Bioresource Technology 222 (2016) 458–469

Contents lists available at ScienceDirect

Bioresource Technology

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

Unravelling the capability of *Pyrenophora phaeocomes* S-1 for the production of ligno-hemicellulolytic enzyme cocktail and simultaneous bio-delignification of rice straw for enhanced enzymatic saccharification

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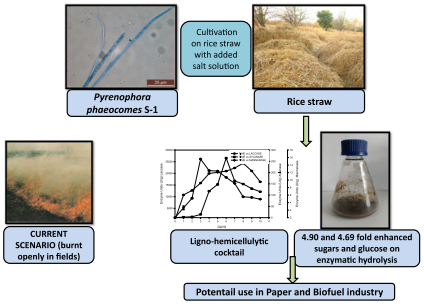
HIGHLIGHTS

- Isolation of a natural fungal variant producing ligno-hemicellulolytic enzymes.
- High co-productivities of laccase, xylanase and mannanase on rice straw under SSF.
- Maximum yield of cocktail after 8 days by *Pyrenophoraphaeocomes* on rice straw.
- Appreciable biodegradation of lignin and hemicellulose of rice straw in 40 days.
- 4.90 fold increase in released sugars upon enzymatic hydrolysis of treated biomass.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 2 October 2016
Accepted 3 October 2016
Available online 5 October 2016

Keywords:
Ligno-hemicellulolytic
Rice straw
Laccase
Xylanase
Mannanase
Biological pretreatment

ABSTRACT

A natural variant of *Pyrenophora phaeocomes* isolated from natural biodiversity was able to grow on various agricultural residues by co-producing laccase, xylanase and mannanase. Solid state fermentation of rice straw induced the highest productivities corresponding to 10,859.51 ± 46.74, 22.01 ± 1.00 and 10.45 ± 0.128 IU gds^{-1} for laccase, xylanase and mannanase respectively after 4 days. Besides producing the ligno-hemicellulolytic enzyme cocktail, 40 days cultivation of *P. phaeocomes* S-1 on rice straw brought about the 63 and 51% degradation of lignin and hemicellulose. These components were further removed with mild alkali extraction revealing the overall losses amounting to 78 and 60% respectively for lignin, and hemicellulose. The biologically pretreated straw upon enzymatic hydrolysis revealed 50% saccharification efficiency releasing 470 mg g^{-1} sugars. Application of this knowledge will lead to efficient management of waste rice straw with low cost production of industrially important enzymes cocktail and its biological delignification for effective enzymatic hydrolysis to free sugars.

1. Introduction

Huge amount of the lignocellulosic residues are released into the environment each day through forestry, agricultural practices, timber and agro-industries which are ultimately burnt in open leading to serious global threats (Howard et al., 2003). These are cheap, renewable and freely accessible substrates for many industries including paper and biofuel. Wheat, rice and maize are the leading food crops in the world supplying 50% of all calories consumed by the human population. Rice is the second largest crop of the world in terms of the harvested area leading to the

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http://dx.doi.org/10.1016/j.biortech.2016.10.012
0960-8524/

production of rice straw, rice husk as wastes which remain underutilized. India contributes 21% while China is on the top with 30% of the world's annual rice production. Out of the total rice straw generated, 23% remains unutilized which is either burnt in open-field or buried according to soil incorporation strategies (Gadde et al., 2009). However, both these strategies lead to significant air pollution via the production of methane gas and green house effect (Singh et al., 2011). This waste cannot be used as fodder due to low digestibility, high silica content and low nutritive value. The proper management for efficient disposal and utilization of rice straw has attracted the researcher's interest. Rice straw contains high amount of cellulose (44%) and hemicellulose (20.1%) bound together with lignin (19%) and non-digestible silica (9.8%) which makes it the least preferred substrate for production in paper and biofuel industry (Devevre and Horwath, 2000).

The efficient use of plant lignocellulose, specifically the rice straw for supporting growth of the fungi capable of co-producing different set of enzymes with simultaneous delignification of the substrate would appear to be a promising and ambitious goal. A key issue for utilization of rice straw in major industrial sector is the disintegration of complex polymers. Thus, development of biological pretreatment method with simultaneous production of industrially important enzymes system can have an edge over other pretreatment methodologies, which requires large amounts of harsh chemicals, adding to the economics of the process. The basic principle involved in this methods is to remove or alter the hemicellulose or lignin with decreased crystallinity of the cellulose. Biological pretreatment is an environmental friendly and low cost method. Among the various microorganisms, white rot fungi can bring about the depolymerization of the lignin with the help of their extracellular lignolytic enzymes (Yamagishi et al., 2011).

Ligno-hemicellulases including laccase, xylanase and mannanase are among the attractive enzymes for paper and biofuel industry. This unique cocktail targets lignin and hemicellulose without affecting cellulose; which in pure form is utilized by a large industrial sector. Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are copper-linked oxidoreductases, with the ability to catalyze non-specific one-electron oxidation of wide variety of aromatic compounds coupled with the concomitant reduction of molecular oxygen to two water molecules (Ntougias et al., 2015). Due to broad spectrum of substrates, laccase can be found in higher plants, bacteria, fungi and some insects with white rot fungi being on top due to their extracellular, non-specific and non-stereoselective enzyme system (Kuhar et al., 2015). *endo*-β-1,4-Xylanase (E.C.3.2.1.8), glycoside hydrolase, catalyzes the hydrolysis of the glycosidic linkage (β-1,4) in the xylan backbone forming sugar hemiacetal and the corresponding free aglycone with reduction in the degree of polymerization (Uday et al., 2016). Endo-β-1,4-mannanases (EC 3.2.1.78), attack the internal glycosidic bonds of mannan in hemicellulose releasing short β-1, 4-manno-oligosaccharides (Soni et al., 2015). These hemicellulolytic enzymes are produced by various microorganisms ranging from bacteria, fungi to yeasts from those filamentous fungi are on top with production of multiple isoforms (Luo et al., 2010).

The major challenges in industrial applications of these enzymes are low yields and expensive media formulations used for their productions. Major studies have been focused on purification and characterisations instead of hyper-production of all the three enzymes on cheaper lignocellulosic substrates, which should be the top priority. Similarly, there is a need to screen natural biodiversity for isolation of novel fungal strains capable of co-producing all the three enzymes of ligno-hemicellulolytic cocktail. Thus, co-production of such a cocktail on cost effective and cheap substrate is a prerequisite for their commercialization.

In view of the above stated demands, the present study was designed with an objective of isolating a fungal strain capable of co-producing a cocktail of laccase, xylanase and mannanase on lignocellulosic residues and can simultaneously de-lignify rice straw for efficient enzymatic hydrolysis.

2. Material and methods

2.1. Microorganism

A natural variant of *P. phaeocomes* S-1, capable of co-producing ligno-hemicellulolytic enzymes, used in the study, was isolated from the biodiversity of the Chandigarh city, India by screening the decaying wooden samples. Laccase producing fungal colonies showed the development of reddish zone on guaiacol containing plates (Kiiskinen et al., 2004). On the other hand, to visualize xylanolytic activities, the xylan containing fungal plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 N NaCl (Apun et al., 2000; Teather and Wood, 1982). The mannanase activity was revealed by a clear zone around the colonies in locust bean gum plates (Phothichittol et al., 2006). The strain was selected on the basis of its ability to co-produce all the three enzymes of the ligno-hemicellulolytic cocktail in appreciable titre after 4 days of solid state fermentation (SSF) on wheat bran.

2.1.1. Solid state fermentation of wheat bran

The SSF was carried out by taking 5 g wheat bran moistened with 7.5 ml of distilled water dispensed in different sets of 250 ml Erlenmeyer flasks as the basal medium. The flasks were sterilized by autoclaving at 15 psi for 30 min, inoculated with the 5 mycelial discs (7 mm), cut from the periphery of actively growing colonies on PDA plates followed by incubation at 28 °C under static condition for 4 days with manual shaking once a day. Enzyme extraction was carried out in 200 ml distilled water in the laboratory blender and the fermented residue was separated by filteration through a metallic sieve followed by centrifugation at 10,000 rpm at 4 °C for 10 min. The mycelium free supernatants thus obtained were used as crude enzyme preparation.

2.1.1.1. Enzyme assays. Laccase was assayed by measuring the change in absorbance of guaiacol at 470 nm upon oxidation at 50 °C in 10 min (Jhadav et al., 2009) and the enzyme activity was expressed in terms of International Units (IU) equivalent to the amount of enzyme required to oxidize 1 μmoles of guaiacol per min. Xylanase and Mannanase activities were determined using oat spelt xylan (Bailey et al., 1992) and guar gum (Stalbrand et al., 1993) as the substrates and measuring the μmoles of xylose and mannose liberated per min respectively at 50 °C using dinitrosalicylic acid reagent (Miller, 1959). The productivities of all the three enzymes have been expressed as IU g^{-1} of dry substrate (gds) used in solid state fermentation.

2.1.2. Identification of the strain

The strain was identified to the genus level based on macroscopic and microscopic characteristics while the complete taxonomic status was established on the basis of molecular analysis by performing 18S rDNA sequencing using the services of Xcelris Labs Ltd, India. Based on sequencing, BLAST and Multialn results, the phylogenetic tree was constructed by using MEGA 4 software (Tamura et al., 2007).