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Purification, characterization and molecular docking study of NADPH dependent xylose reductase from thermotolerant *Kluyveromyces* sp. IIPE453

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ABSTRACT

A NADPH dependent xylose reductase, key enzyme for reducing xylose into xylitol, was isolated from thermotolerant yeast *Kluyveromyces* sp. IIPE453 and characterized in detail. Purified enzyme (37 KDa) demonstrated maximum activity in pH 6.5 and 45 °C temperature. Molecular docking study of *in silico* protein model revealed a set of polar amino acid residues involved in catalytic mechanism.

1. Introduction

Green conversion processes are now-a-days considered for a wide variety of synthetic applications [1]. Biocatalyst mediated chemical conversions are emerging over conventional catalytic processes pertaining to its' ambient reaction conditions with better substrate selectivity and specificity. Development of many powerful analytical techniques has grown interest amongst scientific community for detailed investigation of reaction mechanisms and enzyme characteristics. In-silico approach through computational modelling is further strengthening the study at molecular level [2]. Comprehensive knowledge of active site binding pocket has helped to visualize acceptance of substrates, and biochemical characterization guided in rational design of enzyme variants with altered properties and substrate specificities [3]. By careful redesigning of metabolic pathway and enzyme modification, many desired biocatalytic transformations can be achieved effectively [4–6].

Biocatalytic reduction of D-xylose into xylitol is a promising alternative to the conventional process with high hydrogen input and severe reaction conditions (Raney-Nickel) [7]. However, xylitol production via fermentation process suffers from low conversion rate and productivity [8]. Reduction of D-xylose into xylitol via xylose reductase (XR) is the first step of D-xylose assimilation in microbial system to catalyze reversible reduction of aldehydes and/or ketones to their corresponding alcohols using either NADPH or NADH [9,10]. XR from different organisms have shown substantial degree of amino acid sequence similarity [11]. Crystallographic studies of many aldoketoreductases (AKRs) revealed a common (α/β) 8 barrel fold, with cofactor binding pocket at C-terminus. Analyses of XR gene from different sources have divulged a common specificity for NADPH [12]. Similarly, structural studies of XR from *Candida tenuis* illustrated a dual-specific enzyme with a preference for NADPH, with key residues involved in cofactor interaction and suggested mechanisms for utilization of NADPH as well as NADH [13,14]. Hence, a detailed characterization of XR is required for understanding rationale of the conversion.

In this paper, purification and characterization of a xylose reductase (XR_{KS}) from thermotolerant yeast *Kluyveromyces* sp. IIPE453 capable of fermenting D-xylose into xylitol [15] has been described. A 3D model of XR_{KS} via homology modelling approach has been developed for active site prediction. Attempt has also been made to understand enzyme's detail physicochemical properties and basic principle underlying D-xylose transformation and identification of the functional residues involved in catalysis. This information could

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be further explored for selective enzyme modification via genetic manipulation. We have visualized this work as first step of the entire bio-process with whole cell biocatalysis for improved xylitol production.

2. Materials and methods

2.1. Microorganism and culture condition

Kluyveromyces sp. IIPE453 (MTCC 5314) [16] was used for this experimental purpose. It was grown on lignocellulosic biomass derived acid hydrolysate (xylose concentration of 20 g/L supplemented with salts (composition in g/L): ammonium sulfate, 2; disodium hydrogen phosphate, 0.15; potassium dihydrogen phosphate, 0.15; magnesium sulfate, 0.06) at 45 °C and pH 4.5 in an airlift fermenter (Electrolab, UK; 8 L working volume). Aeration was maintained at 1 vvm (volume of air/volume of medium per unit time). Cell biomass was harvested at different time intervals corresponding to its sugar consumption for optimizing maximum enzyme activity and corresponding xylitol production. Under optimized condition entire cell biomass was harvested for enzyme recovery as well as DNA isolation.

2.2. Recovery & quantification of intracellular XR_{KS}

Harvested cell biomass was separated from the broth by centrifugation (Sorvall Lynx 6000) at 8×10^3 relative centrifugal force (r.c.f) for 10 min at 4 °C. Cell pellet was washed with deionized water and resuspended in 150 mL Lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 20% w/v Glycerol, 1 mM Dithiothreitol) for disruption in high pressure homogenizer (Panda Plus 2000, Gea). Supernatant was used to quantify XR_{KS} and total protein.

XR_{KS} activity was determined as per Zhang et al. [17] with minor modification using a U2900 UV–vis spectrophotometer (Hitachi, Japan) operating at room temperature for 5 min at λ_{340} nm after 1 min delay time to nullify endogenous oxidation of 0.3 mM reductant. Reductant NADPH/NADH (extinction coefficient 6.22×10^3 $L.M^{-1}\ cm^{-1}$) consumption was measured as variance in absorbance for a defined time span. Potassium phosphate buffer (50 mM, pH 7.0) was used and reactions were started by addition of xylose (500 mM). Each activity determination was made in triplicate, being the variation coefficient not higher than 5%. One unit of XR_{KS} activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of NADP/NAD per min under specified conditions.

Total protein was measured by the method of Bradford [18] with bovine serum albumin (BSA) as standard.

2.3. Isolation & sequencing of XR_{KS} gene

Genomic DNA was isolated using protocol of Hoffman and Winston [19] with minor modifications. A set of primers (Forward primer XR_{KS} FP: 5′GGCTGGCATGGAATTGTTAC3′ and reverse primer XR_{KS} RP: 5′CGCCCATCCAATATACAGAG3′) for PCR (Thermocycler master gradient; Eppendorf; Model 5331 gradient) amplification was designed with clone manager professional (v.9.0) using XR_{KS} sequence of *K. marxianus* NBRC1777 (NCBI GU574744). Reaction mixtures were incubated in a thermal cycler (Thermocycler master gradient; Eppendorf; Model 5331 gradient) for an initial denaturation at 95 °C for 5 min followed by 29 cycles of 95 °C for 1 min, 62 °C for 45 sec with gradient of 5 °C, and 72 °C for 2 min. Amplified DNAs were gel excised and purified using GeneJet PCR purification kit™ (Thermo Fischer Scientific) following manufacturer's instruction. DNA was sequenced in Applied Biosystems 3730xl DNA Analyzer via paired end sequencing method. Analysed [20,21] sequence was submitted to NCBI database (GenBank (gb) accession number: KJ563917). Nucleotide sequence of XR_{KS} was analysed to derive the coding sequence (CDS) and protein sequence data (gb AHY04295) through Augustus gene prediction software (v.2.5.5) [22] with parameters of *K. lactis* molecular weight, isoelectric point, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were analysed with Expasy-ProtParam [23] from protein sequence data.

2.4. Purification of XR_{KS}

XR_{KS} was purified to homogeneity in three steps. Crude extract was cooled and ammonium sulfate crystals were slowly added with constant stirring up to 85% saturation. The solution was allowed to stand overnight at 4 °C. Precipitate was collected after centrifugation at 12×10^3 r.c.f. at 4 °C for 15 min and dissolved in 100 mM phosphate buffer (pH 7.0) to achieve a volume of 1/10th of the original crude sample. Precipitated protein was estimated and dialyzed against 100 mM phosphate buffer (pH 7.0) to remove excess salts. Concentrated protein solution was lyophilized and dissolved in minimum volume of 20 mM Tris-chloride buffer (pH 7.0). Protein was loaded in a DEAE macropep (Himedia, India) column (20 cm × 1.85 cm) pre-equilibrated with 20 mM Tris-chloride buffer (pH 7.0). Column was washed with same buffer until absorbance of effluent (λ_{280}) became zero and bound proteins were eluted by NaCl gradient (0–0.5 M, flow rate 0.2 mL min^{-1}). Each fraction of 2 mL was collected and those with high specific activity were pooled and lyophilized. Lyophilized powder was redissolved in minimum volume of same buffer solution and was passed through pre-equilibrated adenosine 2′,5′-bisphosphate agarose (Sigma–Aldrich, USA) column (5.3 cm × 0.80 cm). Column was then washed until 280 nm of effluent reached zero. Bound proteins were eluted by NADP gradient (0–25 mM) in same buffer. Fractions were collected at a flow rate of 20 mL h^{-1} and active fractions were pooled. Each purification step was monitored by SDS-PAGE. High specific activity fractions of purified enzyme were pooled and lyophilized for further characterization.

2.5. Electrophoresis, molecular mass determination and activity staining

SDS-PAGE was carried out according to Laemmli [24] with 8% polyacrylamide. Prestained molecular weight ladders (Puregene and Bio-RAD) were run along with samples to determine molecular mass of the target protein. Protein bands were visualized by sensitive silver staining method. Purified enzyme was evaluated in native PAGE for activity staining with the same gel composition, pH and voltage like SDS-PAGE; only difference being SDS replaced by equivalent amounts of suitable buffer in gel preparation, electrode buffer and sample buffer. β-mercaptoethanol was excluded in the sample buffer and the sample was not boiled prior to application. After electrophoresis, gel was washed with 0.1 M phosphate buffer (pH 7.0) and stained for enzymatic activity.

Activity staining [25] was performed by immersing the gel into a 30 mL Tris-glycine buffer solution (pH 7.0) [5X stock solution containing 0.5 M Xylitol, 75 mg NADP, 24 mg methyl tetrazolium (MTT), 1.2 mg phenazine methosulfate (PMS) was stored in amber colour bottle]. Staining was performed in dark for 3 h to visualize violet colour bands corresponding to XR_{KS} activity on native gel.

2.6. Characterization of enzyme

2.6.1. Kinetic determination

Initial rate of reaction for xylose reduction was calculated by estimating XR_{KS} activity with different concentrations of Xylose (0.1–0.7 M) and cofactor (1–4 mM). Michaelis–Menten constant