Industrial Crops and Products 44 (2013) 488–495

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

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

Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bio-ethanol production

Muhammad Asgher, Zanib Ahmad, Hafiz Muhammad Nasir Iqbal*

Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan

ARTICLE INFO

Article history:
Received 31 July 2012
Received in revised form
30 September 2012
Accepted 3 October 2012

Keywords:
Sugarcane bagasse
Alkali pretreatment
Ligninolytic pretreatment
P. ostreatus IBL-02
Bio-ethanol

ABSTRACT

To expand the range of natural resources researchers have been re-directing their interests in biomass based bio-fuels, which can be obtained from lignocellulosic biomass. Sugarcane bagasse was pretreated with alkali and ligninolytic enzymes extract produced from *Pleurotus ostreatus* IBL-02 to depolymerize lignin and expose the cellulose polymers. The de-lignified bagasse was used as substrate for bio-ethanol production in sequential saccharification and fermentation by an indigenous strain of *Saccharomyces cerevisiae*. Alkali treatment (4% NaOH) and ligninolytic enzymes extract (25 mL) treatment caused 48.7 and 33.6% de-lignification of sugarcane bagasse, respectively. The de-lignified residues were treated with indigenously produced crude cellulase extract from *Trichoderma harzaianum* that resulted in 69.2 and 72.9% cellulose hydrolysis of alkali and ligninolytic enzymes pretreated bagasse, respectively. *S. cerevisea* was grown on the hydrolyzates to produce ethanol at 37 °C and pH 5.5 that produced 18.2 and 16.3 g/L ethanol using alkali and enzyme pretreated substrates, respectively. For maximum ethanol production, different parameters like fermentation time period, pH, temperature, substrate level and inoculum sizes were optimized using both alkali and enzymes pretreated substrates. Under optimum conditions ethanol production of 32.45 g/L and 28.15 g/L was obtained from alkali and ligninolytic enzymes treated sugarcane bagasse, respectively. In conclusion, the results obtained after high-performance liquid chromatography (HPLC) analysis suggesting ligninolytic pretreatment as a promising tool for bio-ethanol production in sequential saccharification and fermentation. Enzymatic treatment of waste biomass could be of particular interest, since it seems an eco-friendly approach for bio-ethanol production.

1. Introduction

Increasing costs of fossil fuels and their greenhouse gases emission effects are creating a dire need to explore cheaper and environment friendly bio-fuels. Presently, ethanol as well as other bio-fuels produced from plant biomass is an alternative to fossil fuels. Ethanol is valuable in many respects, ranked as industrial solvent and employed in the preparation of medicines, resins, flavoring extracts, perfumes, varnishes, and shellac. Ethanol is a natural fuel that burns cleaner and causes less environmental problems than petroleum. This enables preparation of gasoline–ethanol blends resulting in gasohol for internal combustion engines that has higher octane value and replaces lead in gasoline (Ghorbani et al., 2011).

Currently, bio-ethanol is produced on industrial scale from sucrose and starch; however, these bio-ethanol production systems pose concerns about competition with food and feed supplies. Although this ethanol is produced at a competitive cost, the raw material supply will not be sufficient to meet the increasing demand for fuel ethanol (Galbe and Zacchi, 2007). Ethanol made from lignocellulosic biomass is attractive as renewable liquid fuel for transportation. Lignocelluloses materials are most promising feedstock as natural, abundant, and renewable resource and can potentially provide a long term sustainable fuel supply (Alonso et al., 2008; Goh et al., 2010). However, large-scale economical commercial production of fuel ethanol from lignocellulosic materials has still not been implemented.

For designing fuel ethanol production processes, the assessment of the utilization of different feed stocks (i.e. sucrose containing, starchy materials, lignocellulosic biomass) is required considering the big share of raw materials in ethanol costs (Cardona and Sánchez, 2007). The transformation of biological resources as energy-rich crops or lignocellulosic biomass requires pretreatment of the feedstock for fermenting organisms to convert them into ethanol (Cardona and Sánchez, 2007). The cost-effective conversion of various types of lignocellulosic biomass to fermentable sugars with as little toxic inhibitory byproducts as possible remains a challenge for the production of cellulosic ethanol (Dagnino et al., 2013). The lignin component of the biomass material poses pre-treatment challenges because of its nonproductive binding and inactivation

* Corresponding author. Tel.: +92 41 9200161x3312.
E-mail address: nasir_pk99@hotmail.com (H.M.N. Iqbal).

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http://dx.doi.org/10.1016/j.indcrop.2012.10.005

of cellulases. Pre-treatment of lignocellulosic materials to remove lignin and hemicellulose can significantly enhance the hydrolysis of cellulose (Goshadrou et al., 2011). A solution to this problem may be additional enzymatic hydrolysis by complex containing, enzymes like ligninolytic (laccase, MnP, LiP) and cellulases (Pal et al., 2013). In industrial scale ethanol production from lignocellulosic residues, the alkali pretreatment of substrates for removal of lignin barrier is one of the bottle neck problems because it substantially adds to the overall production cost and also contributes to environmental issues. There is a dire need to develop a cheaper biological process for delignification of lignocellulosic biomass.

White rot fungi (WRF) are the most efficient degraders of lignin (Iqbal et al., 2011), and are probably also the most suitable organisms to be utilized in an industrial process that requires delignification of lignocellulosic substrates (Asgher et al., 2012a). Lignin peroxidase (LiP) E.C. 1.11.1.14, manganese peroxidase (MnP) E.C. 1.11.1.13 and laccase E.C. 1.10.3.2 are the major ligninolytic enzymes of WRF that are directly involved in the degradation of lignin in lignocellulosic substrates (Asgher and Iqbal, 2011; Bandounas et al., 2011; Iqbal and Asgher, in press). There are several advantages of using WRF enzyme extracts for delignification of plant biomass including the following:

i. The ligninolytic enzyme extracts from WRF also contain other accessory enzymes and mediators required for the action of these enzymes for complete degradation of lignin
ii. The enzyme extracts may also contain cellulase enzymes that can simultaneously hydrolyze the exposed cellulose fibers to some extent, thus facilitating the subsequent action of cellulases during saccharification of de-lignified biomass
iii. The ligninolytic and celluloytic enzyme extracts can be used in a novel "simultaneous pretreatment, saccharification and fermentation (SPSF)" process configuration that can save time, energy and environment.

The present study involved the use of ligninolytic enzymes extract from *P. ostreatus* IBL-02 for de-lignification of sugarcane bagasse, followed by saccharification using cellulases extract from *T. harzianum* and bio-ethanol production by *S. cerevisea*.

2. Materials and methods

2.1. Chemicals and solid substrates

All the chemicals used were of analytical grade and mainly purchased from Sigma–Aldrich (USA), Merck (Germany) and Scharlau (Spain) and used as such without further purification. The agro-industrial wastes, i.e. wheat straw and sugar cane bagasse were obtained from a local fruit market in Faisalabad, Pakistan. The collected substrates were crushed into pieces, sun and oven dried (at 60 °C), ground to fine particle size (40 mm mesh size) and stored in airtight plastic jars to avoid free moisture effect.

2.2. Production of ligninolytic enzymes and their assays

2.2.1. Preparation of fungal inoculum

An indigenous fungal strain *P. ostreatus* IBL-02 was obtained from National Fungal Culture Collection Pakistan (NFCCP; Strain-IBL-02) and used to produce ligninolytic enzymes. Aqueous spore suspension of *P. ostreatus* IBL-02 was prepared by growing the fungus in inoculum medium for 5 days. The inoculum medium was the Kirk's basal medium supplemented with 1% sterile glucose solution (Tien and Kirk, 1988). The medium was adjusted at pH 4.5 using M HCl/M NaOH and autoclaved (121 °C) for 15 min. A loop full culture of *P. ostreatus* IBL-02 was transferred to the sterilized medium and the flask was incubated for 3 days at 30 °C to get 10^6–10^8 spores/mL.

2.2.2. Production of ligninolytic enzymes under optimum conditions

The pre-optimized solid state fermentation medium of wheat straw was used for the production of ligninolytic enzyme (Aslam and Asgher, 2011). The ligninolytic production medium containing 5 g substrate was moistened with Kirk's basal salts medium (the main constituents of the salt media were: $(NH_4)_2SO_4$, 10 g/L; KH_2PO_4, 4 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L and $CaCl_2$, 0.5 g/L). The flasks were autoclaved, inoculated with 5 mL of the fungal inoculum and incubated at 30 °C for 5 days. To the fermented biomass 100 mL distilled water was added and the flask was kept in shaker at 120 rpm for 30 min (Iqbal et al., 2011). The contents were filtered and filtrates were centrifuged at 3000 × *g* for 5 min. The supernatant was assayed for ligninolytic enzymes and used as enzymes extract for delignification of sugarcane bagasse.

2.2.3. Ligninolytic enzymes assays

Lignin peroxidase (LiP) activity was measured by the method of Tien and Kirk (1988), following the H_2O_2 dependent oxidation of veratryl alcohol to veratraldehyde at 25 °C. Reaction mixture contained 4 mM veratryl alcohol (1 mL) at 25 °C in (1 mL) 100 mM tartarate buffer of pH 3, and the reaction was initiated by 0.2 mM H_2O_2 (0.5 mL). Absorbance of reaction mixture was monitored at 310 nm (ε_{310} = 9300) (Iqbal et al., 2011). Manganese peroxidase (MnP) was assayed by the method of Wariishi et al. (1992), at 25 °C by the H_2O_2 dependent oxidation of oxidized manganic–malonate complex at 270 nm (ε_{270} = 11,570). Reaction mixture contained 1 mL of 1 mM manganese sulfate in 1 mL sodium malonate buffer (50 mM; pH 4.5) and 100 μL of enzyme sample and the reaction was initiated by the addition of H_2O_2 to a final concentration of 0.1 mM (Asgher and Iqbal, 2011). Laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in a reaction mixture containing (1 mL) 1 mM ABTS in (1 mL) 0.1 M sodium acetate buffer (pH 4.5) with a 50 μL enzyme sample (Wolfenden and Willson, 1982). The oxidation was followed at 25 °C and 420 nm (ε_{420} = 36,000) (Asgher et al., 2012b).

2.3. Cellulase production and their assays

2.3.1. Cellulase production under optimum conditions

Sugarcane bagasse was used as substrate for the production of cellulase enzymes by *T. harzianum* under pre-optimized liquid state fermentation conditions. For preparation of inoculum *T. harzianum* was grown in Vogel's medium at pH 4.5. The inoculated flask was incubated at 28 °C for three days to get homogenous inoculum having 10^7–10^8 spores/mL. For the production of cellulases in liquid state fermentation, the fungus was grown in 500 mL Erlenmeyer flask containing 5 g sugarcane bagasse in 100 mL Vogel's medium. The flask was sterilized, inoculated (2 mL inoculum) and it was incubated for 5 days at 28 °C. The contents of the flask were filtered and the filtrate was used as crude cellulase extract for saccharification of alkali and ligninolytic pretreated sugarcane bagasse.

2.3.2. Cellulase assays

Endo 1,4-β glucanases was assayed using carboxymethyl cellulose (CMC) as substrate and 3,5-dinitrosalisylic acid (DNS) as coupling reagent by the method of Gadgil et al. (1995). The assay mixture contained 0.1 mL of enzyme solution with 1 mL of 1% CMC and 1 mL of 0.1 M citrate buffer of pH 4.8. The assay tube was incubated for 30 min at 50 °C and the reaction was then terminated by adding DNS reagent. The reaction mixtures were boiled for 15 min and then cooled in ice. The absorbance was measured at 540 nm against reagent blank. The released glucose was determined by