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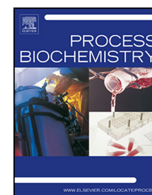

Unraveling the secretome of *Termitomyces clypeatus* grown on agroresidues as a potential source for bioethanol production

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ABSTRACT

Termitomyces clypeatus MTCC 5091 is an edible mushroom and is prized for its nutritional value as well as for harboring plethora of enzymes essential for carbohydrate degradation. *T. clypeatus* when grown on agricultural based carbon sources efficiently induced high quantities of lignocellulolytic enzymes in the secretome. Optimization in tamarind kernal powder (TKP) media through response surface methodology enhanced the enzyme yields by several folds. Correlation between extracellular protein productions of the fungus with respect to its specific growth rate established that secreted proteins were produced most efficiently at low specific growth rates. Proteins released in the *T. clypeatus* secretome were quantified and identified using SDS-PAGE, 2D gel electrophoreses, zymography and matrix-assisted laser desorption mass spectrometry. 36 proteins identified from the protein spots belonged majority to glucosyl hydrolase family, transporters, uncharacterized and hypothetical proteins. The potential synergistic interactions between the cellulases and xylanases in enzyme preparations of *T. clypeatus* during hydrolysis of steam pretreated bagasse (SPB) showed improved hydrolysis efficiency and enhanced rate of hydrolysis as observed in high performance liquid chromatograpy. The changes in the ultra-structure of SPB after 12 h enzymatic hydrolysis were observed by scanning electron microscopy. The hydrolysates obtained produced ~7.2 g/L ethanol after 6 h fermentation determined by gas chromatography.

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1. Introduction

Every year, huge amounts of lignocellulosic wastes generated from agro-industrial practices contribute to a sizeable proportion of biowaste raising concerns over environmental issues. Much of this 'lignocellulosic' waste is often disposed of by burning or left in the fields to rot. This lignocellulosic biomass can be successfully developed into renewable energy as second generation biofuel. The major structural component of lignocellulose is lignin, hemicellulose and cellulose that can be degraded to simple polysaccharides by diverse lignocellulolytic degrading microbial communities. Lignocellulolytic enzymes have significant potential applications in various industries including chemicals, fuel, food, animal feed, brewery and wine, textile and laundry, pulp and paper, and agriculture [1,2].

This study focuses on the possibilities of using tamarind kernal powder (TKP) as a substrate for hemicellulolytic enzymes production by *Termitomyces clypeatus*. TKP is obtained from the seeds of tamarind (*Tamarindus indica* L.) after the pulpy edible part is removed. It is rich in carbohydrate, protein, fibres and oils [3]. TKP has extensive application in jute and textile industries as pasting and sizing material. The excellent gelling and adhesive characteristics of decorticated seed powder possess several applications in food and pharmaceutical industries [4]. The polysaccharide rich kernel powder is composed of variety of neutral sugars, arabinose, xylose, mannose, glucose, and galactose where the fermentable sugars, namely—glucose, xylose and galactose units, are present in the ratio of 2.8:2.25:1.0 [5]. TKP is also a rich source of galactoxyloglucan & was used in the preparation of xyloglucan & ethanol production using the thermotolerant *Ascomycetous* fungi *Debaromyces hansenii* [6]. Different derivatives synthesized from tamarind kernel powder showed high selectivity for metal ions adsorptions & were used as ion exchangers in effluent treatments [7]. The rich polysaccharide content was suitable for production of

Abbreviations: TKP, tamarind kernel powder; 2DG, 2deoxyglucose; HPLC, high performance liquid chromatography; GC, gas chromatography; 2D-PAGE, second dimension polyacrylamide gel electrophoresis; EG, endoglucanse; CBH, cellobiohydrolase; PNP, *p*-nitrophenol; PNPX, *p*-nitrophenyl-β-D-xylopyranoside; PNPC, *p*-nitrophenyl-β-D-cellobioside; PNPAF, *p*-nitrophenyl-α-L-arabionfuranoside; PNPA, *p*-nitrophenyl acetate; HPLC, high performance liquid chromatography; CAZY, carbohydrate-active enzymes; YPD, yeast extract peptone dextrose; DTT, dithiothreitol.

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different enzymes, such as xylanase, cellulase, cellobiose dehydrogenase from filamentous fungi *T. clypeatus* [5,8]. TKP has served as a good substitute for pectin as a gelling agent & could also efficiently produce pectinase in submerged fermentation using *Aspergillus* sp [9,10].

The conversion of lignocellulosic biomass into soluble sugars requires the action of set of synergistically acting large multienzyme complex. This complex includes endoglucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases (cellobiase), hemicellulases, xylanase, β-xylosidase, acetyl xylan esterase, arabinofuranosidase, including certain phenolic esterases, and some accessory enzymes (pectinase, laccase, endo-mannanases) are needed to expose cellulose microfibrils covered by hemicelluloses [11,12]. Production of economically efficient second generation ethanol requires hyper-producing fungi capable of secreting a large array of enzymes in the secretome, a suitable growth medium for maximum enzyme production and optimized fermentation conditions.

It is well-established that culture conditions affect significantly the production of cellulases and hemicellulases. Highest cellulase yields have been obtained on cellulose containing carbon sources [13,14]. However many reports have found that agricultural wastes are superior at inducing biomass degrading enzymes than pure cellulose in many fungi [15,16]. This fungus presented a very interesting model where rate of secretion of carbohydrate degrading enzymes is directly influenced by presence of certain ingredients in the media [17,18]. The fungus produced high titers of β-glucosidase when grown in presence of 2-deoxyglucose in cellobiose medium [19]. β-Glucosidases play a vital role in regulating the cost of bioethanol production since it increases the efficiency of cellulose hydrolysis by preventing accumulation of cellobiose & hydrolyzing it into fermentable glucose units. Commercial *Trichoderma reseei* cellulase cocktail is β-glucosidase-deficient or exhibits low β-glucosidase activity (despite recent developments) and requires supplementation of β-glucosidase from other sources [20]. In this study we successfully formulated a media composition for enhanced production of β-glucosidase and cellulase along with other hemicellulolytic enzymes in equally high titers. To the best of my knowledge this is the first report where such diverse arrays of enzymes are secreted by a fungus in the culture filtrate without any genetic manipulation.

The aim of the current work is to get a deeper understanding of the cascades of enzymes housed by *T. clypeatus* and to screen hydrolytic enzymes and/or enzyme complexes needed for bioethanol production. The identified enzymes provided valuable insight into how *Termitomyces* subsist on agricultural based carbon source and induce lignocellulolytic enzymes production to be used for efficient plant biomass degradation. Most of the fungal proteome reported till date has been performed with ascomycetes and the fruiting bodies. Among the few reports concerning basidiomycetes, mostly *Phanerochaete chrysosporium* is studied since the genome of this organism is available from 2004 [21,22]. Few other secretomes e.g., from *Ganoderma lucidum* on sugarcane bagasse [23] and *Trametes trogii* on Populus wood [24] have been recently reported. This is the first report of proteomic analysis of the secretome of *T. clypeatus* in mycelial culture grown in agroresidue.

2. Material and methods

2.1. Preparation of agro residues

Agroresidues like tamarind kernel powder, corncob, were obtained from local market. Steam pretreated sugarcane bagasse was received as gift from CSIR-National Chemical Laboratory, Pune India. All fine chemicals used in this study were purchased from Sigma. Cellobiose, cellotriose, cellotetrose and xylobiose were purchased from Megazyme International Ireland, Limited (Bray Business Park, Bray, Co., Wicklow, Republic of Ireland) Novozyme188 and Cellulase (catalogue no. C-0901 from *Penicillium funiculosum*, activity 10 $U mg^{-1}$) was purchased from Sigma Chemical Co., USA.

2.2. Strain, media and mycelial growth

T. clypeatus (MTCC 5091) used in this study was maintained in our laboratory as earlier reported [24]. The fungal mycelia was cultured in 100 ml/in 500 ml shake-flasks containing (%, w/v), Glucose—5, Malt extract—5, Potato extract—20%. Other media components used were yeast extract 0.5, ammonium di hydrogen phosphate 2.5, and other micronutrients (%, w/v) such as $CaCl_2{\cdot}2H_2O$, 0.037; KH_2PO_4, 0.087; $MgSO_4{\cdot}7H_2O$, 0.05; Boric acid, 0.057; $FeSO_4{\cdot}7H_2O$, 0.025; $MnCl2{\cdot}4H_2O$, 0.0036; $NaMoO_4{\cdot}4H_2O$, 0.0032; $ZnSO_4{\cdot}7H_2O$ 0.03 and inoculum (10%) was added in the culture. The experiments were carried out in triplicates of 100 ml/500 ml flasks for 7 days at pH 5.0, 30 °C under shaking condition (250 rpm) in orbital shaker.

For determination of dry weight, samples collected were centrifuged at 9000 × *g* for 30 min, and the resulting supernatant was filtered through a 0.45 m membrane filter (Millipore). The dry weight of mycelia was measured after repeated washing of the mycelial pellets with distilled water and drying overnight at 70 °C to a constant weight.

2.3. Measurement of mycelial growth

Mycelial growth was measured by determining the glucosamine concentration after acid hydrolysis of mycelia following the method of Desgranges et al. [25]. Mycelia collected after centrifugation (9000 × *g* for 30 min) and repeated washing were freeze-dried and treated with 10 ml of 10 M HCl for 16 h at 20 °C. Hydrolysis was completed by autoclaving the acid-hydrolyzed mycelia at 121 °C for 2 h and the total volume was adjusted to 50 ml with distilled water. The concentration of glucosamine–HCl released after hydrolysis of the mycelia was measured colorimetrically according to the method of Nilsson and Bjurman [26].

2.4. Assay of enzyme activities

β-Glucosidase (E.C 3.2.1.21) assay was carried out in the reaction mixture containing 2 mM pNPG as substrates in 0.1 M sodium acetate buffer (pH 5.0) and an appropriate amount of the enzyme at 45 °C [19]. The reaction mixture (0.5 ml) was incubated at 50 °C for 30 min, and the reaction was terminated by the addition of 0.25 ml of 1 M Na_2CO_3 solution. One unit of β-glucosidase activity was defined as the amount of enzyme liberating 1 μM of *p*-nitrophenol (*p*NP) per minute under the assay conditions. Similarly β-xylosidase (E.C 3.2.1.37) [17], cellobiohydrolase (CBH) [27], α-L-arabinofuranosidase (EC 3.2.1.55) [28] and acetyl (xylan) esterase (EC 3.1.1.6) [28] were assayed respectively using *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), PNPAF and PNPA as substrates. Carboxymethylcellulase (CMCase) activity was assayed using carboxymethyl cellulose (CMC) as substrates by DNS method [8,29]. For xylanase assay, 0.9 ml xylan (1%, w/v) was incubated with 0.1 ml of enzyme (diluted appropriately) at 50 °C for 10 min, and the reducing sugar was determined by the DNS method at 540 nm with xylose as standard [30]. Filter paper degrading activity (FPA) was assayed according to the method of Ghosh [31] using 1 × 6 cm (50 mg) Whatman No.1 filter paper strips as substrate. Cellobio-dehydrogenase activity was measured according to Saha et al. [5] following the decrease in absorbance of DCIP for 5 min at 520 nm and 25 °C. One unit (U) of enzyme activity

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