Characterization of a β-Xylosidase from *Clostridium clariflavum* and Its Application in Xylan Hydrolysis

Alei Geng,* Hongcheng Wang, Jian Wu, Rongrong Xie, and Jianzhong Sun

A β-xylosidase gene, *xyl43C*, from *Clostridium clariflavum* was heterogeneously expressed in *Escherichia coli* BL21. Xyl43C showed strong activity toward xylobiose, with specific activity of 76.6 U/mg and K_m of 4.97 mM. The optimal pH and temperature of Xyl43C were pH 6.0 and 60 °C, respectively. Xyl43C retained 94.4% activity after incubation at 55 °C for 1 h, and 75.4% at 60 °C for 1 h. It also showed xylose tolerance with IC_{50} (half maximal inhibitory concentration) of approximately 100 mM. It nearly completely hydrolyzed 2 g/L of xylobiose at enzyme load of 2.51 mg/g xylobiose within 30 min and converted 40 g/L of corncob xylan into xylose at enzyme load of 1.48 mg/g xylan, with a yield of 60.9%. In conclusion, Xyl43C is an efficient xylose-tolerant β-xylosidase, with promising application potential in saccharification of xylan in biofuels industry.

Keywords: Beta-xylosidase; Clostridium clariflavum; Xylose tolerant; Corncob xylan

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INTRODUCTION

Xylan is a major component of hemicellulose in plant biomass, and it is a potential source of fermentable sugars for biofuels production. Xylan is composed of β-1,4-linked D-xylopyranose units. These units are converted to xylose *via* hydrolysis by hemicellulases, such as endo-1,4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) (Juturu and Wu 2012). The full utilization of plant biomass for biofuels production requires the simultaneous thorough decomposition of both cellulose and hemicellulose, including xylan, into monosugars. Saccharification enzymes, including xylanases and β-xylosidases, are therefore crucial for the biofuels industry, and they also have accounted for a remarkable proportion of costs in the current lignocellulosic biofuels industry (Klein-Marcuschamer *et al*. 2012). Hence there have been many efforts to develop new efficient enzymes.

β-Xylosidase is the rate-limiting enzyme for the hydrolysis of xylan, and the β-xylosidase reaction consumes xylobiose and xylotriose, which could otherwise severely inhibit xylanase activity. There are remarkable synergistic effects between xylanases and β-xylosidases in xylan degradation (Yang *et al.* 2014; Zhang *et al.* 2014). Natural cellulolytic systems usually contain beta-xylosidases, which nevertheless might not satisfy the industry's requirements. It is necessary to develop efficient β-xylosidases for improving the saccharification levels of biomass, as well as cutting down the enzyme costs. According to the carbohydrate-active enzymes database (Lombard *et al.* 2014), β-xylosidases are distributed in several glycoside hydrolase (GH) families, including GH 1, GH 3, GH 30, GH 39, GH 43, GH 51, GH 52, GH 54, GH 116, and GH 120. Efficient β-xylosidases, with both high specific activities and product tolerance, have been found in GH 3, GH 39, and GH 43, suggesting the potential of discovering new efficient β-xylosidases from these GH

families (Shi *et al.* 2013; Bhalla *et al.* 2014; Yang *et al.* 2014).

Microorganisms are the major source of saccharification enzymes. Various fungi and bacteria have cellulolytic capabilities (Shallom and Shoham 2003). *Clostridium clariflavum* decomposes plant biomass very efficiently, with an even higher yield than the famous cellulolytic bacterium, *Clostridium thermocellum* (Izquierdo *et al.* 2014). The efficient cellulolytic nature of *C. clariflavum* is partially attributed to its elaborate hemicellulase system (Izquierdo *et al.* 2012). It is necessary to explore the hemicellulases from *C. clariflavum*, especially β-xylosidases. According to the genome annotation of *C. clariflavum*, eight genes, *Clocl_0074*, *Clocl_0088*, *Clocl_1869*, *Clocl_2437*, *Clocl_2442*, *Clocl_2443*, *Clocl_2763*, and *Clocl_3058*, are putative β-xylosidases, most of which belong to GH 43 except for *Clocl_2443*, which belongs to GH 39.

In a preliminary study, three of these genes from *C. clariflavum* DSM 19732, *Clocl_0074*, *Clocl_2443*, and *Clocl_2763*, were cloned and expressed in *Escherichia coli* BL21, which is an efficient universal host for expressing prokaryotic genes, including β-xylosidase genes (Shi *et al.* 2013; Bhalla *et al.* 2014). Clocl_2443 (GenBank accession: WP_014255586.1) was found to have relative high activity toward pNPX, but only minor activities toward xylobiose (data not shown). Clocl_2763 (GenBank accession: WP_014255867.1) showed no activity toward either pNPX or xylobiose (data not shown). However, Clocl_0074 (GenBank accession: WP_014253469.1, also named Xyl43C in this work) showed high activity toward xylobiose. In this study, Xyl43C was characterized, and the potential of this enzyme in the saccharification of xylan is discussed.

EXPERIMENTAL

Materials

Gene *xyl43C* was synthesized with codons optimized by Synbio Technologies, Suzhou, China. *p*-Nitrophenyl β-D-xylopyranoside (pNPX) was obtained from Sigma, Beijing, China. Xylobiose, xylotriose, and xylotetraose were purchased from TCI, Shanghai, China. Corncob xylan was from Macklin, Shanghai, China. Vector pET-22b was from Novagen, Madison, WI, USA. *E. coli* BL21 (DE3) was from TransGen, Beijing, China. Enzymes for molecular cloning were from Takara, Dalian, China. Other chemical reagents were analytically pure and obtained from Sinopharm Chemical, Shanghai, China.

Methods

Gene clone

The synthesized *xyl43C* gene (GenBank accession: MF737442) was subcloned into the *Nco*I/*Xho*I site of pET-22b vector according to the manufacturer's instructions (Takara, Dalian, China), and verified by sequencing. The resulting plasmid was subsequently transformed in *E. coli* BL21 for gene expression.

Gene expression and protein purification

The transformant carrying pET-22b-*xyl43C* was inoculated into 4 mL of Luria-Bertani (LB) medium, supplemented with 100 μg/mL ampicillin, and grown at 37 °C overnight. The overnight culture was then inoculated into 1 L of LB, grown to OD 1.2 at 37 °C, and induced by 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C, 120 rpm for 15 h.

Cells were harvested and disrupted using a supersonic-wave disrupting method as described by Yan *et al.* (2013). The Xyl43C protein was heat-treated and purified using a nickel column (HisTrap FF column, GE Healthcare, Piscataway, NJ) according to the previous protocol (Geng *et al.* 2015), and Xyl43C was eluted at 300 mM imidazole. The targeted elution was desalted using a HiTrap desalting column (GE Healthcare). The purified protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue R-250 staining.

Enzymatic activity assay

The optimal pH and temperature of Xyl43C were assayed using pNPX as the substrate at a final concentration of 2 mM at various pHs and temperature conditions, and read at OD_{410}. Buffers used were 200 mM sodium acetate (NaAc) for pH 5.0 to 5.5, 200 mM sodium phosphate for pH 6.0 to 8.0, and 200 mM glycine-NaOH for pH 8.5 to 10.0. The standard activity assays were determined in a total volume of 500 μL and a final buffer concentration of 40 mM under optimal conditions, that is pH 6.0 and 60 °C. The activity of Xyl43C on xylobiose was determined using substrates at 5 g/L and measured by high performance liquid chromatography (HPLC) using a refractive index detector (LC-20A, Shimadzu, Kyoto, Japan), equipped with Aminex HPX-87H column (Bio-Rad, Beijing, China). A unit of enzyme activity (U) was defined as the number of micromoles of pNP released from pNPX or half number of micromoles of xylose produced from xylobiose per minute.

Thermostability of Xyl43C was accessed by calculating the relative residual activities of this enzyme after a heat-treatment process, which was carried out for various time periods and at different temperatures. Xylose tolerance was determined by calculating the relative residual activities of the enzyme toward pNPX, in the presence of various concentration of xylose. The K_m values were measured using the Lineweaver-Burk method under optimal conditions.

Xylan hydrolysis

Xylan from corncob and xylobiose were used as substrates to determine the hydrolysis efficiency of Xyl43C. The release of xylose was accessed by using HPLC to determine the percent conversion of xylan or xylobiose. All results were the average of three replicates, and Student's *t*-test was employed to analyze the significance of differences.

RESULTS AND DISCUSSION

Expression and Purification of Xyl43C

The codon optimized gene, *xyl43C*, was successfully expressed in *E. coli* BL21 (Fig. 1, lane 1). According to the Pfam annotation (http://pfam.xfam.org), Xyl43C belongs to GH 43, and it shares 81% identity with a putative protein (GenBank accession: WP_036941306.1) from *Pseudobacteroides cellulosolvens*. The full length *xyl43C* is 981 bp, however, the apparent molecular weight of the recombinant Xyl43C was approximately 7 kDa larger than the predicted size of 40.3 kDa (including the signal peptide and histag, Fig. 1, lane 3). The increased molecular weight was unusual but had also been observed from some fungal proteins, whose apparent molecular weight were approximately 10 kDa larger than their predicted sizes (Inoue *et al.* 2013; Damasio *et al.* 2017). The increments

in apparent molecular weight of proteins from *E. coli* were also observed from a β-galactosidase and an α subunit of a RNA polymerase (Pedersen *et al*. 1978). The increments in apparent molecular weight of proteins from fungi were attributed to the glycosylation of proteins, while the reason for those from *E. coli* is currently unknown.

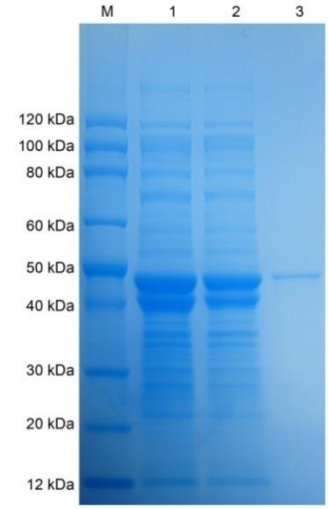

Fig. 1. Expression and purification of Xyl43C as analyzed by SDS-PAGE on a 10% gel. Lane M, protein marker; Lane 1, total soluble protein; lane 2, heat-treated protein at 55 °C for 30 min; and lane 3, purified protein using combination of a nickel column and a desalting column

Optimal pH and Temperature

According to Fig. 2, Xyl43C was active under weak acid, neutral, or weak alkaline conditions (pH 5.5 to pH 8.5), with an optimal pH at 6.0. Glycine buffer seemed to have a simulation effect on the activity of Xyl43C, and the relative activity at pH 8.5 retained approximately 50% of the maximum activity, which was even higher than the relative activities at pH 7.5 and pH 8.0 in phosphate buffer. The activities of Xyl43C gradually increased as the reaction temperature was increased from 25 °C to 60 °C (Fig. 3). As the temperature was further increased from 60 °C to 70 °C, the enzyme activities decreased to less than 50% percent of the maximum. The optimal pH and temperature of Xyl43C were similar to the optimal growth conditions of *C. clariflavum* (Shiratori *et al.* 2009). Under the optimal condition, the specific activities of Xyl43C toward pNPX and xylobiose were 4.4 U/mg and 76.6 U/mg, respectively. The K_m values were 1.16 mM and 4.97 mM for pNPX and xylobiose, respectively.

Both the specific activity and substrate affinity of Xyl43C toward xylobiose were remarkably higher than the β-xylosidases either from *Trichoderma reesei* (25 U/mg, 7.2 mM, respectively) (Semenova *et al.* 2009) or *Aspergillus fumigatus* (15.6 U/mg, 23.8 mM) (Kitpreechavanich *et al.* 1986). There are several β-xylosidases with much higher activities than Xyl43C, based only on the activity toward pNPX (Shi *et al.* 2013; Bhalla *et al.* 2014). The activity toward pNPX might not truly reflect the efficiency of β-xylosidases in xylobiose hydrolysis.

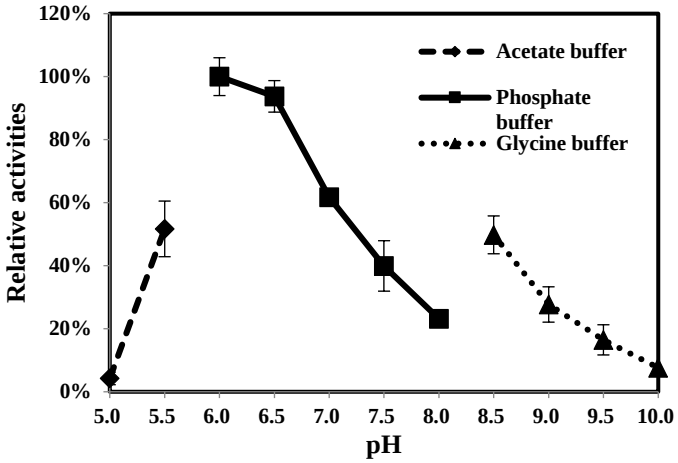

Fig. 2. Optimal pH of Xyl43C. The enzyme activities were determined using pNPX as substrate at 60 °C and various pHs for 5 min. Error bars represent standard deviations (*n* = 3).

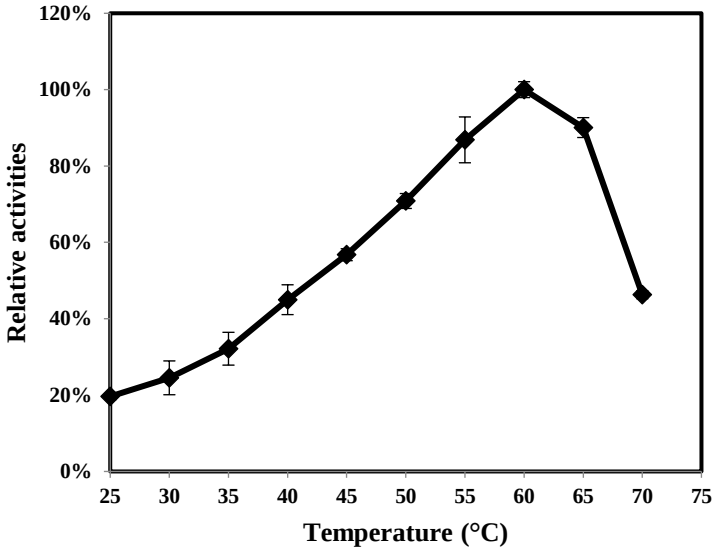

Fig. 3. Optimal temperature of Xyl43C. The enzyme activities were determined using pNPX as substrate at pH 6.0 and different temperature for 5 min. Error bars represent standard deviations (*n* = 3).

Thermostability and Xylose Tolerance

Xyl43C showed relative high thermostability at 55 °C than at 60 °C (Fig. 4). After a 1-h heat-treatment, the residual activities of Xyl43C were 94.4% at 55 °C treatment but 75.4% at 60 °C treatment. After a 15-h heat-treatment, Xyl43C still retained 27.4% or 8.5% activities at 55 °C or 60 °C, respectively. In the xylose tolerance tests (Fig. 5), as the final concentrations of xylose were increased from 0 mM to 200 mM, the relative activities gradually decreased from 100% to 26.8%, with an IC_{50} value (half maximal inhibitory concentration) at approximately 100 mM.

These results suggested that Xyl43C was a relatively thermostable and xylose-tolerant β-xylosidase. The thermostability of Xyl43C was higher than that of β-xylosidases from *Penicillium sclerotiorum* (Knob and Carmona 2009) and *Geobacillus thermoleovorans* (Wagschal *et al.* 2009), which both retained less than 40% activities after incubation at 60 °C for 1 h. The xylose tolerance was much higher than some fungal β-xylosidases from *Arxula adeninivorans*, *Aureobasidium pullulans*, and *T. reesei*, which has

a K_i value of 2 mM to 10 mM (Zanoelo *et al.* 2004), but lower than β-xylosidases from *Geobacillus* sp. strain WSUCF1 (Bhalla *et al.* 2014) and *Thermotoga thermarum* (Shi *et al.* 2013), which both retained more than 80% activity in the presence of 100 mM xylose.

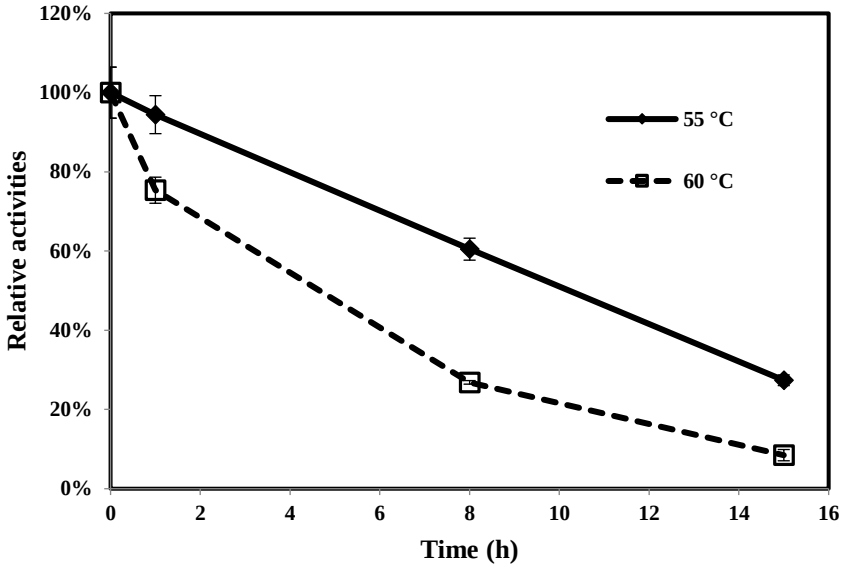

Fig. 4. Thermostability of Xyl43C. The enzyme activities were determined using pNPX as substrate at pH 6.0 and 60 °C for 5 min, after heat treatments at 55 °C or 60 °C for various time spans. Error bars represent standard deviations (*n* = 3).

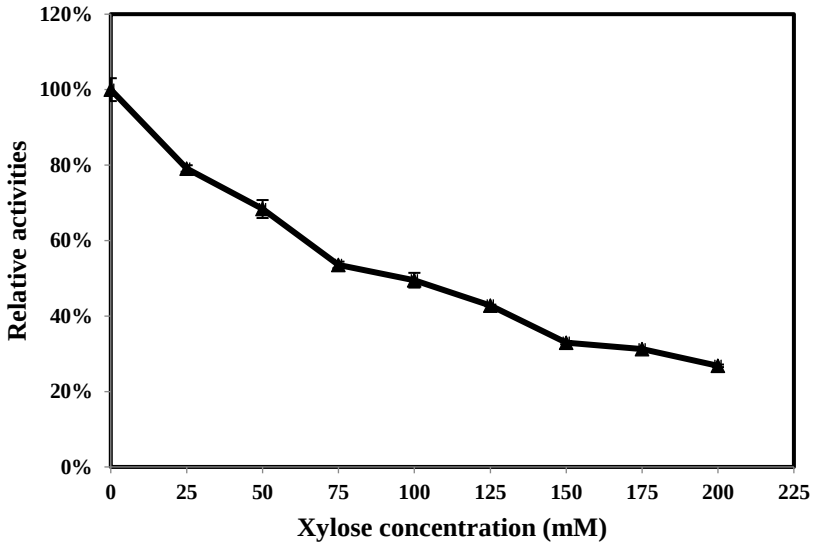

Fig. 5. Xylose tolerance of Xyl43C. The enzyme activities were determined using pNPX as substrate at pH 6.0 and 60 °C for 5 min, in the presence of xylose at different concentrations. Error bars represent standard deviations (*n* = 3).

Hydrolysis of Xylobiose and Xylan

Xyl43C was highly active on xylobiose. As shown in Fig. 6, in the hydrolysis of 2 g/L of xylobiose, as the loads of Xyl43C were increased from 0.36 mg/g xylobiose to 2.51 mg/g xylobiose, the production of xylobiose greatly increased from 14.9% to 97.9%, while the residual xylobiose remarkably decreased from 85.1% to 2.1%. Further increasing the

load of Xyl43C to 3.95 mg/g xylobiose, the yield of xylobiose achieved 99.4%, but without significance (p = 0.15).

Xyl43C was also used to directly hydrolyze corncob xylan, which is rich in xylooligosaccharides. The main components of the corncob xylan were xylobiose, xylotriose, and xylotetraose, which accounted for 85.6% of the total weight of the corncob xylan, as analyzed by HPLC. In the hydrolysis of 40 g/L of corncob xylan, as the loads of Xyl43C was increased from 0 to 1.48 mg/g xylan, xylose gradually accumulated from 0.05 g/L to 24.37 g/L, and the highest yield achieved at 1.48 mg/g xylan, which equaled 60.9% conversion of corncob xylan (Fig. 7). Moreover, after hydrolysis, the concentration of xylobiose, xylotriose, and xylotetraose were all maintained at low levels (2.15, 1.14, and 1.00 g/L, respectively), when the enzyme load was at 1.48 mg/g xylan. Further increasing the enzyme load to 14.8 mg/g xylan appeared to only slightly increase the percent conversion to 62.3%, but without significance (p = 0.2).

Xyl43C hydrolyzed not only xylobiose but also small xylooligosaccharides such as xylotriose and xylotetraose. This versatility was also observed in other β-xylosidases (Lagaert *et al.* 2011; Shi *et al.* 2013). Xyl43C could nearly completely hydrolyze 2 g/L of xylobiose, but could not completely decompose corncob xylan. The incomplete hydrolysis of corncob xylan was probably because of product inhibition, as well as the lack of assistance of other relevant enzymes. For instance, Xue and Shao (2004) reported that a β-xylosidase from *Thermotoga maritima* could achieve nearly 100% conversion of corncob xylan only in the presence of a xylanase, as well as an α-arabinosidase. While, excess of two β-xylosidases from *Bifidobacterium adolescentis* was used separately to hydrolyze 5 mg/mL of xylooligosaccharides from wheat in a 1-h incubation; however, neither of them achieved more than 30% conversion (Lagaert *et al.* 2011). Although xylooligosaccharides are valuable in the field of food and medicine (Mussatto and Mancilha 2007), fully converting xylan into xylose is always the goal so as to raise the overall yield of biofuels in the biofuels industry. Future work might focus on the synergy between Xyl43C and xylanases as well as α-arabinosidases in xylan hydrolysis.

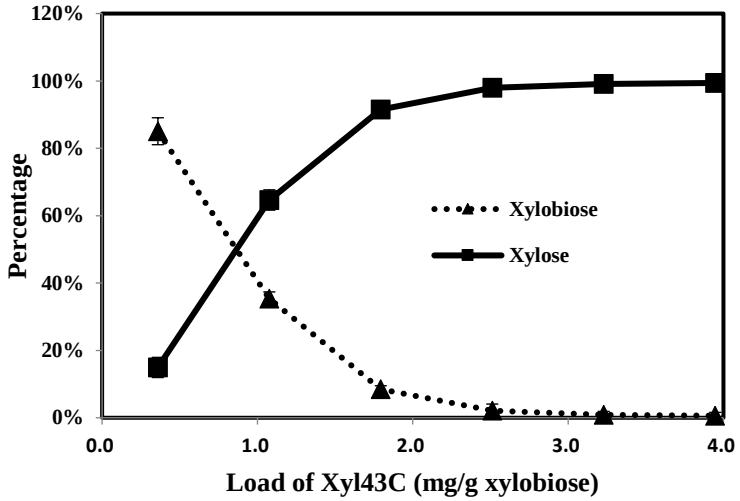

Fig. 6. Effect of different enzyme loads on the hydrolysis of xylobiose. Reaction was performed at 60 °C and pH 6.0 for 30 min. NaAc buffer was used for the better separation of xylobiose during HPLC. Xylobiose was provided at a final concentration of 2 g/L. The total reaction volume was 2 mL.

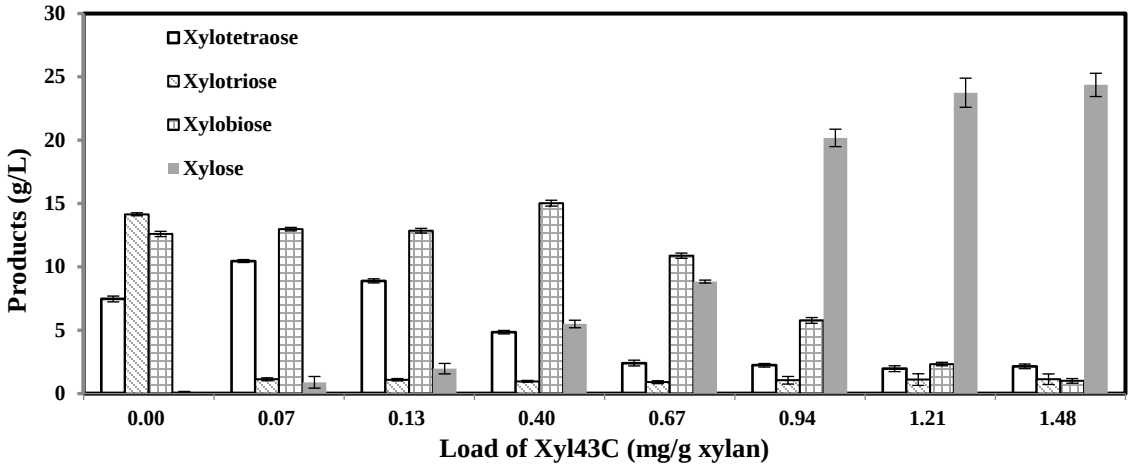

Fig. 7. Effect of different enzyme loads on the hydrolysis of corncob xylan. Reaction was performed at 60 °C and in pH 6.0 NaAc buffer for 30 min. Corncob xylan was supplemented at a final concentration of 40 g/L. The total reaction volume was 2 mL.

CONCLUSIONS

1. Xyl43C is an efficient β-xylosidase from *Clostridium clariflavum*, and it was successfully heterogeneously expressed in *E. coli* BL21.
2. Xyl43C had a high specific activity of 76.6 U/mg toward xylobiose, and it showed xylose tolerance with IC_{50} of approximately 100 mM.
3. Xyl43C could nearly completely convert 2 g/L of xylobiose at enzyme load of 2.51 mg/g xylobiose within 30 minutes.
4. Xyl43C could convert 40 g/L of xylose within 30 min at an enzyme load of 1.48 mg/g xylan, with a yield of 60.9%. The completely hydrolysis of corncob xylan might need the assistance of a xylanase and an α-arabinosidase.
5. Xyl43C is an efficient β-xylosidase, and it should have potential application in saccharification of xylan in biofuels industry.

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