Microbiological Research 192 (2016) 73–83

Contents lists available at ScienceDirect

Microbiological Research

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

Do cultural conditions induce differential protein expression: Profiling of extracellular proteome of *Aspergillus terreus* CM20

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ARTICLE INFO

Article history:
Received 11 December 2015
Received in revised form 7 June 2016
Accepted 17 June 2016
Available online 18 June 2016

Keywords:
Aspergillus terreus
Submerged fermentation
Solid-state fermentation
Extracellular proteome
Glycoside hydrolase
LC-MS/MS

ABSTRACT

The present study reports the diversity in extracellular proteins expressed by the filamentous fungus, *Aspergillus terreus* CM20 with respect to differential hydrolytic enzyme production profiles in submerged fermentation (SmF) and solid-state fermentation (SSF) conditions, and analysis of the extracellular proteome. The SSF method was superior in terms of increase in enzyme activities resulting in 1.5–3 fold enhancement as compared to SmF, which was explained by the difference in growth pattern of the fungus under the two culture conditions. As revealed by zymography, multiple isoforms of *endo*-β-glucanase, β-glucosidase and xylanase were expressed in SSF, but not in SmF. Extracellular proteome profiling of *A. terreus* CM20 under SSF condition using liquid chromatography coupled tandem mass spectrometry (LC–MS/MS) identified 63 proteins. Functional classification revealed the hydrolytic system to be composed of glycoside hydrolases (56%), proteases (16%), oxidases and dehydrogenases (6%), decarboxylases (3%), esterases (3%) and other proteins (16%). Twenty families of glycoside hydrolases (GH) (1, 3, 5, 7, 10, 11, 12, 15, 16, 28, 30, 32, 35, 43, 54, 62, 67, 72, 74 and 125), and one family each of auxiliary activities (AA7) and carbohydrate esterase (CE1) were detected, unveiling the vast diversity of synergistically acting biomass-cleaving enzymes expressed by the fungus. Saccharification of alkali-pretreated paddy straw with *A. terreus* CM20 proteins released high amounts of glucose (439.63 ± 1.50 mg/gds), xylose (121.04 ± 1.25 mg/gds) and arabinose (56.13 ± 0.56 mg/gds), thereby confirming the potential of the enzyme cocktail in bringing about considerable conversion of lignocellulosic polysaccharides to sugar monomers.

1. Introduction

Lignocellulosic biomass is currently the most promising feedstock source for realizing sustainable energy demands of agrarian economies. The current availability of biomass in India is estimated at about 640 MT per year, of which 234 MT is available as surplus for energy generation (Hiloidhari et al., 2014). Lignocellulose, a composite of ~40–50% cellulose, ~25–35% hemicellulose and ~15–20% lignin represents a feasible and sustainable resource for renewable fuel (Holtzapple, 1993; Saritha et al., 2012). For the coherent conversion of the lignin-carbohydrate complex (LCC) to fermentable sugars, efficient methods of biomass pretreatment and biomass-hydrolysing machinery are necessitated (Jørgensen et al., 2007).

The most effective method of biomass hydrolysis involves the concerted action of cellulases, hemicellulases, and other glycoside hydrolases (Harris et al., 2010). The prominent of these are the cellulases, comprising of *exo*-β-glucanases and *endo*-β-glucanases (cleaving β-1,4-glycosidic bonds from chain ends and internally within chains, respectively), and β-glucosidases (cleaving the final β-1,4 linkage of cellobiose or small polysaccharides) (Davies et al., 1995). Hemicellulases are more complex, consisting of an array of xylanases, mannases, arabinases, and their corresponding glycosidases. Their synergistic action brings about hydrolysis of ester bonds and glycosidic bonds, thereby removing the side chains (Campos et al., 2014; Sweeney and Xu, 2012). To achieve complete conversion of the lignocellulose polysaccharides to sugar monomers, enzyme cocktails which are combinations of various glycoside hydrolases that act in synergy are indispensable.

Filamentous fungi are among the microorganisms which degrade lignocellulosic biomass through extracellularly produced glycoside hydrolases. Prominence has been given to the production of enzymes by fungi because of their ability to express ample amounts of hydrolases that are secreted into the medium, thus enabling effortless extraction and purification (de Souza et al.,

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http://dx.doi.org/10.1016/j.micres.2016.06.006
0944-5013/

2015; Mohanram et al., 2013; Polizeli et al., 2005). The production of cellulase and other lignocellulolytic enzymes has been widely studied in submerged culture processes in the laboratory, ranging from shake flask to 15,000 l fermentations (Sukumaran et al., 2005; Xia and Cen, 1999). The ease of handling and greater control of environmental factors of this technology requires special mention (Mrudula and Murugammal, 2011). Solid-state fermentation (SSF) is an alternative process for bioproducts production. The cultivation conditions in SSF are suitable for the growth of fungi which are able to grow at low water activities (Haltrich et al., 1996; Kumar et al., 2007). Commercial level production utilizes lignocellulosic biomass, such as cereal straw, spent hulls of pulses, bagasse, other agricultural residues, and paper industry wastes, as the carbon source (de-Limaa et al., 2005; Reczey et al., 1996; Szijarto et al., 2004). The use of nutrient-rich lignocellulosic materials is advantageous because the use of cheaper substrates can lower the cost of enzyme production (Sadhu and Maiti, 2013).

Recently, the hydrolytic efficiency of *Aspergillus terreus* has been utilized to hydrolyse highly ordered cellulose with high saccharification efficiencies (Narra et al., 2012; Nazir et al., 2012). However, identification and selection of promising targets for biorefinery applications require more insights into the hydrolytic machinery of microorganisms. The culture conditions influence the induction of specific enzyme isoforms leading to considerable variations in secretomes among different organisms and even within the same species (Girard et al., 2013; Hashemi et al., 2013; Li et al., 2013; Nazir et al., 2010). Thus, in the present study, a comprehension on the extracellular proteome of *A. terreus* and the differential expression of proteins under SmF and SSF conditions by the fungus was taken up.

2. Materials and methods

2.1. Isolation of microorganism and inoculum preparation

The fungal isolate CM20 was isolated as part of a bioprospecting survey from the Andaman coastal soils (Andaman and Nicobar Islands, India) by cultivation on Reese's mineral medium (Reese and Mandels, 1963) with acid swollen cellulose (1%) as sole carbon source. The culture was maintained on Potato Dextrose Agar (PDA) slants and was periodically sub-cultured.

2.2. Substrate for fermentation experiments

Dried, chopped straw of the rice variety Pusa Sugandh 5, collected from the farms of ICAR-Indian Agricultural Research Institute, New Delhi, was used as substrate for fermentation experiments. The cellulose, hemicellulose and lignin contents in the paddy straw were determined to be 39.3%, 22% and 16%, respectively, by the Updegraff (1969), the TAPPI (1996) and the NREL LAP-003 (Templeton and Ehrman, 1995) methods.

2.3. Comparative profiling of enzymes and other growth parameters under SmF and SSF conditions

2.3.1. Production of enzymes under SSF and SmF

For solid state fermentation, three grams of chopped substrate was taken in 250 ml Erlenmeyer flasks, to which 15 ml of Reese's mineral medium was added. For submerged fermentation, 30 ml Reese's mineral medium dispensed in Erlenmeyer flasks with 1% (w/v) of the substrate was used. For inoculation of both SSF and SmF cultures, 1 ml of spore suspension (1×10^7 spores/ml) of the fungal strain was used. The SSF cultures were incubated for seven days at 30 °C and the contents of the flasks were mixed manually on alternate days. The SmF cultures were incubated with agitation at 150 rpm in incubator shaker (Kuhner make) at 30 °C for seven days. After the incubation period, the contents of the flasks were extracted and were used as sources of extracellular enzymes.

2.3.2. Quantitative cellulolytic and xylanolytic enzyme assays

Filter paper lyase (FPase; total cellulase) and carboxy methyl cellulase (*endo*-β-1,4-glucanase; CMCase) activities were assayed as described by Ghose (1987). Avicelase (*exo*-β-glucanase) activity was assayed by the method of Zhang et al. (2009) and xylanase activity, by the method described by Ghose and Bisaria (1987). One unit of FPase/CMCase/Avicelase corresponded to 1 μM of glucose formed per minute during hydrolysis and one xylanase unit was expressed as 1 μM of xylose formed per minute during hydrolysis. β-glucosidase assay was performed using *p*-nitrophenyl-β-D-glucopyranoside as substrate (Wood and Bhat, 1988) and the activity was calculated in terms of μM of *p*-nitrophenol produced per ml of culture filtrate per minute. Proteins in the filtrates were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.The detailed protocols used in enzymatic assays have been provided as supplementary information (Supplementary text).

2.3.3. Scanning electron microscopy (SEM) analysis

The morphology and growth pattern of the fungus under SSF and SmF conditions were examined with SEM analysis. The samples were fixed in 2.5% glutaraldehyde overnight and were washed with 0.1 M phosphoric acid. The washed samples were then dehydrated with acetone by gradually increasing the concentration up to 100%. Finally, after palladium coating, SEM studies were done with Zeiss EVOMA10 scanning electron microscope at 20 KV/EHT and 10 Pa between 500× to 5000×.

2.3.4. Molecular weight determination by SDS-PAGE and activity staining by zymography

In order to determine the molecular weights of the various enzyme fractions produced in SSF and SmF, SDS-PAGE of the enzyme extract was carried out according to the method of Laemmli (1970). The crude enzyme extracts were initially concentrated by precipitation with ethanol in a ratio of 1:5 followed by resuspension of the precipitate in 0.05 M citrate buffer (pH 4.8).

For SDS-PAGE, the protein (10 μg) was loaded to 12% (w/v) SDS-PAGE gel and was run at 100 V for approximately 3 h. After electrophoresis, the gel was stained overnight in a solution of 0.1% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) methanol and 10% (v/v) glacial acetic acid. After destaining, the gel was analyzed for bands along with a molecular weight marker. For the detection of cellulase and xylanase by zymography, 10 μg of *A. terreus* protein was boiled with SDS sample buffer (without β-mercaptoethanol) and loaded on zymogram gel made of 12% (w/v) PAGE gel with 1% CMC or birchwood xylan. Following electrophoresis, the zymogram gel was soaked for 1 h in 2.5% (v/v) Triton X 100 for the renaturation of protein and washed thoroughly in distilled water prior to incubation at 50 °C for 30 min with 0.05 M citrate buffer (pH 4.8). The gel was washed with distilled water and stained with 0.1% Congo red for 10 min and washed with 5% NaCl until clear bands became visible. For β-glucosidase zymography, the sample was unboiled and the same amount of protein was loaded onto the gel. After renaturation and washing, the gel was incubated with 0.05 M citrate buffer (pH 4.8) containing 0.1% (w/v) esculin and 0.03% (w/v) ammonium iron (III) citrate for 10 min at 55 °C. The activity staining was visualized under UV light using Gel Doc EZ Imager (BioRad, Hercules, CA).