Two Bifunctional β -Xylosidase / α -L-Arabinofuranosidases From GH43 With Different Structures in the xylan Degradation Strain of *Paenibacillus Physcomitrellae* XB Displayed the Similar xylo-oligosaccharides Degradation Ability

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Research

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Abstract

Background

The strain *Paenibacillus physcomitrellae* XB isolated from moss of *Physcomitrella patens* was found have the xylan degradation ability, but its degradation characteristics and the related mechanism has not been revealed.

Results

In this study, *Paenibacillus physcomitrellae* XB exhibited different xylan degradation ability under the different substrates of corncob xylan (CCX), oat spet xylan (OSX), wheat flour arabinoxylan (AX) and beech wood xylan (BWX). Genomic analysis showed that ~ 38 genes were related to xylan degradation, and quantitative real time RT-PCR showed that two glycoside hydrolase family 43 genes (*Pph_0602* and *Pph_2344*) were up-regulated on 1% CCX and xylose. Substrate-specific experiments with purified proteins Ppxyl43A (*Pph_0602*) and Ppxyl43B (*Pph_2344*) revealed that both of them exhibited β -xylosidase activity toward chromogenic substrate *p*-nitrophenyl-D-xylopyranoside and α -L-arabinofuranosidase activity toward *p*-nitrophenyl- α -L-arabinofuranoside, indicating at least bifunctionality. Combined their degradation features on the natural substrates of different xylans with the hydrolytic products separated by thin-layer chromatography and high-performance anion exchange chromatography profiles, it was found that both Ppxyl43A and Ppxyl43B were with the similar degradation ability on xylo-oligosaccharides (like CCX, OSX, xylohexaose and xylobiose). Both of them even could hydrolyze xylohexaose and xylobiose completely to xylose, but could not hydrolyze BWX and AX to produce xylooligosaccharides or xylose, suggesting they have no endo-xylanase activity and mainly hydrolyze xylo-oligosaccharides by β -xylosidase activity. Moreover, the kinetic parameters of β -xylosidase and α -L-arabinofuranosidase of both two proteins indicated their affinity with all the detected natural substrate (CCX) and chromogenic substrates were nearly similar. In addition, despite having no signal peptides, both of them might export outside the cell by the nonconventional pathways. However, Ppxyl43B exhibited wider temperature and pH ranges, higher pH and thermostability, and was less influenced by metal ions than Ppxyl43A. Given its enzymatic characteristics and predicted structure, it is likely that the C-terminus domain (*GH43_C2*) of Ppxyl43B enhances the stability of the two enzymes and also restricts the substrates' or metal ions' access to the active sites.

Conclusions

Ppxyl43A and Ppxyl43B were β -xylosidase/ α -L-arabinofuranosidase bifunctional enzymes with different structures from *Paenibacillus physcomitrellae* XB and exhibited similar xylo-oligosaccharides hydrolyse ability, which would be useful in the further lignocellulosic biomass conversion.

Background

Lignocellulosic biomass is the most abundant renewable resource and can be obtained from agricultural residues, herbaceous grasses, and forest harvests. Xylan is one of the major polymeric hemicellulosic constituents of lignocellulosic biomass, and its effective utilization is crucial for the economical production of highly-valued substances [1].

Xylan is a heteropolysaccharide consisting of a linear backbone chain of β -1,4-linked xylose units that can be substituted by various residues, such as α -l-arabinosyl, *O*-acetyl, 4-*O*-methyl D-glucurono and feruloyl [2]. Due to the structural complexity, the complete degradation (side chain and backbone cleaving) of xylan is very difficult and requires up to six distinct enzymes [3]. Generally during the degradation of the main xylan backbone, endo- β -1,4-xylanase acts randomly to liberate unbranched xylooligosaccharides (XOSs) that are then further degraded by β -xylosidase into xylose, the involved glycoside hydrolase (GH) enzyme were found in GH10, GH11, GH43, GH5 and GH8 families; while the side chain cleaving is commonly performed by α -glucuronidase, α -L-arabinofuranosidase, acetyl xylan esterase, feruloyl esterase and α -L-galactosidase, which were often found in GH family of GH43, GH51, GH62, GH67, GH115 and carbohydrate esterase (CE) family of CE4, CE1, CE5, CE6 and CE16 [3].

The GH43 family is of particular interest and has many multifunctional xylanolytic enzymes, including bifunctional β -xylosidase/ α -L-arabinofuranosidase from *Clostridium stercoreum* [4], *Paenibacillus woosongensis* [5] and *Thermotoga thermarum* [6]; trifunctional endo-xylanase/ β -xylosidase and α -L-arabinofuranosidase from *Paenibacillus curdlandolyticus* B-6 [7, 8]; and multifunctional β -xylosidase/ α -L-arabinofuranosidase/ β -1,4 lactase/ α -1,6 raffinase/ α -1,6 stachyase/ β -galactosidase/ α -1,4 glucosidase from a cow rumen microbial consortium [9]. Due to different enzymes have different specificities and activity conditions causing practical obstacles for bringing efficient degradation, bi/multifunctional enzymes would be helpful in greatly reducing the production cost and were worthy of much more attention.

Although many biomass-degrading enzymes characterized to date are from fungal secretomes, such as *Thermothelomyces thermophile* [10], more recently bacterial strains have been shown to exhibit strong xylan degradation ability and many related xylanolytic enzymes have been shown to have potential application value in biomass conversion [4]. *Paenibacillus physcomitrellae* strain XB is a strain isolated from moss [11]. Based on its putative xylanolytic gene structure and expression levels in the presence of xylan and xylose, we identified two genes from GH43 that encode bifunctional enzymes and analyzed their characteristics. We speculate they may be important for boosting xylo-oligosaccharide hydrolysis efficiency in biomass conversion.

Results

Xylan degradation characteristics of strain XB

In order to determine the xylan degradation ability of strain XB, four kinds of xylan from different sources were used as substrates in the assay system. The result showed that much more reducing sugars were produced from the crude enzyme when using 1% OSX and CCX as substrate, with the highest value (3.28

mg/mL and 3.02 mg/mL, respectively) on the fifth day. For AX and BWX, there was less reducing sugar detected, and the highest peak appeared on the third (1.59 mg/mL) and fourth day (1.58 mg/mL), respectively (Fig. 1a). Thus, strain XB had the ability to degrade all tested xylans, but its degradation ability was different on different xylan types.

Genomic features and gene prediction of xylanolytic enzyme of strain XB

Genome assembly of strain XB resulted in 4 contigs, which were then combined into one scaffold. The genome was 4.83 Mb in size and the G+C content was 51.87 mol%. The NCBI Prokaryotic Genome Annotation Pipeline identified 4371 genes and 4198 proteins. Annotation with CAZy revealed strain XB had a relatively high proportion of CAZomes for 210 proteins (Additional file 2: Table S1). The glycoside hydrolase (GH) family accounted for more than 50% (107) of them, followed by the glycosyl transferase family (GT, 36), the carbohydrate esterase family (CE, 19) and the carbohydrate binding module family (CBM, 47). Only one axillary activity (AA) family and none of the polysaccharide lyase (PL) families were detected in the CAZomes of strain XB. (Additional file 1: Fig. S1).

When combining CAZy and COG/KEGG/GO/NR results, approximately 38 genes were considered putative xylan degradation-related genes (Table 1). Of these, two genes' (*Pph_0648* and *Pph_1610*) encoded proteins had endo-1,4- β -xylanase activity and belonged to the GH11 and GH10 families, respectively. One gene was annotated as xylanase and assigned to GH8 family (*Pph_0002*). In addition, there were 35 predicted proteins, belonging to GH43 (6), GH51 (3), GH31 (4), CBM 35 (1), GH36 (6), GH54 (1), GH67 (1) and CE4 (13) that encoded β -xylosidase, α -N-arabinofuranosidase, α -glucuronidase, α -galactosidase, α -xylosidase or peptidoglycan/xylan/chitin deacetylase, respectively.

Putative xylan degradation gene expression induced by CCX and xylose

The relative expression levels of genes encoding GH8, GH11, GH10, six of GH43 and three GH51 families were measured via RT-qPCR using *recA* as the reference gene (Additional file 2: Table S2; Fig. 1b). The majority of genes exhibited up-regulation in R2A broth with 1% CCX, and several were over-expressed more than 5-fold ($p < 0.01$), such as *Pph_1610* (GH10), *Pph_0648* (GH11), *Pph_0602*, *Pph_2344*, *Pph_2078* and *Pph_3723* of GH43, *Pph_4538* (GH51). Only two genes (*Pph_0602* and *Pph_2344*) displayed extremely high expression levels when 1% xylose was added to the medium, up-regulating more than 5-fold (p -value < 0.01). Thus, *Pph_0602* and *Pph_2344* not only could be induced to overexpress at high levels in a substrate of CCX but also could be prompted by xylose. They were further analyzed based on protein expression, purification, and enzymatic characteristics.

Gene cloning and purification of recombinant enzymes

The coding region of two GH43 family encoding genes *Pph_0602* and *Pph_2344* were linked with expression vector pET28a (+) based on the two restriction enzymes *Bam* HI and *Xho* I for construction of recombinant proteins Ppxyl43A (pET28a-Pph_0602), and *Eco*R I and *Xho* I for recombinant proteins Ppxyl43B (pET28a- Pph_2344) (Additional file 2:Table S2). Ppxyl43A and Ppxyl43B contained open

reading frames encoding a protein of 329 (~36.9kDa) and 543 (~62.3kDa) amino acids, respectively (Additional file 2:Table S3). The SDS-PAGE showed single bands consistent with the expected sizes of Ppxyl43A and Ppxyl43B after purification and washing with buffer containing 50 and 100 mM imidazole (Fig. 2a and 2b).

In order to understand the reaction patterns of Ppxyl43A and Ppxyl43B, the reducing sugars and xylose amounts produced from 1% CCX assay showed that BL21 with either recombinant Ppxyl43A or Ppxyl43B could hydrolyze CCX and produce the significantly more reducing sugars (Fig.2c) and xylose (Fig. 2d) intracellularly than control ($p<0.01$). Whether for xylose or reducing sugars, recombinant Ppxyl43A exhibited an increasing trend intracellularly with induction time from 2 h to 18 h, and recombinant Ppxyl43B expressed higher activity levels from 2 h-induction and maintained a relatively stable level up to 18-h induction time. Moreover, Ppxyl43A and Ppxyl43B produced nearly similar amounts of reducing sugars (Fig.2c), while recombinant Ppxyl43B produced much more xylose than Ppxyl43A (Fig.2d). In addition, with a longer induction time (6 h and 18 h), some reducing sugars could be detected from both two recombinants and little xylose could be assayed mainly from recombinant Ppxyl43B extracellularly.

Substrate-specific and hydrolytic products analyses

The substrate specificity of purified Ppxyl43A and Ppxyl43B was determined by measuring the amount of reducing sugars toward several polysaccharides and xylose toward *p*NP derivatives, including *p*NPX and *p*NPAf, as substrates. Ppxyl43A and Ppxyl43B displayed similar substrate degradation ability, and both could hydrolyze *p*NPX, *p*NPAf, and various types of polysaccharides, containing CCX, XOS, and OSX. Ppxyl43A was more active toward *p*NPX, and other polysaccharides, such as CCX, XOS, X2, and X6 ($p<0.01$) than Ppxyl43B after 10 min's digestion. However, neither Ppxyl43A nor Ppxyl43B could hydrolyze BWX and AX to reducing sugars (Table 2).

In order to determine the action model of Ppxyl43A and Ppxyl43B, hydrolytic products were further investigated by TLC and HPAEC method. Comparing the TLC profiles of different substrates with their hydrolytic products, CCX and OSX used in this survey mainly contained xylobiose, Xylotriose, Xylotetraose, et cetera—thus, in fact, should belong to xylo-oligosaccharides. When digested with Ppxyl43A and Ppxyl43B, xylose could be produced from CCX and OSX (Figure 3a), and their profiles were similar with those from XOS, but BWX could not be hydrolyzed by either Ppxyl43A or Ppxyl43B to produce xylo-oligosaccharides or xylose (Figure 3b) and only very little xylose was also produced from AX (Additional file1: Fig. S2). The TLC hydrolysis profile of X6 and X2 exhibited that both Ppxyl43A and Ppxyl43B could hydrolyze X6 and X2, even completely, to xylose (Figure 3c). Thus, All TLC profiles showed that Ppxyl43A and Ppxyl43B mainly hydrolyze CCX and OSX via β -xylosidase activity. In addition, although very weak TLC signals of arabinose could be observed from the substrate of *p*NPAf (Additional file1: Fig. S2), it also reflected Ppxyl43A and Ppxyl43B could hydrolyze *p*NPAf to arabinose and supposed that both had α -L-arabinofuranosidase activity. Thus, both Ppxyl43A and Ppxyl43B possess at least bifunctional enzyme activity for β -xylosidase and α -L-arabinofuranosidase. The hydrolytic products separated by HPAEC profiles also confirmed xylose could be produced from X6 (Fig. 4a, 4b and 4c) and

X2 (Additional file1: Fig. S3) and arabinose was produced from *p*NPAf (Fig. 4d, 4e and 4f) after digestion by Ppxyl43A and Ppxyl43B.

In addition, According to the TLC hydrolytic profile at different reaction times, Ppxyl43A displayed more rapid CCX hydrolytic activity than Ppxyl43B. Xylose could be detected at 3 min post-incubation with Ppxyl43A, and the amount increased significantly within 1 h (Fig. 5a). The xylose signal could not be observed until 60 min post-incubation by Ppxyl43B (Fig. 5b).

Effects of pH and temperature on activity and stability

The effects of pH on β -xylosidase and α -L-arabinofuranosidase activity of Ppxyl43A and Ppxyl43B further examined using *p*NPX and *p*NPAf displayed that the optimum pH value was 6.0 for β -xylosidase but 5.0 for α -L-arabinofuranosidase for Ppxyl43A (Fig. 5a). Ppxyl43A retained more than 60% β -xylosidase activity in pH range 3.0 to 8.0 but only retained ~60% of α -L-arabinofuranosidase activity in pH range 4.0-6.0. The pH stability assay showed that highest stability occurred in pH range 5.0-8.0 for β -xylosidase and 6.0-8.0 for α -L-arabinofuranosidase activity (Fig. 5b). However, for Ppxyl43B, the optimal pH for β -xylosidase was 5.0 and 3.0 for α -L-arabinofuranosidase activity (Fig. 5c). β -xylosidase could act in the widest pH range, retaining 80% or more activity over the entire detected pH range of 2.0 to 9.0; whereas, the relative activity of α -L-arabinofuranosidase was only 60% of total within pH range 3.0-6.0. The pH stability assay showed that both enzymes could retain 60% relative activity from pH 3.0-9.0 (Fig. 5d). Thus, there were notable differences in suitable pH ranges for the two enzymes—Ppxyl43B had a wider pH range and better pH stability than Ppxyl43A.

The optimal temperature of purified Ppxyl43A for α -L-arabinofuranosidase activity was 40°C, while that for β -xylosidase was 50°C (Fig. 6a). Thermostability assays showed that Ppxyl43A had a relatively high activity only when the temperature was lower than the optimum and could retain more than 50% activity across the temperature range 10-40°C for α -L-arabinofuranosidase and 20-50°C for β -xylosidase (Fig. 6b). While the optimal reaction temperature of Ppxyl43B was 40°C for both enzymes, β -xylosidase activity level were similar across the entire range of 20-70°C (Fig. 6c), and they displayed high thermostability in temperatures ranging from 10 to 70°C (Fig. 6d). Thus, Ppxyl43B exhibited a wider temperature range and higher thermostability than Ppxyl43A, especially when using *p*NPX as substrate for β -xylosidase activity.

Effects of metal ion on enzyme activity

Among the 10 kinds of metal ions detected, the β -xylosidase activity of Ppxyl43A and Ppxyl43B could be enhanced by 10 mM Ca^{2+} and Mn^{2+} by approximately 10% over the control group, slightly inhibited by Fe^{3+} and Ni^{2+} (10-30% loss of activity) and strongly inhibited by Zn^{2+} and Cu^{2+} by loss of 70-80% activity. In addition, Mg^{2+} and K^{+} enhanced β -xylosidase activity of Ppxyl43B but decreased its activity in Ppxyl43A. However, the α -L-arabinofuranosidase activity of Ppxyl43A could be stimulated by Mg^{2+} , Co^{2+} , Ca^{2+} , K^{+} , Ba^{2+} and Mn^{2+} , especially by Ca^{2+} (increasing by approximately 50% activity), but strongly

inhibited by Fe^{3+} (43% loss of activity), Cu^{2+} , and Zn^{2+} (82-90% decrease of activity); whereas the α -L-arabinofuranosidase activity of Ppxyl43B could only be significantly enhanced by Fe^{3+} , Ni^{2+} , and K^+ , strongly inhibited by Zn^{2+} , and no remarkable differences were observed in activity levels in the presence or absence of other metal ions (Table 3).

Kintetic properties

Kinetic parameters such as V_{max} and K_m were determined from regression lines of Lineweaver-Burk plots using $p\text{NPX}$ and $p\text{NPAf}$, respectively (Table 4). The V_{max} and K_m values obtained for β -xylosidase of Ppxyl43A based on the substrate of $p\text{NPX}$ were 8.64 ± 1.87 U/mg and 2.37 ± 0.54 mM, while those for Ppxyl43B were 13.49 ± 0.27 U/mg and 2.12 ± 0.05 mM, respectively; when using CCX as the substrate, the detected V_{max} and K_m values were 481.48 ± 159.30 U/mg and 88.44 ± 48.16 mg/ml for Ppxyl43A, and 316.80 ± 217.95 U/mg and 75.16 ± 34.35 mg/ml for Ppxyl43B, and there were no significant differences between K_m values of two proteins ($p > 0.05$). In addition, the kinetic parameters for $p\text{NPAf}$ showed that the average V_{max} and K_m values were 5.73 ± 5.35 U/mg and 3.03 ± 1.64 mM for Ppxyl43A, were slightly higher than those of Ppxyl43B (0.86 ± 0.60 U/mg and 0.82 ± 0.28 mM) but not significantly. Thus, these two proteins showed similar affinity to both chemical and natural substrates.

Discussion

In this study we characterized the xylan degradation ability of strain *Paenibacillus physcomitrellae* XB and two β -xylosidase/ α -L-arabinofuranosidase bifunctional enzymes of GH43 family in strain XB were purified and analyzed, this is the first time to determine its xylan degradation characteristics and mechanism of strain XB. The result showed that it had the ability to degrade all tested xylans, but its degradation ability was different on different xylan types. Of them, BWX and AX used in this study were insoluble, and their structure might be much more recalcitrant than CCX and OSX, hence more difficult to hydrolyze.

Screening the whole genome of strain XB, approximately 38 genes might be related to xylan degradation, which contained the putative encoding genes of endo-xylanase, β -xylosidase, α -N-arabinofuranosidase, α -glucuronidase, α -galactosidase, α -xylosidase and peptidoglycan/xylan/chitin deacetylase. Of all these xylanolytic enzymes, β -xylosidase had the highest structural diversity and could hydrolyze more glycosidic bonds than any other xylanolytic enzymes, thus, was considered as a crucial enzyme in xylan degradation. Previous reports revealed that GH43 was the second largest β -xylosidase-containing family, even several of them have been found to be bi/multifunctional [12] and played important roles in the xylan degradation [7, 8, 13]. However, most of the characterized β -xylosidases, to some extent, inhibited themselves by xylose, arabinose, glucose, and/or other monosaccharides [14, 15]. Even the β -xylosidase in some GH43 family members was inhibited 25-66% by D-xylose [12]. However, in this survey both *Pph_0602* and *Pph_2344* were predicted as β -xylosidase of GH43 family and up-regulated in the medium supplemented with xylose and CCX, suggesting that β -xylosidase expression might be improved by

xylose in some cases. Due to this interesting point, their enzymatic characteristics were worthy of further analyses.

Assessment of the reducing sugars and xylose produced by the two recombinants of Ppxyl43A (pET28a-*Pph_0602*) and Ppxyl43B (pET28a-*Pph_2344*) revealed that some reducing sugars and xylose could be detected extracellularly with a longer induction time (6 h and 18 h), which reflects that part of Ppxyl43A or Ppxyl43B could be exported outside the cell. However, the two proteins of Ppxyl43A and Ppxyl43B did not possess N-terminal signal peptides according to SignalP prediction results (Additional file 2: Table S3), and therefore, should only express intracellularly. Whereas previous reports found that some proteins can be exported without a classical N-terminal signal peptide, and this kind of secretion is known as leaderless secretion or non-conventional/non-classical secretory pathway [16]. Further prediction by SecretomeP 2.0a Server (SecP scores were 0.91 and 0.84 for Ppxyl43A and Ppxyl43B, respectively) exceeded the threshold of 0.5 for bacterial sequences (Additional file 2: Table S3) supposed that Ppxyl43A or Ppxyl43B might also secret outside the cell by this kind of leaderless pathway. In this case, these proteins may play important roles in CCX degradation by strain XB by exporting of outside the cells. In addition, whether for xylose or reducing sugars, recombinant Ppxyl43A exhibited an increasing trend intracellularly with induction time longer, and recombinant Ppxyl43B expressed relatively stable and higher activity levels from 2 h- to 18-h induction time; moreover, although recombinant Ppxyl43A and Ppxyl43B produced nearly similar amounts of reducing sugars, recombinant Ppxyl43B could produce much more xylose than Ppxyl43A. All of these results implied that the action patterns of Ppxyl43A and Ppxyl43B were not completely same.

The substrate specific assay showed that both Ppxyl43A and Ppxyl43B possess at least bifunctional enzyme activity for β -xylosidase and α -L-arabinofuranosidase based on the chemical substrate of *p*NPX and *p*NPAf. According to the different kinds of natural xylan substrates and their hydrolysis products, it was confirmed that Ppxyl43A and Ppxyl43B mainly hydrolyze CCX and OSX via β -xylosidase activity and could not hydrolyze BWX by endo-xylanase activity, which also suggests that although strain XB has the ability to degrade BWX and AX, this degradation likely needs endo-xylanase activity expressed by other proteins. Further predicting their structure by SWISS-MODEL and InterPro Scan Server, Ppxyl43A showed 65.09% sequence identity with 5glk.1.A, which is a monomer structure of *CoXyl43* that belongs to the *GH43_AXH-like* subgroup [17]. The *CoXyl43* structure is composed of a single catalytic domain that consists of a five-bladed β -propeller [18] and proved to be with β -xylosidase and α -arabinofuranosidase activity to promote plant biomass saccharification by degrading xylooligosaccharides into xylose [19]; while Ppxyl43B displayed 57.71% sequence identity with the template of 5zqj.1.A [20] and also had a domain with a five-bladed β -propellor belonging to the *GH43_XybB* subgroup with β -1, 4-xylosidase and α -L-arabinofuranosidase activity. In addition, both Ppxyl43A and Ppxyl43B contained three acidic residues (Asp-20, Asp-140 and Glu-227 for Ppxyl43A; Asp-14, Asp-127 and Glu-187 for Ppxyl43B) (Additional file 2: Table S3), which were considered active sites. Thus, the detected bifunctional enzyme activity of Ppxyl43A and Ppxyl43B was generally consistent with the predicted results. Moreover, the kinetic parameters of these two proteins also confirmed their affinity with substrates of bifunctional β -xylosidase/ α -L-arabinofuranosidase.

However, the enzyme characteristics of Ppxyl43B exhibited a wider pH and temperature range, better pH stability and thermostability than Ppxyl43A, especially when using *p*NPX as substrate for β -xylosidase activity. Moreover, the xylose was released much more slowly from CCX when digested by Ppxyl43B (at 60 min post-incubation) than by Ppxyl43A (at only 3 min post-incubation). Further comparing the structure of these two proteins, it was found that besides the domain of a five-bladed β -propellor, Ppxyl43B also contains an additional β -xylosidase C-terminal Concanavalin A-like domain (*GH43_C2*), which has a sandwich structure of β strands with a complex topology. Its structure was considered like a long V-shaped groove, forming a single extended substrate-binding surface across the face of the propeller, which probably restricts access to a portion of the active site forming a pocket [12]. Hence, it might be more difficult for substrates to bind to this active site than those without this C-terminal domain, which may be the reason that why xylose was produced much more slowly by Ppxyl43B. This extra C-terminus domain was also considered as an evolutionary remnant that might be unnecessary for catalytic function but might aid in protein stability [21], which might also be the reason that why there was the relatively higher enzyme stability in Ppxyl43B than Ppxyl43A.

In addition, both two enzyme activities of Ppxyl43A and Ppxyl43B could be influenced by many metal ions, and less of them influenced enzyme activity for Ppxyl43B. In fact, the β -xylosidase and α -arabinofuranosidase activity of recombinant CoXyl43 has been ever reported to be inactivated by zinc and copper ions but were activated by manganese ions, while only β -xylosidase activity was dramatically enhanced by calcium ions [21]. Another GH43 family member of RS223-BX was also found to be enhanced in β -xylosidase activity by calcium ions, cobalt, ferrous, magnesium, manganese and nickel ions [22]. Thus, the effects of cations on the activity of GH43 family have been frequently observed and may be caused by high accessibility through the central tunnel [23]. In this survey, Ppxyl43B had an additional C-terminal domain and less substrate binding sites (Additional file 2: Table S3) than Ppxyl43A might restrict access for substrates to the active site to form a pocket to finish the hydrolysis reaction [12], which might be the reason why cations rarely influenced the enzyme activity for Ppxyl43B.

In summary, although there were some differences between Ppxyl43A and Ppxyl43B, like their structures, and the optimal enzyme activity conditions (pH, temperature, pH stability and thermostability, and metal ions), they also displayed the similar enzymatic features, such as the ability of degrading natural xylo-oligosaccharides and chemical substrates based on β -xylosidases or α -L-arabinofuranosidases activity and exporting outside the cell by non-conventional way. In addition, the encoding genes *Pph_0602* and *Pph_2344* of Ppxyl43A and Ppxyl43B also expressed significantly at high levels under the 1% CCX and xylose suggesting that they might be stimulated by xylose or other xylo-oligosaccharides, which would also be a special advantage in their application for biomass conversion by strain XB in the future. Certainly, much more work is needed to understand their reaction mechanisms in detail, and many more xylanolytic genes in strain XB need to be analyzed for function before the application of strain XB on bioconversion of lignocellulosic biomass would be possible.

Conclusions

Paenibacillus physcomitrellae XB was proven to have the ability to degrade different kinds of xylan. Its genomic features showed that approximately 38 genes might be involved in xylan degradation. Two genes of GH43 family (*Ppxyl43A* and *Ppxyl43B*) were significantly up-regulated under the induction of both xylose and CCX. The enzyme characteristics of purified proteins showed that both of them at least were bifunctional enzymes with the similar affinity with the substrates of β -xylosidase and α -L-arabinofuranosidase. However, *Ppxyl43B* displayed higher stability across pHs, temperatures, and in the presence of metal ions than *Ppxyl43A*, which might be related to an extra C-terminus domain in *Ppxyl43B*. Future research would be helpful in developing the application of these two proteins in the xylan bioconversion process by strain XB.

Materials And Methods

Strain cultivation and chemicals

Paenibacillus physcomitrellae XB was maintained at -80°C in R2A broth with 30% glycerin; for use in this study, it was activated by inoculation into fresh R2A medium and cultured at 30°C in a shaker at 180 rpm.

The main types of xylan or xylo-oligosaccharides used in this study were corncob xylan (CCX, Meklin, China), oat spelt xylan (OSX, Sigma, America), xylooligosaccharide (XOS, Meklin, China), beech wood xylan (BWV, Harvey, China), wheat flour arabinoxylan (AX, Medium Viscosity, Megazyme, Ireland) and xylohexaose (X6, Yuanye, China). The xylobiose (X2), xylose (X1) and arabinose (Ara) were also used and obtained from Yuanye Co. of China.

Xylan degradation assay

Paenibacillusphyscomitrellae XB was cultured in R2A broth (pH 6.0) at 30°C for 24 h and then activated by inoculation into R2A medium with 1% CCX, OSX, BWV, and AX, respectively. In order to test the ability to degrade these xylans, the strain precipitate was collected at 2 and 7 days post incubation, centrifuged at 8000 rcf for 15 min at 4°C, and the supernatant, which was used as the crude enzyme, was transferred to a new tube. The assay was performed in 50 mM acetic acid-sodium acetic buffer (pH 5.0) containing 1% substrate. The reaction system used 100 μ L of different substrate and 100 μ L of each crude enzyme. After 10 min incubation at 50°C, samples were immediately added into 300 μ L of 3, 5-dinitrosalicylic acid (DNS) and boiled on a heating block at 100 °C for 10 min before being cooled on ice for 5 min. Finally, the reducing sugars were determined by DNS colorimetry [24] with xylose as the standard. The absorbance was measured using a microplate reader—Varioskan Flash, [Thermo Scientific](#)—at 540 nm. All reactions were assayed at least in triplicate.

Genomic DNA extraction, sequencing, and annotation for strain XB

To obtain DNA for sequencing libraries, strain XB was first inoculated into 200 mL of liquid R2A broth, incubated in a shaker at 30°C for 24 h, and harvested by centrifugation at 13000 rpm for 2 min. Subsequently, genomic DNA extraction, quality detection, sequencing and raw reads processing were

performed according to the previous method (Wang et al. 2020). Finally, a *de novo* genome was assembled using SOAP version 2.04 [25].

The genome annotation was carried out by using the NCBI Prokaryotic Genome Annotation Pipeline [26] and the gene finding tool GeneMarkS+ Version 4.1 [27]. The databases used for gene/protein annotation were the Cluster of Orthologous Groups (COG) of proteins [28], the Gene Ontology (GO) project (<http://www.geneontology.org/>), non-redundant (NR) database [29] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [30]. Carbon-metabolism related enzymes were annotated according to the Carbohydrate-Active enZymes Database (CAZy) [31]. The xylanolytic genes were predicted by combining all above databases (shown in Table 1). In addition, SignalP -5.0 (<http://www.cbs.dtu.dk/services/SignalP/>) was used to determine signal peptides [32] and SecretomeP 2.0a (<http://www.cbs.dtu.dk/services/SecretomeP/>) used to predict the secretions pathway. TMHMM v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) was used to predict the transmembrane helices in proteins; SWISS-MODEL (<https://swissmodel.expasy.org/>) was used to build the model, and InterPro Scan (<http://www.ebi.ac.uk/interpro>) used to make a classification to the protein family and predict their conserved domain.

Expression analysis of putative xylanolytic genes based on RT-qPCR

Total RNA was extracted from strain XB samples incubated in R2A broth with 1% CCX, 1% xylose and without both of them (control), at 30°C for five days. All the protocols of RNA extraction, reverse transcription, cDNA synthesis and real-time PCR were performed as the previous report [33]. Primers used for the reference gene *recA* and 12 xylanolytic genes are shown in additional file 2 (Table S2). The relative fold change of each gene was calculated based on the of $2^{-\Delta\Delta Cq}$ method.

Amplification, cloning, and differential expression of Pph_0602 and Pph_2344

High fidelity PCR was performed to obtain the full-length of the two highly expressed genes of *Pph_0602* and *Pph_2344* based on primers with restriction enzyme sites (Additional file 2: Table S2). Then, the two gene fragments and vector pET28a (+) were ligated with T4 DNA ligase (Takara Co.) to construct recombinant plasmids, which were then transformed into *E. coli* BL21(DE3) competent cells.

Recombinant enzyme induction was performed with 1 mM IPTG at 37°C for 2 h, 6 h and 18 h, and purification using Ni^{2+} -nitrilotriacetic acid (NTA) metal-chelating affinity chromatography was completed [34]. The purity of the recombinants named Ppxyl43A (pET28a-*Pph_0602*) and Ppxyl43B (pET28a-*Pph_2344*) was evaluated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined according to Bradford's method using bovine serum albumin as the standard [35]. Finally, the reducing sugar in the crude enzyme was assayed in all recombinants and the control (only with empty vector of pET28a (+)) by DNS method [24]. Xylose was detected by using 50 mM acetic acid-sodium acetic buffer (pH 5.0) containing 200 μL of *p*-nitrophenol- β -D-xylopyranoside (*p*NPX) (4 mM) and the crude enzyme. The mixture was incubated at 50°C for 15 min and stopped by 0.4

mL of 2 M Na₂CO₃. The xylose was calculated according to the *p*-nitrophenol (*p*NP) level measured via absorbance at 410 nm and compared with a standard reference [7].

Substrate specific detection of recombinants Ppxyl43A and Ppxyl43B

In order to measure the activity of the two purified proteins of Ppxyl43A and Ppxyl43B, CCX, OSX, BWX, AX, X6, and X2 were used as the substrates. The reaction was performed in 50 mM acetic acid-sodium acetate buffer (pH 5.0) containing 1% substrate. After adding 100 µL purified Ppxyl43A or Ppxyl43B and incubating at 50°C for 10 min, the reducing sugars were detected as aforementioned methods. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol reducing sugar per min. In addition, 4 mM of *p*NPX and *p*-nitrophenol- α -L-arabinofuranoside (*p*NPAf) were also used as substrates to determine the β -xylosidase and α -L-arabinofuranosidase activity respectively, via the amount of *p*NP released [7]. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol *p*NP per min.

Analysis of hydrolysis products.

Hydrolysis products of X2, X6, *p*NPAf, AX, CCX, OSX, BWX and XOS were firstly determined by thin-layer chromatography (TLC) on silica gel 60 F254 plates of 20 cmX20 cm (Sigma) with a mixture of *n*-butanol, acetic acid, and water (2:1:1) as a solvent system, after incubating 1% (w/v) of them with 0.5 units of PpAxy43A and PpAxy43B at 50°C (pH 5.0). Sugar spots were detected by heating the plates to 110°C after spraying them with 95:5 (v/v) of methanol: ammonium sulfate. Further analysis of the hydrolysis products of X6, X2, and *p*-NPAf was also completed with high-performance anion exchange chromatography (HPAEC, Dionex ICS-5000+, Thermo Scientific) with pulsed amperometric detection (PAD) using a CarboPac PA20 column under a gradient elution (mobile phase: 90:10 of 2 mM NaOH and 500 mM NaOH) at 0.3 mL/min.

Effects of pH and temperature on the activity of Ppxyl43A and Ppxyl43B

In order to observe the effects of pH on β -xylosidase and α -L-arabinofuranosidase activity, substrates *p*NPX and *p*NPAf were used to determine the enzymatic activities of Ppxyl43A and Ppxyl43B at different pHs (ranging from 2.0 to 9.0) at 50 °C using aforementioned methods. The pH was adjusted using Sodium Citrate Buffer (pH 3.0, 4.0 and 5.0), Disodium hydrogen phosphate-sodium dihydrogen phosphate buffer (pH 6.0, 7.0 and 8.0), and Tris-HCl buffer (pH 9.0), respectively. Activity level of 100% was defined as optimal pH. The effect of temperature on the activity of Ppxyl43A and Ppxyl43B was measured under optimal pH at 10-80°C. Activity of 100% was defined as the optimal temperature. The pH stability was assessed by incubating the enzyme in 50 mM of each pH buffer at the optimal temperature for 1 h. Thermostability enzyme activities were measured after preincubation of the enzyme at a temperature range from 10°C to 80°C for 1 h without the substrates. All the relative activities were calculated from the ratio of assayed enzyme activity of each treatment and that without treatment.

Effects of metal ions on enzyme activity of Ppxyl43A and Ppxyl43B

The effects of metal ions on the activity of Ppxyl43A and Ppxyl43B were determined by using *p*NPX and *p*NPAf as substrate. The reaction was performed in 50 mM sodium acetic buffer (pH 5.0) containing 1% substrates and 10 mM corresponding metal compounds. The enzyme activity was determined as the method above described. Residual activity was calculated based on the control without metal compounds addition.

Kinetics and thermodynamics

The *K_m* and *V_{max}* values of Ppxyl43A and Ppxyl43B at optimal pH and optimal temperature (section 2.8) were determined by using *p*NPX (0.1 to 8.0 mM), *p*NPAf (0.1 to 8.0 mM) and CCX (0.1 to 2%) as the substrate, respectively. The Michaelis constants were determined from Lineweaver-Burk plots.

Statistical analyses

All above enzyme activity measurements and RT-*q*PCR experiments of related genes were run in at least triplicate. The statistical method of T.TEST in the Microsoft Excel 2010 package was used to determine differences in mean values. For this purpose, *p*-values < 0.05 were considered significant, while *p*-values < 0.01 were considered extremely significant.

Declarations

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Author's contributions

XJZ performed the majority of experiments and data analyses; SW performed the genomic sequencing of strain XB; YHL designed the experiment, wrote and edited the manuscript. Both authors discussed the results, read and approved the final manuscript.

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Availability of data and materials

The whole genome sequence of *P. physcomitellae* strain XB (4.83 Mb) was deposited in the NCBI database with accession number CP022584.1. The amino acids sequences for Ppxyl43A and Ppxyl43B from strain XB were under the accession numbers WP_094092920.1 and WP_094094306.1, respectively. Other data generated or analyzed during the study were included in the manuscript and additional files.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare they have no competing interests.

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Tables

Table 1. Annotated enzymes related to xylan degradation in *Paenibacillus physcomitrellae* XB

Gene ID	Enzyme annotation by KEGG/COG/GO/NR	Annotation by CAZy
<i>Pph_0648</i>	xynA endo-1,4-beta-xylanase	GH11
<i>Pph_1610</i>	xynA endo-1,4-beta-xylanase	GH10-CBM9-CBM22
<i>Pph_0002</i>	Xylanase	GH8
<i>Pph_0602</i>	beta-xylosidase/abfA alpha-N-arabinofuranosidase	GH43
<i>Pph_0706</i>	NA	GH43
<i>Pph_1651</i>	abfA alpha-N-arabinofuranosidase /beta-xylosidase;	GH43
<i>Pph_2078</i>	hydrolase activity	GH43-CBM6-CBM66
<i>Pph_2344</i>	xynB xylan 1,4-beta-xylosidase	GH43
<i>Pph_3723</i>	beta-xylosidase	GH43
<i>Pph_1650</i>	alpha-N-arabinofuranosidase B activity	GH54-CBM42-CBM13
<i>Pph_1668</i>	abfA alpha-N-arabinofuranosidase	GH51
<i>Pph_3259</i>	abfA alpha-N-arabinofuranosidase	GH51
<i>Pph_4538</i>	abfA alpha-N-arabinofuranosidase	GH51
<i>Pph_2137</i>	aguA alpha-glucuronidase	GH67
<i>Pph_0460</i>	Alpha-galactosidase	GH36
<i>Pph_0481</i>	alpha-galactosidase	CBM35
<i>Pph_3729</i>	alpha-xylosidase	GH31
<i>Pph_1544</i>	alpha-xylosidase	GH31
<i>Pph_2116</i>	alpha-xylosidase	GH31
<i>Pph_0705</i>	alpha-xylosidase	GH31
<i>Pph_0712</i>	alpha-galactosidase	GH36
<i>Pph_1263</i>	alpha-galactosidase	GH36
<i>Pph_2173</i>	alpha-galactosidase	GH36
<i>Pph_3041</i>	alpha-galactosidase	GH36
<i>Pph_3857</i>	alpha-galactosidase	GH36
<i>Pph_0147</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_0543</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_0791</i>	Peptidoglycan/xylan/chitin deacetylase	CE4

<i>Pph_0817</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_1357</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_1666</i>	xylanase/chitin deacetylase	CE4
<i>Pph_1773</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_2377</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_3082</i>	hydrolase activity	CE4
<i>Pph_3308</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_3620</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_3732</i>	Peptidoglycan/xylan/chitin deacetylase	CE4
<i>Pph_3863</i>	Peptidoglycan/xylan/chitin deacetylase	CE4

Table 2 Specific activities of the two proteins on various substrates

Substrate	Xylanolytic enzyme activity assessed (U/mg)*		
	Ppxyl43A	Ppxyl43B	<i>p</i> -Value
<i>p</i> NPX	7.99±0.10	3.24±0.03	<i>p</i><0.01
<i>PNPAf</i>	1.52±0.11	1.42±0.03	<i>p</i>>0.05
X2	7.52±0.39	3.36±1.16	<i>p</i> <0.01
X6	21.28±0.78	12.84±0.60	<i>p</i> <0.01
CCX	113.60±1.21	89.05±2.05	<i>p</i> <0.01
XOS	88.14±3.62	74.19±0.96	<i>p</i> <0.01
OSX	5.41±0.41	6.13±1.70	<i>p</i>>0.05
BWX	NA	NA	NA
AX	NA	NA	NA

*, NA, non-available

Table 3 The relative activity (%) of β -xylosidase and α -L-arabinofuranosidase on the substrate of *p*NPX and *p*NPAf after treatment with different metal ions

Metal ion	β -xylosidase		α -L-arabinofuranosidase	
	Ppxyl43A	Ppxyl43B	Ppxyl43A	Ppxyl43B
Cu ²⁺	19.67±0.37	38.39±2.22	10.50±0.45	108.57±3.43
Mg ²⁺	87.64±2.60	99.72±1.77	127.43±2.51	97.42±2.48
Co ²⁺	102.03±1.30	108.25±1.53	119.39±3.06	101.06±1.86
Fe ³⁺	66.36±1.86	98.76±2.32	57.63±2.14	116.59±1.73
Ca ²⁺	112.91±0.75	79.23±1.42	148.51±2.94	109.61±2.45
K ⁺	76.00±1.05	89.44±2.58	126.66±2.18	118.04±3.11
Ni ²⁺	71.69±3.58	108.27±1.94	89.52±5.16	116.56±1.98
Zn ²⁺	21.09±1.15	111.76±2.80	18.40±1.97	11.36±0.88
Ba ²⁺	111.09±2.14	87.03±1.45	126.98±1.98	101.19±2.24
Mn ²⁺	107.91±1.73	112.82±2.82	129.78±1.25	96.37±2.87

Table 4 Kinetic parameters of Ppxyl43A and Ppxyl43B with different substrates

Substrates	V_{max} (U/mg)		K_m	
	Ppxyl43A	Ppxyl43B	Ppxyl43A	Ppxyl43B
pNPX	8.64±1.87	13.49±0.27	2.37±0.54 mM	2.12±0.05 mM
pNPAf	5.73±5.35	0.86±0.60	3.03±1.64 mM	0.82±0.28 mM
CCX	481.48±159.30	316.80±217.95	88.44±48.16 mg/mL	75.16±34.35 mg/mL

Figures

Figure 1

Xylan degradation ability and gene expression changes during xylan degradation by strain *Paenibacillus Phscomitrellae* XB. (a) reducing sugars produced from different xylans substrate; (b) quantitative RT-PCR results of 12 genes showing their gene expression level changes in the crude enzyme derived from those induced by substrate of 1% corncob xylan and xylose.

Figure 2

Purification of Ppxyl43A and Ppxyl43B and their effects on reducing sugars and xylose produced by the strain *E. coli*. BL21(DE3) under the substrate of 1% corncob xylan. (a) and (b) show protein purification. M, Marker; 1, the total protein; 2, the liquid obtained after the total protein flowed through the Ni-NTA column; 3, the protein washed by buffer with 20 mM imidazole; 4 and 5, the protein eluted by buffer with 50 mM and 100 mM imidazole, respectively. (c) and (d) display reducing sugars and xylose amount detected

from the crude enzyme (extracellular) and total protein lysate (intracellular) of strain BL21(DE3), respectively.

Figure 3

TLC separation of products released from different substrates by Ppxyl43A and Ppxyl43B. (a) and (b) TLC result of products released from different substrates. M, Standard sample of xylose (X1) and xylololigosaccharides (X2-X6); CCX and OSX (a), XOS and BWX (b), X6 and X2 (c). +A and +B represented

these substrates were digested 24 h by Ppxyl43A and by Ppxyl43B, respectively. +A1 and +B1 shown the substrate X6 was digested by Ppxyl43A and Ppxyl43B for 1 min and 60 min, respectively.

Figure 4

HPAEC profiles of the hydrolytic products of X6 and pNPAf. (a) merged picture of standards of xylose (X1) and xylohexaose (X6); (b) and (c) display the xylose appearing in the hydrolytic product of X6 by Ppxyl43A and Ppxyl43B, respectively. (d) mixed standards of arabinose (Ara), xylose (X1) and xylobiose (X2); (e) and (f) arabinose appearing in the hydrolytic product of pNPAf by Ppxyl43A and Ppxyl43B, respectively.

Figure 5

TLC profiles of the hydrolytic products of CCX digested for different time (min) by Ppxyl43A (a) and Ppxyl43B (b). M, Standard sample of xylose (X1) and xylololigosaccharides (X2-X6); CK, the CCX control.

Figure 6

Effects of pH and pH stability on the enzyme activity of Ppxyl43A (a and b) and Ppxyl43B (c and d).

Figure 7

Effects of temperature and thermostability on the enzyme activity of Ppxyl43A (a and b) and Ppxyl43B (c and d).

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