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Potential halophilic cellulases for *in situ* enzymatic saccharification of ionic liquids pretreated lignocelluloses

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HIGHLIGHTS

- *Aspergillus terreus* UniMAP AA-6: a newly isolated halophilic cellulases-producing strain.
- Thermotolerant properties of halophilic cellulases are improved with the increment of salt concentration.
- Halophilic cellulases exhibits compatibility with ionic liquids.
- Halophilic cellulases of *A. terreus* UniMAP AA-6 promise a single pot system for saccharification of ionic liquid pretreated lignocelluloses.

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ABSTRACT

Ionic liquids (ILs) have been used as an alternative green solvent for lignocelluloses pretreatment. However, being a salt, ILs exhibit an inhibitory effect on cellulases activity, thus making the subsequent saccharification inefficient. The aim of the present study is to produce salt-tolerant cellulases, with the rationale that the enzyme also tolerant to the presence of ILs. The enzyme was produced from a locally isolated halophilic strain and was characterized and assessed for its tolerance to different types of ionic liquids. The results showed that halophilic cellulases produced from *Aspergillus terreus* UniMAP AA-6 exhibited higher tolerance to ILs and enhanced thermo stability in the presence of high saline conditions.

1. Introduction

A substantial amount of lignocellulosic materials such as agricultural residues, agricultural by-products, woody biomass are produced annually worldwide. These materials are essentially potential raw materials for the production of various renewable products such are biofuel, biodegradable plastics, biosurfactants, enzymes, etc. (Pandey et al., 2000).

In general, the production of the renewable products from lignocellulose biomass requires three steps; first, the pretreatment steps for removal of lignin and other waxy materials to expose cellulose. Second, the hydrolysis of the exposed cellulose to simple sugar by cellulases. Finally, the fermentation of simple sugar to various renewable products.

The pretreatment step is normally performed by using a strong acid or alkaline solution. However, they are not preferable due to cost and environmental issues. Ionic liquids (ILs), the liquid form of salt at room temperature have been recognized as an alternative green solvent for lignocelluloses pretreatment process (Mäki-Arvela et al., 2010; Wang et al., 2012; Gunny and Arbain, 2013). This type of salt has been shown to be effective in the liberation of cellulose from the complex structure of lignocellulose materials (Li et al., 2009; Xu et al., 2012; Moniruzzaman and Ono, 2012). While, ILs are effective for breaking down lignocelluloses, they can also inhibit cellulase enzymes used in the subsequent saccharification, thereby making the overall saccharification of cellulose inefficient. The inhibition might be related to the high salinity of ILs, which has the capacity to inactivate the enzymes by interfering with the polypeptides folding of the enzymes (Turner et al., 2003; Zhao et al., 2009; Salvador et al., 2010).

Due to the inhibition effect of ILs on enzyme activity, a washing process is required to remove residual ILs after the pretreatment process. Large scale washing requires extra energy, thus incurring

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more processing cost (Engel et al., 2010; Zhang et al., 2011). To deal with this, some authors (Kamiya et al., 2008) have introduced the idea of *in situ* saccharification of cellulose in aqueous-ionic liquid media, whereby the pretreatment and saccharification process is performed in a single-pot, thus skipping the expensive cellulose regeneration step. This work can be effectively performed if cellulases tolerant to ILs are used.

In view of the above discussion, it is important to produce cellulases which are compatible with the saline condition of ILs. The enzyme can be produced from halophilic microorganisms relying on their capability to secrete enzymes which are active in a high saline environment (Oren, 2010). Due to the above facts, the aim of the present study is to produce cellulases from halophilic microorganism which are compatible with ionic liquids. In this study, 1-ethyl-3-methylimidazolium acetate [EMIM][Ac], 1-butyl-3-methylimidazolium acetate [BMIM][Ac] and 1-butyl-3-methylimidazolium chloride [BMIM][Cl] were used for preliminary experiments on the compatibility of halophilic cellulases with ionic liquids.

The approach taken in the present study comprises of four major steps, first, the isolation and screening of cellulase producing microorganism from coastal areas. Second, on evaluation of the capability of salt-tolerant microbes for producing salt-tolerant cellulases. Third, characterization of the enzyme focusing on the stability at different salt and temperature conditions. Finally, on investigation of enzyme stability in the presence of different concentrations of ILs. These studies were conducted with the hypothesis that the newly-isolated halophilic strain would produce ILs-tolerant cellulases.

2. Methods

2.1. Isolation and screening of cellulases-producing microbes

Cellulases-producing microbes were isolated using filter paper as carbon source in a modified way as explained by Shahriarinour et al. (2011). The positive microbes were screened for plate screening using Gram's Iodine agar media (Kasana et al., 2008). Colonies showing discoloration upon addition of Gram's Iodine were taken as positive cellulases-producing strains and selected for further studies.

2.2. Molecular identification of fungi

Fungal samples were identified by the sequencing of the internal transcribed spacer (ITS) regions. To determine the identity of the two fungal samples, the amplified ITS PCR products obtained from total genomic DNA using primer set ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) Gardes and Bruns (1993) and ITS4 (5′-TC CTCCGCTTATTGATATGC-3′) White et al. (1990) were sequenced. The sequences obtained were compared to sequences in the GenBank database (http://www.ncbi.nlm.nih.gov). A homology search was performed using bioinformatics tools available online; BLAST (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997). The ITS sequence data for fungal sample AA-3 and AA-6 was deposited into the GenBank database under the accession numbers of KF364667 and KF364668, respectively.

2.3. Capability evaluation of salt-tolerant microbes for producing salt-tolerant cellulases

The positive microbial strains were isolated and inoculated separately on agar medium containing 0–30% (w/v) salt concentrations (NaCl). The ability of the microbial strains to degrade cellulose under different salt concentrations were qualitatively estimated using the hydrolysis capacity (HC) value, i.e. the diameter ratio of the clearing zone and colony/sample (Taechapoempol et al., 2011). *Trichoderma reesei* RUT C-30 and cellulases (commercial cellulases purified from *Aspergillus niger*) were used as a positive control. The best cellulases-producing microbe was chosen on the basis of highest salt tolerance activity.

2.4. Growth media and conditions

The best isolates were grown at pH 7.1, temperature 30 °C and a rotation speed of 150 rpm using the media compositions in g/l: KH_2PO_4,13.61; KOH, 4.21; Yeast extract, 1.98; $MgSO_4{\cdot}7H_2O$, 0.25; $FeSO_4{\cdot}7H_2O$, 0.0017; NaCl, 30 and Peptone, 1.98. The carbon source in the medium was CMC (5 g/l) (Shivanand et al., 2012). Crude enzyme was prepared through removal of the cell by centrifugation at 10,000 rpm for 10 min at 4 °C. The harvested supernatant was assayed for cellulase activity.

2.5. Cellulase assay

The total cellulase activity was determined by filter paper assay (FPase) using Whatman No. 1 filter paper strip with dimensions 1.0 × 6.0 cm equivalent to 50 mg as a substrate. They were assayed according to standard International Union of Pure and Applied Chemistry (IUPAC) procedures recommended by Ghose (1987) and expressed as international unit (IU). One FPA is the concentration of cellulase that can release 2.0 mg of glucose from 50 mg of cellulose over a 60 min period. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugar per minute under the assay conditions. The amount of reducing sugar was determined by dinitrosalicylic acid (DNS) according to the standard method (Miller et al., 1960), and glucose was used as a standard.

2.6. Characterization of halophilic cellulases

2.6.1. Effect of salt concentration on cellulase activity

For the study of halostability, the cellulase was pre-incubated in 0.05 M citrate buffer (pH 4.8) with different concentrations of NaCl (0–20%, w/v) at 30 °C. Cellulase activity was determined for 1 h and 24 h incubation times respectively.

2.6.2. Effect of temperature on cellulase activity

For the study of thermal stability, the enzyme was pre-incubated at a different temperature (4–90 °C) in saline conditions (1–3 M). Cellulase activity was determined after a 1 h incubation period.

2.6.3. Stability of halophilic cellulases in the presence of ionic liquids

Cellulase from the newly-isolated strain was used to check its stability in ionic liquids. The relative enzyme activity was determined at a different ILs concentration (0–20%, v/v) in 0.05 M citrate buffer (pH 4.8). The mixtures were incubated at 30 °C for 1 h.

3. Results and discussion

3.1. Isolation and screening of cellulases-producing microbes

Cellulases-producing microbes were enriched and isolated by using filter paper as carbon sources. The two cultured samples showed positive results as the medium turned cloudy and the filter paper became degraded. The positive microbial strains were isolated and screened using Gram's Iodine agar media. The strains formed a hydrolysis zone around their colonies indicating their ability to secrete extracellular cellulases. Based on the morphology