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Evaluation of sawdust hemicellulosic hydrolysate for bioproduction of xylitol by enzyme xylose reductase

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ABSTRACT

Sawdust hemicellulosic hydrolysate can be used as a promising xylose source for enzymatic conversion to xylitol, a specialty product. The aim of this study was to design various factors by observing their effects on xylitol production from hemicellulosic hydrolysate by xylose reductase (XR) enzyme. The one-factor-at-a-time (OFAT) strategy was adopted to determine the effective levels of process factors influencing xylitol yield. Enzymatic xylitol production was conducted in batch mode using different levels of reaction time (2–18 h), temperature (20–70 °C), pH (4.0–9.0), xylose concentration (9.4–37.6 g/L), NADPH concentration (1.17–5.32 g/L), enzyme concentration (2–6%), and agitation (50–150 rpm). Among the factors examined, time, temperature, pH, NADPH, and enzyme concentration significantly influenced the biosynthesis of xylitol with the suitable level of 10 h, 30 °C, 7.0, 3.66 g/L, and 3% (v/v), respectively. Xylitol yield obtained was 56% (w/w) under these observed optimum conditions. This is the first report on the bioproduction of xylitol from lignocellulosic substrate by XR.
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1. Introduction

Xylitol, a natural pentahydroxy polyol ($C_5H_{12}O_5$), has a similar sweetening power to sucrose. The global demand of xylitol is ever increasing because of its unique functional properties like insulin independent metabolism, low caloric value (2.4 cal/g), anticariogenicity, and bacterial growth inhibiting capability (Albuquerque et al., 2014; Rafiqul and Sakinah, 2013). Moreover, xylitol is identified as one of the twelve high value products that can be manufactured from lignocellulosic materials (LCMs) (Li et al., 2013). Xylitol is industrially manufactured by catalytic reduction of pure xylose, and can also be manufactured by biotechnological approaches. The chemical process is laborious, cost- and energy-intensive, and also poses environmental hazards as it utilizes a toxic Raney nickel catalyst, and high temperature and pressure (Rafiqul and Sakinah, 2013). The resultant product is very costly mainly because of the extensive separation and purification procedures. Biotechnological methods for xylitol production are based on the use of microbes or isolated enzymes. The microbial production of xylitol has been studied extensively as an alternative to the chemical process (Deng et al., 2014; Misra et al., 2013; Wang et al., 2012). The major advantage of the microbial process over chemical procedures is its lower cost due to the nonnecessity of extensive xylose purification (Deng et al., 2014; Rafiqul and Sakinah, 2013).

Yeast strains are still the primary focus of studies that aim to produce xylitol from hemicellulosic hydrolysate. These organisms can produce xylitol as an intermediate metabolite during xylose metabolism in the cells (Albuquerque et al., 2014). Xylose-fermenting yeasts have a metabolic system with NAD(P)H-dependent xylose reductase (XR; EC 1.1.1.21) and $NAD(P)^+$-dependent xylitol dehydrogenase (XDH; EC 1.1.1.9) and these enzymes are induced by xylose. The metabolism of xylose inside the microbial cells occurs mostly in two steps (Albuquerque et al., 2014; Arruda et al., 2011): (i) xylose is reduced to xylitol by XR, and (ii) the produced xylitol is either secreted from the cell or oxidized to xylulose by XDH. These two reactions are

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considered to be rate-limiting for xylose fermentation and xylitol production. Xylulose is then phosphorylated by xylulokinase (EC 2.7.1.17) to xylulose 5-phosphate, which can be catabolized by pentose phosphate, glycolytic, or by phosphoketolase pathways (Albuquerque et al., 2014; Rafiqul and Sakinah, 2013). One of the major bottlenecks for the commercialization of xylitol bioproduction is the downstream process for recovering xylitol from fermented broth because of the low product concentration, fermentation byproducts, and the complex composition of the fermentation medium (Faveri et al., 2004; Rafiqul and Sakinah, 2013). Although *Candida* sp. has been identified as the best xylitol producer among yeasts, these strains are also suspected to be human pathogenic in nature under opportunistic conditions (Jacques and Casaregola, 2008). However, the microbial process has not yet been able to accumulate the advantages of the chemical process mainly because of the low productivity of xylitol and downstream processing problem.

The current trends in fermentative xylitol production are directed toward the development of genetically engineered microbes, particularly bacteria, to tackle the problems encountered by the wild strains. Although xylitol production has been greatly improved by fermentation technology, there are still challenges that need further investigations. These challenges include construction of robust and novel strains with hydrolysate inhibitor tolerance, maintaining a stable performance of the engineered organisms in industrial-scale operations, developing more efficient downstream technologies for the recovery of products, and enhancing xylitol yield and productivity based on the cost effective biotransformation of LCM to xylitol. Taking into account the bottleneck of microbial process, it is important to emphasize on the development of xylose reductase (XR)-dependent biotransformation of xylose from LCM to xylitol. There are scarce reports on the enzymatic production of xylitol from commercial xylose (Neuhauser et al., 1998; Nidetzky et al., 1996), or from a mixture of pure xylose and hemicellulosic hydrolysate (Branco et al., 2011) by XR. The enzymatic approach to xylitol production from xylose present in the LCMs might provide an alternative for the chemical process. In addition, this method is safe and environment friendly because xylitol production can be achieved without high temperature, pressure, toxic catalyst, or xylose purification.

LCM, including wood and agricultural residues, is a promising sugar source for the production of specialty chemicals, biofuels, and pharmaceutical ingredients because it is regenerable, abundant, and uncompetitive with food resources. *Meranti* wood sawdust (MWS) is a lignocellulosic waste of sawmill. Its high content of hemicellulose (30.64%) makes it adequate for xylitol bioproduction. The hemicellulose fraction of MWS is selectively hydrolyzed by dilute acid to produce a xylose-rich hydrolysate (Rafiqul and Sakinah, 2012a,b; Rafiqul et al., 2014a) that can be used as a potential and economical substrate for bioconversion to a variety of value-added products mainly xylitol. A common problem associated with efficient biotransformation of xylose is that the hydrolysate contains various byproducts (e.g., furfural, hydroxymethylfurfural, and acetic acid) that are inhibitory to organisms or enzymes (Misra et al., 2013; Rafiqul et al., 2014b). Misra et al. (2013) demonstrated that adaptation of yeast cells to hemicellulosic hydrolysate is an inexpensive and effective technique to alleviate the inhibitory effects of toxic components on the conversion of xylose to xylitol. XR is an intracellular enzyme commonly found in yeast and fungi. It has potential application in production of xylitol and ethanol from xylose (Rafiqul et al., 2014b). XR was prepared in this study from an adapted strain of *Candida tropicalis*. It was revealed that the reduction of xylose to xylitol by XR depended on the type and concentration of substrate, and experimental operating conditions. The reaction pH was considered to be a potential parameter that affects the ionization of functional groups on the enzyme (Nidetzky et al., 2003). For a bioprocess, it is, therefore, important to design the process parameters that influence the product yield for further optimization study.

Classically, parameter design has been carried out by monitoring the influence of one variable at a time on an experimental response. While only one process variable is changed, others are maintained at a constant value. This optimization procedure is called one-factor-at-a-time (OFAT) (Bezerra et al., 2008). Montgomery (2001) pointed out that OFAT is the most extensively used experimental strategy for process optimization, which does not require advanced statistical knowledge. However, the main disadvantage of this method is that it is time-consuming and incapable of detecting the true optimum conditions due to the absence of the interaction effects among the factors (Bezerra et al., 2008; Montgomery, 2001). The OFAT approach was used to determine the observed optimum conditions for maximum xylitol yield due to the lack of information on the in vitro enzyme-based production of xylitol from lignocellulosic substrate. This work was undertaken to explore the impacts of parameters namely reaction time, temperature, pH, xylose, NADPH and enzyme concentration, and agitation rate on xylitol production from MWS hemicellulosic hydrolysate (MWSHH) by XR and to design parameters for further optimization.

2. Materials and methods

2.1. *Hydrolysis of Meranti wood sawdust (MWS)*

Meranti wood sawdust (MWS) was collected from a local sawmill (Seng Peng Sawmills Sdn Bhd, Malaysia), and prepared for subsequent hydrolysis. It was hydrolyzed at 124 °C with 3.26% (w/w) H_2SO_4 for 80 min using a liquid to solid ratio of 8 g/g. These hydrolytic conditions were chosen based on previous studies reported by the authors (Rafiqul and Sakinah, 2012a; Rafiqul et al., 2014a). The solid and liquid phases were separated by filtration. The resulting filtrate, MWS hemicellulosic hydrolysate (MWSHH), was neutralized with CaO powder to pH 6.0 and its composition was determined by analytical methods. The MWSHH was stored at 4 °C, and used in subsequent XR and xylitol production experiments.

2.2. *Microbial strain, preparation and maintenance of adapted yeast*

The microbial strain, *C. tropicalis* IFO 0618, was obtained from the American Type Culture Collection (ATCC), USA. The yeast strain was routinely cultured on YPD agar plate and maintained at 4 °C. The yeast extract peptone dextrose (YPD) agar medium having the following composition: 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 water. Adapted yeasts were prepared via six successive batch cultures of *C. tropicalis* cells with fresh hydrolysate growth medium (HGM) as described in the previous report (Rafiqul and Sakinah, 2014). They were maintained on agar plates made from YP-hydrolysate (YPH) agar medium and utilized in subsequent XR preparation experiment. The YPH agar 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 above. The pH of all the media was adjusted to 6.0 with 1 M HCl before autoclaving at 120 °C and 15 psi for 20 min. To prevent undesired reactions, the sugar solutions (such as glucose or xylose in distilled water) and MWSHH were autoclaved separately from other medium ingredients. The sterilized carbon sources were then mixed together with other components before use.

2.3. *Inoculum preparation and growth conditions*

The inoculum was prepared from a fresh culture grown at 30 °C for 36 h on YPH agar plate by transferring a single colony of adapted *C. tropicalis* to an Erlenmeyer flask (250 mL) containing 50 mL of HGM (MWSHH containing 1.88% xylose, 0.3% yeast extract, 0.3% K_2HPO_4 and 0.1% $MgSO_4{\cdot}7H_2O$). The flask was incubated at 30 °C in a rotary shaker incubator for 24 h at 150 rpm.

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