Biocatalysis and Agricultural Biotechnology 3 (2014) 227–235

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Original Research Paper

Production of xylose reductase from adapted *Candida tropicalis* grown in sawdust hydrolysate

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ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form
19 May 2014
Accepted 21 May 2014
Available online 2 June 2014

Keywords:
Xylose
Xylose reductase (XR)
Xylitol
Meranti wood sawdust (MWS)
Hemicellulosic hydrolysate
Adapted *Candida tropicalis*

ABSTRACT

Xylose, recovered from *Meranti* wood sawdust (MWS), can be used as a promising and alternative carbon source for yeast growth as well as for the production of xylose reductase (XR). This enzyme has potential applications in the bioproduction of various high value products, especially xylitol. The aim of this study was to isolate XR from adapted yeast *Candida tropicalis* and to characterize it. The XR enzyme was prepared from *C. tropicalis* strain, grown in MWS hydrolysate-based medium, by ultrasonic homogenization. The isolated XR was characterized based on enzyme activity, stability, and kinetic constants. The activity of NADPH-dependent crude XR measured was 11.16 U/mL. It was stable at pH 5.0–7.0 and temperature of 25–40 °C for 24 h, and retained above 95% of its original activity after 4 months of storage at −80 °C. The apparent K_m values of XR for xylose and NADPH were 81.78 mM and 7.29 μM while the V_{max} for xylose and NADPH were 178.57 and 12.5 μM/min, respectively. The low $K_{m,app}$ and high $V_{max,app}$ values of XR for xylose as a substrate indicates a strong binding affinity for xylose and good productivity of the reaction.

1. Introduction

Meranti (Shorea sp.*)* wood sawdust (MWS) is a lignocellulosic waste of sawmill that is available at low cost throughout the year in Malaysia. It contained cellulose (41.06%), hemicellulose (30.64%) and lignin (22.23%) as the major biopolymers (Rafiqul and Sakinah, 2012). The high amount of xylan (29.22%) in MWS makes this biomass adequate for xylose extraction. The hemicellulosic fraction of MWS is easily hydrolyzed by dilute acid to produce xylose-rich MWS hemicellulosic hydrolysate (MWSHH) that can be used as a potential substrate for microbial growth and for the production of specialty chemicals. The use of MWSHH as carbon and energy source for the growth of microorganisms has a dual benefit, the reduction of commercial xylose utilization and the preparation of industrially important enzyme XR that enhances the economy of the bioprocess. The efficient utilization of xylose is, therefore, important in a bioprocess for the conversion of lignocellulosic material (LCM) to various high value bioproducts, especially xylitol. Xylitol, a natural five carbon sugar alcohol, is a functional sweetener that has raised commercial demand because of its potential application in the food, pharmaceutical, health, and cosmetic sectors (Rafiqul and Sakinah, 2013). The most important application of xylitol is its use as an ideal sweetener for diabetic patients because of its insulin independent metabolism. Other potential applications of xylitol are as thin coatings on pharmaceutical tablets, as an anticariogenic agent in toothpaste formulations, in mouthwashes, beverages, ice cream, chewing gum, jams, jellies, marmalades, desserts, and in bakery products (Rafiqul and Sakinah, 2013; Winkelhausen and Kuzmanova, 1998).

Xylose reductase (XR; EC 1.1.1.21) is an intracellular enzyme commonly found in yeast and filamentous fungi. This enzyme occurs in the cytoplasm of microorganisms, where it catalyzes the first step of xylose metabolism by reducing xylose to xylitol with the concomitant oxidation of NAD(P)H to $NAD(P)^+$ (Ronzon et al., 2012). It has potential applications in the biotechnological production of xylitol, sorbitol, and ethanol from xylose (Rawat and Rao, 1996; Tomotani et al., 2009), which make the enzyme a focus of interest. The utilization of high priced commercial xylose limits the large-scale production of XR as well as its industrial application for manufacturing xylitol and other value added bioproducts. This issue has encouraged the authors to work toward the development of improved techniques to lower the costs of XR preparation. Thereby, the use of hemicellulosic hydrolysate as xylose source for XR preparation from yeast strains can be interesting from an economic point of view. XR is not commercially available despite a large number of reports found in the literature on the important use of this enzyme, as well as a description of downstream processing to separate it from yeasts (Rawat and Rao, 1996; Tomotani et al., 2009).

Xylose-fermenting yeast under the genus *Candida* is still regarded as the best source of XR among the microorganisms. As a result, XR

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http://dx.doi.org/10.1016/j.bcab.2014.05.003
1878-8181/

from *Candida* yeast has been isolated and characterized in the last few decades (Cortez et al., 2006; Lee et al., 2003; Mayr et al., 2000; Nidetzky et al., 2003; Ronzon et al., 2012; Tamburini et al., 2010; Verduyn et al., 1985; Yokoyama et al., 1995). XR normally prefers NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) as coenzyme (Yokoyama et al., 1995), but in some cases it utilizes both NADH and NADPH (Mayr et al., 2000; Neuhauser et al., 1997). The variation in coenzyme specificities is proposed to regulate the redox potential balance among nicotinamide coenzymes under different growth conditions of yeasts (Nidetzky et al., 2003). XR is a member of the aldose reductase family of enzymes on the basis of sequence and structural similarities (Lee et al., 2003). Most of XR enzyme is monomeric with a molecular weight of about 33–40 kDa. The functional enzymes from *Pichia stipitis* (Bengtsson et al., 2009), *Neurospora crassa* (Rawat and Rao, 1996), and *C. tropicalis* (Yokoyama et al., 1995) consist of two identical subunits. In this study, XR was isolated from yeast *C. tropicalis* IFO 0618 because it is one of the most efficient xylitol producers (Horitsu et al., 1992; Rafiqul and Sakinah, 2013). The activity of an inhibitor or antimicrobial agent against enzymes or microorganisms is typically measured by minimum inhibitory concentration (MIC) assay. The MIC is referred to as the lowest concentration of a compound required to inhibit the activity of an enzyme *in vitro* over a defined time interval relative to the highest activity (Nickavar and Yousefian, 2011; Sabitha et al., 2012). It was concluded that MIC is successfully used to evaluate whether a specific compound has any influence on the inhibition associated with an enzyme activity.

It is necessary and important to prepare and characterize XR from the potential xylose-fermenting yeast in order to explore its maximum efficiency on xylose to xylitol bioconversion. In addition, to understand the relative roles of XR in xylose conversion, the functional properties of it must be known in detail. The application of XR may offer an economic interest over the chemical and microbial reduction of xylose to xylitol. A simple schematic diagram of *in vitro* xylose to xylitol reduction by NAD(P)H-dependent XR is shown in Fig. 1. Therefore, easily obtainable and highly active XR is desirable for *in vitro* enzyme-based production of xylitol. Although XR from different yeasts was characterized, the enzyme has not yet been isolated and studied from yeast grown in lignocellulosic hydrolysate-based medium. The purpose of this study was to prepare XR from adapted yeast *C. tropicalis* grown in MWS hydrolysate-based medium and to characterize it. The specific purposes of this research are: to test the potential of MWSHH as a carbon source for the production of XR enzyme, and to characterize XR produced by adapted *C. tropicalis* cultivated in MWSHH-based medium.

2. Materials and methods

2.1. Raw material and hydrolysis of MWS

MWS was collected from local sawmill (Seng Peng Sawmills Sdn Bhd, Malaysia), and its sample was prepared for experiments as described in the previous report (Rafiqul and Sakinah, 2012). Oven dried MWS was mixed with the required amount of sulfuric acid solution (%, w/w) in screw capped Erlenmeyer flask, and the batch hydrolysis was performed in an autoclave at 124 °C with 3.26% H_2SO_4 for 80 min using a liquid to solid ratio of 8 g/g. These operating conditions were chosen based on the results of another study conducted by the authors. The liquid and solid phases were separated by filtration, and the resulting filtrate was named as MWS hemicellulosic hydrolysate (MWSHH). MWSHH was neutralized with CaO powder to a pH 6.0 and its composition was determined by analytical methods. It contained 18.8 g/L xylose, 4.64 g/L glucose, 2.55 g/L arabinose, 4.14 g/L acetic acid, 0.55 g/L furfural, 0.08 g/L hydroxymethylfurfural (HMF), and 1.55 g/L lignin degradation products (LDPs). The MWSHH was stored at 4 °C and used in subsequent adapted yeast cultivation and XR preparation experiments.

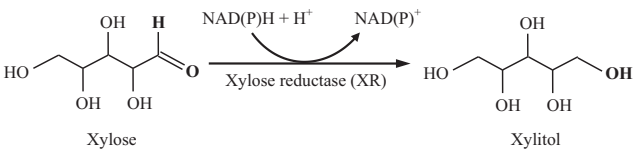

Fig. 1. Reduction of xylose to xylitol by NAD(P)H-dependent XR.

2.2. Microbial strain and maintenance

The microbial strain used in this study was *C. tropicalis* IFO 0618 (ATCC® 96745™) obtained from the American Type Culture Collection (ATCC), Virginia, USA. The yeast strain was incubated on YPD agar plate at 30 °C for 30 h and maintained at 4 °C in a refrigerator. The yeast extract peptone dextrose (YPD) agar medium having the following composition as formulated by Yokoyama et al. (1995): 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, and 2% agar (w/v) in ultrapure water. The synthetic growth medium (SGM) composed of 3% xylose, 0.3% yeast extract, 0.3% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ (w/v) in water (Rao et al., 2006). The pH of all the media used was adjusted to 6.0 with 1 M HC1 before autoclaving.

2.3. Preparation and maintenance of adapted yeast strain

Adapted yeasts were prepared by sequentially transferring and growing cells in media containing increasing concentrations (10, 20, 40, 60, 80, and 100% (v/v)) of crude MWSHH supplemented with (%, w/v): required amount of xylose, 0.3 yeast extract, 0.3 K_2HPO_4 and 0.1 $MgSO_4 \cdot 7H_2O$ to yield the same composition used for SGM. The cells were collected by centrifugation at 2000*g* for 10 min at 4 °C (Eppendorf centrifuge 5810 R, Germany) and then resuspended in fresh hydrolysate growth medium (HGM). Six successive batch cultures were carried out with hydrolysate media at 30 °C for 24 h at 150 rpm in a rotary shaker incubator to obtain adapted yeast *C. tropicalis*. The adapted strains were maintained in agar plates made from YP-hydrolysate agar medium and utilized in subsequent XR preparation experiments. This medium was prepared from MWSHH containing 1.88% xylose instead of using glucose, and the rest of the media ingredients were the same as the YPD agar medium as mentioned before.

2.4. Inoculum preparation and growth conditions

The inoculum was prepared from fresh culture grown at 30 °C for 36 h on YP-hydrolysate agar plate by transferring a single colony of adapted *C. tropicalis* to a 250 mL flask containing 50 mL of HGM (MWSHH containing 1.88% xylose, 0.3% yeast extract, 0.3% K_2HPO_4 and 0.1% $MgSO_4 \cdot 7H_2O$) as outlined by Yokoyama et al. (1995). The inoculated flask was incubated at 30 °C in an incubator shaker (Infors HT Ecotron, Swizerland) for 24 h at 150 rpm.

2.5. Growth profile of C. tropicalis

Growth profile experiments were carried out to test the potential of MWSHH as a carbon source for yeast cultivation and for the production of XR enzyme. Ten percent (v/v) of the adapted *C. tropicalis* inoculum (3.34 ± 0.40 g/L DCW) from a 24 h culture was aseptically added to the HGM for initiating growth profile experiments. The cells were continuously cultivated in 1 L loosely cotton plugged Erlenmeyer flasks containing 250 mL of culture at