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Improved production of reducing sugars from rice husk and rice straw using bacterial cellulase and xylanase activated with hydroxyapatite nanoparticles

Nalok Dutta, Arka Mukhopadhyay, Anjan Kr. Dasgupta, Krishanu Chakrabarti *

Department of Biochemistry, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, India

HIGHLIGHTS

- NP increased the activity and thermostability of extracellular cel and xyl.
- NP-activated xyl and cel enhanced the production of reducing sugars from RH and RS.
- An enzymatic procedure used as an alternative to bioadverse chemical methods.

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ABSTRACT

Purified bacterial cellulase and xylanase were activated in the presence of calcium hydroxyapatite nanoparticles (NP) with concomitant increase in thermostability about 35% increment in production of D-xylose and reducing sugars from rice husk and rice straw was obtained at 80 °C by the sequential treatment of xylanase and cellulase enzymes in the presence of NP compared to the untreated enzyme sets. Our findings suggested that if the rice husk and the rice straw samples were pre-treated with xylanase prior to treatment with cellulase, the percentage increase of reducing sugar per 100 g of substrate (starting material) was enhanced by about 29% and 41%, respectively. These findings can be utilized for the extraction of reducing sugars from cellulose and xylan containing waste material. The purely enzymatic extraction procedure can be substituted for the harsh and bio-adverse chemical methods.

1. Introduction

Biomass is the most abundant renewable energy resource in the world. Agricultural residues, such as rice straw contain ≈25% of hemicellulose, a heteropolymer composed mainly of xylose (El-Masry, 1983; Kuhad and Singh, 1993). This pentose can be used as a substrate to produce a wide variety of compounds or fuels by chemical or biotechnological processes. Cellulosic biomass is uniquely suited for sustainable production of liquid transportation fuels, and the power of modern biotechnology promises competitive advantages. Many reasons have been offered for the loss in enzyme effectiveness with time and the consequent high doses required for good yields including end-product inhibition, enzyme deactivation with time and temperature, drop in substrate reactivity with conversion due to initial removal of more easily hydrolyzed material, and nonproductive binding of enzyme to lignin (Converse et al., 1988; Holtzapple et al., 1990; Scheiding et al., 1984). Although the exact cause is still uncertain and multiple factors are likely responsible, substrate and end-product inhibition are believed to be very important (Xiao et al., 2004), with glucose and cellobiose identified as the principle cellulase inhibitors that bind to cellulase active sites regardless of the inhibition pattern (Holtzapple et al., 1990). However, other hemicellulose sugars, such as mannose, xylose, and galactose, have also been shown to inhibit cellulase (Xiao et al., 2004).

Straw as agricultural waste biomass could be a source of alternative energy to substitute fossil energy for reducing greenhouse gas emission as well as avoid the local pollution problems from open burning. Rice straw is attractive as a fuel because it is renewable and considered to be carbon dioxide neutral but has not yet been commercially used as a feedstock for heat and energy because of insufficient incentives or benefit for farmers to collect rice straw instead of field burning. Rice husk (RH) is an agricultural by-product abundant in all rice producing countries.

Abbreviations: NP, nanoparticle; NP-xyl, nanoparticle-activated xylanase; NP-cel, nanoparticle-activated cellulase; RH, rice husk; RS, rice straw.

* Corresponding author. Tel.: +91 33 9831535059; fax: +91 33 24614849.
E-mail address: kcbioc@gmail.com (K. Chakrabarti).

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The annual output of RH of world is about 80 million tons (Li and Wang, 2008). RH is rich in xylan and amorphous silica, which makes it possible to utilize RH effectively. The contents of xylan and amorphous silica in RH are each around 20% (wt.%), varying with the variety, climate and geographic location of the growth of rice. The xylan in RH is one kind of good raw materials to produce D-xylose. D-Xylose is widely used in fields related to the food and pharmaceutical industries for its low calorific value and acceptable organoleptic properties. According to the research of Toshiyuki et al. (1983), the xylan in RH is made up of substituted arabinoxylan. Xylan in RH can be easily converted into soluble product (Vegas et al., 2008). Pretreatment methods for lignocellulosic materials have been extensively studied (Gharpuray et al., 1983; Grethlein and Converse, 1991; Weil et al., 1994; Kumar et al., 2013). The processes for bioconversion of crops and residues to fuels and chemicals are receiving increased attention due to the apparent need for reduction in consumption of petroleum fuels. These wastes (crop residues) can be hydrolysed by acids or enzymes to lower molecular weight carbohydrates and finally to monomeric sugars (Yoonan and Kongkiattikajorn, 2004). Waste materials from a wide range of agro-industrial processes may also be used as substrates for microbial growth, thereby resulting in waste utilization for the synthesis of valuable by-products. In the current literature, we have a devised a purely enzyme based methodology comprising of sequential treatment of purified xylanase and cellulase enzymes on rice straw (RS) and rice husk (RH) and estimating the amount of D-xylose and reducing sugars generated in the process. In our study, it has been seen that the production of reducing sugars has been increased in the presence of hydroxyapatite nanoparticles (HAp) when the RH and the RS samples have been pre-treated with xylanase prior to treatment with cellulase. The xylanase pretreatment is necessary as it breaks down the substrate to be further accessible to cellulase. The novelty of this process lies in the fact that here, we have employed only pure enzyme in the form of xylanase to break down xylan, a deviation from the chemical processes which employ ammonia (Zhu et al., 2004) to achieve this feat.

2. Methods

2.1. Materials

The hydroxyapatite nanoparticle dispersion, 10 wt.% in H_2O <200 nm (Acc. No. 702153) was supplied by Sigma–Aldrich. PCR primers (forward and reverse) and silver nitrate for silver staining were obtained from Sigma–Aldrich. (Germany) α-Cellulose, Birchwood xylan, agar powder and glutaraldehyde were purchased from Himedia, India. All other reagents for bacterial culture media such as agar, and salts were purchased from SRL (India) and were of analytical grade. 10–200 kDa protein markers were obtained from Fermentas (Germany) (#SM0661) and 20–120 kDa protein markers were obtained from Thermoscientific (USA) (#26612).

2.2. Isolation of cellulase and xylanase secreting strain

Termite soil was collected from an agricultural tract in North 24-Parganas, West Bengal. Cellulolytic bacterial strains were isolated from the soil by using enrichment and serial dilutions technique. The medium used for isolation of cellulolytic bacteria contained 1.0% peptone, 1.0% carboxymethylcellulose (CMC), 0.2% K_2HPO_4, 1% agar, 0.03% $MgSO_4{\cdot}7H_2O$, 0.25% $(NH_4)_2SO_4$ and at pH 8.0 for 48 h of incubation at 37 °C. Bacterial colonies were purified by repeated streaking. Pure cultures of bacterial isolates were individually transferred to CMC agar plates. The CMC agar plates were incubated at 37 °C for 5 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with a solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity. The best producer was designated as NAKC.

For screening of xylanase secreting strains, soil samples were incubated in nutrient broth {beef extract (1.0%), yeast (1.1%) NaCl (0.5%), and peptone (0.5%)} at pH 8 and 37 °C with constant shaking at 200 rpm for 72 h. Periodically, the samples from enriched broth were withdrawn, appropriately diluted and plated on basal salt agar medium containing birchwood xylan (0.5% w/v). The plates were incubated at 37 °C for 48 h and colonies developed were assayed for xylanase production by flooding with a solution of Congo red (0.1%) for 15 min and then washed with 1 M NaCl. The colonies showing halos around them were picked and maintained on nutrient agar slants and the treatment was repeated to give the best producer as described for cellulase. The best producer was designated as NAKX.

2.3. Ribotyping

To identify strains NAKC and NAKX, 16S rDNA gene sequencing was carried out for both the strains. Genomic DNA was prepared from all different isolates by the sodium dodecyl sulfate proteinase K cetyltrimethylammonium bromide (CTAB) method (Mukhopadhyay et al., 2012). Partial amplification of the 16S rRNA gene was performed with the thermal cycler ABI 9700 (ABI, Foster City, USA). The amplified and gel-eluted PCR fragments were sequenced with an ABI 3100 Genetic Analyzer. Sequencing reaction was performed by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA). The nucleotide sequences for strains NAKC and NAKX were deposited in the GenBank under the accession number's KF214093 and KF214094, respectively.

2.4. Purification of cellulase and xylanase from NAKC and NAKX strains

Cellulase and xylanase enzymes were purified from 100 ml cultures of NAKC and NAKX, respectively. Cell free supernatant was precipitated with 80% saturation of ammonium sulfate followed by dialysis. All steps of the purification procedure were performed at 4 °C. The dialysed proteins were loaded onto a CM-Sepharose column that had been pre-equilibrated with 10 mM sodium phosphate buffer (pH 8.0) and allowed to equilibrate overnight. After washing the column with 10 mM sodium phosphate buffer, a 60 ml increasing discontinuous gradient (0–200 mM) of NaCl dissolved in 10 mM sodium phosphate buffer (pH 8.0) was applied to the column. Proteins were eluted in fractions of 1 ml. The fractions showing cellulase and xylanase activity (assayed as below) were concentrated using a Macrosep 10 K unit and loaded onto a glass column packed with Sephadex G-100 and Sephadex G-75, respectively (bed volume 30 ml) and equilibrated with 10 mM sodium phosphate buffer. Elution of the proteins was done using the same buffer. The collection of the fractions and assay of enzyme activity were as described below. Fractions were run on 12% SDS polyacrylamide according to Swain and Ross (1994) using Bio-Rad electrophoresis apparatus. The amount of protein that was loaded on SDS–PAGE gel lanes was 0.22 mg/ml for both cellulase and xylanase enzymes. Protein markers and the protein bands were stained by silver staining (Liao et al., 1997).