Biocatalysis and Agricultural Biotechnology 6 (2016) 195–201

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid-state fermentation

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form
9 April 2016
Accepted 11 April 2016
Available online 12 April 2016

Keywords:
Schizophyllum commune
Lignocellulosic materials
Ligninases
Process optimization
Delignification
Enzymatic saccharification

ABSTRACT

The modification of lignin is recognized as an important aspect of the successful refining of lignocellulosic biomass. *Schizophyllum commune*, a white rot basidiomycete was studied for ligninolytic enzymes (manganese peroxidase, lignin peroxidase and laccase) production in solid-state fermentation (SSF) of rice straw. Various physiological factors such as incubation time, culture pH, incubation temperature, C:N ratio and addition of mediators were optimized to enhance enzymes productivity. Maximum enzyme recoveries were obtained at pH, 5.0; temperature, 35 °C; C:N ratio, 20:1; mediator, $MnSO_4$; inoculum size, 4 mL after incubation time of 144 h. The crude ligninolytic extract thus produced was used for delignification of various agro-industrial residues. The enzyme extract caused 61.7%, 47.5%, 72.3% and 67.2% lignin removal from banana stalk, corn cobs, sugarcane bagasse, and wheat straw, respectively. The optimally delignified substrate was enzymatically digested by crude cellulase extract from *Trichoderma harzaianum* that resulted 47.3% and 69.4% cellulose hydrolysis from the native and pretreated bagasse, respectively. The results suggested that lignocellulosic waste could be utilized as low-cost substrate for the production of enzymes which play significant role in many industrial and biotechnological sectors.

1. Introduction

The microbial decomposition of lignin and plant cell-wall polysaccharides has become crucial for the development of innovative biotechnological processes in various industries such as pulp and paper, textile and chemical synthesis (El-Shishtawy et al., 2015; Garcia-Torreiro et al., 2016). The removal of encrusted lignin from the lignocellulosic biomass is quite challenging to accomplish and obviously a specific feature of filamentous fungi belonging to phylum Basidiomycota (Liers et al., 2011; Munir et al., 2015). In nature, lignocellulose transformation is predominantly attributed to the synergistic action of several oxidoreductases actively secreted by wood-rotting fungi accompanying with low-molecular-mass mediators (Hatakka and Hammel, 2010). Among them are the lignin-modifying peroxidases, including manganese-dependent peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (LiP, EC 1.11.1.14) and versatile peroxidase (VP, EC 1.11.1.16) (Hofrichter et al., 2010). Laccases (phenol oxidases, EC 1.10.3.2) are another group of multi-copper oxidative biocatalysts which take part in biomass de-polymerization by oxidizing a variety of phenolic compounds and aromatic diamines (lignin, melanin and humic substances) (Baldrian, 2006).

Agricultural wastes, forestry wastes and agro-industrial residues generally accumulated in the environment have ecological disadvantages (Asgher et al., 2014). However, these lignocellulosic wastes could be harnessed as potential raw materials for economic production of high added value products such as biocatalysts, fuel ethanol, single-cell protein, organic acids, secondary metabolites and other fine chemicals that currently remains the subject of considerable attention (Iqbal and Kamal, 2012). Delignification of biomass is regarded as the most important step in this regard (Asgher et al., 2016a, 2016b). Significant efforts were being made to convert this lignocellulose either as bio-fuel or as a valuable starting material for commodity chemical synthesis (Howard et al., 2003; Garcia-Torreiro et al., 2016). Since the cost of lignocellulosic substrates play central role in determining the economy of delignification process, lot of research focus had been given to the usage of low-price substrates and, therefore screening of agricultural wastes for release of sugars. Various agro-wastes have been delignified by other researchers employing individual and co-cultures of white-rots (Asgher et al., 2013a, 2013b).

The solid-state fermentation (SSF) processes have been appeared particularly suitable route for the production of enzymes by filamentous fungi since they offer natural habitats on which fungus grows better (Bilal et al., 2015). In alternative to traditional submerged fermentation (SmF), SSF present advantages of improved yields, cost competitive, easier products recovery, and lack

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http://dx.doi.org/10.1016/j.bcab.2016.04.003
1878-8181/

of foam formation. Furthermore, due to low water contents, contamination risks were significantly eliminated and, therefore the volume of residual wastes also decreases (Yasmeen et al., 2013). The industrial scale enzyme synthesis through medium optimization and selection of appropriate growth promoting substrate plays a noteworthy role in the design of an efficient biotechnology (Moldes et al., 2004: Bilal and Asgher, 2015a, 2015b).

Schizophyllum commune is a ubiquitous white-rot fungus with a worldwide distribution that can degrade complex plant biomass, including the recalcitrant lignin (Irshad and Asgher, 2011; Horisawa et al., 2015). Since the genome of *Schizophyllum commune* encodes an extensive catalog of genes implicated in lignocellulose degradation, its lignocellulolytic enzyme pool is expected to provide a prospective enzyme source for biotechnological applications (Zhu et al., 2016). In fact, *S. commune* has the most complete polysaccharide breakdown machinery of all basidiomycetes examined. This complete machinery is consistent with the wide variety of substrates that support growth of *S. commune* (Ohm et al., 2010). The current study was aimed to optimize the culturing conditions for enhanced lignin-modifying enzymes (LMEs) production in SSF by *Schizophyllum commune* IBL-06. The potential of enzyme extract for the delignification of different agricultural wastes followed by saccharification using cellulases extract from *T. harzianum* was also investigated.

2. Materials and methods

2.1. Agricultural waste

Rice straw collected from students Research Farms, University of Agriculture, Faisalabad was chosen as the nutrient source for production of LMEs through SSF. Before use, the substrate was oven dried at 60 °C, crushed in a commercial mill (Ashraf Herbal Laboratories limited, Faisalabad) and sieved to 40 mesh particle size.

2.2. Fungal strain, medium and inoculum development

S. commune IBL-06 was obtained from culture stock of the Industrial Biotechnology Laboratory, Department of Biochemistry, University of Agriculture; Faisalabad. The fungal culture was maintained on potato dextrose agar (PDA) slants for 3–5 days at pH 4.5 and 28 °C and preserved at 4 °C. Inoculum was prepared by growing the strain on a rotary shaker (150 rpm and 30 °C) in 250-mL sterilized culture flask filled with 100-mL of Kirk's basal medium with additionally 1.0% (w/v) glucose solution (sterilized through filtration). The basal medium contained; ammonium tartrate (0.22 g L^{-1}), KH_2PO_4 (0.21 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.05 g L^{-1}), $CaCl_2$ (0.01 g L^{-1}), Thiamine (0.001 g L^{-1}), 10 mL Tween 80 (10%), 10 mL 100 mM veratryl alcohol and 10 mL trace-metal solution. The traced element solution was prepared by mixing the salts of $CuSO_4$, (0.08 g L^{-1}); $NaMoO_4$, (0.05 g L^{-1}), $MnSO_4$. H_20, (0.07 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.043 g L^{-1}) and $FeSO_4$, (0.05 g L^{-1}). Prior to sterilization, pH of the medium was adjusted to pH 4.5 (WTW pH-meter; InoLab pH 730). After 5–7 days of cultivation, homogeneous spore suspension (1×10^6–1×10^8 spores/mL) was attained and used as inoculum (Yasmeen et al., 2013).

2.3. Optimization of culture conditions for enzyme production

The effect of various physicochemical parameters including incubation time (48, 96, 144, 192, 240 h), incubation pH (3, 4, 5, 6, 7), temperature (20, 25, 30, 35, 40 °C), carbon (molasses, glucose, fructose, starch) and nitrogen sources (ammonium sulphate, peptone, urea, and ammonium chloride), carbon/nitrogen ratio (5:1, 10:1, 15:1, 20:1, 25:1) and effect of mediators ($MnSO_4$, oxalate, ABTS, veratryl alcohol and H_2O_2) were optimized to achieve maximum ligninolytic enzyme production. Classical optimization strategy i.e., by varying one variable at a time and keeping the previously optimized factors at optimum level was employed for optimization study.

2.4. Production and extraction of ligninolytic enzyme

The SSF was carried out in triplicate Erlenmeyer flasks (250-mL) containing 5 g of rice straw pre-moistened with sterilized Kirk's basal salts medium (66% w/v) without glucose. After maintaining the medium pH to 5.0, the fermentation flasks were inoculated with 4 mL homogeneous mycelium suspension and subjected to fermentation at 35 °C for 144 h, using a C:N ratio of 20:1 (molasse:ammonium sulphate) and $MnSO_4$ as mediator. After designated time, the fermented biomass was harvested for extracellular enzymes with 100 mL of distilled water. The extracts in the flasks were shaken (120 rpm for 30 min) followed by centrifugation at 4000 rpm for 10 min. The clear filtrate was used as crude enzyme extract for activity assays (Bilal and Asgher, 2015a, 2015b).

2.5. Ligninolytic enzyme assays

Activities of all the oxidoreductases were measured spectrophotometrically (HALO-DB 20). MnP activity was specifically assayed as described previously by monitoring the formation of manganic-malonate complexes at 270 nm (Wariishi et al., 1992). A 2.6-mL of assay mixture comprised $MnSO_4$ (1 mL; 1 mM), 1 mL of 50 mM Na-malonate buffer pH 4.5, 500 μL of H_2O_2 and 100 μL of crude enzyme solution. Activity of LiP 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. Reactive mixture (2.6 mL) contained 1 mL tartrate buffer (100 mM) of pH 3, 1 mL of 4 mM veratryl alcohol, 500 μL of H_2O_2 and 100 μL of enzyme aliquots. Laccase was assayed by monitoring 2, 2 azinobis (3-ethylbenzthiazoline)−6 sulphonate (ABTS) oxidation in Na-malonate buffer at 436 nm (Wolfenden and Willson, 1982). A 2.1-mL of reaction mixture containing 1 mL of 50 mM Na-malonate buffer (pH 4.0), 1 mL of ABTS and 100 μL of enzyme solution. Blank contained 100 μL of distilled water instead of enzyme solution. Enzyme activities were represented in units per gram of dry substrate (U/gds), with one unit of enzyme activity defined as the amount of enzyme that catalyzed the formation of 1 μmol of corresponding products in one min under the given assay conditions.

2.6. Pretreatment with ligninolytic enzymes extract

Varying volumes of ligninoytic enzymes extract containing LiP, MnP and laccase was applied at different doze levels (5, 10, 15, 20, and 25 mL) for 24 and 48 h at 35 °C. The volume of each flask containing 30 g substrate was made to 200 mL mark with 50 mM Na-malonate buffer of pH 5.0. The percentage of lignin was calculated before and after ligninolytic enzymes treatment in order to determine the enzyme efficiency and percentage lignin removal from the substrate.

2.7. Determination of lignin and percent delignification

Untreated and ligninolytic enzymes treated substrates were analyzed for lignin content and percent delignification according to Johnson et al. (1961), with slight modifications. The sample was dissolved in 10 mL 25% (w/v) acetyl bromide in re-distilled glacial acetic acid (GAA $\geq$ 99%) by heating at 70 °C ± 2 °C in a water bath for 30 min. The sample was stored in a special digester tube with a