Development of a new method for D-xylose detection and quantification in urine, based on the use of recombinant xylose dehydrogenase from *Caulobacter crescentus*

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Highlights

- Lactose intolerance is a digestive disorder widespread in more than a half of the world's population.
- Enzymatic detection using recombinant xylose dehydrogenase was found to be an excellent method for quantification of D-xylose in both buffer and urine samples.
- Limits of detection (LoD) and quantification (LoQ) of the developed method in buffer were 0.568 mg/dL, and 1.89 mg/dL respectively.
- Recombinant XylB-wt showed exceptional stability when freeze-dried. The enzyme retained 88 % of activity after one year stored at 4 °C and about 70 % when stored at room temperature
- This method can easily be incorporated into a new test for the diagnosis of hypolactasia.

Abstract

The gene *xyl*B from *Caulobacter crescentus* has been cloned and expressed in *Escherichia coli* providing a high yield of xylose dehydrogenase (XylB) production and excellent purity (97 %). Purified recombinant XylB showed an absolute dependence on the cofactor NAD^+ and a strong preference for D-xylose against other assayed mono and disaccharides. Additionally, XylB showed strong stability when stored as freeze-dried powder at least 250 days both at 4 °C and room temperature. In addition, more than 80 % of the initial activity of rehydrated freeze-dried enzyme remained after 150 days of incubation at 4 °C. Based on these characteristics, the capability of XylB in D-xylose detection and quantification was studied. The linearity of the method was maintained up to concentrations of D-xylose of 10 mg/dL and the calculated limits of detection (LoD) and quantification (LoQ) of xylose in buffer were 0.568 mg/dL and 1.89 mg/dL respectively. Thus, enzymatic detection was found to be an excellent method for quantification of D-xylose in both buffer and urine samples. This method can easily be incorporated in a new test for the diagnosis of hypolactasia through the measurement of intestinal lactase activity.

Keywords: Enzymatic detection / Gaxilose / Hypolactasia / Xylose quantification / Xylose dehydrogenase / Intestinal Lactase Activity

1. Introduction

Lactose intolerance is the symptomatic response to low levels of intestinal lactase (hypolactasia), the enzyme responsible for hydrolysis of ingested lactose (Semenza et al., 2006). The most common symptoms associated with lactose intolerance are: abdominal pain, abdominal swelling, bloating, wind and diarrhea (Arola, 1994; Bohmer and Tuynman, 2001; Jarvis and Miller, 2002; Campbell et al., 2009). A simple test for the diagnosis of hypolactasia (LacTEST®) was recently developed (Hermida et al., 2013; Aragón et al., 2014). This test is based on the use of gaxilose (4-*O*-β-D-galactopyranosyl-D-xylose), a synthetic lactose analogue. After oral administration, gaxilose is cleaved by intestinal lactase to D-galactose and D-xylose (Scheme 1).

((Insert here Scheme 1))

Galactose is transformed into glucose in the liver while xylose, which is passively absorbed, is partially metabolized, appearing in the blood and being finally excreted in urine. The levels of D-xylose in blood or urine correlate with the total *in vivo* lactase activity, representing a direct measurement of the hypolactasia degree. Detection of xylose is currently carried out using a colorimetric method based on the chemical reagent phloroglucinol. This method has been proved to be sensitive and reliable for the detection of xylose in biological samples (Hermida et al., 2014). However, the use of an acid medium and high temperature makes this analytical method difficult to use with routine clinical automated analyzers. In order to facilitate xylose quantification in highly automated clinical laboratories, the development of an enzymatic method working under mild conditions, should prove extremely useful.

An attractive candidate for enzymatic detection of D-xylose is the family of xylose dehydrogenases (XDHs), which catalyze the oxidation of D-xylose to D-xylonolactone (Scheme 2). This reaction requires a concomitant reduction of a cofactor which can be NAD^+ or $NADP^+$.

((Insert here Scheme 2))

Many D-XDH from different organisms have been identified and studied in the last decades (Aoki et al., 2001; Berghäll et al., 2007; Johnsen et al., 2009; Mihasan et al., 2013). Most of them display their highest activities using $NADP^+$ as cofactor, but they are also able to utilize NAD^+ at acceptable activity ratios. Even though D-xylose is the main substrate for all of them, they have also shown moderate to good activity with other sugars, especially D-glucose. This fact represents an important drawback for the enzymatic detection of D-xylose in biological samples such as blood, due to the presence of significant concentrations of D-glucose. Interestingly, it has also been described a subgroup of D-XDH enzymes, which display a higher selectivity towards D-xylose, i. e. the XylB enzymes group (Scheme 2). This group is absolutely dependent on NAD^+ as cofactor, showing no activity when $NADP^+$ is used. XylB has been identified in three microorganisms; *Pseudomona sp* (Dahms and Russo, 1982) *Arthrobacter sp* (Yamanaka et al., 1977) and *Caulobacter crescentus* (Stephens et al., 2007; Toivari et al., 2012) however, only the latter has been isolated and characterized.

In the last past few years, some D-xylose detection methods involving XylB from *C. crescentus* have been described. A XylB cell-surface displaying system has been developed, in which dehydrogenase was expressed on the outer membrane of *E. coli* cells fused to the ice nucleation protein from *Pseudomonas borealis* DL7 as an anchoring motif (Liang et al., 2012). This enzyme-surface-displayed bacteria modified bioanode, has also been used for the development of xylose based biofuel cells (Xia et al., 2013). By immobilization of the engineered *E. coli* cells on multi-walled carbon nanotube electrodes, the same authors developed a selective biosensor (Li et al., 2012). This biosensor was subsequently improved by co-immobilization of a glucose dehydrogenase for simultaneous voltammetric detection of D-xylose and D-glucose (Li et al., 2013). In addition, a D-xylose detection kit based on a XDH is commercially available from Megazyme International (Wicklow, Ireland). This kit was primarily designed for and used in the determination of glucan and xylan fractions in lignocelluloses (Selig et al., 2011).

In this paper, we describe the heterologous expression in *E. coli* of xylose deshydrogenase from *C. crescentus*, the functional characterization of the recombinant enzyme and its application to the development of a sensitive and reliable method for xylose detection and quantification in urine samples.

2. Materials and methods

2.1. General Remarks

SDS-PAGE was performed using 10% and 5% acrylamide in the resolving and stacking gels, respectively. Gels were stained with Coomassie brilliant blue R-250 (Applichem GmBH). Electrophoresis was always run under reducing conditions, in the presence of 5% β-mercaptoethanol. Protein and DNA gels were quantified by densitometry. The *E. coli* BL21(DE3) competent cells were purchased from Stratagene Co. The pET28b(+) expression vector was purchased from Invitrogen Co. Plasmids and DNA purification kits were from Sigma-Aldrich. All other chemicals were purchased from commercial sources as reagent grade.

2.2. Protein expression and purification

The sequence of the gene *xyl*B from *C. crescentus* strain NA1000 was codon optimized for expression in *E. coli* (OptimumGene^{TM} algorithm), chemically synthesized and cloned into pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). Sites for three restriction enzymes were included in the designed synthetic gene: *Hin*dIII in the 3' terminal position and both *Nde*I and *Nco*I in the 5' terminal position. Two different cloning strategies were followed in order to get two alternative XylB protein constructions: a wild-type enzyme (XylB-wt) and a N-terminal six histidine-tagged enzyme (XylB-6His). The XylB-wt construction was obtained by digestion of the optimized *xyl*B gene with *Nco*I and *Hind*III and cloning in the expression vector pET28b(+). The resultant plasmid pET-*xyl*B was transformed into *E. coli* BL21(DE3) competent cells. Alternatively, the synthetic gene *xyl*B was digested with the endonucleases *Nde*I and *Hind*III and sub-cloned in pET28b(+), in order to build the XylB-6His protein construction. Expression colonies of BL21(DE3) *E. coli* containing the plasmids pET-*xyl*B-

wt/pET-*xyl*B-6His were cultured in Luria-Bertani (LB) broth containing kanamycin (26.3 μg/mL) at 37 ºC with shaking. When the culture reached an $O.D_{600nm}$ of 0.5-0.6, XylB expression was induced with IPTG (1 mM) and the temperature was dropped to 30 ºC. The cultures were maintained overnight. After that, cells were harvested by centrifugation and suspended in 50 mM NaH_2PO_4 buffer pH 8.0. Cell suspension was disrupted by sonication and the cell lysate was centrifuged at 10,000 xg for 20 min. The resulting soluble cell extract was treated with DNase I (10 μg/mL, 20 min at 4 ºC) for nucleotide elimination. XylB-wt enzyme was purified by size-exclusion chromatography loading 5 mL of cell free extract (CFE) on a HiLoad 26/60 Superdex 200 PG column controlled by the AKTA-FPLC system (GE Healthcare Life Science). The purification was carried out in 50 mM NaH_2PO_4 buffer pH 7.2 containing NaCl (0.15 M) at a constant flow rate of 1.0 mL/min. The purity of fractions was assessed by SDS-PAGE. Eluted fractions containing pure protein were pooled together, dialyzed against 5 mM NaH_2PO_4 buffer pH 7.2 and freeze-dried.

2.3. Protein analysis

Peptide mass fingerprint analysis from the SDS-PAGE band corresponding to the XylB-wt was performed at the Proteomic Unit of the Spanish National Center of Biotechnology (CSIC). Samples were digested with sequencing grade trypsin overnight at 37 ºC. The analysis by MALDI-TOF mass spectrometry produces peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. Data were collected in the *m/z* range of 800-3600.

2.4. Enzyme activity assays

XylB catalyzes the oxidation of D-xylose with concomitant reduction of its cofactor NAD^+. Activity was measured by spectrophotometric monitoring of the NADH formed from cofactor reduction. Variations of absorbance at 340 nm are proportional to consumed D-xylose concentration in the reaction (ε_{NADH} = 6220 $cm^{-1}M^{-1}$). Reaction mixtures of 1 mL contain NaH_2PO_4 (50 mM, pH 8.0), NAD^+ (0.5 μmol), D-xylose (5 μmol), and enzyme sample (0.2-0.5

μg of protein). When kinetics or substrate specificity assays were carried out, activities were measured in 96-well titration microplates. Thus, reagents were scaled down to a final volume of 0.3 mL maintaining their final concentrations. Activity assays were performed at 25 °C. One unit of XylB activity was defined as the amount that produces 1 μmol of D-xylonolactone per min under the above conditions.

2.5. Steady-state kinetic assays

Steady-state kinetic assays for XylB activity were measured in triplicate at 25 ºC in 96-well plates (total volume of 0.3 mL), using the previously described activity assay. Measurements of kinetic parameters for D-xylose were performed with purified XylB (0.25 μg) at eight different D-xylose concentrations and saturating concentration of NAD^+ (0.5 mM, 0.17 μmol). Assays to determine the kinetic parameters for D-glucose were carried out using 2.4 μg of purified XylB at ten concentrations of substrate and 0.5 mM of NAD^+ (0.17 μmol). Kinetic assays for NAD^+ were performed at eleven concentrations of the cofactor and saturating D-xylose (5 mM, 1.7 μmol), employing 0.25 μg of purified XylB. Kinetic constants were calculated using the built-in nonlinear regression tools in *SigmaPlot 12.0* (Systat Software Inc).

2.6. Optimum pH and stability studies

XylB optimum pH was calculated measuring its activity at pH values between 5.5 and 9.5. Activity was determined as described above using three buffers at different pH: Bis-Tris buffer (pH 5.5, 6.0, 6.5, and 7.0), Tris-HCl buffer (pH 7.0, 7.5, and 8.0), and NaH_2PO_4 buffer (pH 8.0, 8.5, 9.0, and 9.5). Ionic strength of each buffer was adjusted to 0.1 M with NaCl.

Stability of purified enzyme was measured after freeze-dried at intervals of storage at 4 °C and room temperature and once rehydrated with buffer pH 8.0 and stored at 4 °C. Finally, the stability of the enzyme along different cycles of freezing and thawing was also examined.

2.7. Substrate specificity

Substrate specificity of XylB was evaluated by measuring its activity with seven monosaccharides (D-xylose, L-arabinose, D-glucose, D-ribose, D-galactose, D-mannose, and D-fructose) and two disaccharides (gaxilose and maltose). XylB activity was measured in triplicate at 25 ºC in 96-well plates (total volume of reaction 0.3 mL), using the previously described activity assay. When substrates different from D-xylose or L-arabinose were assayed, concentration was increased to 100 mM.

2.8. Detection and quantification of D-xylose

Quantification of D-xylose in buffer or urine samples was carried out in 10 min reactions with a total volume of 0.2 mL (96-well plates). A typical assay contains: NaH_2PO_4 buffer (50 mM, pH 8.0), NAD^+ (0.72 μmol), and D-xylose sample (35 μL, 1:5 v/v). After incubation of the initial mix for 5 min, reaction was initiated by addition of XylB (0.15 U, 5 μg of protein). Variation of absorbance at 340 nm (ΔAbs_{340nm}) before and after XylB addition was used to calculate the D-xylose concentration of the sample. Linearity of the enzymatic quantification method was evaluated measuring 10 serial dilutions of a standard D-xylose (200 mg/dL) in buffer or urine samples. Limit of detection (LoD) and limit of quantification (LoQ) of the technique were calculated from the regression parameters by the statistical formula: LoD = 3x*RSD/a*; LoQ = $10 \cdot$x*RSD/a* where RSD is the Residual Standard Deviation (Shrivastava and Gupta, 2011; Ott and Longnecker, 2010).

3. Results and Discussion

3.1. Heterologous expression and purification of XylB from Caulobacter crescentus

The sequence of the gene *xyl*B (GeneBank Accession Number: CP001340.1) was analyzed using the OptimumGene™ algorithm (GenScritp) in order to optimize a variety of parameters such as codon usage bias and GC content which are critical to the efficiency of gene expression in *E. coli* BL21(DE3) and modified accordingly. The optimized *xyl*B gene was sub-cloned in the expression vector pET28b(+). We followed two different cloning strategies in order to get two

alternative XylB protein constructions: a wild-type enzyme (XylB-wt) and a N-terminal six histidine-tagged enzyme (XylB-6His). Expression of recombinant XylB as a His-tagged protein allows for a simple purification process by ion metallic affinity chromatography (IMAC). However, it has previously been described that the activity and stability of XylB can be affected by the presence of the His-tag (Stephens et al., 2007). The vector pET28b(+) allowed us to get the two constructions (with and without 6His-tag) via digestion with specific restriction endonucleases. Thus, the XylB-wt construction was performed by digestion with the endonucleases combination *Nco*I/*Hin*dIII, while the construction XylB-6His was made by digestion with the endonucleases *Nde*I/*Hin*dIII. Sites for the three restriction enzymes were included in the designed synthetic gene. In this way, it was possible to build the two different XylB constructions using a unique synthetic gene. The strategy designed for achieving the XylB-wt construction involves the insertion of three additional amino acids in the N-terminal position: Met, Gly and His. These extra amino acids are absolutely required for retaining the open reading frame after ligation into pET28b(+). Due to its short length, this tag should not interfere in the folding and activity of the enzyme. Both constructions were transformed into *E. coli* DH5α cells for conservation and into *E. coli* BL21(DE3) strain for expression.

SDS-PAGE analysis of XylB expression showed two soluble proteins matching the expected molecular weight of the wild type protein and the XylB-6His (Figure 1). The amount of expressed protein was analyzed by densitometry and showed that XylB-wt represented 81 % of the total soluble protein in both checked strains. However, the percentage of XylB-6His was much lower i. e. between 24 % and 27 % (Figure 1).

((Insert here Figure 1))

Xylose dehydrogenase activity in each CFE was also measured (Table 1). Both samples of XylB-wt displayed activity between 15-20 U per mg of total protein. However, specific activity in XylB-6His samples was 5-7 times lower than those detected in CFEs with wt enzyme (Table 1). Control CFE did not show XDH activity, even increasing the amount of sample in the assay

to10 μL, which was five times more than in the XylB-6His assay and 50 times more than in the XylB-wt assay.

((Insert here Table 1))

Since XylB-wt showed higher expression levels and greater specific activity than XylB-6His, further work was carried out only with XylB-wt. Thus, recombinant XylB-wt was purified by size-exclusion chromatography. The elution profile of one of the performed purifications is shown in Figure 2. Only the fractions with highest absorbance and activity values were taken, discarding the rest of the peak in order to avoid possible interfering proteins and to increase the purity degree. To prevent an excessive salts concentration in freeze-dried samples (Superdex columns required high concentrations of NaCl in the purification buffer to avoid unspecific protein interactions) a dialysis step was included. Specific activity of the purified protein was 30 U/mg.

((Insert here Figure 2))

To unambiguously identify the overexpressed protein as the *xylB* gene product, peptide-mass fingerprinting of the recombinant protein was obtained. Sixteen predicted tryptic peptides with molecular masses in the analyzed m/z range were found in the peptide mass fingerprint of the XylB-wt sample, verifying that the recombinant protein had the expected features of XylB from *C. crescentus* NA1000 (Figure 3).

((Insert here Figure 3))

3.2. Stability studies

The optimum pH of XylB from *C. crescentus* was determined to be 7.5 (Figure 4). This pH was consistent with that previously found in the literature for this enzyme (Stephens et al., 2007). However, the enzyme was more stable at pH 8.0. Soluble samples of the recombinant protein stored at pH 8.0 and 4 °C maintained their activity without detectable losses for more than two weeks. A similar result was found in rehydrated freeze-dried samples at pH 8.0, however, when pH was increased to 8.5, the half-life of the enzyme decreased dramatically. Recently, the

thermostability of this enzyme has been improved by rational design mutagenesis for its application in the development of an efficient enzymatic biofuel cell (Feng et al., 2016). Thus, pH 8.0 was chosen for storage and reaction conditions.

((Insert here Figure 4))

XylB-wt showed exceptional stability when freeze-dried. After one year stored at 4 °C, the enzyme retained 88 % of its initial activity and about 70 % when stored at room temperature. This fact is especially interesting from the perspective of future commercialization of an enzymatic method. In addition, the high stability of the rehydrated freeze-dried enzyme in aqueous solution was especially remarkable (Figure 5A). When an aliquot of enzyme powder diluted in a pH 8.0 buffer was stored at 4 °C, the enzyme retained 85 % of its initial activity after 150 days. Finally, the stability of the enzyme along different cycles of freezing and thawing was examined. Three aliquots of rehydrated enzyme from three different freeze-dried batches were frozen at -20 °C; they were thawed and their activity analyzed along 4-5 cycles of freezing and thawing over different time periods. The resulting data showed that the enzyme did not lose activity during freezing, even when this process was repeated five times (Figure 5B).

((Insert here Figure 5))

3.3. Substrate scope

As noted above, D-xylose is the main substrate for all XDHs. However, they are also able to catalyze the oxidation of other sugars, with higher or lower activity ratios depending on the enzyme and the sugar substrate. This fact represents an important drawback for the detection of D-xylose in biological fluids due to the presence of other sugars which will interfere in xylose determination. Therefore, the substrate scope of recombinant XylB-wt was studied with nine different sugars that included both, mono- and disaccharides (Table 2).

((Insert here Table 2))

Only with D-xylose and D-arabinose did XylB show activity at low substrate concentrations (5 mM) however, the activity measured with D-arabinose was six-fold lower than with D-xylose.

Since arabinose is rare in biological samples, especially those from fasting patients, it should not be a problem as possible interfering compound in D-xylose detection. The recombinant enzyme also showed activity with D-ribose, D-glucose and D-galactose, but only when the substrate concentration was increased 20 times from 5 to 100 mM (Table 2). Regarding the assayed disaccharides, only gaxilose was found to be substrate of XylB-wt, but only at high concentration and with a poor relative activity of 2 % with respect to D-xylose.

Although D-glucose is not a good substrate of XylB-wt, this sugar can interfere in the determination of D-xylose, due to its presence at significant concentrations in biological samples such as blood. For this reason, a more detailed study was carried out to calculate the kinetic parameters for D-xylose and D-glucose as substrates of XylB-wt (Table 3).

((Insert here Table 3))

Interestingly, the catalytic efficiency (k_{cat}/K_M) of XylB-wt for D-xylose was four orders of magnitude higher than for D-glucose (Table 3). Thus, the selectivity of the recombinant enzyme for xylose, defined as the ratio between the catalytic efficiency with D-xylose and D-glucose ($(k_{cat}/K_M)_{xyl}/(k_{cat}/K_M)_{glu}$), was 15,000, which clearly indicates a higher specificity of the enzyme for the first substrate in the assayed conditions. This result supports the use of XylB-wt in D-xylose detection, even in samples where D-glucose is also present. XylB-wt showed a K_M value one order of magnitude lower than previously described for other XylB constructions (Toivari et al., 2012).

3.4. Development of a detection and quantification method for D-xylose based on the use of XylB-wt

The proposed method is based on the reaction depicted in Scheme 2. The enzymatic oxidation of xylose comes at the expense of NAD^+ reduction to NADH and the emergence of this compound can be quantified by absorbance at 340 nm (ε_{NADH} = 6220 $M^{-1}\cdot cm^{-1}$). Typically, activity assays were carried out in two steps: in the first step the enzyme and its cofactor were solved in buffer, and the unspecific background was recorded; in the second step, reaction was initiated by

addition of the substrate, being the slope of the increase of absorbance with time directly proportional to the enzyme activity. In a preliminary study, the linearity of the analytical procedure was stated by measuring serial dilutions of D-xylose ranging from 0.1 to 19 mg/dL in buffer. Linearity was observed in the concentration range of 0.1-15 mg/dL with a correlation coefficient r^2 of 0.9992 (Figure 6). Limits of detection (LoD) and quantification (LoQ) of the method were calculated from the regression parameters with the equations described in the Materials and Method section of this paper. Thus, calculated LoD was 0.568 mg/dL, and LoQ was 1.89 mg/dL in buffer.

((Insert here Figure 6))

Since this method was extremely efficient and sensitive for the quantitation of xylose in buffer, we decided to study whether it could be valid for the measurement of D-xylose in two human fluids: urine and blood serum. The urine negatively affected the basic parameters of the method: linearity, LoD and LoQ. Three urine samples were obtained from volunteers who did not have any digestive disorders or diseases that could interfere in the normal composition of urine, such as diabetes or abnormal kidney function. Urine samples were primed with D-xylose concentrations up to 15 mg/dL. It was immediately discovered that the color of urine interfered with the assay background; therefore, it was necessary to use a smaller volume of the sample than in the case of the buffer, resulting in a 1:6 dilution in the reaction. Under these conditions, linearity was not affected when urine samples were employed and linear regression parameters were quite similar to those obtained when urine was substituted by a buffer. Urine color in concentrated samples slightly affected y_0 but this interference was not high enough to interfere in D-xylose quantification. Linear regression parameters were calculated in triplicate and are summarized in Table 4.

((Insert here Table 4))

When the enzymatic method was compared with the phloroglucinol method, a linear correlation was found (Figure 7A). In addition, the unspecific background in D-xylose detection was

considerably reduced when the enzymatic method was employed, due to the lower effect that urine's yellow color produces compared to colorimetric detection. This can be observed in Figure 7B which represents a urine sample primed with increasing concentrations of xylose. Figure 7B also shows that the enzymatic method was more accurate than the colorimetric method for quantifying the concentration of xylose. Nevertheless, these results were consistent with those obtained during the first LacTEST® phase I clinical trial. In this study, quantification of xylose amounts in urine after the administration of placebo (water) to 12 healthy volunteers yielded a mean value of 12.92 ± 5.26 mg (Hermida et al., 2013), suggesting the presence of an interfering component in some urine samples. On the other hand, the linear range for the colorimetric method using phloroglucinol was found to be 0.5 to 20 mg/dL, being slightly wider than that obtained with the enzymatic method (0.1 to 15 mg/dL) (Hermida et al., 2014).

((Insert here Figure 7))

Finally, we conducted a preliminary set of studies with three samples from healthy volunteers to evaluate the accuracy of the new enzymatic method in the detection of xylose in blood serum. Since xylose concentration in blood following gaxilose ingestion is lower than in urine (Hermida et al., 2013), serum samples were primed with xylose concentrations up to 1 mg/dL, which is close to the calculated cut-off (0.97 mg/dL) for categorizing between normo and hypolactasic patients (Aragón et al., 2014; Hermida et al., 2013). When xylose in serum samples was measured under the described conditions for urine samples, the results were not consistent with those obtained in buffer (Figure 8).

((Insert here Figure 8))

A relatively high absorbance value was obtained in the absence of xylose (the y_0 value increases) and increasing concentrations of xylose resulted in a smaller increase in absorbance than was expected (the slope of the regression plot was lower than with buffer). Therefore, some component of the blood serum made it impossible to quantify xylose in these samples.
Work is in progress to overcome these limitations.

4. Conclusion

Two constructions of the enzyme xylose dehydrogenase were expressed in *E. coli*: a six histidine-tagged enzyme (XylB-6His) and a wild-type form (XylB-wt). Expression of the wild-type form was three times higher than that of the His-tagged counterpart and therefore, XylB-wt was selected as a reference enzyme for the development of the assay. It should be noted that the produced protein is the first wild-type form of a xylose dehydrogenase from *C. crescentus* described to date.

The developed enzymatic method, based on the use of recombinant XylB, provides a reliable tool for D-xylose quantification in urine and represents an alternative to the use of phloroglucinol. When the enzymatic method was compared with the phloroglucinol method (reference method for D-xylose detection), a linear correlation was found. In addition, the unspecific background observed with some urine samples was considerably reduced when employing the enzymatic method, increasing its accuracy and preventing the possible overestimation of xylose concentration.

To conclude, this new method can be stated as highly sensitive, robust and easily implementable for use in automated clinical analyzers.

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Figure Legends

Scheme 1. Hydrolysis of gaxilose catalyzed by intestinal lactase.

Scheme 2. Reaction catalyzed by different members of the xylose dehydrogenase family.

Figure 1. SDS-PAGE analysis of XylB expression. MW: molecular weight marker; wt1 and wt2: fractions of the two XylB-wt expression strains; 6His1 and 6His2: fractions of the two XylB-6His expression strains; control: fractions of the control culture from BL21(DE3)/pET28b(+) strain. Percentages of expressed XylB over the total protein content are showed under each lane.

Figure 2. Size-exclusion purification of the recombinant XylB-wt. Insert shows the elution profile of XylB-wt activity. SDS-PAGE shows: low molecular weight markers (MW); CFE; fractions containing XylB activity (lanes 1-4). Percentages of XylB over the total protein content are showed under each lane.

Figure 3. Sequence of XylB-wt, indicating the theoretical trypsin cleavage sites (↓). The sequence for the identified peptides is shaded. Molecular mass of each peptide (under or over lined) is indicated in Da.

Figure 4. Effect of pH on XylB from *C. crescentus*. Enzyme activity was measured using three buffers, each at a different pH: Bis-Tris buffer (●), NaH_2PO_4 buffer (Δ) and Tris-HCl buffer (■). The ionic strength of each buffer was adjusted to 0.1 M with NaCl. The error bars represent the standard deviation (SD) of the measurements.

Figure 5. A) Stability of XylB-wt during a period of time at different temperatures. Freeze-dried enzyme was stored at 4 °C (●) and RT (▼). Stability at 4 ºC of a rehydrated freeze-dried aliquot in a pH 8.0 buffer was also analyzed (■). B) Stability of XylB-wt along different cycles of freezing and thawing. Three different batches of freeze-dried enzyme were rehydrated with phosphate buffer (pH 8.0), and their activity was examined at 37 °C after consecutive freezing cycles: sample 1 (●), sample 2 (▼) and sample 3 (■). The error bars represent the SD of the measurements.

Figure 6. D-Xylose concentration in buffer against absorbance at 340 nm. The linearity of the method in the case of the buffer was excellent with an r^2 of 0.9992 up to a xylose concentration of 10 mg/dL. The error bars represent the SD of the measurements.

Figure 7. A) Correlation between the measurements of xylose performed with the colorimetric method of phloroglucinol and the novel enzymatic method. B) One urine sample primed with increasing concentrations of xylose and measured with both the colorimetric and the enzymatic method. The error bars represent the SD of the measurements.

Figure 8. Xylose quantification in blood serum samples. (●) Xylose in buffer; (○) xylose in serum 1; (▼) xylose in serum 2; (△) xylose in serum 3. The error bars represent the SD of the measurements.

Table 1

Xylose dehydrogenase activity measured in the cell free extracts of the recombinant *E. coli* cells.

Sample	Activity (U/mL)	[Protein] (mg/mL)	Specific Activity (U/mg)
Control	ND[a]	7.60	ND[a]
XylB-wt1	126.5	8.58	14.73
XylB-wt2	127.7	6.18	19.85
XylB-6His1	18.45	6.06	3.04
XylB-6His2	14.10	5.93	2.38

[a] ND: no detected.

Table 2

Specific and relative activity of recombinant XylB-wt with different mono and disaccharides substrate.

Sugar	Concentration (mM)	$Activity_{spec}$ (U/mg)	$Activity_{rel}$ (%)
D-Xylose	5	30.0	100
L-Arabinose	5	4.8	16
D-Ribose	100	4.4	15
D-Glucose	100	3.9	13
D-Galactose	100	0.5	2
D-Mannose	100	0.0	0
D-Fructose	100	0.0	0
Gaxilose	100	0.7	2
Maltose	100	0.0	0

Table 3.

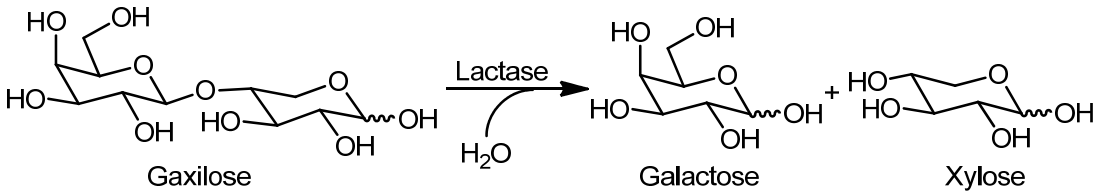
Kinetic constants of XylB-wt for D-xylose, D-glucose and NAD+.

Substrate	V_{max}+SD ($U \cdot mg^{-1}$)	k_{cat}+SD (min^{-1})	K_M+SD (mM)	k_{cat}/K_M+SD ($mM^{-1} \cdot min^{-1}$)
D-Xylose	30.2 ±1.0	$4.9x10^3$ ±0.035	0.041 ±0.005	$11.9x10^4$
D-Glucose	10.7 ±0.1	$1.7x10^3$ ±0.05	216 ±30	7.96
NAD+	30.1 ±1.0	$4.8x10^3$ ±0.065	0.078 ±0.01	$6.2x10^4$

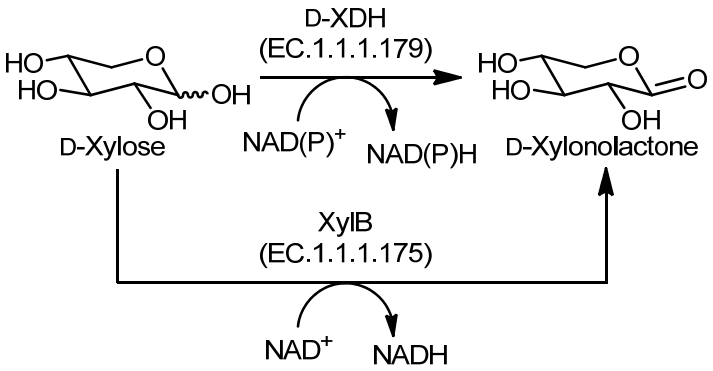
Table 4.

Linear regression coefficients and detection-quantification limits obtained for different urines primed with D-xylose Buffer containing D-xylose was included as control.

Sample	y_0	a	r^2	RSD	LoD (mg/dL)	LoQ (mg/dL)
Urine 1	0.0083	0.0462	0.9994	0.0073	0.474	1.58
Urine 2	-0.0119	0.0473	0.9980	0.0137	0.868	2.90
Urine 3	0.0069	0.0464	0.9994	0.0077	0.498	1.66
Buffer + D-xylose	-0.0070	0.0475	0.9992	0.0090	0.568	1.89


HO
OH
O
HO
O
O
HO
OH
OH
OH
Lactase
H_2O
HO
OH
O
HO
OH
OH
+
HO
O
HO
OH
OH
Gaxilose
Galactose
Xylose

Scheme 1


D-XDH
(EC.1.1.1.179)
HO
HO
O
OH
OH
D-Xylose
NAD(P)+
NAD(P)H
HO
HO
O
O
OH
D-Xylonolactone
XylB
(EC.1.1.1.175)
NAD+
NADH

Scheme 2

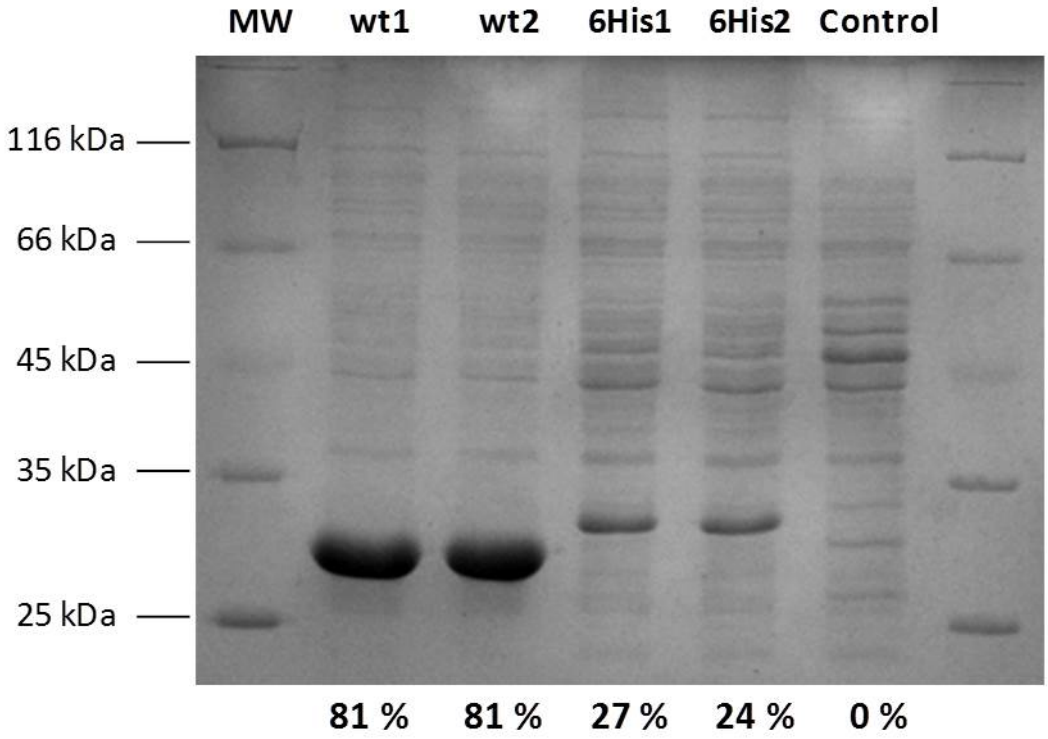
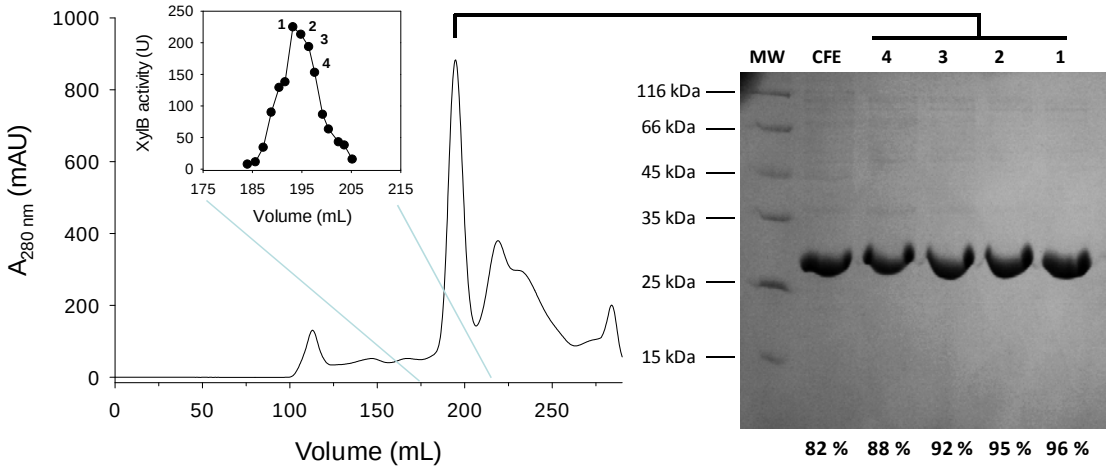

MW
wt1
wt2
6His1
6His2
Control
116 kDa
66 kDa
45 kDa
35 kDa
25 kDa
81 %
81 %
27 %
24 %
0 %

Figure 1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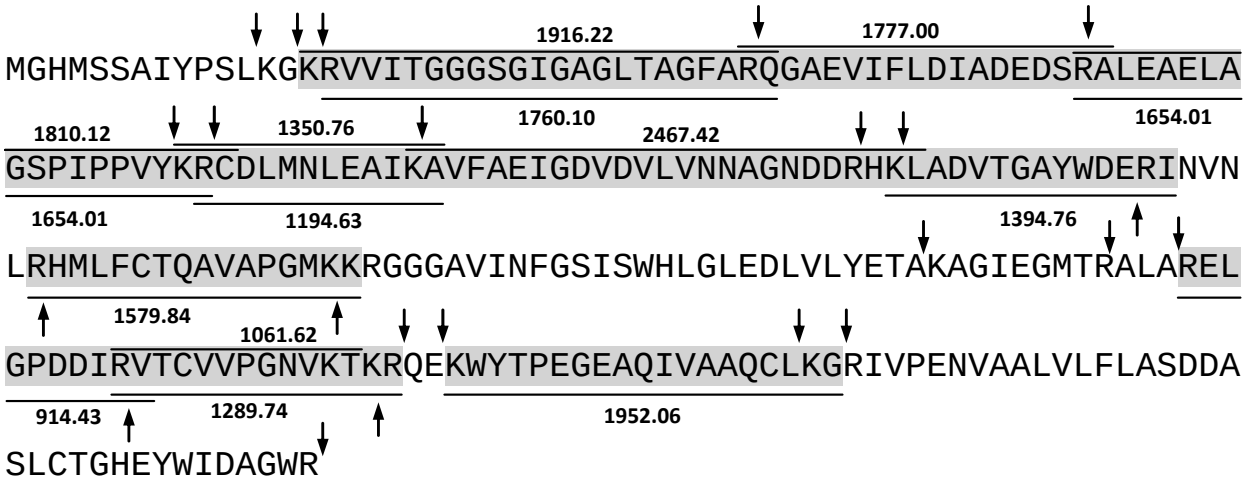
800
600
400
200
0
A280 nm (mAU)
0
50
100
150
200
250
Volume (mL)
250
200
150
100
50
0
XylB activity (U)
175
185
195
205
215
Volume (mL)
1
2
3
4
MW
CFE
4
3
2
1
116 kDa
66 kDa
45 kDa
35 kDa
25 kDa
15 kDa
82 %
88 %
92 %
95 %
96 %

Figure 2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1777.00
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1654.01
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1350.76
1760.10
2467.42
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1654.01
1194.63
1394.76
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1061.62
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1952.06
SLCTGHEYWIDAGWR

Figure 3

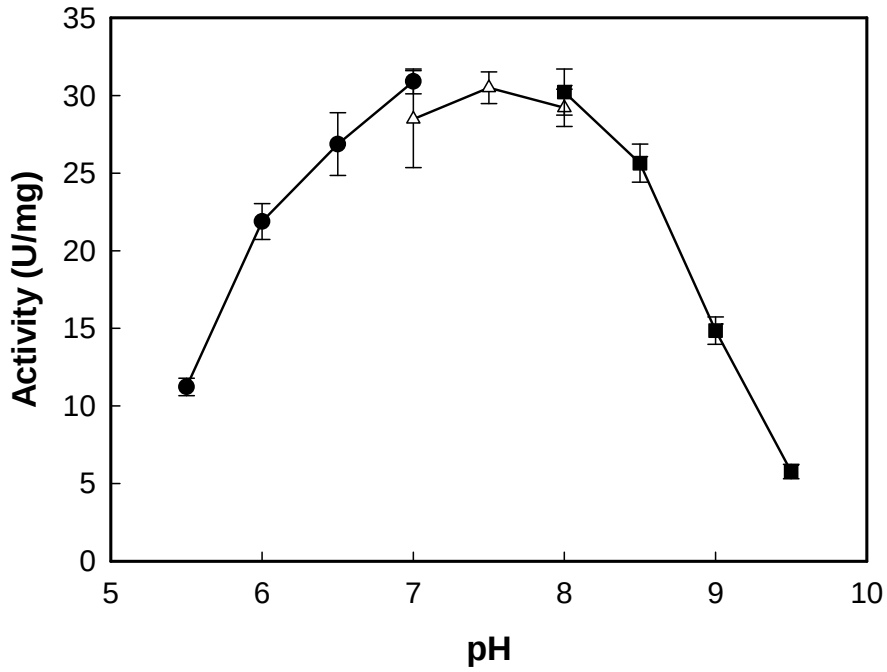


Figure 4

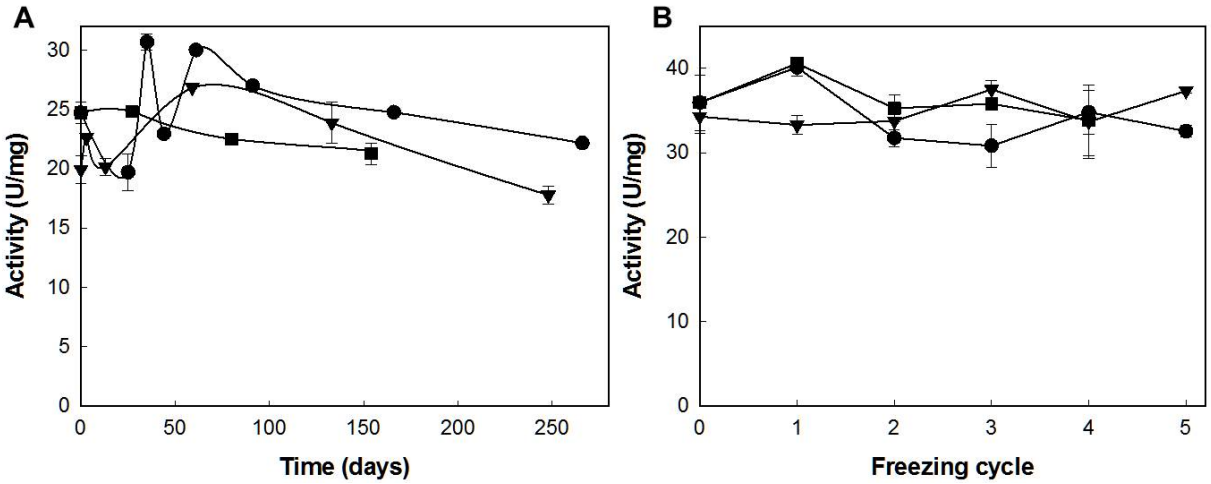
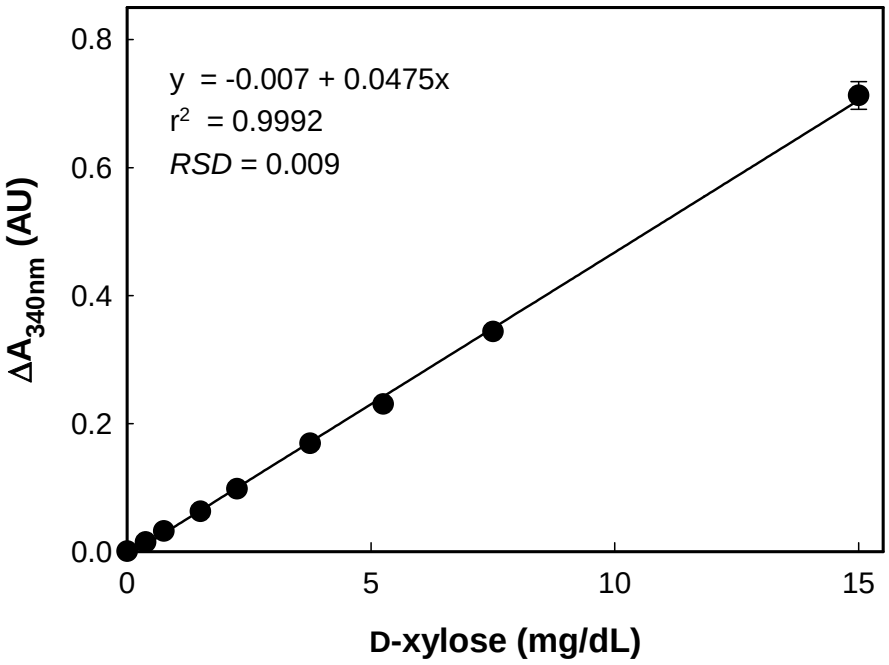


Figure 5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r2 = 0.9992
RSD = 0.009
ΔA340nm (AU)
D-xylose (mg/dL)
0.0
0.2
0.4
0.6
0.8
0
5
10
15

Figure 6

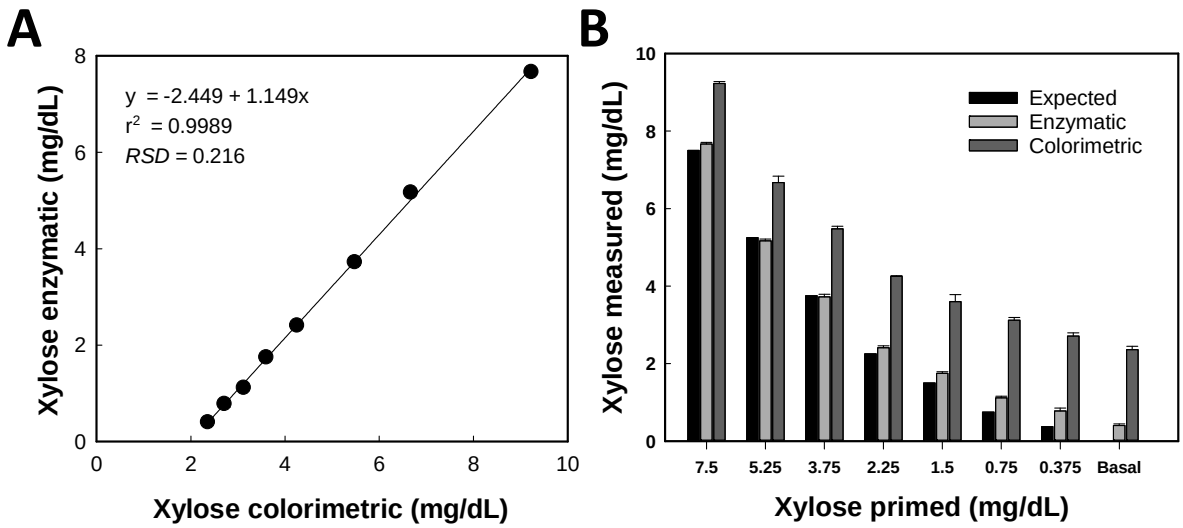
A
y = -2.449 + 1.149x
r2 = 0.9989
RSD = 0.216
Xylose enzymatic (mg/dL)
Xylose colorimetric (mg/dL)
B
Expected
Enzymatic
Colorimetric
Xylose measured (mg/dL)
7.5
5.25
3.75
2.25
1.5
0.75
0.375
Basal
Xylose primed (mg/dL)

Figure 7

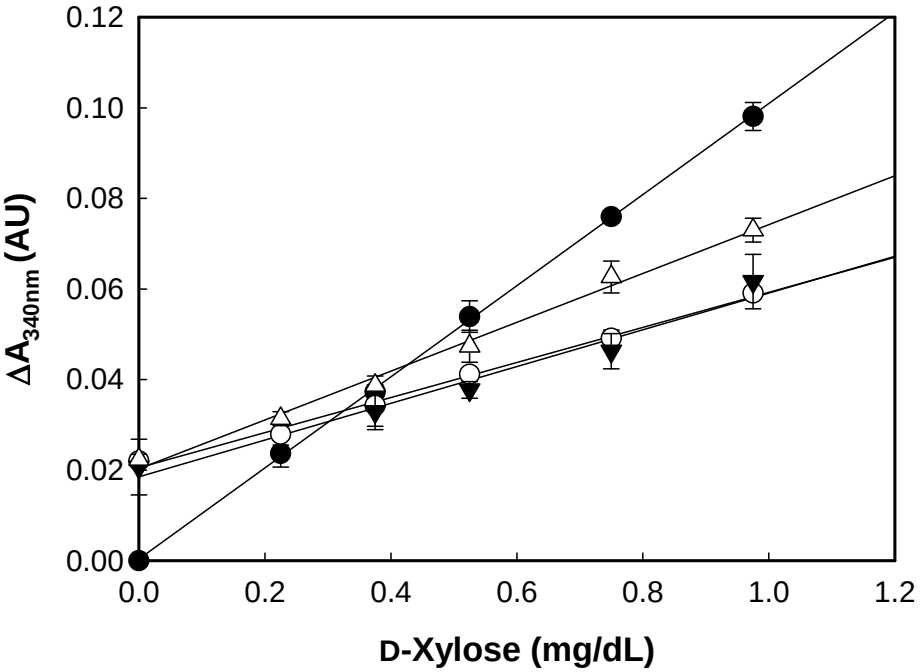
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ΔA340nm (AU)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
D-Xylose (mg/dL)

Figure 8