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Inhibition of *Debaryomyces nepalensis* xylose reductase by lignocellulose derived by-products

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ABSTRACT

Xylose reductase (XR) is a biocatalyst that converts xylose to xylitol and it has a potential application in the enzyme based production of xylitol from lignocellulosic hydrolysates. However, the development of a successful XR based bioprocess for xylitol production is challenging, due to the presence of inhibitory lignocellulose derived by-products (LDBs) in hydrolysates. Though various methods have been developed to mitigate the toxic effects of LDBs, none of them were promising. One of the reasons for this, could be the lack of knowledge on the enzyme inhibition mechanisms. Therefore, for the first time, here we investigated mechanisms of XR inhibition by major LDBs using XR from *Debaryomyces nepalensis*. We found that phenol showed competitive inhibition of XR whereas gallic acid, vanillin, furfural, 5-hydroxymethylfurfural (HMF) and acetate exhibited mixed inhibition. The inhibitory constants (K_I) of vanillin, phenol, gallic acid, furfural, HMF and acetate were found to be 0.1, 11, 32, 54, 45 and 100 mM respectively. Moreover, the enzyme stability was drastically affected in the presence of phenols. In addition, molecular docking simulations performed using AutoDock 4.2.6 program revealed the putative binding sites of LDBs on XR and corroborated the experimental data.

1. Introduction

Lignocelluloses constitute cellulose, hemicellulose and lignin, which are abundant, inexpensive and renewable energy sources with a great scope of exploitation for the production of biofuels and biochemicals [1]. The acid hydrolysis of lignocellulose, disrupts hemicellulose and releases xylose, a pentose sugar which comprises more than 30% w/w of lignocellulose [1,2]. This plant-derived xylose is promising to exploit for the production of a value-added product named xylitol [3]. Xylitol is chemically a pentose alcohol that has been proven as a multi-beneficial, natural sugar substitute with applications in sugar-free foods, oral hygiene, pharma and cosmetic products [4].

However, the current chemical and microbial processes for xylitol production are non-ecofriendly and uneconomical respectively [5]. The chemical processes require toxic nickel catalyst, high pressure and temperatures whereas downstream processing is cumbersome in biological processes making it uneconomical [5,6]. Therefore, the alternative to this is, the employment of enzymatic strategy [6]. Since only one enzyme xylose reductase (XR) (EC 1.1.1.21) is required for converting xylose to xylitol, the development of a successful enzyme based bioprocess could surpass the shortcomings associated with the current processes [6,7]. However, the lack of knowledge on the effect of major lignocellulose derived by-products (LDBs) that generated while preparation of lignocellulosic hydrolysates, on enzyme, leading to end up with failures [8,9]. These by-products include phenol (a typical structural moiety of plant phenols), gallic acid, vanillin, furfural, 5-hydroxymethylfurfural (HMF) and acetate which are also known as lignocellulose inhibitors of microbial fermentation [9–11]. Albeit various methods have been developed to mitigate the inhibitory effects of LDBs, none of them were promising [12,13]. One of the reasons for this is the overlook ing of mechanisms of inhibition of XR by LDBs. To study this, we used xylose reductase from *Debaryomyces nepalensis* (*Dn*XR) which has been well characterized [14].

In the present study, for the first time, we explored the inhibitory mechanisms of *Dn*XR by phenol, gallic acid, vanillin, furfural, HMF and acetate. In addition, we studied the effect of LDBs on the enzyme stability.

2. Methods

2.1. Strains and materials

The strain *E. coli* Rosetta (Novagen, USA) was used as expression host strain. Previously constructed expression vector pET28a-*Dn*XR

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was used for protein expression (unpublished data). NADH and NADPH (both ≥97% pure) (Sigma, USA) were used for enzyme activity measurements. Ni-NTA agarose (Qiagen, Germany) was used as affinity matrix for purification. The LDBs namely, phenol, HMF, furfural, acetate and other chemicals of analytical grade were purchased either from Himedia or Sisco Research laboratories (India).

2.2. Expression and purification of recombinant DnXR

Plasmid pET28a-*Dn*XR transformed *E. coli* Rosetta was grown to A_{600} = 0.7–0.9 in the LB medium containing 34 μg/ml chloramphenicol and 50 μg/ml kanamycin. Then, IPTG was added to a final concentration of 0.3 mM for induction and incubated at 30 °C, 180 rpm for 8 h. After the cells were collected, lysed by sonication and supernatant was taken for checking expression by SDS-PAGE. To obtain the purified protein, the supernatant was subjected to an immobilized metal ion affinity chromatography (IMAC) using a Ni^{2+} affinity column [15]. All of the fractions (Flow through, 20, 100, 200, 300 mM imidazole elutions) flowing through the column were analyzed by SDS-PAGE. The fractions containing pure *Dn*XR were collected and used for the enzymatic assays. The protein concentrations were estimated by bicinchoninic acid (BCA) assay with bovine serum albumin as a standard [16].

2.3. Enzyme activity assay

The recombinant *Dn*XR activity was measured spectrophotometrically (Lamda 25 UV/VIS spectrophotometer, Perkin Elmer, USA) by monitoring the change in A_{340} in 500 μl reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.3 mM NADPH as a cofactor and 300 mM xylose as a substrate at optimal temperature 45 °C. The change in absorbance at 340 nm was measured continuously at least for 1 min at every 1 s time point. It was ensured by the addition of a specific amount of enzyme that gives a linear curve. One enzyme unit (U) was defined as the amount of enzyme that caused the oxidation of 1 μmol of NADPH per minute [14]. Either heat inactivated enzyme or sodium phosphate buffer (pH 7.0) was taken for measuring the auto hydrolysis of NADPH in the standard reaction mixture and considered as a reference.

2.4. Determination of inhibitory concentrations of LDBs

The enzyme activity was measured at standard assay conditions in the presence of increasing concentrations of each LDB (0–100 mM) and obtained data were used to calculate half maximal inhibitory concentration (IC_{50}) and minimal inhibitory concentration (MIC) using linear regression function of Graphpad prism 5.

2.5. Enzyme kinetics and stability

In the presence of a particular concentration of each LDB, the xylose kinetic parameters of enzyme were determined by measuring the initial velocities at different xylose concentrations (5–700 mM). The obtained data were used to calculate the kinetic parameters by fitting to the various kinetic plots such as Michaelis–Menten, Lineweaver-Burk, Eadie-Hofstee and Eisenthal & Cornish-Bowden plot [17]. The Michaelis–Menten equation for competitive inhibition model used was

$$v_o = \frac{V_{max}[S]}{\left(1 + \frac{[I]}{K_I}\right) K_M + [S]} \quad (1)$$

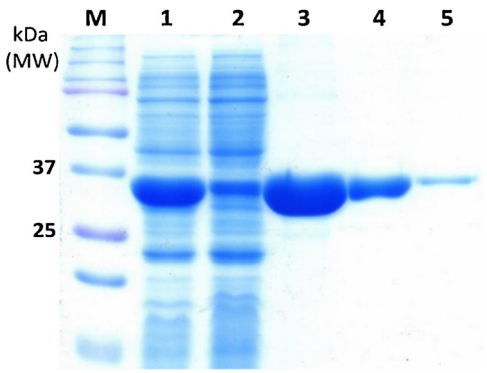

Fig. 1. SDS-PAGE analysis of recombinant *Dn*XR. Lane M: molecular weight (in kDa) marker; lane 1: soluble fraction of the induced cells; lane 2: flow through during the purification; lanes 3, 4, 5: 100, 200, 300 mM imidazole elutions respectively. The samples run on a 12% acrylamide gel stained with coomassie brilliant blue R-250. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The Michaelis–Menten equation for mixed inhibition model used was

$$v_o = \frac{V_{max}[S]}{\left(1 + \frac{[I]}{K_I}\right) K_M + \left(1 + \frac{[I]}{K'_I}\right)[S]} \quad (2)$$

With the plot of K'_M vs [I], we obtained a linear trend by linear regression analyses, values with intercept K_M and slope K_M/K_I and was used for calculation of K_I.

The stability of *Dn*XR was measured at 35 °C by incubating enzyme in the presence of a particular concentration of LDB in 50 mM sodium phosphate buffer (pH 7.0) for 8 h. Aliquots of enzyme at different time points were taken and specific activities were measured under standard assay conditions. The half-life of the enzyme was calculated by considering the first-order deactivation kinetics as described previously [18].

2.6. Statistical analysis

All experiments were performed in triplicates and results were expressed as average ± S.D. Regression analysis was performed for various kinetic plots and R^2 values were determined. The differences in mean values were evaluated by analysis of variance (two-way ANOVA) method and statistical significance was considered as $p < 0.05$. GraphPad Prism 5 was used for all statistical analysis.

2.7. Homology modelling and docking simulations

The protein sequence of *D. nepalensis* xylose reductase (accession no: KT239024) was retrieved from NCBI database and used for the homology modelling [19]. A homology model of *Dn*XR was built with SWISS-MODEL (v8.05) automated mode [20] using the available crystal structure of *Candida tenius* XR (PDB ID: 1K8C) as a template [21]. Further the *Dn*XR homology model was used for docking simulations using the program AutoDock 4.2.6 [22].

The macromolecule *Dn*XR was prepared for docking by removal of water molecules, addition of Gasteiger charges for all *Dn*XR atoms and addition of all hydrogens using AutoDockTools 1.5.6 [22]. The ligands such as phenol, gallic acid, vanillin, furfural, HMF and acetate were retrieved from either PDB or PubChem in their available sdf format and converted to pdb format using the molecular graphics package PyMOL. Using this pdb format, each ligand was prepared for docking using AutoDockTools 1.5.6 by converting it to pdbqt format as described in the provided tutorial [22]. The pdbqt format provides the possible rotatable bonds, the torsions