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Expression and characterization of cold-adapted xylanase Xyl-L in *Pichia pastoris* for xylooligosaccharide (XOS) preparation

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Abstract

Background Xylan, the second most abundant polysaccharide in plant biomass, requires endoxylanases for its hydrolysis into xylooligosaccharides (XOS). Xylanases have been widely used in industries such as animal feed, bakery, juice production, and paper pulp. Recently, XOS have gained attention for their health benefits, including improved digestion, reduced cholesterol, and antioxidant effects. The cold-adapted GH10 xylanase of Antarctic origin Xyl-L was previously expressed in *Escherichia coli*, showing promising low-temperature activity. However, *Pichia pastoris* is currently a preferred host for industrial xylanase production due to its ability to express complex proteins and secrete them into the culture medium. This study explored the expression of Xyl-L in *P. pastoris* and evaluated its potential for XOS production using common flours as substrates, aiming for applications in the food and nutraceutical industry.

Results Comparison between AOX1 (P_{AOX1}) and GAP (P_{GAP}) promoters for recombinant Xyl-L production in *P. pastoris* showed that the P_{AOX1} promoter resulted in higher activity per wet-cell weight. Co-transforming P_{AOX1}-Xyl strains with plasmids encoding genes aiding in protein folding (*HAC1* or *PDI1*) did not enhance Xyl-L catalytic activity compared to the parental P_{AOX1} strain. Thus, P_{AOX1}-Xyl was cultivated in 3 L bioreactors in fed-batch cultures; it is presumed that the enzyme is produced with glycosylations within its structure, given its migration within the SDS-PAGE gels. The produced Xyl-L was purified from the culture supernatant, resulting in peak xylanase activity after 90 h, with specific activity of 5.10 ± 0.21 U/mg, at pH 7.5 and 25°C, using beechwood xylan. It also showed a *Km* of 3.5 mg/mL and a *kcat* of 9.16 s^{-1}. Xyl-L maintained over 80% of relative activity between pH 5.6—8.6 and 37 — 44° C, and was activated by $CaCl_2$ and $MgCl_2$, but inhibited by $MnCl_2$. Xyl-L was tested using several flours (whole wheat, rye, oatmeal and all-purpose) as substrates, where XOS with a polymerization degree (DP) of 2 were obtained from each substrate, whole wheat flour generated XOS with DP 3, and XOS with DP 2, 3 and 4 were produced when beechwood xylan was used as substrate.

Conclusions The xylanase Xyl-L was successfully expressed in *P. pastoris* and proved to be able to degrade various flour substrates, producing XOS with DP ranging from 2 to 4, indicating its potential applications in the nutraceutical and food industries. Further studies must be performed to optimize its production in bioreactors.

Keywords Xylanase, Cold-adapted, Xylooligosaccharides

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Background

After cellulose, xylan is the second most abundant polysaccharide in plant biomass and a major component of the plant cell wall [1]. This carbohydrate has a complex structure, and its hydrolysis needs cooperative action of a variety of enzymes. The most relevant enzymes involved in this process are the xylanolytic enzymes, including endoxylanases (EC 3.2.1.8), mainly grouped in the glycosyl hydrolase families 10 (GH10) and 11 (GH11). These enzymes catalyze the internal β-1,4-glycosidic linkages of the xylan backbone to produce xylooligosaccharides (XOS) [2]. These XOS can be decorated with different side groups (e.g. α-d-glucopyranosyl uronic acid or its 4-O-methyl derivative, acetyl groups, or arabinofuranosyl residues) [3].

In industry, xylanases are widely used. They are incorporated in animal feed to improve digestion and for reducing unwanted mineral residues in the excreta, which could aid in reducing environmental issues related to animal breeding [2]. In the bakery industry, they are used to improve dough properties [4], while in other sectors, they serve purposes such as clarifying fruit juices, prebleaching pulp for paper production to reduce the use of harsh chemicals, among others [5].

On the other hand, over the last decade XOS have gained attention given their prebiotic effect in humans and animals at low doses [6, 3]. The documented health benefits of XOS include a reduction in blood cholesterol, increase in calcium absorption, antioxidant effects, maintenance of gastrointestinal health, reduced colon cancer risk, cytotoxic effect on human leukaemic cells and benefits to patients with type two diabetes mellitus [7]. Currently, the vast majority of the proposed methods for XOS production use xylan extracted from lignocellulosic raw material, as a way of utilizing waste produced by other industries [8].

Considering the above, the interest in producing xylanases has been increasing. Acevedo et al. [9] successfully cloned a GH10 xylanase of Antarctic origin (Xyl-L) [9], and later expressed it in *E. coli*, where it proved to have promising activity at low temperatures [10]. Cold-active enzymes tend to have a broader substrate specificity, compared to their thermophilic counterparts, and are also energy efficient, as they have lower optimal temperatures [11]. An example of cold-active xylanase application is its use in bread dough for improving dough stability and flexibility, as well as for increasing bread volume and crumb structure [12–14]. Additionally, they are highly preferred as they can avoid the occurrence of undesirable chemical reactions at higher temperatures due to their rapid heat-inactivation, derived from their structural thermolability [10, 11, 15].

As xylanases have a large potential for application within the food and nutraceutical industry, the host organism used for their heterologous production becomes a relevant factor, mainly because of final product safety. Furthermore, for industrial production purposes, certain variables must also be considered, such as production yields and required purification steps, in order to establish an economically viable process. Thus, different expression hosts have been considered for the production of recombinant xylanases, such as *Aspergillus* and *Pichia pastoris* (syn *Komagataella spp*). While products obtained from both of them have received GRAS (Generally Recognized as Safe) certification, the latter tends to be chosen for large-scale recombinant protein production, due to its easier handling. *P. pastoris* has been used successfully to produce different recombinant active and functional xylanolytic enzymes. In addition, it is able to secrete proteins into the culture supernatant, which is a helpful feature in large-scale protein production [8, 16, 17].

Regarding all of the above, this study explored the secretory production of the cold-adapted xylanase Xyl-L, using *P. pastoris* as host. The ability of this enzyme to produce XOS was measured under different conditions, and the production of XOS with commonly used flours as substrates was also tested.

Materials and methods

Materials

Beechwood xylan (purity > 95%), and XOS standards (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose) were purchased from Megazymes (Wicklow, Ireland). Bradford reagent (Protein Assay Dye) was purchased from Bio-Rad (Hercules, USA). Anti-foam Itapon DG160 was kindly donated by Química Italquim (Santiago, Chile). Whole wheat flour, rye flour, oatmeal flour (La Fuente Natural, Santiago, Chile) and all-purpose flour (Selecta, Santiago, Chile) were purchased at a local supermarket. All other materials used were purchased from Merck (Darmstadt, Germany).

Media

Synthetic screening medium ASMv6 contained per liter: 22 g citric acid monohydrate, 6.30 g $(NH_4)_2HPO_4$, 0.49 g $MgSO_4 \cdot 7H_2O$, 2.64 g KCl, 0.0535 g $CaCl_2 \cdot 2H_2O$, 1.47 mL PTM0 trace metals without biotin, 0.4 mg biotin; pH was set to 6.5 with KOH.

PTM0 trace metals contain per liter: 6.0 g $CuSO_4 \cdot 5H_2O$, 0.09 g KI, 3 g $MnSO_4 \cdot H2O$, 0.2 g $Na_2 MoO_4 \cdot 2H_2O$, 0.02 g $H_3 BO_3$, 0.5 g $CoCl_2$, 42.2 g $ZnSO_4 \cdot 7H_2O$, 65.0 g $FeSO_4 \cdot 7H_2O$, and 5.0 mL $H_2 SO_4$ (95% -98%).

Strain constructions

The ORF encoding secreted Xyl-L gene (US 8679814 B2 [18]) from *Psycrobacter sp.* (without its predicted native 25 aa N-terminal signal peptide) was synthesized with codon optimization for expression in *P. pastoris* by GeneArt (Thermo Fisher Scientific, Germany). A C-terminal His6-tag was added. Molecular cloning was performed using Golden Gate Assembly (GGA) following the methodology of Prielhofer et al. (2017) [19]. Briefly, on the first GGA round, the synthetic Xyl-L gene was ligated in frame with the N-terminal *Saccharomyces cerevisiae* alpha-mating factor leader for secretion into the BB1 backbone plasmid, using the restriction enzyme BsaI. Then, *E. coli DH10B* cells were transformed with the plasmid and selected on LB agar containing 50 μg/mL kanamycin. In the second GGA round, the promoter, the ORF and the terminator were ligated into the BB3aZ vector backbone, using the enzyme BpiI. Two different promoters were used, the constitutive GAP promoter and the strong methanol-inducible AOX1 promoter. The plasmid was transformed into *E. coli DH10B* and positive transformants were selected using 50 μg/mL Zeocin. The final constructions were checked by PCR and sequencing, using the J77 (5'-GAGGTATGTAGGCGGTGCTA-3') and bb3zeorv (5'-TTAGTATGCTGTGCTTGGGTG-3') primers.

Then, 5 μg of correctly assembled plasmid were linearized with AscI enzyme, eluted in 15 μL of water, and used to transform 50 μL of electro-competent *P. pastoris* BT3445(CBS7435 MutS). Transformants were selected and isolated on YPD plates containing 50 μg/mL zeocin.

Selected *P. pastoris* P_{AOX1}-XyL strains were transformed with a plasmid encoding for *P. pastoris* protein disulfide isomerase (*PDI1*) or the Hac1p transcription activator [20]. Transformants were selected using 100 μg/mL nourseothricin and 200 μg/mL hygromycin, respectively.

Small scale screening cultures

Small scale screening cultures were carried out in 24 deep-well plates (24-DWP) following the protocol by Zahrl et al. [20]. Per construct at least 7 *P. pastoris* transformants were tested [21]. Briefly, 2 mL of YPD + Zeocin were inoculated with a single *P. pastoris* transformant and incubated at 25°C and 280 rpm for 24 h. Then, the plate was centrifuged at 2000 × *g* for 5 min and the resulting cell pellet was resuspended in 1 mL of 2X ASM media by shaking for 2 min. The OD_{600} of each pre-culture was measured in a microtiter plate, for calculating the inoculum volume so the induced culture could start with an OD_{600} equal to 4.

The calculated volume was added to 1 mL of 2X ASM media in a new 24-DWP, followed by 1 mL of polysaccharide (PS) with glucose-releasing enzyme (PSE) in a 2-fold concentration. Depending on the promoter, each media had variations: for methanol-induced screenings there was a final concentration of 0.35% enzyme, 25 g/L PS plus methanol shots of 0.5%, 1%, 1% and 1% after 3, 19, 27 and 43 h, respectively [50 mL PSE for P_{AOX1}: 25 mL PS + 25 mL water + 0.35 mL enzyme (2-fold concentrated)]; for P_{GAP}, a final concentration of 0.7% enzyme and 50 g/L PS [50 mL PSE for P_{GAP}: 50 mL PS + 0.7 mL enzyme (2-fold concentrated)] was used.

After 48 h of cultivation at 25°C and 280 rpm, cultures were harvested by taking a 1 mL sample from the culture broth and putting it into a pre-weighted 1.5 mL Eppendorf tube. Centrifugation was performed at 13,000 rpm for 5 min. The supernatant was transferred into a fresh tube and the recombinant protein production was checked by SDS-PAGE and activity assays as described in "Activity assay" Section, while the pellet in the initial tube was weighted to determine the wet-cell weight (WCW).

Fed-batch bioreactor cultures

Fed-batch bioreactor cultivations were carried out using a mixed glycerol/methanol feeding strategy as described in Zahrl et al. [22]. Thus, each strain was inoculated into wide-necked, baffled, covered 250 mL shake flasks filled with 50 mL of YPG [per liter: 20 g peptone, 10 g yeast extract, 20 g glycerol] and incubated at 28°C and shaken at 150 rpm, overnight (Pre-culture 1). In the morning, the Pre-culture 2 was made from 100 mL YPG in a 1 L wide-necked, baffled shake flask, which was inoculated with the appropriate volume from Pre-Culture 1, so it reached an OD_{600} ranging from 0.95 to 1.05. This way, over a 10 h incubation period at 28°C with agitation of 150 rpm, the Pre-Culture 2 reached an OD_{600} of 20, which could be used for the inoculation of the bioreactor.

For the fed-batches, bioreactors with a working volume of 3 L were used (Minifors, Infors, Switzerland), and each culture was performed in duplicate. All the bioreactors were filled with 700 mL BSM-media at a pH of approximately 5.5, and then were individually inoculated with 70 mL of the Pre-Culture 2 to reach an OD_{600} ranging from 1 to 2.

The batch phase on glycerol used for biomass generation usually lasted around 14 h. During the initial glycerol

fed-batch phase, glycerol was fed during 8 h at a rate defined by the equation 4.55 + 0.525·t (g/h). The next phase lasted 18 h in total, where a mixed feed of glycerol/methanol was supplied; the glycerol feed rate was set by the equation 4.375 + 0.2275·t (g/h), while the methanol feed was defined by the equation 1.26 + 0.0875·t (g/h). The final phase lasted around 72 h and the feeding was changed to only methanol, at a rate set by the equation 3.85 + 0.028·t (g/h).

During the initial batch phase the temperature was set to 28°C, in the last 2 h (before initiating the production phase) it was decreased to 24°C and kept at this level throughout the remaining process. The pH dropped to 5.0 and was controlled to be maintained at this level using 25% ammonium. Oxygen saturation was set to 30% throughout the whole process (cascade control: stirrer, flow, oxygen supplementation); stirring was applied between 1000 and 1200 rpm, with an air flow range of 2–4 vvm. Foaming was controlled by the addition of antifoam agent Itapon DG 160 on demand.

Samples were taken from the bioreactors twice a day, which were used to measure OD_{600} using a spectrophotometer (Biochrom Libra S50 UV) and then centrifuged for 5 min at 15,000 rpm. The supernatant was collected for analyzing Xyl-L production using SDS-PAGE and activity assays as described in "Activity assay" Section. Protein concentration was also measured as described in "Protein quantification" Section. The cell pellet was weighted and used to register wet-cell weight.

Purification of recombinant Xyl-L

Cultures were centrifuged at 5000 rpm for 10 min at 4°C (Sorvall, Fxy rotor). The supernatant was collected and filtered using 0.2 nm filters and mixed with an equal volume of Binding Buffer (Tris–HCl 50 mM pH 7.0, NaCl 500 mM, Imidazole 15 mM), following the methodology of Lehuedé et al. [23]. Chromatographic steps were carried out on FPLC system AKTA™ Avant 25, where 50 mL of the samples were loaded in a Histrap® FF 1 mL column, which was preequilibrated with Binding Buffer. Then, the column was washed with 20 mL of Binding Buffer. The elution was carried out in 8 mL of a linear gradient from 0 to 100% at a 0.5 mL/min flow of Elution Buffer (Tris–HCl 50 mM pH 7.0, NaCl 500 mM, Imidazole 500 mM) and collected in a 96 deep-well plate in 1 mL fractions.

For desalting, the total sample was loaded on a 52 mL Hiprep 26/10 desalting column preequilibrated with Tris–HCl pH 7.5 20 mM buffer. The elution step was performed by circulating 1.5 column volumes at 10 mL/min of the mentioned buffer. The elution was collected in 2 mL fractions and its absorbance at 280 nm was recorded. The fraction corresponding to the first 280 nm absorbance peak was placed together and stored at 4°C for further analysis.

Enzyme characterization

Protein quantification

The quantification of protein in solution was made using the Bradford Method [24], where 200 μL of Bradford reagent (Bio-rad) were mixed with 5 μL of the enzyme containing solution. After 10 min, the absorbance at 595 nm was measured using a microplate reader (SPECTROstar Omega) and then was associated with protein concentration through a BSA standard curve.

Activity assay

The assays to determine Xyl-L activity were based on Bailey et al. [25], following the dinitrosalicylic acid (DNS) method using xylose as a standard. Thus, the assays were performed in 1.5 mL tubes in triplicate, where 950 μL of reaction buffer (beechwood xylan 1%, Tris–HCl 50 mM pH 8.0, $CaCl_2$ 4 mM) were pre-incubated at 20°C for 15 min. The enzymatic reaction was initiated when 50 μL of the enzymatic sample were added, followed by incubation at 25°C for 30 min in a thermo shaker (MB100-4A, Hangzhou Allsheng Instruments). As a negative control, one assay triplicate replaced the enzyme for the same volume of Milli-Q water.

Then, 100 μL were taken from the reaction solution and immediately mixed with 100 μL of DNS and incubated for 5 min at 95°C in a thermocycler (Bioer GeneExplorer®). After cooling at room temperature, 100 μL of the reaction volume were placed in a 96 well plate to measure its absorbance at 550 nm using a microplate reader (SPECTROstar Omega). The reducing sugars generated by the enzymatic reaction were estimated using xylose as a standard curve. One enzymatic unit (U) was defined as the μmol of reducing sugars released per minute by the action of the enzyme, under given pH and temperature conditions, using xylose as the standard.

Effect of temperature on Xyl-L activity

The effect of temperature on the enzyme activity was evaluated by carrying out activity assays as previously described, but varying the temperature used, ranging from 4°C to 64°C, using a thermocycler Bioer GeneExplorer®). Relative activity was calculated using the optimal temperature as 100%.

Thermal stability of Xyl-L

The remaining enzyme activity was measured at 25°C as previously described, after incubating Xyl-L during

10 min at a set temperature, ranging between 30°C and 95°C, in a thermocycler (Bioer GeneExplorer®).

Effect of pH on Xyl-L activity

To evaluate optimal pH, different buffers were used. Acetate 50 mM buffer was used for pH from 3.7 to 5.6, Tris–HCl 50 mM for pH ranging from 5.8 to 8.0 and Glycine-NaOH 50 mM for pH between 8.6 and 10.6. Every enzymatic reaction was performed at 25°C. Relative activity was calculated using the optimal pH as 100%.

Kinetic parameters

Activity was calculated varying the concentration of beechwood xylan from 0.16 to 40 mg/mL. Activity assay were performed at 25°C and pH 7.5. The kinetic parameters were calculated in a linear regression using the GraphPad Prism 8® software.

Effect of different metal ions on Xyl-L activity

To evaluate how the presence of different ions affected the enzyme catalytic capacity, an ionic compound ($CaCl_2$, $MnCl_2$, KCl, KI, $MgCl_2$ or NaCl) was added to the enzymatic reaction mixture at 5 and 10 mM. A positive control was also performed by adding the same volume of MilliQ water. The activity assays were carried out as described earlier.

Enzymatic production of XOS from different flours

Several enzymatic reactions were performed using the recombinant XyL-L produced, varying the substrate used, so later the production of XOS could be compared using the HPLC system Shimadzu Nexera LC40. Activity assays were performed in triplicate; in each case, 4 mL of reaction buffer were added, containing 2% of the respective flour (whole wheat flour, rye flour, oatmeal flour or all-purpose flour) in Tris–HCl 50 mM pH 8.0. A positive control was performed using xylan as substrate, using the same reaction buffer previously described in "Activity assay" Section, and a negative control (without including Xyl-L) was also performed for each substrate. The enzymatic reaction was initiated when 1.5 mL of Xyl-L were added and the whole solution was incubated at 25°C for 24 h.

Afterwards, a 1 mL sample was taken and centrifuged at 14,000 rpm for 20 min. The supernatant was filtered using 0.22 nm syringe filters for HPLC analysis. Briefly, 10 μL of samples were injected into the Rezek RSO oligosaccharide Ag+ 200 × 10 mm chromatography column at 85°C, using MilliQ water as mobile phase, at a 0.3 mL/min flow. Refraction index (IR) was measured

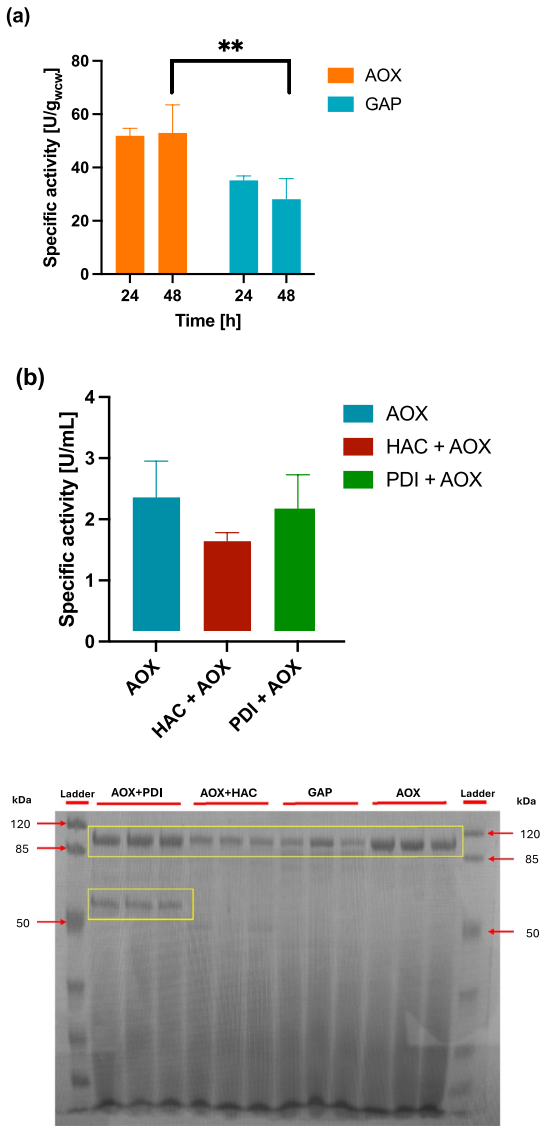


Fig. 1 Xyl-L in small scale screenings.**a** Xyl-L activity in the supernatant normalized over biomass produced by either P_{AOX1} or P_{GAP} based *P. pastoris* strains. 15 individual transformants were cultivated in glucoselimited small scale screenings with (P_{AOX1}) or without (P_{GAP}) methanol induction. ANOVA test was performed to the results; ** indicates that there are significant differences between the corresponding data ($p \leq 0.05$). **b** Influence of potential secretion promoting factors on Xyl-L activity of the P_{AOX1}-XyL parent. **c** SDS-PAGE analysis of Xyl-L strains with different promoters, and PAOX1 strains co-transformed with *HAC1* and *PDI1*. The lanes titles indicate: Ladder for Molecular Weight Protein Ladder; AOX+PDI, for the P_{AOX1}-XyL strain co-transformed with PDI1;AOX+HAC, for the P_{AOX1}-XyL strain co-transformed with HAC1; GAP, for the Xyl-L strain containing P_{GAP} ; GAP, for the Xyl-L strain containing PGAP ; AOX, for the Xyl-L strain containing P_{AOX}. Xyl-L band is inside the yellow box at about 90 kDa, while PDI1 is inside the yellow box around 52 kDa

during the run to identify the degradation products. Xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose (Megazyme) were used as standards.

Statistical analysis

All experiments were performed in triplicate, except for the bioreactors, which were made in duplicates. The experimental data were analyzed using the Analysis of Variance (ANOVA) and Tukey test with a 95% confidence level, in the GraphPad Prism 8® software.

Results

Xyl-L strains comparison

Xyl-L, from the GH10 family, could be successfully produced and secreted by *P. pastoris*, using the *S. cerevisiae* alpha-mating factor leader for secretion. Figure 1a shows that a higher activity per WCW ratio was obtained by the P_{AOX1} based strains in methanol-induced screening cultures, compared to the P_{GAP} based strain, at both 24 h and 48 h of cultivation according to the activity assay.

Given these results, the P_{AOX1}-XyL parental strain was selected to be co-transformed with potential folding and secretion promoting factors that had shown to increase heterologous protein production in previous studies [26]. Either the *HAC1* gene, which encodes the induced version of the unfolded protein response (UPR) transcription factor Hac1 [26, 27] or protein disulfide isomerase (*PDI1*), responsible for the formation of disulfide bonds in eukaryotic cells [28] were chosen.

However, as shown in Fig. 1b, no significant effect was detected when testing and comparing the specific activity of the produced Xyl-L between the engineered P_{AOX1} -XyL strains and the parental strain, in screening cultures. In contrast, Pdi1 even seems to get secreted by itself when overexpressed as an additional band at the molecular weight of approximately 52 kDa appeared, which was confirmed to be Pdi1 by LC-MS/MS. Xyl-L was confirmed to be the band at the molecular weight of approximately 90 kDa, although its predicted molecular weight was 80 kDa. The differences in size could be attributed to glycosylations made by *P. pastoris* during the Xyl-L expression inside the cell [29]. Both of these bands can be seen in Fig. 1c.

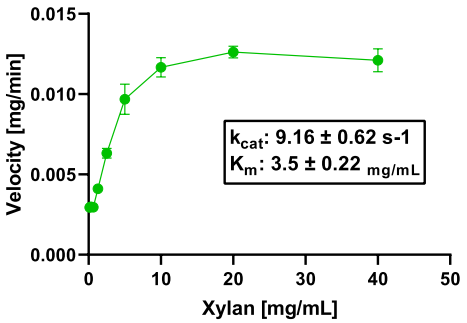


Fig. 3 Kinetic parameters for recombinant Xyl-L, obtained at 25°C, pH 7.5. The kinetic parameters were calculated by Lineweaver Burke plot using soluble xylan as a substrate

Fed-batch bioreactor Xyl-L production

The parental strain P_{AOX1}-XyL was chosen for fed-batch cultivation in bioreactors, using a previously established protocol for methanol/glycerol co-feeding that leads to high productivity of secreted proteins [20]. The bioreactor cultures were divided into four different phases: batch, glycerol fed-batch, glycerol/methanol fed-batch, and methanol fed-batch. As shown in Fig. 2, when analyzing the cultures supernatant, xylanase activity starts to appear after 27 h, when methanol induction starts, and reaches its peak right before the cultures were stopped.

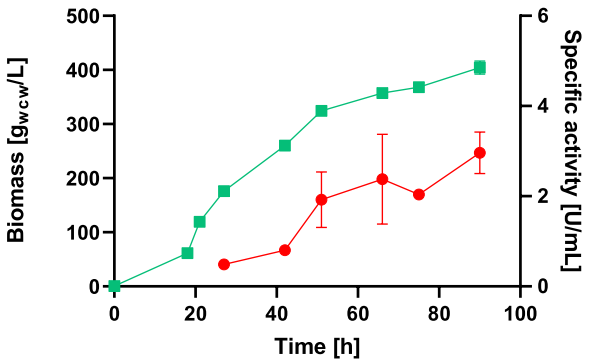


Fig. 2 Bioreactor performance for *P. pastoris* P_{AOX1}-XyL. Biomass formation (green circles) and volumetric xylanase activity profile (red square) of the culture

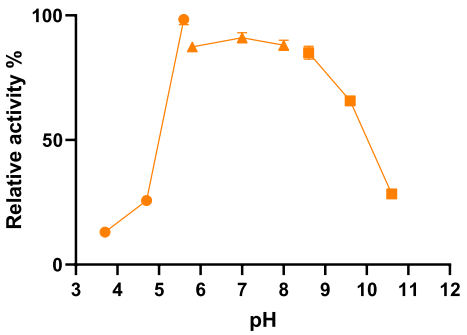
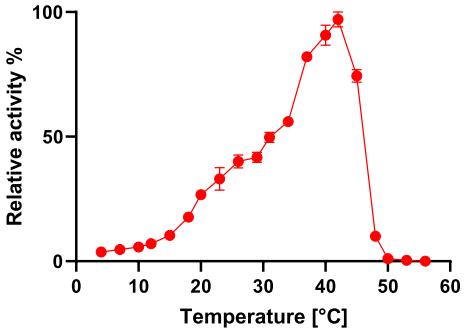
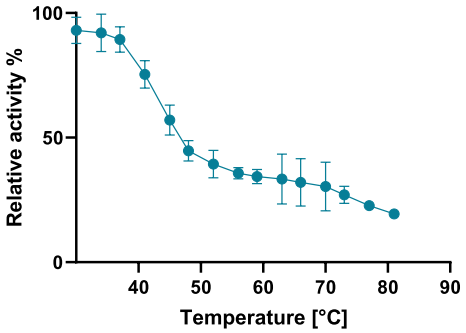


Fig. 4 Effect of pH on the activity of recombinant Xyl-L. The purified enzyme was incubated in different buffers (acetate 50 mM pH 3,7–5,6; phosphate 50 mM pH 5,8–8 and glycine NaOH 50 mM pH 8,6–10,6) for 30 min at 25°C



(a)



(b)

Fig. 5 Influence of temperature on Xyl-L activity and Xyl-L thermal stability. **a** Effect of temperature on activity. The activity assay was performed at different temperatures and relative activity was calculated using the optimal temperature as 100%. **b** Thermal stability of recombinant Xyl-L, where the purified enzyme was incubated for 10 min at different temperatures and then the activity assay was performed. Relative activity was calculated using the measured activity at 25°C as 100%

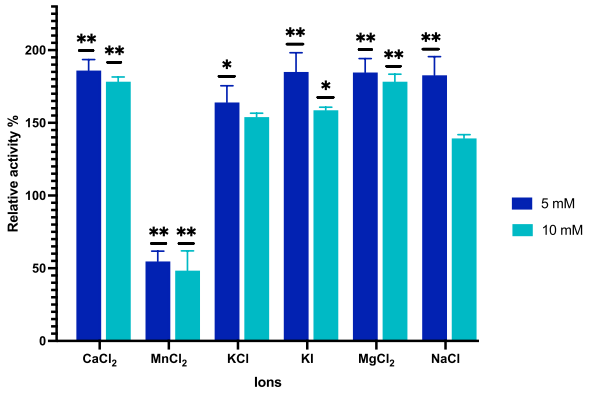


Fig. 6 Effect of different salts on activity of recombinant Xyl-L. Relative activity was calculated using no salt as 100%. ANOVA test was performed to the results; ** indicates that there are significant differences between the corresponding ion concentration and its counterpart with no salt added ($p \leq 0.05$); * indicates that said difference comes from $p \leq 0,1$; bars without * indicates that said difference is not significant

Characterization of Xyl-L

The produced Xyl-L was purified and used to perform activity assays as previously described, and protein concentration was also measured. With this, the specific activity obtained for Xyl-L was 5.101 ± 0.216 U/mg.

Figure 3 shows how the enzymatic rate changed when varying the substrate (xylan) concentration in the reaction mixture for Xyl-L, at pH 7.5 and 25°C. Thus, the kinetic parameters were obtained resulting in a *Km* value of 3.5 mg/mL, *kcat* of 9.16 s^{-1} and therefore a catalytic efficiency (*kcat/Km*) of 2.62 mL mg^{-1} s−1.

The effect of pH is shown in Fig. 4, measured at 25°C. The enzyme had a relative activity of over 80% (with 100% being the highest measured activity) at pH between 5.6 and 8.6, and dropped drastically in pH ranges under 5 and over 10.

Figure 5a shows how temperature affects relative activity of Xyl-L, measured at pH 7.5. The enzyme showed maximum activity at 42°C, but remained with over 80% of its relative activity between 37°C and 44°C. Outside said range, the relative activity begins to drop, but still remains over 40% between 26°C and 45°C, while over 48°C the enzyme loses almost all its catalytic capacity.

Meanwhile, Fig. 5b presents the thermal stability of Xyl-L, showing that relative activity remained around 90% after heating the enzyme at temperatures between 30°C and 37°C, and then performing activity assays at 25°C. When heating to temperatures over 37°C, the relative activity dropped continuously, registering around 20% activity at temperatures above 80°C.

Figure 6 shows how the activity of Xyl-L was significantly enhanced by $CaCl_2$, $MgCl_2$ and KI at 5 mM and 10 mM concentrations, and also by KCl and NaCl at 5 mM concentration. Simultaneously, the enzyme is

Table 1 Xylo-oligosaccharides concentration found by HPLC analysis after enzymatic reaction of Xyl-L with different flours as substrate

SUBSTRATE	Xylo-oligosaccharides [mg/mL]		
	X4	X3	X2
Beechwood xylan	0.083 ± 0.01	1.022 ± 0.063	1.974 ± 0.133
Whole wheat flour	nd	0.036 ± 0.002	0.119 ± 0.006
Rye flour	nd	nd	0.118 ± 0.002
Oatmeal flour	nd	nd	0.059 ± 0.005
All-purpose flour	nd	nd	0.300 ± 0.009

shown to be strongly inhibited with $MnCl_2$ at 5 mM and 10 mM concentrations.

Analysis of hydrolysis products using different flour and beechwood xylan as a substrate

Different types of flours commonly used in the food and feed industry were tested as xylan sources and added to the reaction mixture as substrates, while xylane from beechwood was use as a representative of a purified substrate from hardwood. Interestingly, xylo-based oligosacaride with a depolymerization degree (DP) of 2 (X2) was detected and quantified in whole wheat flour, rye flour, oatmeal flour, and all-purpose flour, while XOS with DP 3 was additionally obtained in whole wheat flour, as shown in Table 1. When beechwood xylan was used as substrate it resulted in the production of XOS with a DP 2, 3 and 4 (X2, X3, X4).

Discussion

Cold-adapted xylanase Xyl-L was successfully produced in a secreted form in *P. pastoris* in small scale screenings and fed-batch bioreactor cultivations. The P_{AOX1} based methanol-induced expression strategy outperformed constitutive P_{GAP}, which agrees with previous studies comparing production of β-xylanase from *Penicillium citrinum* in *P. pastoris* [30].

Regarding the addition of genes encoding proteins that could aid in the expression of Xyl-L in *P. pastoris* (Fig. 1b), overexpression of either *PDI1* or *HAC1* failed to improve the productivity of secreted Xyl-L. This indicates that protein folding and secretion seem not to be a bottleneck for Xyl-L production in the selected best-performing *P. pastoris* strain. Contrarily, it was noted that additional bands or lower amounts of Xyl-L were detected in the supernatant of both *PDI1* and *HAC1* transformed strains, making their overexpression detrimental for further purification of the Xyl-L enzyme (Fig. 1c).

Considering these results, the P_{AOX1}-XyL strain was chosen to perform bioreactor cultures. While the predicted molecular weight of Xyl-L was set in 80 kDa, the band associated to the enzyme in Fig. 1c is found around 90 kDa. The differences in size between Xyl-L produced in *E. coli* and in *P. pastoris*, could be attributed to glycosylations made by the latter during the expression of the enzyme within the cell [29]. During fed- batch cultivation, the peak of the activity was obtained after 90 hrs. Following this time point, Xyl-L began to be degraded, observed in SDS-PAGE (data not shown), which could be attributed to the endogenous secretion of proteases in the latest stage of the culture [31]. Considering the specific activity of the purified XyL-L, a product concentration of 0.59 g/L was obtained from the fermentation.

Regarding the kinetic parameters obtained, generally *Km* values for xylanases are between 0.5 and 5 mg/mL, which positions the *Km* of Xyl-L (3.5 mg/mL) within the expected range [32]. In addition, previously reported values for cold-adapted xylanases vary greatly considering their origin: r-XynA from *Sorangium cellulosum* showed values of 25.77 mg/mL for *Km* and 6.84 s−1 for *kcat* [32], for the enzymatic reaction over beechwood xylan at 30°C, while XynA from *Glaciecola mesophila* registered values of 1.22 mg/mL for *Km* and 69 s−1 for *kcat*, for the same substrate at same said temperature [33]. Given this data, making accurate comparisons becomes difficult, but it is still possible to argue that *kcat* for Xyl-L (9.16 s−1 at 25°C) is similar to the *kcat* reported value for *S. cellulosum*.

The measurement of the effect of pH on Xyl-L activity showed that its optimal pH range is more extended compared to previously reported pH ranges for xylanases of bacterial origin, which tend to be optimal in neutral to alkaline pHs [34]. Thus, the optimal pH range of Xyl-L (which is found between pH 5.6 to 8.6) makes it suitable for most industrial applications, given most of them are performed at these pH values [34].

In relation to the temperature effect on Xyl-L activity, compared to its previous expression in *E. coli*, several changes regarding the optimal temperature range were observed. In *P. pastoris*, Xyl-L lost over 30% of relative activity at 15°C (compared to the result in *E. coli*), while the optimum temperature went up from 35°C to 42. Moreover, compared to the xylanse Xyl-L produce in *E. coli* the thermostability of Xyl-L produced in *P. pastoris* seem to be improved, this could be the effect of the glycosylation present in the Xyl-L produced in *P. pastoris* as in some cases glycosylations have been shown to improve the thermostability of reported enzymes produced in *P. pastoris* [35, 36].

Furthermore, it has been proven that similar cold-adapted enzymes have their optimal temperature range under 30°C, suggesting that the expression of Xyl-L in *P. pastoris* produces an enzyme with an optimal temperature range higher than the average. This feature makes Xyl-L a candidate in the bread making processes for generating XOS within the bread, considering that the temperatures used in the leavening stages are usually around 36°C [37] or under 35°C [38].

The loss of activity observed when the enzyme is exposed to temperatures above 45°C (Fig. 5b) is likely due to enzyme denaturation, as heat tends to break the three-dimensional structure of the protein [39]. Xyl-L exhibits a thermal stability behavior similar to other cold-adapted enzymes, which tend to have high specific activity at room temperature, but poor thermal stability. This is believed to be due to the high flexibility that the enzyme

adopts to catalyze reactions in cold environments [15, 38]. This feature also makes Xyl-L ideal for bread making processes, as it rapidly loses most of its activity after temperatures begin to rise, preventing unwanted reactions due to prolonged activity, such as changing the crumb structure and altering the consistency of bread [15].

The Ca^{2+} activation observed for Xyl-L in Fig. 6 is consistent with what has been previously described for other xylanases from the GH10 family [40]. Inactivation of Xyl-L by Mn^{2+} also has backing data, as it has proven to be an inhibitor of a certain group of xylanases, particularly in xylanases of marine origin [33, 41].

Given that xylan is an abundant natural polymer found in various types of plants, the use of Xyl-L for the production of XOS using edible raw materials was explored, using commercial beechwood xylan as standard. While grain flours such as rye, wheat and oatmeal are complex substrates, beechwood xylan is extracted and purified from hardwood, making it chemically and structurally different than the mentioned flours, which mainly contain xylan in the form of arabinoxylan [1]. Moreover, grains contain xylanase inhibitors [13], that can interfere with xylanase activity. However, the results obtained (Table 1) show that Xyl-L is able to degrade these complex substrates and also produce XOS directly from them. This results suggest that Xyl-L can be potentially used to generate food-grade prebiotics employing certain raw materials used in food production.

Furthermore, compared to the control, Xyl-L released other products into the soluble fraction of the enzymatic digestion, especially when a high fiber flour was used as a substrate A1. Some of these products were eluted at a time point similar to those of the xylotetraose and xylopentaose standards. The diversified number of substitution patterns in XOS, such as arabinose, acetyl groups and D-glucuronic acid, could explain the differences in the retention time obtained by HPLC [42], since these heteroxylans are commonly found in complex substrates [2, 8].

Currently, most of the enzymatic methods implemented to produce XOS, use extracted and purified xylan from agroindustrial by-products [43]. However, novel proposed processes use recombinant xylanase producing microorganisms in fermentative steps, in order to generate XOS directly from residues of the food and brewages industry [42, 44, 45]. To date, studies concerning the utilization of xylanases to produce XOS directly from a grain to make functional food, remain sparse [46, 47]. Nevertheless, the results shown in this study suggest that XOS can be obtained from flours used in the food and feed industry, using the recombinant xylanase Xyl-L expressed in *P. pastoris*, showing the potential for its application in the nutraceutical and food industries. Further improvements are being studied for the industrial applications of Xyl-L.

Conclusions

Enzymes have a long history of safe use in food processing and animal feed. The host used for expressing proteins at the industrial level becomes relevant for the affordable and safe production of recombinant enzymes that can be used as food additives. In this study, it was shown that Xyl-L, a cold-adapted GH10 xylanase of antarctic origin, can be successfully expressed in *P pastoris*. Its characterization resulted in a specific activity of 5.10 ± 0.21 U/mg, at pH 7.5 and 25°C, using beechwood xylan, with a *Km* of 3.5 mg/mL and a *kcat* of 9.16 s−1. The raise in the optimal temperature of Xyl-L produced in *P. pastoris* compared to its *E. coli* counterpart (from 37 to 42° C), might be due to the tendency of *P. pastoris* to add glycosylations to the three-dimensional structure of the recombinant produced proteins.

In addition, Xyl-L showed activity against different flour substrates used in the food industry, producing XOS with a DP ranging from 2 to 4. All these results suggest the potential of Xyl-L produced in *P. pastoris* for its application in the nutraceutical and food industries. Further studies focusing on bioprocesses optimization might still be necessary to achieve higher yields and specific xylanase activity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02690-4.

Supplementary Material 1.

Acknowledgements

The use of the facilities of the BOKU Mass Spectrometry Core Facility is gratefully acknowledged and the authors thank Dr. Clemens Grünwald-Gruber and Mr. Daniel Maresch for performing the mass spectrometry measurements. Special thanks go to Aitor Gonzalez Titos, Marina Jecmenica, Victor Mendes Honorato, Gabriele Wilt and Viktoria Kowarz from BOKU/ACIB, and Cristóbal Falcon, for their help during cloning, strain generation and data analysis. Also, special thanks to Dr. Oriana Salazar for her advising throughout this work.

Author contributions

Juan Asenjo: Supervision, Validation, Review editing, Resources. Barbara Andrews: Supervision, Validation, Review editing. Brigitte Gasser: Supervision, Validation, Writing, Review editing, Resources. Sebastián Rodríguez: Investigation, Methodology, Formal analysis, Conceptualization, Writing. Carolina González: Investigation, Methodology, Formal analysis, Writing. José Pablo Reyes-Godoy: Investigation, Methodology, Formal analysis, Writing.

Funding

This work was supported by the Centre for Biotechnology and Bioengineering (CeBiB) [PIA FB0001] and the ANID Doctorate Fellowship (21221519). Further support was obtained by the Austrian Federal Ministry of Labour and Economy (BMAW), the Austrian Federal Ministry of Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, the Government of Lower

Austria, the Business Agency Vienna and BOKU through the COMET Funding Program managed by the Austrian Research Promotion Agency FFG.

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare no Competing interests.

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Received: 4 October 2024 Accepted: 10 March 2025
Published online: 05 April 2025

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