Process Biochemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Full Length Article

Synergistic effect of syringic acid and gallic acid supplements in fungal pretreatment of sweet sorghum bagasse for improved lignin degradation and enzymatic saccharification

Vartika Mishra [a], Asim K. Jana [a,*], Mithu Maiti Jana [b], Antriksh Gupta [a]

[a] Department of Biotechnology, Dr B R A National Institute of Technology Jalandhar, 144011, Punjab, India
[b] Department of Chemistry, Dr B R A National Institute of Technology Jalandhar, 144011, Punjab, India

ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form 7 February 2017
Accepted 9 February 2017
Available online xxx

Keywords:
Sweet sorghum bagasse
Coriolus versicolor
Biological pretreatment
Supplements
Delignification
Enzymatic hydrolysis

ABSTRACT

Sweet Sorghum Bagasse (SSB) has potential for uses in production of ligninocellulosic biofuel and other fermentation products due to higher biomass yield. Pretreatment of SSB by *Coriolus versicolor* with synergistic syringic acid-gallic acid supplements for enhanced ligninolytic enzyme production, lignin degradation, selectivity value (SV) and enzymatic saccharification have been studied. *C. versicolor* was selected due to its high ligninolytic and low cellulolytic enzyme activities in SSF with $19.3 \pm 0.8\%$ w w^{-1} of lignin degradation in 20 days and SV 0.65. Supplement gallic acid increased the production of MnP; while syringic acid increased the production of LiP, AAO and laccase with enhanced lignin degradation and SV. Combined supplements increased the production of multiple ligninolytic enzymes further than the effect of individual supplements due to synergistic interactions. Activities of laccase, LiP, MnP, PPO and AAO increased by 9.1, 14.6, 2.6, 1.9 and 2.2 folds respectively, which resulted in highest lignin degradation (1.56 times) and SV (3.87 times). Enzymatic hydrolysis of SSB pretreated with combined supplements yielded higher fermentable sugar (~2.18 times). Combined supplements could be used for improvement in pretreatment by SSF. XRD, SEM, FTIR and TGA/DTG analysis of SSB confirmed the results.

1. Introduction

Sweet Sorghum Bagasse (SSB), an agri-process industries waste of high biomass yield is a promising candidate for use in biofuel production and other fermentation products [1]. It is mainly composed of lignin, cellulose and hemicelluloses; but only 20% of theoretical yield of fermentable sugar from its cellulose can be obtained by enzymatic hydrolysis [2]. Lignocellulosic biomass is often subjected to pretreatment processes to increase its enzymatic hydrolysis. Physicochemical pretreatment processes such as high temperature treatment with acid or alkali before enzymatic hydrolysis resulted in the production of inhibitory byproducts. Therefore, selective degradation of lignin and hemicelluloses using the microorganisms have been explored in biological pretreatment [3]. The most promising microorganisms used for biological pretreatment are white rot fungi (WRF). Ligninolytic ability of WRF by the enzymes laccase (phenol oxidase), lignin peroxidase (LiP), manganese peroxidase (MnP), arylalcohol oxidase (AAO) and polyphenol oxidase (PPO) can demineralize the lignin into CO_2 and H_2O [4,5].

WRF vary widely in their ability to degrade lignin and polysaccharide in ligninocellulosic biomass during pretreatment. In the past few years, WRF such as *Ganoderma* sp., *Pleurotus ostreatus*, *Trametes* sp. and *Polyporus brumalis* have been studied for lignin degradation abilities during pretreatment of sugarcane bagasse [2,6] and wheat straw [7,8] etc. Although WRF are the best lignin degraders, problems of pretreatment are non-selective lignin degradation [9], long pretreatment time [3] and cellulolytic enzymes expression during pretreatment. Selectivity value (SV), defined as the ratio of lignin degradation to cellulose consumption is an important parameter of fungal strain for obtaining high lignin degradation with low cellulose consumption [10–12]. WRF *Ganoderma* sp. [2], *Phanerochaete chrysosporium* [13] and *Trametes versicolor* [14,15] reported to have high SV.

Kamcharoen et al. [15] reported that optimization of physical parameters of solid state fermentation (SSF), couldn't significantly enhance SV. But, optimization of particles sizes, moisture content in SSF etc. increased the production ligninolytic enzymes, lignin degradation and SV with reduction in the pretreatment time [16]. The production of ligninolytic enzymes including both oxi-

* Corresponding author at: Department of Biotechnology, Dr B R A National Institute of Technology, G T Road Bye Pass, Jalandhar 144011, India.
E-mail addresses: asimkumarjana@gmail.com, janaak@nitj.ac.in (A.K. Jana).

http://dx.doi.org/10.1016/j.procbio.2017.02.011
1359-5113/

dase and peroxidases can be controlled by carbon/nitrogen sources, inorganic compounds, phenolic and aromatic compounds [17,18]. Addition of phenolic supplements (ferulic acid, xylidine, veratric acid, vanillic acid, cinnamic acid and guaiacol) have been reported to enhance the production of laccase [19], while veratryl alcohol reported to enhance the production of LiP [20]. Enhancement of ligninolytic enzymes production in presence of phenolic compounds in fungal culture have been reported recently [21,22]. Although effect of individual supplements have been reported on specific enzyme [17–22], but supplements in combination to enhance the production of multiple ligninolytic enzymes and lignin degradation has not been reported.

The objective of the present study was to develop a fungal pretreatment process of SSB with high SV using combined effects of two supplements. Suitable fungal strain with high SV was screened initially from different strains reported for the pretreatment in literature [7,12,14,23,24]. The effect of the combination of two phenolic supplements; syringic acid and gallic acid on production of ligninolytic enzymes and cellulolytic enzymes, and selective lignin degradation compared to the effect of individual supplements has been reported for the first time to the best of our knowledge.

2. Materials and methods

2.1. Materials

2,2′azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from MP Biomedical (Mumbai, India). Cellulase (SaccariSEB EG) and β-glucosidase (SaccariSEB BG) were gifted by Advanced Enzyme Technologies Ltd (Thane, India). *p*-nitrophenyl-β-D-glucopyranoside, birchwood xylan, lignin sulfonic acid, catechol, vanillin and verataryl alcohol were purchased from Himedia Laboratory Pvt Ltd (Mumbai, India). Cellulose and syringic acid were purchased from Sigma Aldrich (Mumbai, India). Water was purified and deionized (DI) by Milli-Q purification system (minimum resistivity 18 MΩ cm; Millipore, Billerica, MA, USA). Rest of chemicals used were analytical grade chemicals made by S.D. fine chem. Ltd (Mumbai, India).

2.2. Biomass

Sweet Sorghum Bagasse (SSB) was collected from the local agricultural farm in Jalandhar, Punjab, India. The collected SSB was dried at 40 °C for 72 h and grounded to pass through 5 mm sieve using a wiley mill. SSB (about 3% moisture) was stored in air tight container at room temperature.

2.3. Fungal strain and maintenance

Coriolus versicolor (MTCC 138), *Daedalea flavida* (MTCC 145), *Ganoderma lucidum* (MTCC 1039), *Phlebia radiata* (MTCC 2791), *Pleurotus ostreatus* (MTCC 1801), *Stereum hirsutum* (MTCC 1099) were procured from Microbial Type Culture Collection (MTCC) Chandigarh, India and *Pycnoporus cinnabarinus* (NCIM 1181) from the National Collection of Industrial Microorganisms (NCIM) Pune, India. *Coriolus versicolor*, *Ganoderma lucidum, Phlebia radiata* and *Pycnoporus cinnabarinus* were grown and maintained in 2% (w v^{-1}) malt extract agar media in petri plates at pH 4.5, 25 °C. *Pleurotus ostreatus* and *Stereum hirsutum* were grown and maintained in potato dextrose agar media (potato infusion 20%, dextrose 2% and agar 1.5% w v^{-1}) in petri plates at pH 5.5, 25 °C. All strains were sub-cultured periodically after every 15 days.

2.4. Lignocellulolytic abilities

2.4.1. Ligninolytic ability

Basal medium (BM) was prepared containing (g L^{-1}) ammonium tartrate 5.0, yeast extract 0.1, potassium phosphate monobasic 1.0, calcium chloride 0.001, magnesium sulphate 0.5 and 1.6% (w v^{-1}) agar (pH 4.5). Substrates i.e. 0.1% (w v^{-1}) ABTS, 0.1% (v v^{-1}) guaiacol, 0.01% (w v^{-1}) tannic acid, 0.25% (w v^{-1}) lignin sulfonic acid and 0.01% (w v^{-1}) azure-B were used to determine the extent of ligninolytic ability [25]. Single disc taken from actively growing 7 day old cultured fungal plate using sterile cock borer (10 mm diameter) was used as inoculums. Sterilized petri plates containing BM and supplemented with substrates were inoculated with test strains, and incubated in incubator (Innova 42R, Eppendorf ltd, USA) at 27.5 °C for 5 days. The colourless agar medium turned green due to the oxidation of ABTS to ABTS-azine in the presence of laccase. Colorless medium containing guaiacol in petri plate turned to brick red in the presence of laccase produced by fungi. The appearance of a brown oxidation zone around colonies in petri plates containing tannic acid signified the polyphenol oxidase production. In lignin sulfonic acid plate, 1% w v^{-1} aqueous solution of ferric chloride and 1% w v^{-1} potassium ferricyanide was flooded. Plates were washed with DI water after 10 min and observed for clear zones of degraded lignin around colonies against blue green colour of undegraded lignin. In the petri plate containing azure B, clearance of blue colour of agar showed the ability of lignin peroxidases enzyme production by fungal strain. Measurement of the diameter of these colour zones around the colony were measured as the ligninolytic ability of the strains.

2.4.2. Cellulolytic and hemicellulolytic (xylanolytic) ability

The cellulolytic ability of fungal strains were detected by the Congo red test [26]. Petri plates containing BM with carboxy methyl cellulose (1.1% w v^{-1}) as substrate were sterilized. Single disc taken from actively growing 7 day old cultured fungal plate using sterile cock borer (10 mm diameter) was inoculated and incubated at 27.5 °C for 5 days. Cultivated plates were flooded with aqueous Congo red solution (1 mg mL^{-1}). After 15 min of exposure, dye was drained and plates were washed three times with 1 M NaCl and observed for production of yellow zones around the colonies. For xylanolytic ability, xylan (4% w v^{-1}) was supplemented with BM in petri plates [25]. Sterile plates were inoculated with test strains and incubated at 27.5 °C for 5 days. Plates were flooded with iodine stain (0.25% w v^{-1} aqueous I_2 and 0.25% w v^{-1} KI). After 5 min of exposure, dye was drained and plates were washed with DI water. Plates were observed for appearance of a yellow-opaque area against a blue/reddish purple colour for undegraded xylan (signifying the release of xylanase). Measurement of the diameter of coloured zones around fungal disc were measured as cellulolytic and hemicellulolytic ability of strains.

2.5. Pretreatment of SSB

Ten discs (using sterile cock borer with 10 mm in diameter) of the actively growing 7 day old cultured plates, were inoculated into a 500 mL capacity cotton plugged Erlenmeyer flask containing 50 mL of 2% (w v^{-1}) malt extract medium. After 7 day incubation at 27.5 °C under static conditions, the liquid culture was aseptically homogenized in a sterilized blender for 15 s × 3 cycles. Two mL (~2.4×10^6 spore mL^{-1}) of blended culture was used as inoculum for 5 g of SSB for pretreatment by SSF experiments.

Five grams of grounded SSB (~3% moisture) was taken in 250 mL cotton plugged Erlenmeyer flasks and 11.5 mL of distilled water was added with it to make 75% moisture initially, for solid state fermentation (SSF). Moistened SSB in flasks were autoclaved at 121 °C for 15 min. Sterilized flasks were inoculated with 2 mL inoculum

Please cite this article in press as: V. Mishra, et al., Synergistic effect of syringic acid and gallic acid supplements in fungal pretreatment of sweet sorghum bagasse for improved lignin degradation and enzymatic saccharification, Process Biochem (2017), http://dx.doi.org/10.1016/j.procbio.2017.02.011