J. Microbiol. Biotechnol. (2019), 29(12), 1882–1893
https://doi.org/10.4014/jmb.1807.07020

jmb

Highly Efficient Biotransformation of Astragaloside IV to Cycloastragenol by Sugar-Stimulated β-Glucosidase and β-Xylosidase from *Dictyoglomus thermophilum*

Qi Li[1,2], Tao Wu[1], Linguo Zhao[1,2*], Jianjun Pei[1,2], Zhenzhong Wang[3], and Wei Xiao[3*]

[1]*College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, P.R. China*
[2]*Jiangsu Key Lab for the Chemistry and Utilization of Agricultural and Forest Biomass, Nanjing 210037, P.R. China*
[3]*Jiangsu Kanion Pharmaceutical Co., Ltd., Lianyungang 222001, Jiangsu Province, P.R. China*

Received: July 10, 2018
Revised: August 7, 2018
Accepted: August 9, 2018

First published online:
August 10, 2018

*Corresponding authors
L.Z.
Phone: +86-025-85427962
E-mail: njfu2304@163.com
W.X.
Phone: +86-0518-81152227
E-mail: xw@kanion.com

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2019 by
The Korean Society for Microbiology
and Biotechnology

β-Glucosidases and β-xylosidases are two categories of enzymes that could cleave out non-reducing, terminal β-D-glucosyl and β-D-xylosyl residues with release of D-glucose and D-xylose, respectively. In this paper, two functional β-glucosidase Dth3 and β-xylosidase Xln-DT from *Dictyoglomus thermophilum* were heterologously expressed in *E.coli* BL21 (DE3). Dth3 and Xln-DT were relatively stable at 75ºC and were tolerant or even stimulated by glucose and xylose. Dth3 was highly tolerant to glucose with a K_i value of approximately 3 M. Meanwhile, it was not affected by xylose in high concentration. The activity of Xln-DT was stimulated 2.13-fold by 1 M glucose and 1.29-fold by 0.3 M xylose, respectively. Furthermore, the β-glucosidase Dth3 and β-xylosidase Xln-DT showed excellent selectivity to cleave the outer C-6 and C-3 sugar moieties of ASI, which established an effective and green method to produce the more pharmacologically active CAG, an exclusive telomerase activator. We measured temperature, pH and dosage of enzyme using a single-factor experiment in ASI biotransformation. After optimization, the optimal reaction conditions were as follows: 75ºC, pH 5.5, 1 U of Dth3 and 0.2 U of Xln-DT, respectively. Under the optimized conditions, 1 g/l ASI was transformed into 0.63 g/l CAG with a corresponding molar conversion of 94.5% within 3 h. This is the first report to use the purified thermostable and sugar-tolerant enzymes from *Dictyoglomus thermophilum* to hydrolyze ASI synergistically, which provides a specific, environment-friendly and cost-effective way to produce CAG.

Keywords: Glycosidases, sugar-tolerant, biotransformation, astragaloside IV, cycloastragenol

Introduction

Radix Astragali (a type of Chinese traditional tonic herb), which can enhance immunity over the long term, is widely used in China [1, 2]. There are many active ingredients isolated and identified from astragalus including polysaccharides, saponins, flavonoids, alkaloids and trace elements [3, 4]. Among them, polysaccharides, saponins and flavonoids are the main active components [5]. As one of the main active ingredients in radix astragali saponins, the bioactivities of 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl-cycloastragenol (ASI) have been widely investigated, and many pharmacological activities including anti-inflammatory, anti-virus, anti-oxidation and cardioprotection were reported [6, 7]. As the triterpene aglycone of ASI, cycloastragenol (CAG) shows better pharmacological effects, although the concentration of CAG was much lower than ASI. Except for the pharmacological effects given above, the most outstanding function of CAG was anti-aging [8]. By activating telomerase, the cell division number was increased, which can prolong life [9, 10]. However, the low concentration of CAG in *Radix Astragali* from nature, represents a major obstacle in the separation and purification of CAG from *Radix Astragali*. Methods, including Smith degradation,

acid hydrolysis, microbial transformation and enzymatic biotransformation have been used to hydrolyze ASI into CAG [11−13]. Compared with these methods, there are many disadvantages to Smith degradation and acid hydrolysis, such as complicated reaction conditions, expensive reagents, low purity and inevitably having more by-products. In contrast, microbial transformation and enzymatic biotransformation has greater potential due to high selectivity and mild conditions [14−17]. To date, only one paper has reported that CAG could be obtained by microbial transformation of ASI by *Bacillus sp.* LG-502 with the final conversion rate of 84% after 6 days [11]. However, the complex crude enzyme expressed by the *Bacillus* was not only time-consuming but limited because of its comparatively low selectivity in its transformation ability to convert ASI to CAG. Therefore, direct enzymatic biotransformation with the purified enzymes was considered as a green and efficient technology.

As we all know, β-glucosidases and β-xylosidases are two categories of enzymes that can cleave out non-reducing, terminal β-D-glucosyl and β-D-xylosyl residues while releasing D-glucose and D-xylose. These enzymes are rooted in all kinds of organisms, such as bacteria, yeasts and archaea and play many vital roles in various industrial applications [18−21]. Among these enzymes, some are sensitive to glucose and xylose, and some others are tolerant and even facilitated by glucose and xylose. Altogether, β-glucosidase and β-xylosidase with sugar tolerance can increase the efficiency of substrate hydrolysis and lower the cost in most of industrial processes. As key parts of the cellulose and hemicellulose metabolizing enzymes, classical β-glucosidases and β-xylosidases could finally hydrolyzed short oligosaccharides and xylooligosaccharides into glucose and xylose, respectively, then they are further transformed into alternative energy sources (bioethanol and other fuel products) [22, 23]. Recently, more and more studies have been focused on hydrolyzing natural and flavor glucosylated and xylosylated compounds such as ginsenoside, radix astragali saponins and flavonoids by β-glucosidases and β-xylosidases in many biotransformation applications [24, 25].

Previously, we cloned and expressed one β-glucosidase Dth3 and one β-xylosidase Xln-DT from *Dictyoglomus thermophilum* [26]. In this study, Dth3 and Xln-DT from *Dictyoglomus thermophilum* exhibited high selective hydrolysis for the outer C-6 position glucose and C-3 position xylose in ASI with the final conversion rate of over 90% in 3 h. Meanwhile, Dth3 and Xln-DT displayed high glucose and xylose tolerance, which make the recombinant Dth3 and Xln-DT more suitable for producing CAG and other aglycones in some industrial and pharmaceutical applications.

Materials and Methods

Bacterial Strains, Medium and Materials

The recombinant strains Dth3-pet28a and Xln-DT-pet28a were preserved in Microbial Technology Research Laboratory, Nanjing Forestry University (NJFU). The purified recombinant β-glucosidases (BGL3T, Tpebgl3, Tpebgl1, Sibgl1) and β-xylosidases (Tth XyB3, Tpxy3, XlnD, Tth Xyl) were prepared by Microbial Technology Research Laboratory and Jiangsu Key Laboratory for the Chemistry & Utilization of Agricultural and Forest Biomass, NJFU.

LB (Lysogeny broth) medium (yeast extract 5 g/l, peptone 10 g/l, NaCl 10 g/l, and agar 15 g/l) containing kanamycin (100 mg/ml) was used for bacterial culture.

The substrates *p*NP-β-D-xyloside (*p*NPX), *p*NP-β-D-glucopyranoside (*p*NPGlu), *o*-Nitrophenyl-β-D-glucopyranoside (*o*NPGlu), *p*NP-β-D-galactopyranoside (*p*NPGal), *p*NP-β-D-rhamnopyranoside (*p*NPR), *p*NP-α-L-arabinofuranoside (*p*NPAF) and *p*NP-α-L-arabinopyranoside (*p*NPAP) were purchased from Sigma-Aldrich (USA). Standards of ASI (>98% Purity, HPLC), 3-O-β-D-xylopyranosyl-cycloastragenol (Cyc B, 80% Purity, HPLC) and CAG (>98% Purity, HPLC) were purchased from Chendu Institute of Biology (China), CAS (www.cdmust.com).

Protein Expression and Purification

The recombinant strains Dth3-pet28a and Xln-DT-pet28a were transformed into *E. coli* BL21 (DE3). For the expression of β-glucosidase Dth3 and β-xylosidase Xln-DT, cells were grown in LB medium containing 100 mg/ml of kanamycin at 37°C and induced to express recombinant Dth3 and Xln-DT by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to an optical density at OD_{600} value of 0.6-0.8, and the bacteria were further incubated at 28°C for about 14 h. The cultures were harvested by centrifugation at 8,000 ×*g* (4°C) for 10 min, washed with distilled water several times and resuspended in 1× binding buffer (pH 7.9, 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl). After sonication, the cell extracts were heat treated at 75°C for 30 min and then cooled in ice-bath and centrifuged at 12,000 ×g (4°C) for 30 min. Finally, the supernatants were loaded and purified on a 2 ml Ni^{2+}-NTA affinity chromatography column (Novagen, USA), and the enzyme protein was collected by eluting with Tris-HCl buffer containing 100 mM imidazole. After dialysis and ultrafiltration, the proteins were examined by SDS-PAGE gel and the protein bands were analyzed by image analysis system (Bio-Rad, USA) [27]. Purified protein concentration was measured by the Bradford method using BSA (albumin from bovine serum) as a standard.

Enzyme Assay

Substrates *p*NPGul and *p*NPX were used for β-glucosidase and β-xylosidase activity assay. The reaction mixture contained 5 μl of

20 mM substrate *p*NPGul or *p*NPX, 90 μl of sodium phosphate buffer (50 mM, pH 6.0) and 5 μl of purified enzyme. After 10 min (75°C), the reaction was stopped by adding 300 μl of Na_2CO_3 (1 M). The absorbance of the mixture was immediately measured at 405 nm. The definition of one unit of β-glucosidase and β-xylosidase activity (1 U) was consistent with the literature references [15, 28]. For every sample, the activity was measured three separate times.

The substrate specific activities of the purified enzymes Dth3 and Xln-DT were tested by using *p*NPX, NPGlu, NPGlu, NPGal, *p*NPR, *p*NPAF and *p*NPAP.

The effects of methanol on β-glucosidase and β-xylosidase activity of purified enzyme Dth3 and Xln-DT were measured by adding 5%, 10%, 15%, 20%, 25%, 30%, 40%, and 50% methanol in the mixture. The β-glucosidase and β-xylosidase enzyme Dth3 and Xln-DT were mixed with each solvent for 30 min at 75°C before adding *p*NPGul or *p*NPX to initiate the enzyme reaction. The influence of various glucose and xylose concentrations (100, 200, 300, 400, 500, 800, 1,000, 1,500, 2,000, 2,500, and 3,000 mM) on the β-glucosidase and β-xylosidase activity of purified enzyme Dth3 and Xln-DT was measured. The activity of the enzyme without the methanol or sugar was defined as 100%. Every experiment was performed in triplicate.

Enzymatic Transformation of ASI

For ASI as substrate, five purified β-glucosidases (Tpebgl1, Tpebgl3, BGL3T, Dth3, and Sibgl1) and five purified β-xylosidases (Xln-DT, Tth xynB3, Tpxy3, Tth xyl, and XlnD) were selected to hydrolyze the ASI with the reaction mixture (200 μl) containing 1 g/l ASI (methanol as a solvent), 1 U of β-glucosidase or 1 U of β-xylosidase and sodium phosphate buffer (50 mM, pH 6.0), and it was incubated at 75°C for 1 h and stopped by the ice bath, then terminated by adding 400 μl of methanol. The methanol extract of this material was assayed by HPLC-ELSD. Control samples were created using only substrate without enzyme and using enzyme without substrate.

To select suitable hydrolyzation conditions, the effects of the main factors were investigated. The main factors and the variation ranges were as follows: temperature (70°C, 75°C, 80°C, 85°C, 90°C, and 95°C), pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0), enzymatic hydrolysis time (30, 60, 90, 120, 150, and 180 min) and enzyme dosage (0.1 U, 0.2 U, 0.3 U, 0.4 U, 0.6 U, 0.8 U, 1 U, 1.2 U for Dth3 and 0.05 U, 0.1 U, 0.12 U, 0.16 U, 0.2 U, 0.24 U, 0.3 U, 0.4 U, 0.5 U for Xln-DT added to the total 200 μl of the reaction system).

To confirm the influence of the ASI on β-glucosidase Dth3 and β-xylosidase Xln-DT, different concentrations of ASI (1, 2.5, 5, 7.5, 10, 12.5, and 15 g/l) were incubated with Dth3 and Xln-DT.

The molar conversion rate of ASI to CAG, expressed as hydrolyzation (%), was calculated by means of the following formula (as given by Eq. 1):

$$\text{Hydrolyzation (\%)} = [C_i/M_i]/\,[C_t/M_t] \times 100 \qquad \text{(Eq. 1)}$$

where C_i is the initial concentration of the substrate ASI, M_i is the molar mass of the substrate ASI, C_t is the product concentration of CAG after time *t* and M_t is the molar mass of CAG.

The concentrations of the substrate ASI and product CAG were calculated according to the standard equations (y=1.4394x+2.9095, R^2=0.9989 for ASI and y=1.1663x+3.0684, R^2=0.9966 for CAG). The structures and proposed transformation pathway are shown in Fig. 1.

Assay of ASI and CAG by HPLC and ELSD

The ASI and CAG were analyzed using an Agilent HPLC 1260 system (USA) and a C18 column (4.6 × 250 mm; i.d., 5 μm; Ser. No. USNH017518, USA) with distilled water (A) and acetonitrile (B) as the mobile phase, with the A/B ratios of 40:60 from 0 to 16 min and detection was performed by monitoring the ELSD. The injection volume was 10 μl for each sample; the flow rate was 1 ml/min; the gas flow rate was 2.1 L/min and ELSD drift tube temperature was 90°C.

Results

Expression and Purification of Recombinant Dth3 and Xln-DT

The β-glucosidase Dth3 and β-xylosidase Xln-DT belonging to GH family 3 and family 39 from *Dictyoglomus thermophilum* DSM 3960 were heterologously expressed in *E. coli* BL21 (DE3) after incubation with 0.1 mM IPTG for 14 h. After

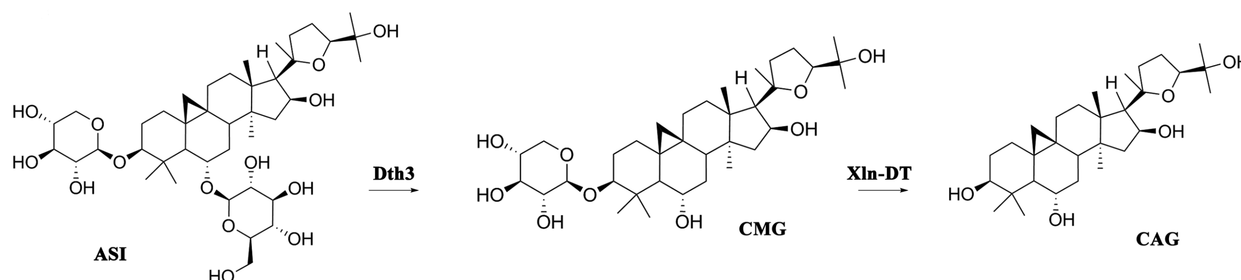

Fig. 1. The chemical structures and transformation pathway of ASI.

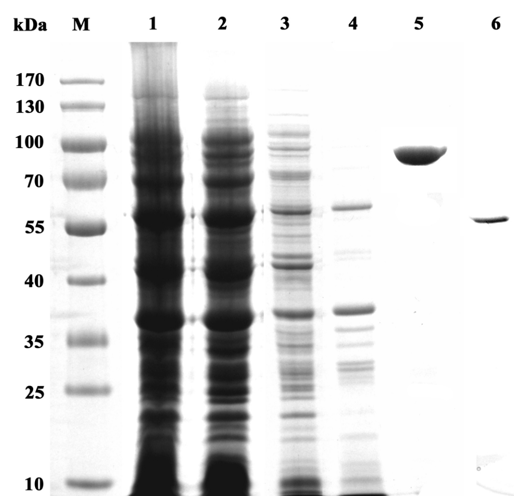

Fig. 2. SDS-PAGE analysis of recombinant glucosidase Dth3 and β-xylosidase Xln-DT expressed in *E. coli* BL21 (DE3).
Lane M, protein marker; Lane 1, crude β-glucosidase Dth3; Lane 2, crude β-xylosidase Xln-DT; Lane 3, supernatant of Dth3 after treatment at 75°C for 1 h; Lane 4, supernatant of Xln-DT after treatment at 75°C for 1 h; Lane 5, purified glucosidase Dth3; Lane 6, purified β-xylosidase Xln-DT.

sonication, almost all the recombinant Dth3 and Xln-DT were found in the soluble fraction, and the activities of the recombinant β-glucosidase and β-xylosidase were 6 U/ml and 1 U/ml, respectively. Then, the soluble fractions were heated treat at 75°C for 30 min, followed by a Ni^{2+}-NTA affinity chromatography. The purified β-glucosidase Dth3 and β-xylosidase Xln-DT showed a single band on the SDS-PAGE gel (Fig. 2). The recombinant β-glucosidase Dth3 and β-xylosidase Xln-DT showed a molecular mass of approximately 88 kDa and 55 kDa on 12% SDS-PAGE, without undesired bands.

Enzyme Characterization of Recombinant Dth3 and Xln-DT

The enzymatic characterization of the recombinant Dth3 and Xln-DT were characterized by using the purified enzymes. The recombinant Dth3 and Xln-DT had optimum pH in 50 mM sodium phosphate buffer at pH 5.5 and 6.0 using the *p*NPGlu and *p*NPX as the substrates, respectively (data were not shown) [26]. The optimum temperatures were 90°C and 75°C for Dth3 and Xln-DT, respectively (data were not shown) [26].

1 mM *p*NP and *o*NP glycosides with α and β configurations detected the substrate specificity of recombinant Dth3 and Xln-DT. All the data were shown in Table 1, which illustrated that recombinant β-glucosidase Dth3 had high activity towards *p*NP-β-D-glucopyranoside and less activity towards *p*NP-β-D-xylopyranoside without any activity towards *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-L-rhamnopyranoside or *o*NP-β-D-glucopyranoside. The recombinant β-xylosidase Xln-DT had the highest activity towards *p*NP-β-D-xylopyranoside, followed by *p*NP-β-D-glucopyranoside and *p*NP-α-L-arabinopyranoside, while showing no enzyme activity towards *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-rhamnopyranoside or *o*NP-β-D-glucopyranoside. All these results indicate that Dth3 and Xln-DT have high specificity on residual glucose and xylose, respectively.

We also detected the effect of methanol on recombinant Dth3 and Xln-DT (shown in Table 2). The residual activity of Dth3 was over 100% by the concentration of methanol below 50%, which indicated that methanol has no inhibitory effect on the recombinant Dth3. For Xln-DT, the residual activity was completely unaffected by low concentration of methanol and even enhanced by the concentration of methanol below 25%. In addition, we

Table 1. Substrate specificity of the recombinant protein Dth3 and Xln-DT.

Substrate[a]	Relative activity (mean% ± SD)	
	Recombinant β-glucosidase Dth3	Recombinant β-xylosidase Xln-DT
p-Nitrophenyl-β-D-xylopyranoside	9.46 ± 0.34	100 ± 2.08[d]
p-Nitrophenyl-β-D-galactopyranoside	ND[b]	ND
p-Nitrophenyl-α-L-arabinofuranoside	ND	ND
p-Nitrophenyl-α-L-arabinopyranoside	ND	10.69 ± 0.88
p-Nitrophenyl-α-L-rhamnopyranoside	ND	ND
p-Nitrophenyl-β-D-glucopyranoside	100 ± 1.18[c]	25.91 ± 1.61
o-Nitrophenyl-β-D-glucopyranoside	ND	ND

[a]Final concentration of each was 1.0 mM.

[b]Not determined, specific activity is not determined by the analytical methods used in this study.

[c]The relative enzyme activity against *p*-Nitrophenyl-β-D-glucopyranoside was assumed to be 100%.

[d]The relative enzyme activity against *p*-Nitrophenyl-β-D-xylopyranoside was assumed to be 100%.

Table 2. The effects of different concentrations of methanol for the recombinant Dth3 and Xln-DT activity.

Final concentration of methanol (%)	Relative enzyme activity (mean%±SD)	
	Recombinant β-glucosidase Dth3	Recombinant β-xylosidase Xln-DT
0	100 ± 2.24[a]	100 ± 4.03
5	101.8 ± 0.74	110.3 ± 1.25
10	100.1 ± 2.29	118.8 ± 1.61
15	102.3 ± 0.98	122.2 ± 5.24
20	101.3 ± 4.23	112.0 ± 2.02
25	103.6 ± 1.07	89.8 ± 4.47
30	102.2 ± 0.81	57.8 ± 5.11
40	100.7 ± 3.32	22.4 ± 2.86
50	96.9 ± 4.32	13.6 ± 1.05

[a]The relative enzyme activity against methanol free was assumed to be 100%.

compared the effects of three different organic reagents (methanol, ethanol and DMSO) on the tolerance of recombinant Dth3 and Xln-DT. The results showed that the effects of three organic reagents on the tolerance of Dth3 and Xln-DT were followed by methanol, ethanol and DMSO. As we all know, some natural products such as ginsensides and radix astragali saponins have inferior water solubility, which is needed to increase the solubilization through high temperature and the addition of hydrophilic organic solvents, such as methanol [16, 29]. Bio-catalysis of radix astragali saponins such as ASI in a two-phase system (water-methanol) can improve solubility of non-polar substrates. Therefore, the recombinant Dth3 and Xln-DT with excellent methanol tolerance indicated that these two recombinant enzymes could be effectively used for biotransformation of ASI in the presence of methanol, which would provide a new development direction to use these novel enzymes for applications in the field of organic phase bio-catalysis of traditional Chinese medicines.

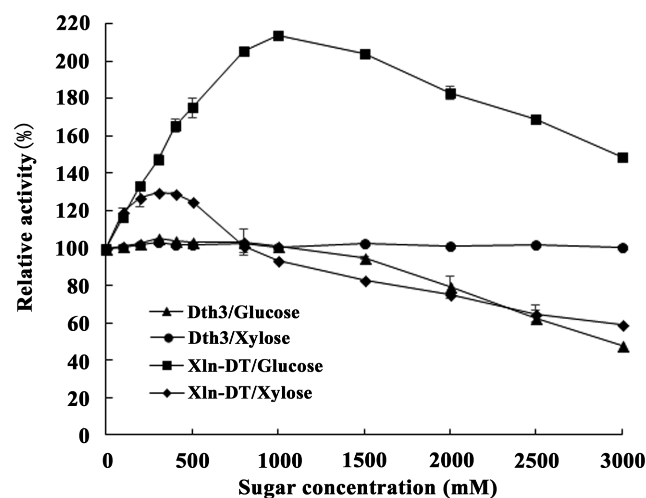

Fig. 3. The effects of sugars on recombinant glucosidase Dth3 and β-xylosidase Xln-DT activity.

Triangle, the effect of glucose on Dth3; cycle, the effect of xylose on Dth3; square, the effect of glucose on Xln-DT; rhombus, the effect of xylose on Xln-DT.

Effect of Sugars on the Activities of Recombinant Dth3 and Xln-DT

Recently, most β-glucosidases and β-xylosidases are sensitive to glucose and xylose, and few β-glucosidases and β-xylosidases are tolerant and stimulated by both glucose and xylose [30, 31]. As we all know, in most industries, application of β-glucosidases and β-xylosidases with glucose and xylose tolerance and stimulation can improve the efficiency of substrate hydrolyzation [32-34]. The effects of glucose and xylose with different concentrations on recombinant Dth3 and Xln-DT activities were determined (Fig. 3). Surprisingly, when the concentration of sugar was below 1 M, there was no inhibition on the relative activities of these two purified enzymes. When the concentration of glucose was up to 1 M, the activity of Dth3 was not affected at all, and 60% of relative activity was reserved in 2.5 M glucose. Xylose showed no any inhibition of Dth3. The relative activity of Dth3 remained at 100% when the concentration of xylose was 3 M. The Xln-DT activity was significantly stimulated by glucose, with a maximal 2.1-fold stimulation by 1 M glucose and 1.3-fold stimulation by glucose at 3 M. In addition, the Xln-DT displayed high xylose tolerance, and was activated by xylose with the concentration below 0.8 M. When xylose was further increased, the enzyme activity of Xln-DT was gradually inhibited. All the results suggest these two glycoside hydrolases are more beneficial to industrial applications without the product feedback inhibition.

Optimization of the Biotransformation

β-Glucosidases and β-xylosidases are known to have the ability to bio-convert ginsenosides, flavonoids and astragalosides [16, 35, 36]. Different β-glucosidases and β-xylosidases from various sources are known to be diverse in structure and specificity of biotransformation of a certain natural active material. In this study, we studied the specificity of five β-glucosidases and five β-xylosidases from different organisms to bio-transform ASI to CAG. All the information about the enzymes was shown in Table 3.

Table 3. Sequence homology of several recombinant β-glucosidases and β-xylosidases from different GH families.

Enzyme type	Name	Source	GH Family	Sequence homology (%)	Reference
β-glucosidase	Dth3	*Dictyoglomus thermophilum* DSM 3960	GH3	100[a]	This study
	BGL3T	*Thermotoga thermarum* DSM 5069^T	GH3	34.3	[15]
	Tpebgl3	*Thermotoga petrophlia* DSM 13995	GH3	45.6	[47]
	Tpebgl1	*Thermotoga petrophlia* DSM 13995	GH1	23.6	[16]
	Sibgl1	*Sulfolobus islandicus*	GH1	25.2	This study
β-xylosidase	Xln-DT	*Dictyoglomus thermophilum* DSM 3960	GH39	100[b]	This study
	Tth xynB3	*Thermotoga thermarum* DSM 5069^T	GH3	27.3	[18]
	Tpxy3	*Thermotoga petrophlia* DSM 13995	GH3	26.2	This study
	XlnD	*Aspergillus niger NL-1*	GH3	28.3	This study
	Tth xyl	*Thermoanaerobacterium thermosaccharolyticum* DSM571	GH120	32.1	This study

[a]The relative sequence homology against recombinant β-glucosidase Dth3 from *Dictyoglomus thermophilum* DSM 3960 was assumed to be 100%.
[b]The relative sequence homology against recombinant β-xylosidase Xln-DT from *Dictyoglomus thermophilum* DSM 3960 was assumed to be 100%.

The recombinant Dth3 belongs to Glycoside Hydrolase (GH) family 3 and had less than 50% sequence homology with the other two GH3 family β-glucosidases and two GH1 family β-glucosidases. The recombinant Xln-DT belongs to GH39 family and had approximately 30% sequence homology with the other three GH3 family β-xylosidases and one GH120 family β-xylosidase. As shown in Fig. 4A, using the following enzyme conditions, specifically, ASI concentration: 1 g/l, enzyme dosage: 1 U, pH: 6.0, temperature: 75°C, and reaction time: 1 h, all five β-glucosidases could remove the outer C-6 glucose of ASI to produce 3-O-β-D-xylopyranosyl-cycloastragenol (Cyc B). The transformation rates of three β-glucosidases from GH3 family reached to over 80%, which were higher than GH1 family β-glucosidases Tpebgl1 and Sibgl1. Among three β-glucosidases from GH3 family, Dth3 could transform almost 100% ASI to Cyc B in 1 h, which was a little more than the other two enzymes. In addition, compared with the other three GH3 family β-xylosidases and one GH120 family β-xylosidase, only Xln-DT could remove the outer C-3 xylose of the ASI with the biotransformation rate of 62% in 1 h (Fig. 4B).

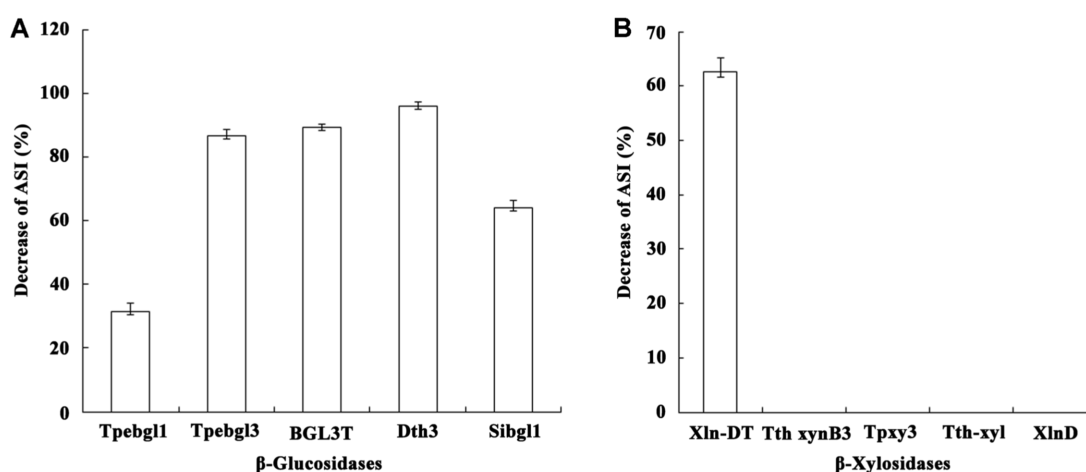

Fig. 4. Biotransformation rate of ASI by different enzymes.
(**A**) Dth3 from *Dictyoglomus thermophilum* DSM 3960; BGL3T from *Thermotoga thermarum* DSM 5069^T; Tpebgl3 and Tpebgl1 from *Thermotoga petrophlia* DSM 13995; Sibgl1 from *Sulfolobus islandicus*; (**B**) Xln-DT from *Dictyoglomus thermophilum* DSM 3960; Tth xynB3 from *Thermotoga thermarum* DSM 5069^T; Tpxy3 from *Thermotoga petrophlia* DSM 13995; XlnD from *Aspergillus niger NL-1*; Tth xyl from *Thermoanaerobacterium thermosaccharolyticum* DSM571.

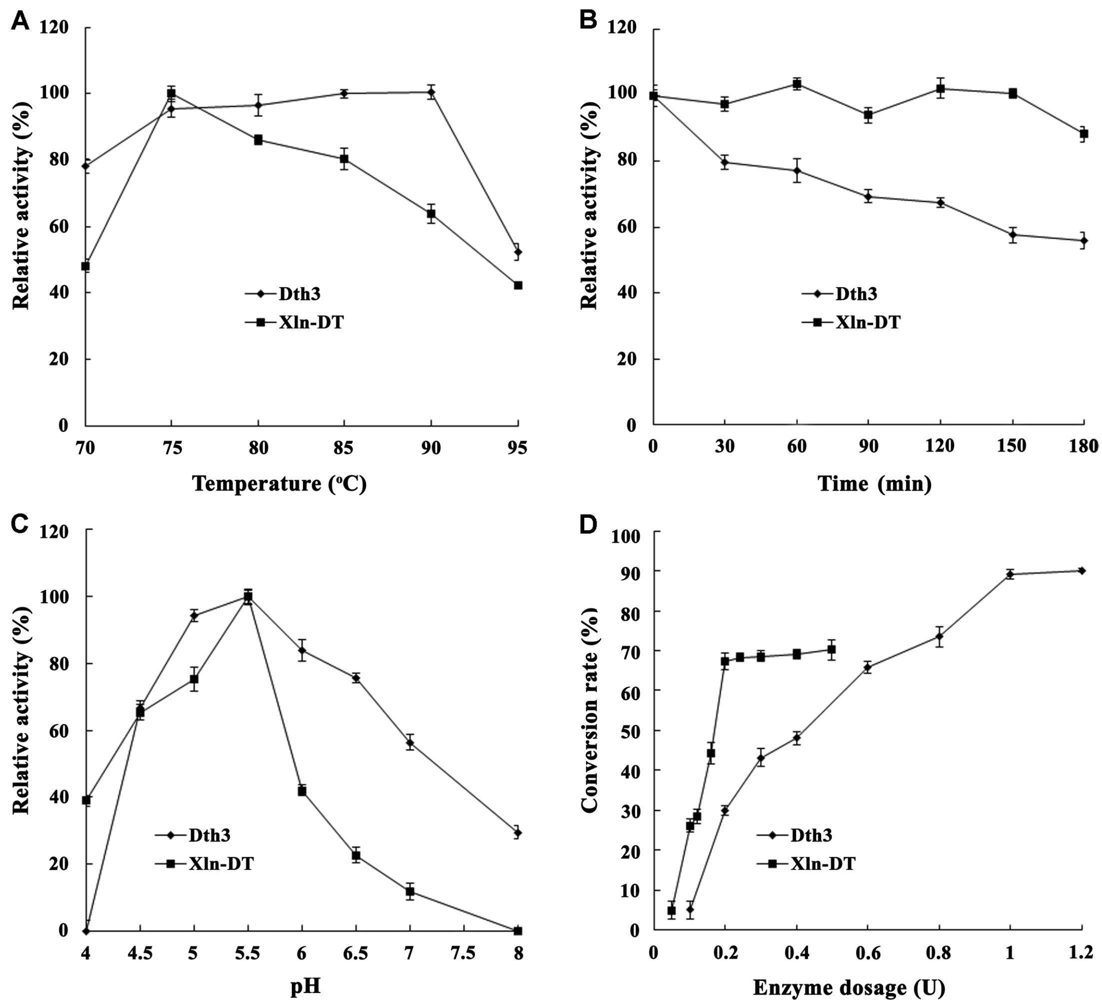

Fig. 5. Optimization of the biotransformation by recombinant glucosidase Dth3 and β-xylosidase Xln-DT

(**A**) The effect of temperature on the biotransformation; (**B**) the effect of temperature on enzyme stability (75°C); (**C**) the effect of pH on the biotransformation; **D**, the effect of enzyme dosage on the biotransformation; rhombus, Dth3; square, Xln-DT.

Therefore, in this paper, the combination of the purified recombinant β-glucosidase Dth3 and β-xylosidase Xln-DT from *Dictyoglomus thermophilum* was selected for the biotransformation of ASI, and the temperature, pH and enzyme dosage were optimized by the single-factor experiments (Fig. 5). The optimum temperature of ASI biotransformation by β-glucosidase Dth3 and β-xylosidase Xln-DT was depicted in Fig. 5A. The biotransformation rate was improved within a temperature range of 70−90°C for Dth3 and 70−75°C for Xln-DT, and the optimal pH was 5.5 for Dth3 and Xln-DT (Fig. 5C). Considering the cost of the enzyme used in the industrial application, the temperature of the reaction should be minimized. Hence, we set the temperature of the reaction at 75°C and examined the effects of temperature (75°C) on the stability of the two recombinant enzymes at the same time. As shown in Fig. 5B, the residual activity of Xln-DT was over 90% after being incubated at 75°C for 180 min, and Dth3 residual activity was about 80% after being incubated at 75°C for 60 min. With the extension of the reaction time, the residual activity of Dth3 was down to 60%. However, in the pre-experiment, the ASI could be degraded by Dth3 in 60 min completely. In addition, the more the dosage of the enzymes, the higher the rate for completing the process of the biotransformation, which was shown in Fig. 5D. The optimum enzyme dosages of the two recombinant enzymes were 1 U and 0.2 U for Dth3 and Xln-DT in the 200 μl system, respectively. The biotransformation efficiency for 1 h of reaction was detected to be more than 90% for ASI in 75°C, pH 5.5 by Dth3 and about 70% by Xln-DT. With the

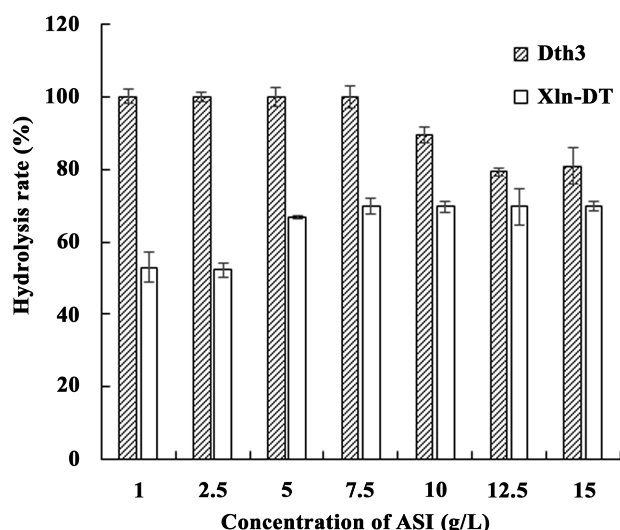

Fig. 6. The influence of ASI on the hydrolysis rate (shadow, Dth3; blank, Xln-DT).

increase of enzyme dosage of Dth3 (over 1 U) and Xln-DT (over 0.2 U), the conversion rate did not increase significantly, which indicated that excessive enzyme could not improve the efficiency of substrate conversion.

The concentration of substrate was also a critical factor affecting the biotransformation efficiency. To examine the effect of ASI on Dth3 and Xln-DT, different concentrations of ASI (from 1 to 15 g/l) were incubated with Dth3 and Xln-DT with the dosage of 1 U and 0.2 U, respectively at 75°C, pH 5.5 in 1 h (Fig. 6). Compared with concentration 1 g/l and 15 g/l, the activity of Xln-DT was not inhibited by ASI. For Dth3, the hydrolyzation rate was inhibited slightly on the high concentration of ASI. When the concentration of ASI was below 7.5 g/l, the hydrolyzation rate was approximately 100%. With the increasing of ASI concentration (15 g/l), the hydrolyzation rate dropped down to 80%.

Biotransformation of ASI by Recombinant Dth3 and Xln-DT

According to the previous study, Dth3 and Xln-DT exhibited substrate specificity for ASI with glucose moiety at C-6 and xylose moiety at C-3 positions. The biotransformation pathway of the two purified enzymes from *Dictyoglomus thermophilum* is ASI-Cyc B by Dth3 and then Cyc B-CAG by Xln-DT, by cleaving the outer glucose and xylose at position C-6 and C-3 of ASI, respectively (Fig. 1). The time-course biotransformation analysis of the enzymatic reaction by recombinant Dth3 and Xln-DT was shown in Fig. 7A. In the first 1 h, a total of 1 g/l ASI was bio-transformed into 0.79 g/l Cyc B with a corresponding molar conversion productivity of 100%. In the next 2 h, the transformed substrate (Cyc B) was bio-transformed into CAG continually, with the concentration of CAG increased gradually. Finally, the concentration of CAG was 0.63 g/l with a corresponding molar conversion rate of 94.5% at the end of the reaction. As shown in Fig. 7B, approximately all the ASI was converted into CAG, which could be identified and examined in the HPLC.

Discussion

β-Glucosidase and β-xylosidase are important ingredients of the cellulose and hemicellulose enzyme systems [37, 38]. Although many β-glucosidases and β-xylosidases have been cloned and characterized from plant, yeast and bacteria, only a few studies about highly thermotolerant and sugar-tolerant β-glucosidases and β-xylosidases have been reported. Thermophilic bacteria, such as *Thermotoga thermarum*, *Thermotoga petrophila* and *Thermoanaerobacterium saccharolyticum*, are well known to produce plentiful glycoside hydrolases with competitive advantages, such as great catalytic ability and excellent pH and temperature stability [39−41]. In the previous study, we identified β-glucosidase of GH3, Dth3, and β-xylosidase with GH39, Xln-DT, from *Dictyoglomus thermophilum*, a kind of thermophilic bacteria. In this study, the purified enzyme exhibited outstanding thermostability under 75°C. After 3 h, the activity of Xln-DT was almost never lost and Dth3 showed that its residual activity was over 60%.

To our knowledge, glucose and xylose are strong inhibitors of β-glucosidases and β-xylosidases [42]. According to the influence of glucose on the β-glucosidase activity, β-glucosidases can be divided into three groups; the glucose-sensitive β-glucosidase, the glucose-tolerant β-glucosidase and the glucose-stimulated β-glucosidase [43]. Similarly, β-xylosidases can be classified as above. Unfortunately, most of the β-glucosidases and β-xylosidases are sugar-sensitive, which inhibits the usage of β-glucosidases and β-xylosidases in the industrial applications. Therefore, the search for β-glucosidases and β-xylosidases with high tolerance for sugars has attracted more interest in recent years since these have great potential in the conversion of cellulose, hemicellulose and other natural and flavor glucosylated and xylosylated compounds. Surprisingly, we heterologously expressed the highly thermostable and sugar-tolerant β-glucosidase Dth3 and β-xylosidase Xln-DT from *Dictyoglomus thermophilum* in *E.coli* BL21 (DE3) in this study. The recombinant purified β-glucosidase Dth3 showed excellent

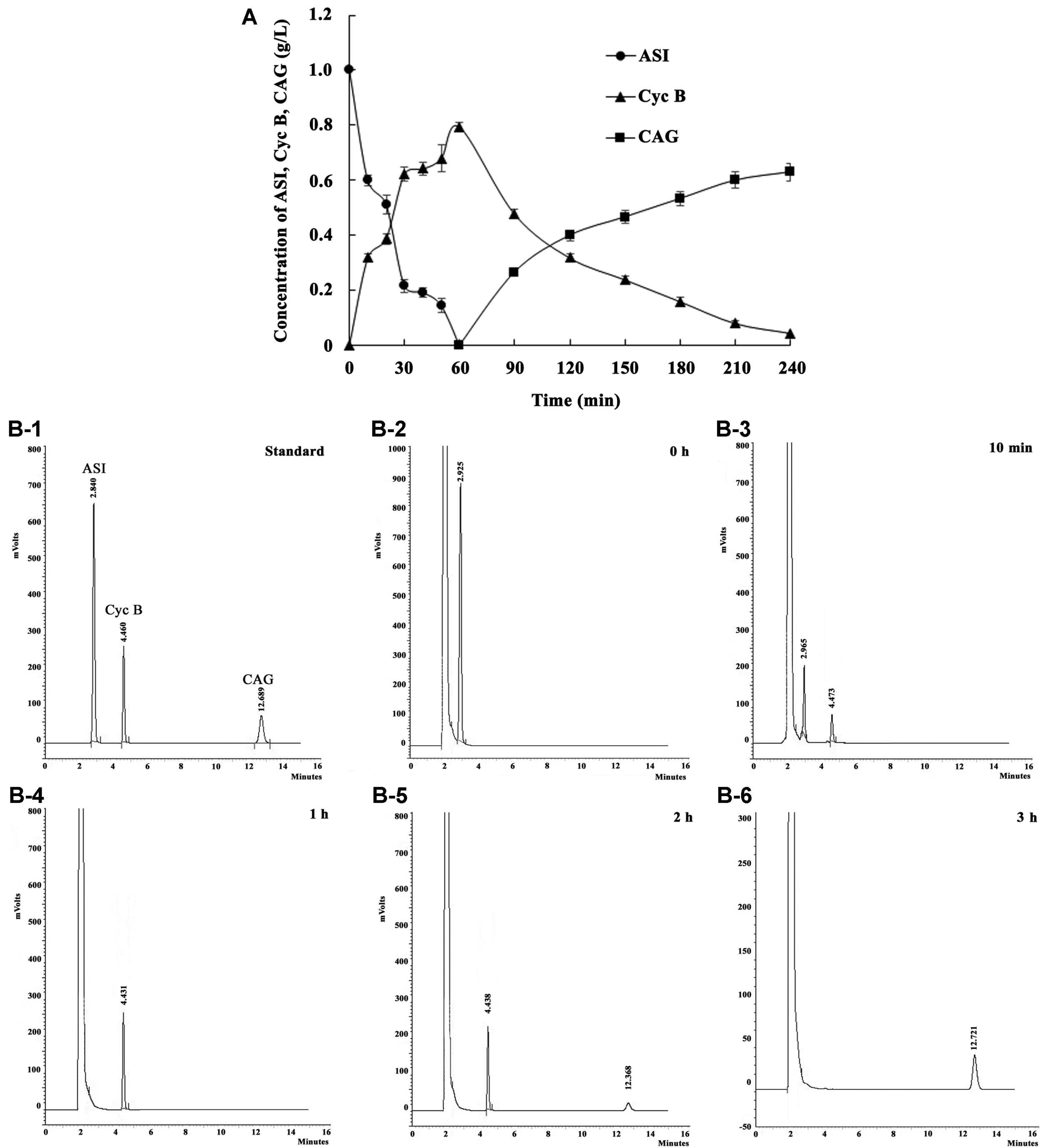

Fig. 7. HPLC analysis of ASI hydrolysis by recombinant glucosidase Dth3 and β-xylosidase Xln-DT.

(**A**) The time course of bioconversion of ASI to CAG by recombinant glucosidase Dth3 and β-xylosidase Xln-DT (cycle, ASI; triangle, Cyc B; square, CAG); (**B-1**) standard sample of ASI (2.840 min), Cyc B (4.460 min) and CAG (12.689 min); (**B-2–B-6**) ASI (1 g/l) incubated with Dth3 (1 U) and Xln-DT (0.2 U) for 0 min, 10 min, 1 h, 2 h, and 3 h.

sugar tolerance. When concentration of glucose was up to 1 M, the β-glucosidase activity was not decreased. With the increasing of glucose, the enzyme activity of Dth3 was gradually inhibited, with a K_i of over 3 M glucose, which was higher than that from *Anoxybacillus flavithermus* subsp. *yunnanensis*, *Thermotoga thermarum,* and other reported β-

glucosidases [31, 44, 45]. In addition, the activity of Dth3 was not affected by xylose. The purified β-xylosidase Xln-DT was also tolerant to xylose inhibition with a K_i of over 3 M xylose. Especially, the β-xylosidase activity was promoted under low concentration of xylose, with the 1.29-fold relative activity by 0.3 M xylose. Moreover, the Xln-DT was activated by glucose at concentration up to 3 M, with the 2.13-fold relative activity by 1 M glucose and 1.48-fold by 3 M glucose. Till now, β-xylosidase stimulated by both glucose and xylose has not been reported yet. Furthermore, this is the first reported β-glucosidase and β-xylosidase from thermophilic *Dictyoglomus thermophilum* that can be so resistant to a high concentration of sugar. During the ASI hydrolysis by β-glucosidases and β-xylosidases, besides the product CAG, the higher concentration is xylose and glucose. Thus, the glucose- and xylose- tolerant and stimulated β-glucosidases and β-xylosidases are more attractive to enhance the ASI hydrolysis. Compared with other glycoside hydrolases, Dth3 and Xln-DT exhibited higher sugar tolerance and stimulation levels and better thermostability, which also indicated greater potential for biotransformation.

Recent research has focused on establishing methods to efficiently convert ASI into CAG. Methods such as Smith degradation, acid hydrolysis two-phase acid hydrolysis and microbial biotransformation have been used to degrade the ASI into CAG [11, 12, 46]. It is well known that chemical reactions such as acid hydrolysis and two-phase acid hydrolysis require a temperature as high as 85°C and are accompanied by a lot of pollution. In addition, because of its low selectivity and yield (48.0%), the chemical method has been abandoned. Although, the CAG yield was over 84.4% by Smith degradation, the process was too complex, requiring four complicated steps, and the cost of the reagent was too high. Wang and Chen reported that the ASI could be bio-converted into CAG with the yield of 84% by *Bacillus sp.* LG-502 after 6 days [11]. Although the specificity has been improved, the biotransformation time is too long and it is difficult to purify product subsequently. Compared with the methods above, biotransformation with purified enzyme has the advantages of greatly strengthened selectivity, high catalytic efficiency, mild reaction conditions and no pollution. In this study, we optimized a method for bio-converting ASI into CAG, as well as established a green and efficient biotransformation method for ASI using β-glucosidase Dth3 and β-xylosidase Xln-DT. The capacities of the β-glucosidase Dth3 and β-xylosidase Xln-DT to hydrolyze ASI synergistically were investigated by using 1 g/l of ASI incubated with the purified enzymes at 75°C. We established a suitable process for the biotransformation of ASI using 1 U of Dth3 and 0.2 U of Xln-DT, pH 5.5, 75°C for 3 h. In the first 1 h, the ASI was bio-transformed into Cyc B by Dth3 100%. Remarkably, in the next 2 h, along with the effect of the β-xylosidase Xln-DT, the Cyc B could be bio-transformed into CAG with the final molar conversion rate of 94.5% without any by-products. All the above results show that our method can efficiently and safely use β-glucosidase Dth3 and β-xylosidase Xln-DT in removal of outer C-6 glucose and C-3 xylose in ASI, thereby serving as a novel method for the biotransformation of ASI. Therefore, the recombinant Dth3 and Xln-DT would be suitable for producing CAG in pharmaceutical and industrial applications, which presents great potential for producing natural medicines.

Acknowledgements

This research was supported by the National Key R&D Program of China (2016YFD0600805), the Jiangsu Provincial Policy Guidance Program (Grant No. SZ-XZ2017023), the Qing Lan Project, and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. He ZQ, Findlay JA. 1991. Constituents of Astragalus membranaceus. *J. Nat. Prod.* **54:** 810-815.
2. Choudhary MI, Jan S, Abbaskhan A, Musharraf SG, Samreen, Sattar SA, *et al.* 2008. Cycloartane triterpenoids from Astragalus bicuspis *J. Nat. Prod.* **71:** 1557-1560.
3. Ma XQ, Shi Q, Duan JA, Dong TT, Tsim KW. 2002. Chemical analysis of Radix Astragali (Huangqi) in China: a comparison with its adulterants and seasonal variations. *J. Agric. Food Chem.* **50:** 4861-4866.
4. Qi LW, Yu QT, Li P, Li SL, Wang YX, Sheng LH, *et al.* 2006. Quality evaluation of Radix Astragali through a simultaneous determination of six major active isoflavonoids and four main saponins by high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors. *J. Chromatogr. A.* **1134:** 162-169.
5. Peng J, Dong F, Qi Y, Han X, Xu Y, Xu L, *et al.* 2008. Preparative separation of four triterpene saponins from radix astragali by high-speed counter. *Phytochem. Anal.* **19:** 212-217.

6. Li H, Zhang Y, Min J, Gao L, Zhang R, Yang Y. 2018. Astragaloside IV attenuates orbital inflammation in Graves' orbitopathy through suppression of autophagy. *Inflamm. Res.* **67:** 117-127.
7. Lei L, Hou X, Xu R, Chang L, Tu M. 2017. Research review on the pharmacological effects of astragaloside IV. *Fundam Clin. Pharmacol.* **31:** 17-36.
8. Cao YL, Wen-Lan LI, Wei LY, Liu XY, Zhi-Hui LI, Wei-Guo HE, *et al.* 2012. Anti-aging function of Cycloastragenol in aging mice induced by D-galactose. *Zhongguo Shiyan Fangjixue Zazhi.* **18:** 208-211.
9. Ip FC, Ng YP, An HJ, Dai Y, Pang HH, Hu YQ, *et al.* 2014. Cycloastragenol is a potent telomerase activator in neuronal cells: implications for depression management. *Neurosignals* **22:** 52-63.
10. Valenzuela HF, Fuller T, Edwards J, Finger D, Molgora B. 2009. Cycloastragenol extends T cell proliferation by increasing telomerase activity. *J. Immunol.* **182:** 90-30.
11. Wang L, Chen Y. 2017. Efficient biotransformation of Astragaloside IV to Cycloastragenol by *Bacillus sp.* LG-502. *Appl. Biochem. Biotechnol.* **183:** 1488-1502.
12. Feng LM, Lin XH, Huang FX, Cao J, Qiao X, Guo DA, *et al.* 2014. Smith degradation, an efficient method for the preparation of cycloastragenol from astragaloside IV. *Fitoterapia* **95:** 42-50.
13. Zhang SD, Jun-Kun LU, Yan JZ, Hui Z. 2016. Research progress of the preparation technology and pharmacological effect of cycloastragenol. *Chinese J. New Drugs* **16:** 1872-1875.
14. And SD, Rosazza JPN. 2006. Microbial and enzymatic transformations of flavonoids. *J. Nat. Prod.* **69:** 499-508.
15. Pei J, Xie J, Yin R, Zhao L, Ding G, Wang Z, *et al.* 2015. Enzymatic transformation of ginsenoside Rb1 to ginsenoside 20(S)-Rg3 by GH3 β-glucosidase from *Thermotoga thermarum* DSM 5069^T. *J. Mol. Catal. B: Enzymatic.* **113:** 104-109.
16. Xie J, Zhao D, Zhao L, Pei J, Xiao W, Ding G, *et al.* 2015. Overexpression and characterization of a Ca^{2+} activated thermostable β-glucosidase with high ginsenoside Rb1 to ginsenoside 20(S)-Rg3 bioconversion productivity. *J. Ind. Microbiol. Biotechnol.* **42:** 839-850.
17. Shah S, Tan H, Sultan S, Faridz M, Shah M, Nurfazilah S, *et al.* 2014. Microbial-catalyzed biotransformation of multifunctional triterpenoids derived from phytonutrients. *Int. J. Mol. Sci.* **15:** 12027-12060.
18. Shi H, Li X, Gu HX, Zhang Y, Huang YJ, Wang LL, *et al.* 2013. Biochemical properties of a novel thermostable and highly xylose-tolerant beta-xylosidase/alpha-arabinosidase from *Thermotoga thermarum*. *Biotechnol. Biofuels* **6:** 27.
19. Benassi VM, de Lucas RC, Jorge JA, Polizeli MDTD. 2014. Screening of thermotolerant and thermophilic fungi aiming beta-xylosidase and arabinanase production. *Braz. J. Microbiol.* **45:** 1459-1467.
20. Patel H, Kumar AK, Shah A. 2018. Purification and characterization of novel bi-functional GH3 family β-xylosidase/β-glucosidase from *Aspergillus niger* ADH-11. *Int. J. Biol. Macromol.* **109:** 1260-1269.
21. Zhao L, Xie J, Zhang X, Cao F, Pei J. 2013. Overexpression and characterization of a glucose-tolerant β-glucosidase from *Thermotoga thermarum* DSM 5069^T with high catalytic efficiency of ginsenoside Rb1 to Rd. *J. Mol. Catal. B: Enzym.* **95:** 62-69.
22. Jonathan MC, Demartini J, Thans SVS, Hommes R, Kabel MA. 2017. Characterisation of non-degraded oligosaccharides in enzymatically hydrolysed and fermented, dilute ammonia-pretreated corn stover for ethanol production. *Biotechnol. Biofuels* **10:** 112.
23. Consolación Á, Manuel RSF, Bruno D. 2016. Enzymatic hydrolysis of biomass from wood. *Microb. Biotechnol.* **9:** 149-156.
24. Shin KC, Seo MJ, Oh DK. 2014. Characterization of β-xylosidase from *Thermoanaerobacterium thermosaccharolyticum* and its application to the production of ginsenosides Rg1 and Rh1 from notoginsenosides R1 and R2. *Biotechnol. Lett.* **36:** 2275-2281.
25. Zhong FL, Ma R, Jiang M, Dong WW, Jiang J, Wu S, *et al.* 2016. Cloning and characterization of ginsenoside-hydrolyzing β-glucosidase from *Lactobacillus brevis* that transforms Ginsenosides Rb1 and F2 into Ginsenoside Rd and Compound K. *J. Microbiol. Biotechnol.* **26:** 1661-1667.
26. Li Q, Wu T, Qi Z, Zhao L, Pei J, Tang F. 2018. Characterization of a novel thermostable and xylose-tolerant GH 39 β-xylosidase from *Dictyoglomus thermophilum*. *BMC Biotechnol.* **18:** 29.
27. Laemmli UK. 1970. Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature* **227:** 680-688.
28. Huang CH, Sun Y, Ko TP, Chen CC, Zheng Y, Chan HC, *et al.* 2012. The substrate/product-binding modes of a novel GH120 β-xylosidase (XylC) from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Biochem. J.* **448:** 401-407.
29. Wan HD, Li D. 2015. Highly efficient biotransformation of ginsenoside Rb1 and Rg3 using β-galactosidase from *Aspergillus sp*. *RSC Adv.* **5:** 78874-78879.
30. Bhatia Y, Mishra S, Bisaria VS. 2002. Microbial β-glucosidases: cloning, properties, and applications. *Crit. Rev. Biotechnol.* **22:** 375-407.
31. Liu Y, Li R, Wang J, Zhang X, Jia R, Gao Y, *et al.* 2017. Increased enzymatic hydrolysis of sugarcane bagasse by a novel glucose- and xylose-stimulated β-glucosidase from *Anoxybacillus flavithermus subsp. yunnanensis* E13T. *BMC Biochem.* **18:** 4.
32. Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresour. Technol.* **127:** 500-507.
33. Xiao Z, Zhang X, Gregg DJ, Saddler JN. 2004. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. *Appl. Biochem. Biotechnol.* **116:** 1115-1126.

34. Yang X, Shi P, Huang H, Luo H, Wang Y, Zhang W, *et al.* 2014. Two xylose-tolerant GH43 bifunctional β-xylosidase/ α-arabinosidases and one GH11 xylanase from *Humicola insolens* and their synergy in the degradation of xylan. *Food Chem.* **148:** 381-387.
35. Pei J, Wu T, Yao T, Zhao L, Ding G, Wang Z, *et al.* 2017. Biotransformation of Ginsenosides Re and Rg 1 into Rg 2 and Rh 1 by thermostable β-glucosidase from *Thermotoga thermarum*. *Chem. Nat. Compd.* **53:** 1-6.
36. Ge L, Chen A, Pei J, Zhao L, Fang X, Gang D, *et al.* 2017. Enhancing the thermostability of α-L-rhamnosidase from *Aspergillus terreus* and the enzymatic conversion of rutin to isoquercitrin by adding sorbitol. *BMC Biotechnol.* **17:** 21.
37. Cao LC, Wang ZJ, Ren GH, Kong W, Li L, Xie W, *et al.* 2015. Engineering a novel glucose-tolerant β-glucosidase as supplementation to enhance the hydrolysis of sugarcane bagasse at high glucose concentration. *Biotechnol. Biofuels* **8:** 202.
38. Zimbardi A, Sehn C, Meleiro L, Souza F, Masui D, Nozawa M, *et al.* 2013. Optimization of β-glucosidase, β-xylosidase and xylanase production by *Colletotrichum graminicola* under solid-state fermentation and application in raw sugarcane trash saccharification. *Int. J. Mol. Sci.* **14:** 2875-2902.
39. Long L, Shi H, Li X, Zhang Y, Hu J, Wang F. 2016. Cloning, purification, and characterization of a thermostable β-glucosidase from *Thermotoga thermarum* DSM 5069. *Bioresources* **11:** 3165-3177.
40. Yang JK, Yoon HJ, Ahn HJ, Lee BI, Pedelacq JD, Liong EC, *et al.* 2004. Crystal structure of beta-D-xylosidase from *Thermoanaerobacterium saccharolyticum*, a family 39 glycoside hydrolase. *J. Mol. Biol.* **335:** 155-165.
41. Colussi F, Silva VMD, Miller I, Cota J, Oliveira LCD, Neto MDO, *et al.* 2015. Oligomeric state and structural stability of two hyperthermophilic β-glucosidases from *Thermotoga petrophila*. *Amino Acids* **47:** 937-948.
42. Souza FHM, Inocentes RF, Ward RJ, Jorge JA, Furriel RPM. 2013. Glucose and xylose stimulation of a β-glucosidase from the thermophilic fungus *Humicola insolens*: a kinetic and biophysical study. *J. Mol. Catal. B Enzym.* **94:** 119-128.
43. Yang Y, Zhang X, Yin Q, Fang W, Fang Z, Wang X, *et al.* 2015. A mechanism of glucose tolerance and stimulation of GH1 β-glucosidases. *Sci. Rep.* **5:** 17296.
44. Yao J, Chen QL, Shen AX, Cao W, Liu YH. 2013. A novel feruloyl esterase from a soil metagenomic library with tannase activity. *J. Mol. Catal. B Enzym.* **95:** 55-61.
45. 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:** 967-975.
46. Kitagawa I, Wang H, Takagi A, Fuchida M, Miura I, Yoshikawa M. 2008. Saponin and Sapogenol. XXXIV. Chemical Constituents of Astragali Radix, the Root of Astragalus membranaceus BUNGE. (1). Cycloastragenol, the 9,19-Cycloanostane-type Aglycone of Astragalosides, and the Artifact Aglycone Astragenol. *Chem. Pharm. Bull.* **31:** 689-697.
47. Shi X, Xie J, Liao S, Wu T, Zhao LG, Ding G, *et al.* 2017. High-level expression of recombinant thermostable β-glucosidase in *Escherichia coli* by regulating acetic acid. *Bioresour. Technol.* **241:** 795-801.