobiose, oligoglucosaccharides as well as in several other glycoconjugates and are often involved in transglycosylation reactions [1]. Based on the substrate specificity the β-glucosidases are classified in Class I, that hydrolyze the aryl β-glycosides (i.e. salicin), Class II, that show a strong preference towards cellobiose and cello-oligosaccharides, Class III, that display a broad substrate specificity hydrolysing the β-glycosidic bond between glycoside residues and a wide variety of aglycone molecules [2]. On the other end, based on their amino acid sequences and well-conserved sequence motifs, β-glucosidases have been classified into glycoside hydrolase (GH) families GH1, GH2, GH3, GH5, GH9, GH30 and GH116, according to CAZY web server (http://www. cazy.org) [3,4]. β-glucosidases are widely distributed and have important roles in many biological pathways, such as degradation of structural and storage polysaccharides, cellular signaling, oncogenesis, host-pathogen interactions, as well as in several biotechnological applications [5,6]. In this regard, β-glucosidases are commonly used in the biorefinery for biomass conversion, taking part in the final step of cellulose breakdown that produces glucose [7], in the food industry to enhance the quality of the beverages and foods [8]. Moreover, β-glucosidases can hydrolyse isoflavonoid-glucosides present in fruits, tea and vegetables, particularly soybeans, generating the isoflavones, well-knowns as phytohormones. These molecules are used in different fields of medicine as antitumor agents or for the treatment and prevention of cardiovascular diseases and osteoporosis [9]. In addition to hydrolytic activity, many β-glucosidases can catalyze the formation of glycosidic bonds by either thermodynamically controlled reverse hydrolysis, or kinetically controlled transglycosilation. This feature, makes β-glucosidases promising biocatalysts for the synthesis of stereo- and regiospecific glycosides or oligosaccharides which are potentially useful as functional materials, nutraceuticals, or pharmaceuticals [10,11]. With the growing importance of β-glucosidases and their application in different biotechnological fields, many efforts have been made to isolate and characterize new enzymes from different sources to obtain better performance. Thermostable β-glucosidases offer several advantages in industrial applications over mesophilic counterparts, promoting faster reactions, high solubility of the substrate, a lower risk of contamination, and also lowering the solution viscosity and increasing the miscibility of the solvent [12]. In this context, the hyperthermophilic bacterium, *Dictyoglomus turgidum*, represents an appealing microorganism both for its hyperthermophilicity [13] and because it grows on a wide range of substrates including starch, cellulose, pectin, carboxymethylcellulose and

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lignin [14]. Its genome was sequenced and analyzed for carbohydrases and contains six annotated β-glucosidases (Dtur_0219; Dtur_0289; Dtur_0321; Dtur_0462; Dtur_1723, and Dtur_1799) [15] and one, Dtur_0219, well characterized belonging to GH3 family [16,17]. With the aim to investigate a new thermostable β-glucosidase, we have chosen Dtur_0462 [15] belonging to GH1 family encoding the enzyme herein named *Dtur*βGlu. The gene was synthetically produced and codon-adapted to *Escherichia coli*. The recombinant protein was biochemically characterized also regarding different substrates hydrolysis and physical-chemical parameters.

2. Materials and methods

2.1. Expression of DturβGlu in E. coli

Dturβglu (Dtur_0462) from *D. turgidum* DSM 6724 genome (GenBank: NC_011661.1) was synthesized by Genewiz (GENEWIZ LLC 115 Corporate Blvd.South Plainfield, NJ USA) with the following changes: 1) the codon usage of the gene was optimized for the expression in *E. coli*; 2) at 5′ and 3′ends of the gene the *Nde*I and *Xho*I restriction sites, respectively, were inserted to allow the cloning in pET-30b (+) vector (Novagen). The recombinant vector pET30/*Dturβglu* was used to transform *E. coli* strain BL21(DE3)-RIL. The transformants were selected on LB plates containing ampicillin 50 μg/ml and chloramphenicol 33 μg/ml at 37 °C and grown in same condition in LB until 0.5 OD_{600nm}. Gene expression was then induced by the addition of 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were harvested by centrifugation 2 h later.

2.2. Enzyme purification

Pellets from 1 l cultures were re-suspended in 50 mM Tris-HCl pH 8.0 supplemented with an protease inhibitors cocktail and disrupted by sonication; then the suspension was clarified by ultracentrifugation [18]. The harvested soluble fraction was heated at 70 °C for 15 min, and therefore centrifuged to remove protein aggregates [19].

*Dtur*βGlu was purified almost to homogeneity in a one stage process using affinity chromatography on HisTrapHP column (GE Healthcare) [20]. Proteins concentration was determined using BSA as the standard [21] and protein homogeneity was estimated by SDS-PAGE (12%).

2.3. Size-exclusion chromatography

Purified *Dtur*βGlu protein was analyzed by size exclusion chromatography. 100 μg of sample was loaded on BIOsep-SEC-S4000 column (Phenomenex) (300 × 7.8 mm) equilibrated in 50 mM Sodium Phosphate pH 7.2. A constant flow rate of 0.5 ml/min was applied. The column was calibrated with albumin (75 kD), aldolase (158 kD), ferritin (440 kD), and thyroglobulin (669 kD).

2.4. β-glucosidase assay

β-glucosidase assay was performed using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu) as substrate. The reaction mixture containing 4 mM *p*NPGlu, 50 mM citrate phosphate buffer pH 5.4, 3.4 nM *Dtur*βGlu was incubated at 80 °C for 10 min. The reaction was stopped by addition of 100 μl of cold 0.2 M Na_2CO_3. The concentration of the released *p*-nitrophenol (millimolar extinction coefficient, 18.5 $mM^{-1}\,cm^{-1}$) was determined by measuring A_{405nm}, using microplate reader (Synergy H4 Biotek). One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol (*p*NP) in a minute under the assay conditions. All assays were performed in triplicate.

2.5. Substrate specificity and kinetic parameters

The hydrolytic activity of *Dtur*βGlu was determined on several substrates [6] as: *p*NPGlu, *o*NP-β-D-glucopyranoside (*o*NPGlu), *p*NP-β-D-xylopyranoside (*p*NPXyl), *p*NP-β-D-galactopyranoside (*p*NPGal), pNP-α-galactopyranoside (*p*NPαGal), *o*NP-β-D-galactopyranoside (*o*NPGal), cellobiose and salicin. Aliquots of *Dtur*βGlu were incubated with saturating concentrations of substrate in 50 mM citrate/phosphate buffer (pH 5.4) for 10 min at 80 °C (standard assay conditions) and the activity was measured by the release of *p*NP and *o*-nitrophenol (*o*Np). The concentration of the released *o*NP (millimolar extinction coefficient, 21.3 $mM^{-1}\,cm^{-1}$) was determined by measuring A_{420nm}. When cellobiose and salicin were used as substrate, the amount of glucose was determined with D-Glucose Assay Kit (GOPOD Format) (Megazyme) according to the manufacturer's protocol and 1 unit (U) of activity is defined as the amount of enzyme which is required to release 1 μmol of glucose per min under the assay conditions.

To determine kinetic parameters of *Dtur*βGlu different concentrations of various substrates were used in the following ranges: 0.1–5 mM for *p*NPGlu and *p*NPGal, 0.1–6.5 mM for *o*NPGlu and *o*NPGal, 0.5–12 mM for *p*NPXyl and 0.5–40 mM for salicin. The enzymatic activity was determined as reported above. The enzyme kinetic parameters, K_m, V_{max}, k_{cat} and k_{cat}/K_m, were calculated by non-linear regression analysis (GraphPad 6.0 Prism software).

2.6. Effect of pH and temperature on enzyme activity

The optimal pH value was determined at 80 °C performing the β-glucosidase assay in the following buffers: 50 mM glycine-HCl for range pH 2.0–3.0, 50 mM citrate phosphate buffer for pH 3.0–6.0, 50 mM phosphate buffer for pH 7.0–8.0, and 50 mM glycine-NaOH for pH 9.0. All the buffers were measured at indicated temperatures.

The pH stability was determined performing the assay after pre-incubation of the enzyme in buffers ranging from pH 3.0 to 9.0 at 37 °C for 1 h. The residual enzymatic activity was determined under the standard conditions (pH 5.4, 80 °C, 10 min).

The optimal temperature was examined by measuring the enzyme activity ranging from 30 °C to 100 °C at its optimal pH. The thermostability assay was carried out by incubating the enzyme at temperatures ranging from 70 °C to 100 °C for different times (30–240 min), under optimal pH conditions. The residual activity was determined under assay conditions reported previously.

2.7. Effect of chemicals on enzyme activity

The effects of different chemicals were tested on *Dtur*βGlu activity. Metal ions (Li^+, Cu^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+} Co^{2+}, Zn^{2+}) were added in the reaction mix as LiCl, $CuCl_2$, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CoSO_4$, $ZnSO_4$ at final concentration of 1 mM; the EDTA, chelating agent, was added at the same concentration. Non-ionic detergents, (Triton X-100 and Tween-20) ionic detergent (SDS), and dimethyl sulfoxide (DMSO) were supplemented in the assay mixture at 0.5% concentration. The monosaccharides (glucose, galactose, xylose and arabinose) were added at final concentration of 2.5, 5, 10, 50 or 100 mM. Primary and secondary alcohols were also tested: ethanol in a range from 5% to 25% and methanol, propanol and 2-propanol in a range from 0.4 M to 2.0 M. The residual activity was determined under the standard condition.

2.8. Hydrolysis of genistin to genistein

The hydrolytic reaction was performed in 200 μl of mixture containing 0.25 mM genistin (dissolved in ethanol 70%/0.1% acetic acid), 0.2 μg of enzyme under standard conditions for 60 min. The enzyme was inactivated for 10 min at 100 °C, the mix was centrifuged at 16000 g for 15 min and the supernatant was loaded on a Vydac C18 reverse

phase column (7 μm, 4.6 × 250 mm) connected to a HPLC system (Perkin Elmer series 200) equipped with an UV detector at 254 nm. The sample was eluted in a mixture of acetonitrile and 100 mM citrate-phosphate buffer pH 3.0 (70:30). The genistin and genistein, used as standard, were detected with retention times of 9.1 and 10.2 min respectively.

```
Dtur_0462          MVKYKFPEGFLWGTATASHQIEGDNFYN-----DWWEFEK-QGKVKNGQV
TmBglA             MNVKKFPEGFLWGVATASYQIEGSPLADGAGMSIWHTFSHTPGNVKNGDT
DtGH               MAKLVFPKEFLWGAATASYQIEGAWNEDGKGESIWDRFAHTPGTIYENQN
TeBglA             -MEVKFPKDFLWGTATSSYQIEGAVNEDGRTPSIWDTFSKTEGKTYNGHT
                        **: ****.**:*:****    :      *  * :  *.  :..

Dtur_0462          SGKACDSWNRYEEDFDLIEKLNNNAYRFSIEWSRIEPE-EGRFDESALER
TmBglA             GDVACDHYNRWKEDIEIIEKLGVKAYRFSISWPRILPEGTGRVNQKGLDF
DtGH               GDIACDHYHRYEEDVELMAEIGLKAYRFSISWPRIFPEGRGKLNPKGVYF
TeBglA             GDVACDHYHRYKEDVEILKEIGVKAYRFSIAWPRIFPE-EGKYNSKGMDF
                   .. *** ::*::**.::: ::. :****** *.** **  *: : ..:

Dtur_0462          YRSMLISLRRRNIEPFVTLHHFTNPLWMAKRGG-WLNPDIIDYYLRYVKK
TmBglA             YNRIIDTLLEKGITPFVTIYHWDLPFALQLKGG-WANREIADWFAEYSRV
DtGH               YEKLIDKLLEKNIKPAITLYHWDLPQALEDKGG-WLNRDTAKYFSEYANF
TeBglA             YKRLVDELLKKDIMPTATIYHWDLPQWAYDKGGGWLNRDSVKWYVEYATK
                   *. ::  * .:.* *  *::*:  *     :** * * :  .:: .*

Dtur_0462          IVSEFKDLVNYWMTINEPNAYAFMAYLYGQFPPQGKSLIKMLRVLNNMAK
TmBglA             LFENFGDRVKNWITLNEPWVVAIVGHLYGVHAPGMRDIYVAFRAVHNLLR
DtGH               MFYKFGDVVPIWITLNEPFVSAFLGYAWGWHAPGKKDMKGAFVAGHNMLL
TeBglA             LFEELGDVIPLWITHNEPWCASILSYGIGEHAPGHKNYREALIAAHHILL
                   :. :: * :  *:* ***   :::.:  * ..*  :.    : . :::

Dtur_0462          AHAKAYEVIHQISPDAK-VSIAYNVIYFEPKNPNSFIDRKFANFGDRIYN
TmBglA             AHARAVKVFRETVKDGK-IGIVFNNGYFEPAS-EKEEDIRAVRFMHQFNN
DtGH               AHGLAVQAYRDGGYKGN-IGITINVATVYPET-NSEEDLKAAEKQDAFGN
TeBglA             SHGEAVKAFREMNIKGSKIGITLNLTPAYPAS-EKEEDKLAAQYADGFAN
                   :*. * :. ::   ... :.*. *     * . :.  *   ..  . : *

Dtur_0462          R-VFIETLLTGKFSSPFIKEEIP--------------YAKNTLDYLGINY
TmBglA             YPLFLNPIYRGDYPELVLEFAREYLPENYKD---DMSEIQEKIDFVGLNY
DtGH               R-WFIDPIFKRKYPEIIWRILEENNWSFVFP-ASDFDIISSPIDFMGINY
TeBglA             R-WFLDPIFKGNYPEDMMELYSKIIGEFDFIKEGDLKTISVPIDFLGVNY
                      *::.:   .:.. . .                    .  :*::*:**

Dtur_0462          YTRILMGLKMGSPE---------GETSDFGWEIYPEGIYKVVKRFYGLT-K
TmBglA             YSGHLVKFDPDAPAKVSFVERDLPKTAMGWEIVPEGIYWILKKVKEEYNP
DtGH               YTRNIVAYDKNSHLGVKRVEGPNEHTDMGWEVYPDGLYDILIQLYRDY-K
TeBglA             YTRSIVKYNEDSMLKAENVPGPGKRTEMGWEISPESLYDLLKRLDREYTK
                   *:  ::  . .:             : :***: *:.:* :: :.

Dtur_0462          KPIYITENGIS---------DAKDEKRPKYLISHLIQLHRAIEEGVDVRG
TmBglA             PEVYITENGAAFDDVVSEDGRVHDQNRIDYLKAHIGQAWKAIQEGVPLKG
DtGH               IPIYITENGAAYNDTVE-DGRIRDINRINYLKEHIKRAYFAIRDGVDLRG
TeBglA             LPMYITENGAAFKDEVTEDGRVHDDERIEYIKEHLKAAAKFIGERGNLKG
                     :****** :          :* :* .*:  *:      * :   ::*

Dtur_0462          YFHWSLMDNFEWAEGFLQRFGLFETDFNTFERKWRESARIYSEIAKNNGI
TmBglA             YFVWSLLDNFEWAEGYSKRFGIVYVDYSTQKRIVKDSGYWYSNVVKNNGL
DtGH               YFVWSLMDNFEWAHGYSKRFGIIYVDYNTQKRILKDSAYFYKKIIEENGV
TeBglA             YFVWSLMDNFEWAHGYSKRFGIVYVDYETQKRILKDSALWYKEVIQKNSM
                   ** ***:******.*: :***:. .*:.* :*  ::*.  *.:: ::*.:

Dtur_0462          TEAMEEKFLK
TmBglA             ED-------- (37%)
DtGH               DG-------- (36%)
TeBglA             S--------- (34%)
```

Fig. 1. Multiple alignment of *Dtur*βGlu with other GHs 1 from *Thermotoga maritima D. thermophilum* and *Thermoanaerobacter ethanolicus*. The conserved motifs NEP and ITENG are boxed. The two conserved glutamate residues, the general acid/base (E159) and the catalytic nucleophile (E324), involved in the catalysis are highlighted in bold.

Table 1
Purification steps of *Dtur*βGlu.

Purification steps	Total proteins (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield
Cellular extract	152.8	3702.6	24.2	1	100%
Heat treatment	54.94	1502.23	40.7	1.68	40.5%
Affinity chromatography	1	160	160	6.61	4.32%

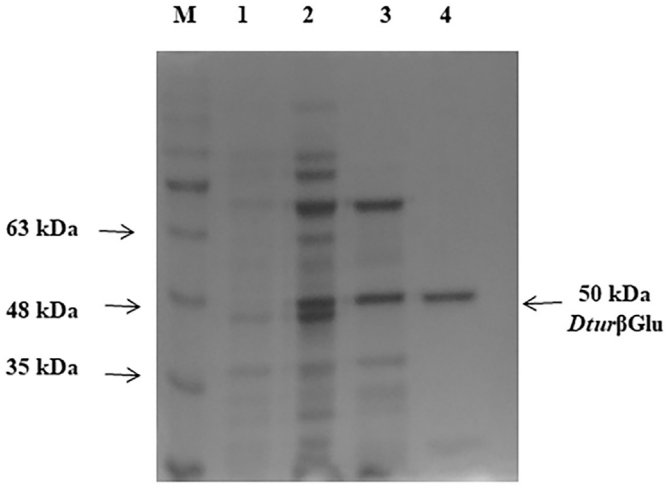

Fig. 2. SDS-PAGE analysis of the recombinant *Dtur*βGlu. M) Protein Marker; 1) cellular extract from not induced cells; 2) cellular extract from IPTG -induced cells; 3) heat-treated sample; 4) affinity chromatography by His-trap.

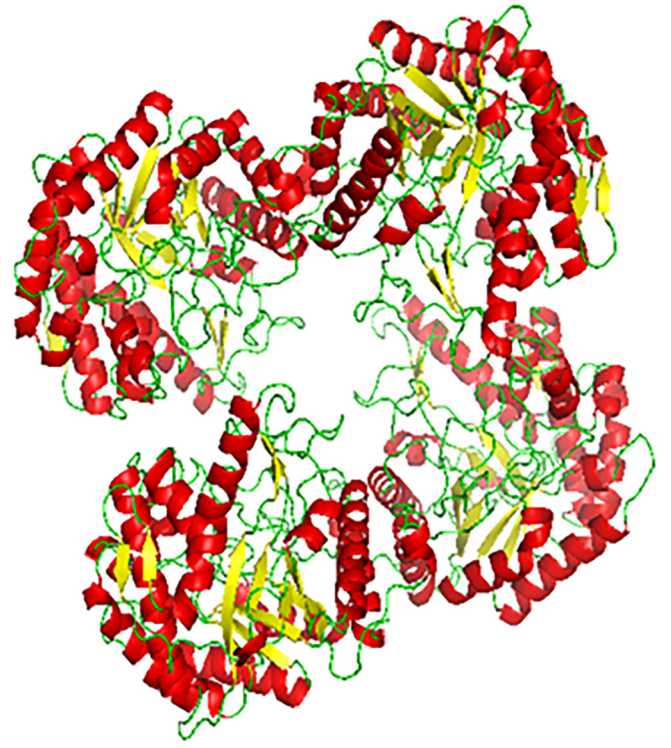

Fig. 3. Homology model of *Dtur*βGlu: The structural model was performed by Swiss Model server using BGLPf from *P furiosus*, TnBgl1A from *T. neapolitana* and Ssβ-Glc1 from *S. solfataricus* as templates.

Table 2
Substrate specificity of *Dtur*βGlu.

Substrate	Specific activity (U/mg)	Relative activity (%)
*p*NPGlu	160	100
*o*NPGlu	21	13
*p*NPGal	155	96.87
*o*NPGal	23	14
*p*NPXyl	10	6.2
Salicin	67	42
Cellobiose	2	1.25
*p*NPαGal	N.D	N.D

3. Results and discussion

3.1. Bioinformatic analysis of DturβGlu

The genome of *D. turgidum* DSM 6724 was recently sequenced [15] and a depth analysis of CAZy database (http://www.cazy.org/) has revealed a great number of potentially interesting enzymes. We have chosen to study a new β-glucosidase classified as GH1 encoded by Dtur_0462. The putative protein, consisting of 418 aa, was analyzed by BlastP and aligned by ClustalW with different GH1 proteins from thermophilic bacteria previously characterized. In details, the putative protein shows 37% identity with *Tm*-BglA (CAA52276.1) from *Thermotoga maritima* [22], 36% with *Dt*GH (ACI19973.1) from *D. thermophilum* [23], and 34% with *Te*-BglA (ADD25173.1) from *Thermoanaerobacter ethanolicus* [24] (Fig. 1). Further bioinformatic analyses performed with Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2) showed that the glutamate residues, E159 and E324, in NEP and ITENG conserved motifs have been identified as potential residues acting as the acid/base and nucleophile catalyst for the GH1 respectively [5].

3.2. Expression and purification of DturβGlu

Dtur_0462 was synthetically produced and codon-adapted to *E. coli* genetic system. The gene was cloned in the expression vector pET30b (+) and the recombinant protein, with His tagged at C-terminal, named *Dtur*βGlu, was expressed in *E. coli* BL21(DE3)-RIL strain. The protein was purified by heat-treatment and His-trap affinity chromatography as summarized in Table 1.

Table 3
Kinetic parameters of *Dtur*βGlu.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*p*NPGlu	0.84	8710	1×10^4
*o*NPGlu	12.08	3417	282
*p*NPGal	1.36	9924	7.3×10^3
*o*NPGal	1.63	1394	855
*p*NPXyl	2.79	651	233
Salicin	8.12	659	81

Table 4
Comparison of kinetic parameters of *Dtur*βGlu with some thermophilic β-glucosidases.

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Reference
*p*NPGlu	*Dtur*βGlu	0.84	8710	1×10^4	This work
	Tm-BglA	0.63	268.1	425	[24]
	Te-BglA	0.79	58.8	74.43	[24]
	*Dt*GH	1.15	238	207	[23]
	Sβ-gly	0.60	634	1058	[29]
	Ttβ-gly	0.1	23.4	227	[30]
	Pk-gly	1.79	1650	922	[31]
Salicin	*Dtur*βGlu	8.12	659	81	This work
	Tm-BglA	12	189.7	15.8	[24]
	Te-BglA	4.7	110.4	23.4	[24]

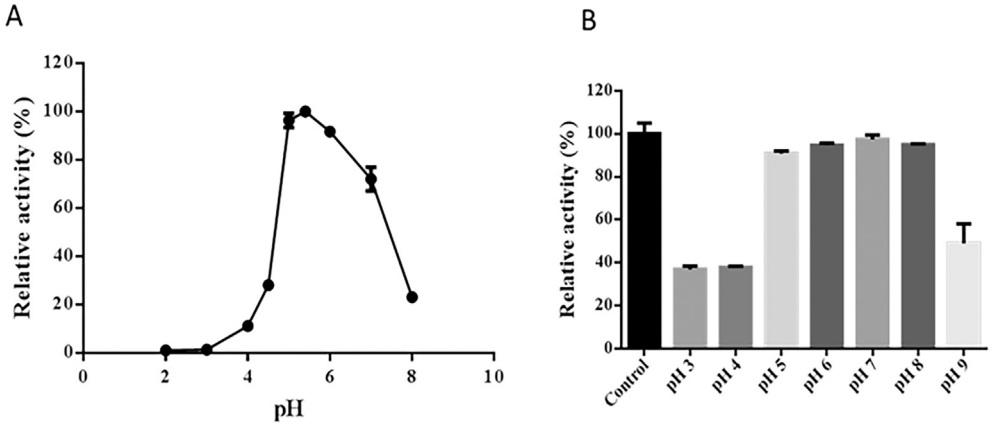

Fig. 4. Effect of pH on enzymatic activity of *Dtur*βGlu. A) pH optimum was measured in buffers ranging from pH 2.0 to pH 8.0. B) The enzyme was incubated in various buffers (pH 3.0–9.0) for 1 h and assayed for residual activity at the optimal conditions.

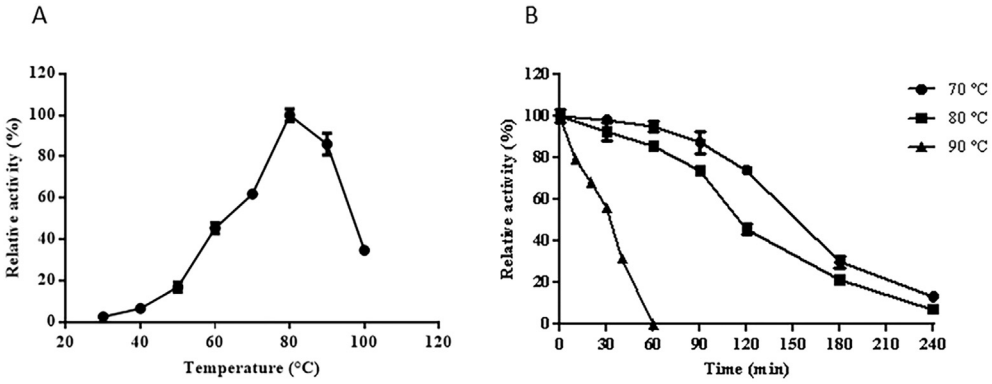

Fig. 5. Effect of temperature on enzymatic activity of *Dtur*βGlu. A) Temperature optimum was determined in the range 30–100 °C. B) Thermostability: the enzyme was incubated at 70 °C, 80 °C, 90 °C, 100 °C for different times and then assayed for residual activity at the optimal conditions.

As revealed by SDS-PAGE analysis (Fig. 2), recombinant *Dtur*βGlu showed a single band with a molecular mass of ~50 kDa, according with the predicted molecular weight of 50,615 Da corresponding to the theoretical value. The yield of the purified protein was about 1 mg for a liter of culture.

To gain insight into the quaternary structure of the enzyme, a homology-based model (SWISS-MODEL) of *Dtur*βGlu was obtained using BGLPf from *Pyrococcus furiosus* [25,26], TnBgl1A from *T. neapolitana* [27] and Ssβ-Glc1 [28] from *Sulfolobus solfataricus* as templates. The model created of *Dtur*βglu indicated a possible tetrameric structure of the enzyme (Fig. 3). The 3D structure was confirmed by size exclusion chromatography analysis that showed a molecular weight of about 200 kDa.

3.3. Substrate specificity of DturβGlu

The hydrolytic activity of *Dtur*βGlu was tested on different substrates both synthetic, as reported below, and natural like salicin, and cellobiose. The results (Table 2) showed that the enzyme displays the highest specific activity towards *p*NPGlu (160 U/mg), *p*NPGal (155 U/mg) and salicin (67 U/mg). It was less active on *o*NPGal (23 U/mg), *o*NPGlu (21 U/mg), *p*NPXyl (10 U/mg) and cellobiose (2 U/mg).

The values of enzymatic activity obtained were also compared with those of another characterized β-glucosidase of *D. turgidum* belonging to GH3 (*Dt*GH3) [16,17]. From this analysis, *Dtur*βGlu turned out to be five and two times more active towards *p*NPGlu and *o*NpGlu than *Dt*GH3; furthermore, *Dtur*βGlu displayed higher specific activity vs *p*NPGal and *o*PNGal whereas *Dt*GH3 did not show any activity on these substrates.

The kinetic parameters of *Dtur*βGlu were determined for different substrates (Table 3), and those towards *p*NPGlu and Salicin were compared with thermophilic β-glucosidases previously characterized (Table 4). *Dtur*βGlu has K_m value towards *p*NPGlu comparable to other reported enzymes showing similar affinity, while it has the highest k_{cat}/K_m value towards both substrates, showing the best catalytic efficiency [23,24,29–31]. Altogether these features make *Dtur*βGlu as a good candidate for possible applications.

Table 5
Effect of metal ions and chemicals on *Dtur*βGlu activity.

Metal ion or chemical agent	Concentration	Relative activity
None	1 mM	100%
$CuCl_2$	1 mM	44.23%
$ZnSO_4$	1 mM	46%
LiCl	1 mM	98.5%
$MgCl_2$	1 mM	53.90%
$CaCl_2$	1 mM	56.79%
$MnCl_2$	1 mM	38.23%
$CoSO_4$	1 mM	47.66%
EDTA	1 mM	100%
SDS	0.5%	0.66%
Triton X-100	0.5%	130%
Tween 20	0.5%	173%

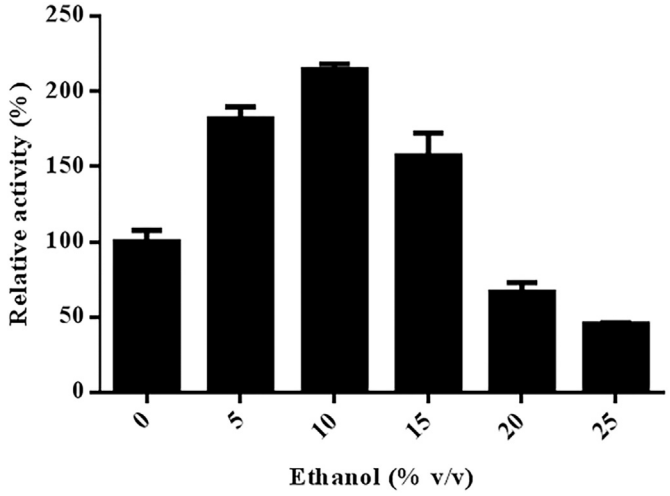

Fig. 6. Effect of ethanol on enzymatic activity of *Dtur*βGlu. The purified enzyme was assayed in the presence of different concentration of ethanol under optimal conditions.

3.4. Effect of pH and temperature on enzyme activity

To evaluate the dependence of *Dtur*βGlu activity from pH, the enzyme was tested over a range of 2.0–8.0 using *p*NPGlu as substrate. The maximal activity was observed at pH 5.4 (Fig. 4A). At pH values of 5.0 and 7.0 the enzymatic activity was approximatively 90% and 72% respectively, like several β-glucosidases from thermophilic bacteria such as *Thermus thermophilus* [32] *Caldicellulosiruptor saccharolyticus* [33] and *Thermoanaerobacter brockii* [34]. On the contrary, the *Dt*GH of *D. thermophilus* showed the optimal value at pH 7.0 and activity dropped at pH 5.0 [23].

The stability of the enzyme was determined at different pH, ranging 3.0–8.0, after 1 h of incubation.

The results showed that *Dtur*βGlu was fairly stable in the pH range of 5.0–8.0, retaining over 90% of its activity (Fig. 4B).

The effect of temperature on enzymatic activity was also measured. The maximal activity was observed at 80 °C (Fig. 5A), showing that the optimal temperature is slightly higher than that of other β-glucosidases from thermophilic microorganisms such as *Halothermothrix orenii* [35], *Scytalidium thermophilum* [36], *Talaromyces thermophilus* [37] and *Myceliophthora thermophila* [38].

Thermostability of the enzyme at various temperatures was monitored by measuring its residual activity for different incubation times. After 90 min at 80 °C the relative activity was about 80%, while after 2 h of incubation at 70 or 80 °C the residual activity of *Dtur*βGlu was still 70% and 50% respectively (Fig. 5B) showing a good thermostability compared with other thermophilic β-glucosidases. In detail β-glucosidases from fungi such as *Scytalidium thermophilum* [36] and *Fusarium oxysporum* [39] showed an half-life of 20 min at 55 °C and about 120 min at 80 °C respectively; in thermophilic bacterium such as *Thermus* sp. IB-21, the BglA and BglB showed an half-life <0.4 h and 2.7 h at 80 °C respectively [40] while in *T. thermophilus* HB27 the thermostability of *Tt*βGly was 10 min at 90 °C [41].

In conclusion, the results show that *Dtur*βGlu, due to the high thermostability and pH tolerance in a wide pH range, might be exploited in various industrial fields.

3.5. Effect of metal ions and chemicals on enzyme activity

The effect of various metal ions, chemicals and detergents on *Dtur*βGlu activity was studied and the results are reported in Table 5. Cu^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} inhibited the activity of *Dtur*βGlu by ~50%, Ca^{2+} and Mg^{2+} ~30%, while Li^{+} didn't affect the enzyme activity. The interaction with metal ions could determine alterations of protein's secondary and/or tertiary structure responsible for the decrease of the enzymatic activity.

*Dtur*βGlu activity did not change in the presence of EDTA, indicating that this enzyme does not require metal ions as cofactors.

The influence of various surfactants such as SDS, Tween-20 and Triton X-100 was also determined. The presence of the ionic detergent affected totally *Dtur*βGlu activity while Tween-20, Triton X-100, increased the enzymatic activity of 180% and 120% respectively. The enzymatic activity of other β-glucosidases, such as the β-glucosidase of *Fervidobacterium islandicum*, is reported to be improved by non-ionic detergents [42]. Interestingly, as also reported in several studies, the addition of surfactants plays a role in thermal stabilization of the enzymes thus potentially increasing-conversion yields [43,44], furthermore the non-ionic detergents can help the enzymes against the shear-induced deactivation during biomass treatment.

3.6. Effect of organic solvents and monosaccharides on DturβGlu activity

Various organic solvents were tested to verify their effect on enzymatic activity: DMSO had only a moderate effect on *Dtur*βGlu activity (66% of relative activity at 0.5% DMSO), while various alcohols such as methanol, or 2-propanol or propanol increased the enzymatic activity;

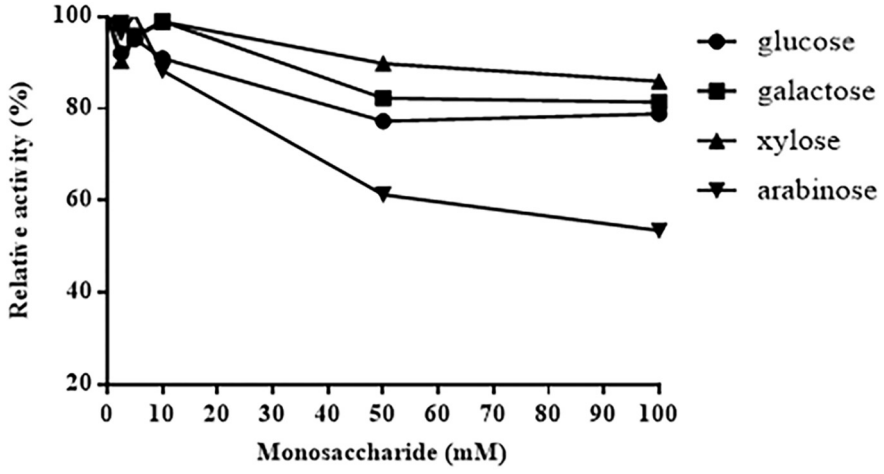

Fig. 7. Effect of monosaccharides on enzymatic activity of *Dtur*βGlu. The purified enzyme was assayed in the presence of different concentration of various monosaccharides under optimal conditions.

in particular 1.6 M 2-propanol or propanol increased the activity of 25% while methanol, at the same concentration, enhanced the activity of 50% (data not shown). A greater effect was achieved in the presence of ethanol as shown in Fig. 6 where *Dtur*βGlu activity was determined in a range 5% - 20%. of ethanol. The enhancement of enzymatic activity could be due to additional hydrogen bonds that may contribute to the stabilization of the enzyme, but further information must be obtained from structural studies. Most of β-glucosidases show low activity in presence of alcohols because of reduced flexibility and destabilization of the enzyme [45]. The discovery of several β-glucosidases, naturally tolerant to organic solvents, is still limited, and to improve this capacity various enzymes were submitted to mutagenesis [46]. Many examples, reported in literature, describe as the change of some residues or the presence of specific motifs in the aminoacidic sequence can enhance the tolerance of the β glucosidases over ethanol and organic solvents [45–47]. Furthermore, we investigated the inhibition of β-glucosidases by various monosaccharides, since their accumulation in many biotechnological processes represents a serious bottleneck [48]. The results showed that supplementation of different concentrations of glucose, galactose, xylose and arabinose did not lead to a strong inhibition of *p*NPGlu hydrolysis, in fact, *Dtur*βGlu retained 80% of its activity in presence of 100 mM glucose, galactose and xylose and 60% in presence of

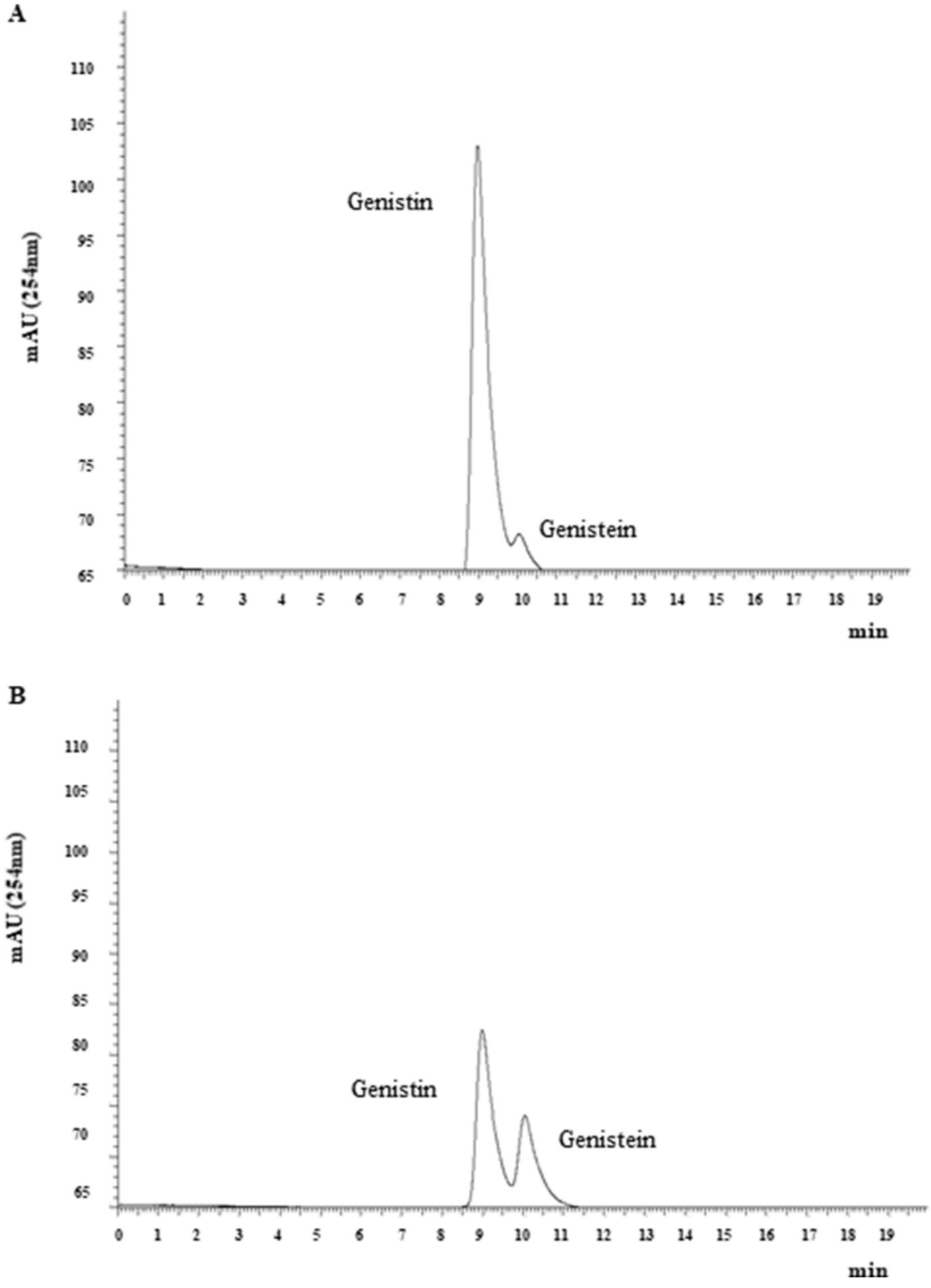

Fig. 8. HPLC profile for conversion of genistin into genistein. The reaction mixture containing 50 mM citrate phosphate buffer pH 5.4, 0.25 mM genistin, was incubated for 60 min at 80 °C without (A) or with *Dtur*βGlu(B).

100 mM arabinose (Fig. 7). The inhibitory effect of glucose on *Dtur*βGlu, in a range from 50 to 500 mM, was tested with different concentrations of *p*NPGlu (0.4 and 0.8 mM).

K_i value of 750 mM, determined by the Dixon plot, was 3.5 and 2.5 fold greater than the thermophilic β-glucosidases from *F. islandicum* and from *P. furiosus* respectively [2,42–47,49], showing, hence, a good glucose tolerance.

3.7. Enzymatic hydrolysis of genistin

Isoflavones are actually exploited as new prebiotic with health-promoting activity. In nature they are primarily present in many soy-based food products as isoflavone-glycosides and can be obtained by enzymatic hydrolysis in alternative to chemical processes. Water is a poor solvent for isoflavones that are generally soluble in acetonitrile, or ethanol or methanol mixed with water, consequently to extract the isoflavones from natural source ethanol often was used. Since *Dtur*βGlu showed high tolerance to ethanol, we assayed the ability of the enzyme to hydrolyze genistin liberating glucose and genistein. The releasing of aglycone molecule, was determined by HPLC after 60 min of incubation of the reaction mixture under the optimal conditions (Fig. 8). In this contest the new thermophilic β-glucosidase, *Dtur*βGlu, could be a starting point to develop new biological drugs highly resistant to temperatures and pH that could be used in spore surface display an emerging technique with a wide range of potential applications [50].

4. Conclusion

In this study, we have biochemically characterized a novel recombinant thermostable β-glucosidase, *Dtur*βGlu, from the anaerobic hyperthermophilic bacterium *D. turgidum*. *Dtur*βGlu was expressed in *E. coli* and purified to homogeneity. Differently from the general monomeric structure of GH1 enzymes, *Dtur*βGlu showed a tetrameric structure according to many hyperthermophilic archaeal β-glucosidases previously characterized [26,51].

The enzyme exhibited good β-glucosidase and β-galactosidase activities on synthetic substrates; furthermore, it was also able to hydrolyse natural substrates such as salicin, with a catalytic efficiency higher than other thermophilic β-glucosidases, and the isoflavone glycoside, genistin. Moreover, *Dtur*βGlu is stable and active at high temperature and in a wide range of pH. The addition of surfactants and alcohols enhanced the activity of *Dtur*βGlu, while the metal ions did not significantly hinder it; furthermore, the enzyme showed a good tolerance to monosaccharides. Finally, the comparison of *Dtur*βGlu with the previously characterized *Dt*GH from *D. thermophilum*, underlined the complementarity of these thermophilic enzymes that could be used in a mixture for the hydrolysis of different substrates. These results, finally, highlight the interest towards this microorganism as good source of GH stimulating the characterization of new enzymes to be used for biotechnological applications.

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Declaration of conflict of interest

The authors declare that there is not conflict of interest.

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