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RESEARCH ARTICLE

Evaluation of 18 isolates of basidiomycetes for Lignocellulose degrading enzymes

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Abstract: Since fossil fuel resources are limited there is a necessity to produce alternative types of fuel that are renewable and eco-friendly. Basidiomycetes are potential sources of enzymes that can be used for biofuel production. The current study aimed to isolate basidiomycetes from Sri Lanka, screen them for lignocellulose degrading enzymes, namely cellulase, xylanase, laccase, Mn peroxidase and lignin peroxidase and study the effect of potential inducers of laccase production. Among the eighteen basidiomycetes isolated, *Pycnoporus* sp. produced the highest cellulase activity (0.23 FPU/ml) whereas *Phlebiopsis* sp. produced the highest xylanase activity (5.4 U/ml). *Earliella scabrosa* produced the highest laccase (91.2 U/l) and Mn peroxidase (17.5 U/l) activities. Lignin peroxidase activity was not detected from the isolates. Effect of alkali lignin, Cu^{2+} and rice bran, three potential inducers, on laccase production by *E. scabrosa*, *Pycnoporus* sp. and *Trametes hirsuta* (M40) was studied. Results indicated that alkali lignin (2 g/l) significantly increased laccase production from *Pycnoporus* sp. and *T. hirsuta* (M40) while Cu^{2+} increased laccase production from *E. scabrosa* and *T. hirsuta* at 200 µM. Use of rice bran (10 g/l) resulted in higher laccase production from *E. scabrosa* and *Pycnoporus sp.* High laccase activity (79600 U/l) was obtained from *E. scabrosa* by using 50 g/l of rice bran and by extending the incubation period to 18 days. The study concluded that some of the basidiomycetes isolated can produce significant lignocellulose degrading enzyme activities.

Keywords: Basidiomycetes, Cellulase, Xylanase, Laccase, Mn peroxidase.

INTRODUCTION

Fossil fuel resources are limited and their utilization leads to global warming and other environmental problems. Hence, it is necessary to produce alternative types of fuel that are renewable and eco-friendly. Biofuel production from plant cell wall polysaccharides is an attractive alternative to fossil fuel. It involves saccharification of the polysaccharides and fermentation of the resultant sugars to the desired biofuel. Due to lignification of plant cell walls, treatment with enzymes alone results in slow and incomplete saccharification (Zhang *et al*., 2007). Hence, currently the plant materials are subjected to thermo-chemical pre-treatment prior to enzymatic hydrolysis (Margeot *et al*., 2009). The costs of pre-treatment and saccharifying enzymes add to the overall cost of production, thereby negatively affecting its economic viability. Biological pre-treatment with lignin degrading enzymes is a potential alternative that may be carried out under milder conditions at lower cost.

The enzymes mainly required for the saccharification of plant polysaccharides are cellulases and xylanases (Bischof *et al.*, 2016). Lignin degrading enzymes include laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) (Pollegioni *et al.*, 2015). While laccase requires oxygen for its activity, MnP and LiP require hydrogen peroxide. Auxiliary enzymes such as aryl alcohol oxidase and glyoxal oxidase produce the hydrogen peroxide required for their activity (Daou *et al.*, 2016). Since many basidiomycetes are known to degrade both cellulose and lignin efficiently (Zhu *et al.*, 2016), they may be used as sources of enzymes for both biological pre-treatment and saccharification.

Degradation products of lignin act as the natural inducers of laccase (Janshekar *et al*., 1981). Cu^{2+} is an integral component of laccase and induces laccase production in many fungal species (Piscitelli *et al*., 2011). Rice bran contains 0.9 - 2.9% of γ-oryzanols, which are predominantly esters of trans-ferulic acid with

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phytosterols (Lerma-Garcia *et al*., 2009). Ferulic acid also induces laccase in certain basidiomycetes (Piscitelli *et al*., 2011).

The objective of the current study was to isolate basidiomycetes from the Central Province of Sri Lanka and screen them for the production of lignocellulose degrading enzymes, namely cellulase, xylanase, laccase, Mn peroxidase and lignin peroxidase, under submerged culture conditions. Further, effect of three potential inducers, namely alkali lignin, Cu^{2+} and rice bran, on laccase production by three selected basidiomycetes was investigated. It was hypothesized that the potential inducers may significantly increase laccase production by the selected isolates.

MATERIALS AND METHODS

Reagents and Instrumentation

Alkali lignin, $CuSO_4.5H_2O$ and the salts used for media preparation were from Sigma (USA). Potato dextrose agar and yeast extract were from HiMedia (India). ITS1 and ITS4 oligonucleotide primers were from Integrated DNA Technologies Inc. Mytaq™ DNA polymerase and Mytaq™ reaction buffer (5x) used for the polymerase chain reaction were from Bioline (Australia). UV-Vis spectrophotometry was carried out by using Shimadzu UV-2450 UV-Vis spectrophotometer.

Isolation and preservation of basidiomycetes

Fruiting bodies of basidiomycetes were collected from the Central Province of Sri Lanka (5° 54' N - 9° 52' N latitude and 79° 39' E - 81° 53' E longitude). A piece of tissue was removed from the stipe of the basidiocarp and surface-sterilized with 70% ethanol for one minute followed by 3% H_2O_2 for 30 seconds. The tissue was subsequently washed with sterile distilled water and placed on potato dextrose yeast extract agar (PDYA) with gentamicin (50 mg/l), chloramphenicol (50 mg/l) and carbendazim (1 mg/l). The basidiomycetes were further purified by successive plate transfers, maintained on PDYA slopes at 2 – 8 °C and preserved in sterile distilled water in airtight screw capped tubes at room temperature (de Capriles *et al.*, 1989).

Identification of the basidiomycetes

The basidiomycetes were identified based on morphological characteristics and/or DNA sequencing of the ITS region. Genomic DNA was extracted from the basidiomycetes according to Cenis (1992), with an additional step of freezing and thawing prior to homogenization of the cells. PCR amplification was performed with ITS1 and ITS4 primers (White *et al.*, 1990). The PCR products were sequenced at Macrogen Inc. (South Korea). Consensus sequences were made from the forward and reverse sequences and were analysed for similarity with existing nucleotide sequences in the NCBI and ENA databases by using the BLAST tool.

Enzyme production for screening

Cellulase and xylanase activities were evaluated in a medium modified from Peláez *et al.* (1995). It contained (per litre): cellulose, 10 g; glycerol, 0.5 g; KNa tartrate tetrahydrate, 3 g; $(NH_4)_2HPO_4$, 1 g; yeast extract, 1 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.3 g; $FeSO_4.7H_2O$, 5 mg; trace element solution, 1 ml. Trace element solution contained [per 100 ml]: $ZnSO_4.7H_2O$, 100 mg; $CuSO_4.5H_2O$, 25 mg; $MnSO_4.H_2O$, 100 mg; $CoCl_2.6H_2O$, 100 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 10 mg; $Na_2B_4O_7.10H_2O$, 10 mg. pH of the medium was adjusted to 5.6. Laccase, MnP and LiP activities were evaluated in a medium modified from the afore-mentioned medium by replacing cellulose and glycerol with glucose (10 g/l). The basidiomycetes were grown on PDYA and circular discs of growth (diameter = 5 mm) were cut from near the edge of each colony by using a sterile cork borer. Each disc was radially cut into 8 equal pieces with a sterile scalpel, in order to reduce the pellet size of growth under submerged condition. Growth from three discs from each basidiomycete was inoculated to 20 ml of the medium in 100 ml Erlenmeyer flasks and incubated at 28 °C in the dark with shaking at 100 rpm. After 7 days, the cultures were filtered with Whatman No.1 filter paper and the filtrates were used for the enzyme assays.

Enzyme assays

Total cellulase assay was performed with Whatman No.1 filter paper as the substrate (Mandels *et al.*, 1976). Reducing sugars formed were measured by using 3,5-dinitro salicylic acid (DNS) reagent (Miller, 1959) with glucose standards. The total cellulase activity was

expressed as filter paper units per ml (FPU/ml) which is the amount of reducing sugar, in micromoles, released by 1 ml of undiluted enzyme per minute. Xylanase activity was measured according to Gottschalk *et al.* (2010) with beechwood xylan (Sigma) as the substrate and the reducing sugars formed were measured by using DNS reagent with xylose standards. Laccase activity was measured by using ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonate) as the substrate (Bourbonnais *et al.*, 1995). The assay mixture (3 ml) contained 0.5 mM ABTS, 100 mM sodium acetate (pH 5.0) and 300 µl of culture supernatant. The oxidation of ABTS was followed by measuring the absorbance at 420 nm at one second intervals for 60 seconds at room temperature. The absorbance readings were plotted against time as shown in Figure 1. If the absorbance readings became non-linear within 60 seconds, then the culture supernatant was diluted in distilled water to obtain tenfold dilutions and the diluted supernatant was used for the assay. The enzyme activity of undiluted supernatant was calculated by multiplying the activity of the diluted supernatant by the dilution factor. The amount of oxidized product formed was calculated by using the Beer-Lambert equation $A = \varepsilon cl$, where A= absorbance, ε= molar absorptivity, c = concentration of the analyte and l= path length of the cuvette. Molar absorptivity of 36 mM^{-1} cm^{-1} (Bourbonnais *et al.* 1995) was used for the calculations. Manganese peroxidase (MnP) activity was measured by using phenol red as the substrate (Peláez *et al.*, 1995). The reaction mixture (3 ml) contained 0.1 mM H_2O_2, 0.1 mM $MnSO_4$, 0.1 mg/ml phenol red, 100 mM sodium tartrate buffer (pH 5.0) and 1 ml of crude enzyme. Reaction was stopped by the addition of 120 µl of 2 M NaOH after 30 minutes. The increase in absorption at 610 nm was measured against the corresponding enzyme blank (to which 120 µl of 2 M NaOH was added prior to the addition of the reaction mixture). The amount of phenol red oxidized was calculated by using the Beer-Lambert equation and the molar absorptivity ε610 = 4460 $M^{-1}cm^{-1}$ (Peláez *et al.*, 1995). Lignin peroxidase activity was measured by using methylene blue as the substrate (Magalhães *et al.*, 1996). The assay mixture (3 ml) contained 0.1 ml of 1.2 mM methylene blue, 0.6 ml of 0.5 M sodium tartrate (pH 4.0) and 2.2 ml of enzyme. Reaction was started by the addition of 0.1 ml of 2.7 mM H_2O_2. Conversion of the dye to Azure C was determined by measuring the absorbance at 664 nm immediately after the addition of H_2O_2 and again after 30 minutes. Enzyme activity was expressed as the decrease in absorbance at 664 nm per minute.

Effect of potential inducers on laccase production

To determine the effect of alkali lignin and Cu^{2+} on laccase production, alkali lignin (0.5 g/l, 2 g/l) or $CuSO_4.5H_2O$ (50µM, 200µM) were included in the medium. To determine the effect of rice bran on laccase production, glucose in the medium was reduced to 1 g/l and finely ground and sieved (80 µm) rice bran was included at 10 g/l. Glucose was included in the medium at 1 g/l in order to shorten the possible lag phase in the growth of the isolates.

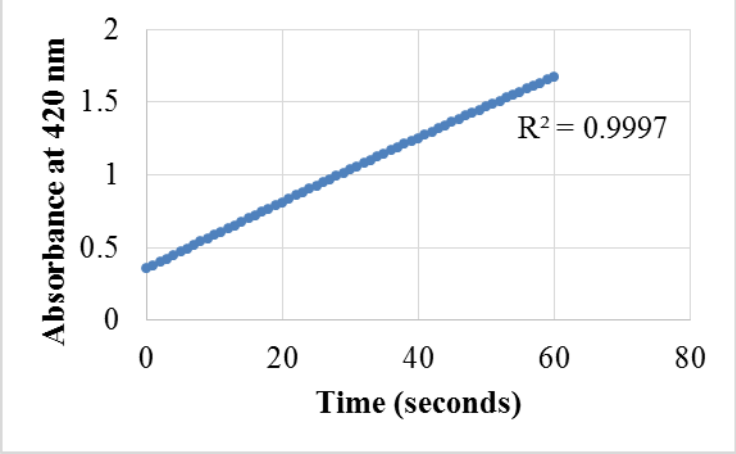

Figure 1: A plot of absorbance at 420 nm against time during oxidation of ABTS by laccase present in a culture supernatant.

Statistical analysis

Statistical analyses were carried out by using Minitab®17 statistical software. Comparisons were made by ANOVA with Tukey's method or by two sample t-test ($\alpha = 0.05$). Culturing of basidiomycetes and enzymatic degradations were carried out in triplicates.

RESULTS AND DISCUSSION

Isolation, identification and screening of enzyme activities of the basidiomycetes

In total, 18 basidiomycetes were isolated (Figure 2). Their identities and the enzyme activities when grown in the screening media are shown in Table 1. Cellulase activity was detected from 16 out of the 18 basidiomycetes, while xylanase activity was detected in all. *Pycnoporus* sp. produced the highest cellulase activity of 0.23 FPU/ml, which is comparable to the activities produced by the wild type filamentous fungus *Trichoderma reesei* QM6a (Mandels, 1975), which is the parent strain of the mutant strain *Trichoderma reesei* RUT-C30, used for industrial production of cellulases (Peterson and and Nevalainen, 2012). The highest xylanase activity of 5.4 U/ml was produced by *Phlebiopsis* sp. Other isolates that produced high xylanase activities included *Schizophyllum commune* (M1) (5.12 U/ml) and *Pycnoporus* sp. (4.59 U/ml).

Laccase activities greater than 10 U/l were produced by *Earliella scabrosa*, *Polyporus* sp. (M20), *Pycnoporus* sp., *Trametes hirsuta* (M29), *T. hirsuta* (M36) and *T. hirsuta* (M40). Mn peroxidase (MnP) activities greater than 10 U/l were produced by *Coprinopsis* sp., *E. scabrosa* and *T. hirsuta* (M40). The highest laccase (91.2 U/l) and MnP activities (17.5 U/l) were both produced by *E. scabrosa*. LiP activity was not detected from any of the isolates. In a similar study, Peláez *et al.* (1995) also found no LiP activity within a larger collection of basidiomycetes. Thus, it appears LiP activity is rare among the basidiomycetes and limited to a few species such as *Phanerochaete chrysosporium* (Janusz *et al.*, 2013).

Effect of alkali lignin, Cu^{2+} and rice bran on laccase production

The effect of alkali lignin, Cu^{2+} and rice bran on laccase production by three selected basidiomycetes (*E. scabrosa*, *Pycnoporus* sp. and *T. hirsuta* (M40)) was studied. The three basidiomycetes were selected based on their high laccase and MnP activities during the initial screening (Table 1).

Alkali lignin significantly increased laccase production by *Pycnoporus* sp. and *T. hirsuta* (M40) at 2 g/l but not at 0.5 g/l (Figure 3). It had no significant effect on laccase production by *E. scabrosa*. *Pycnoporus* sp. appeared to degrade alkali lignin in culture, as indicated by the reduction in brown colour of the medium. However, it could not be verified by spectrophotometry according to Janshekar *et al.* (1981) due to interference by pigments, proteins and other compounds secreted by the fungus.

(a) (b) (c) (d)

Figure 2: Some of the isolated basidiomycetes (not to scale). (a) *Schizophyllum commune* (M1) (~ 1/2 x), (b) *Pycnoporus* sp. (M21) (~ 1/4 x) (c) *Trametes hirsuta* (M29) (~ 1/10 x), (d) *Microporus xanthopus* (M25) (~ 1/10 x).

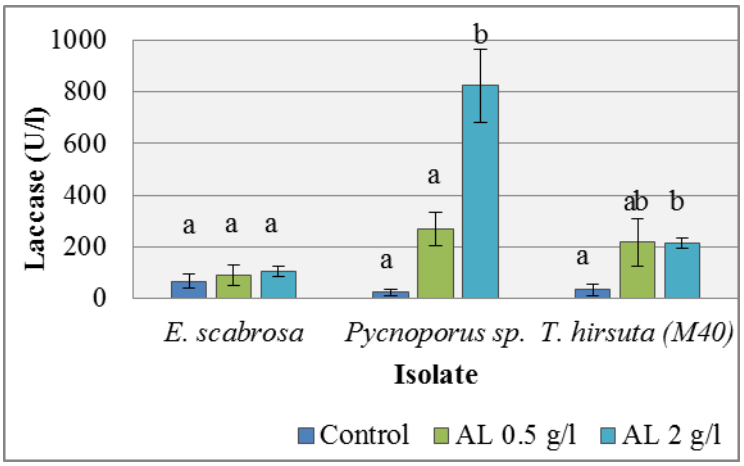

Figure 3: Production of laccase by the three selected basidiomycetes with different concentrations of alkali lignin. AL – Alkali lignin. Error bars indicate standard errors of the means of triplicates. For individual isolates, values not sharing the same letter are significantly different.

Table 1: Identities and enzyme activities of the basidiomycetes collected and grown *in vitro.*

Basidiomycete	Cellulase (FPU/ml)	Xylanase (U/ml)	Laccase (U/l)	MnP (U/l)
*Schizophyllum commune** (M1)[a]	0.025	5.12	0.6	0
*Schizophyllum commune** (M3)[a]	0.016	0.88	0.4	0
Coprinopsis sp.* (M5)	0	0.08	2.7	10.45
Phlebiopsis sp.* (M7)	0.074	5.41	0	0
Marasmius sp. (M12)	0.054	0.70	0.8	0.29
*Earliella scabrosa** (M14)	0	0.16	91.2	17.5
Trametes sp. (M15)	0.082	0.97	0.5	0
Unidentified (M17)	0.020	2.50	0.5	0
Polyporus sp. (M20)	0.024	0.30	79.9	0.91
Pycnoporus sp.* (M21)	0.232	4.59	33.1	0.64
Microporus xanthopus (M25)	0.073	2.60	0	0
*Trametes hirsuta** (M29)[b]	0.082	0.93	60.4	0.58
*Annulohypoxylon stygium** (M31)	0.033	3.80	0.5	0
*Schizophyllum commune** (M33)[a]	0.029	1.60	0	0.08
*Trametes hirsuta** (M36)[b]	0.074	1.67	15.1	0
Lentinus sp.* (M37)	0.018	0.10	0.6	0.16
Polyporus sp. (M39)	0.018	0.16	0.2	0.19
*Trametes hirsuta** (M40)[b]	0.059	1.24	69.3	14.87

* Identifications made with the aid of the DNA sequence of ITS region. Scientific names are followed by laboratory codes shown within brackets. a, b - Isolated from different locations (Knuckles, Gannoruwa and Pilimathalawa and hence presumed to be different strains of the same species.

Presence of Cu^{2+} in the medium resulted in significantly higher laccase production by *E. scabrosa* and *T. hirsuta* at 200 µM, but not at 50 µM (Figure 4). No effect of Cu^{2+} was observed on *Pycnoporus* sp. Cu^{2+} is known to inhibit fungal growth at higher concentrations.

Laccase production by *E. scabrosa* and *Pycnoporus* sp. was significantly higher when rice bran (10 g/l) was used as the main source of carbon (Figure 5). The increase was profound with *E. scabrosa*, which produced a mean laccase activity of 13060 U l^{-1} after 7 days of cultivation. In order to determine whether higher

concentration of rice bran would lead to higher laccase production by *E. scabrosa*, the fungus was grown in the medium with 50 g/l rice bran and 1 g/l of glucose. After 7 days, a mean laccase activity of 4 U/l was obtained from the culture. However, the mean laccase activity increased to 79600 U/l after 18 days of culturing. Laccase biosynthesis is often regulated by nutrient carbon and nitrogen levels (Piscitelli *et al*., 2011). Hence, the delay in laccase production by *E. scabrosa* with 50 g/l rice bran may possibly be due to higher initial carbon and nitrogen levels.

During the study, it was observed that higher pellet sizes of the fungi in submerged cultures were associated with lower laccase production. It was probably due to reduced oxygen transfer to the interior of the larger pellets. Reports on parameters affecting laccase production by other basidiomycetes support this notion (Tinoco-Valencia *et al.*, 2014). Hence, laccase production by *E. scabrosa* with rice bran may be further enhanced by optimizing the pellet size, aeration and other parameters of submerged cultivation, such as rotations per minute and incubation temperature.

In addition to biofuel production, cellulase and laccase have other industrial applications such as bio-stone washing of denim and clarification of fruit juices.

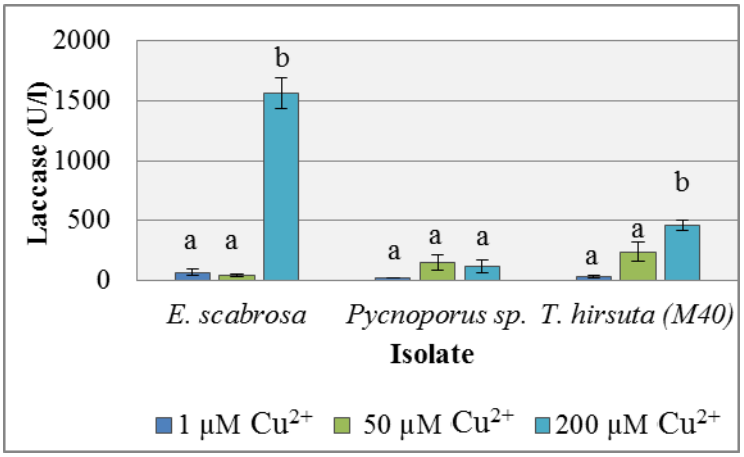

Figure 4: Production of laccase by the three selected basidiomycetes with different concentrations of Cu^{2+}. Error bars indicate standard errors of the means of triplicates. For individual isolates, values not sharing the same letter are significantly different.

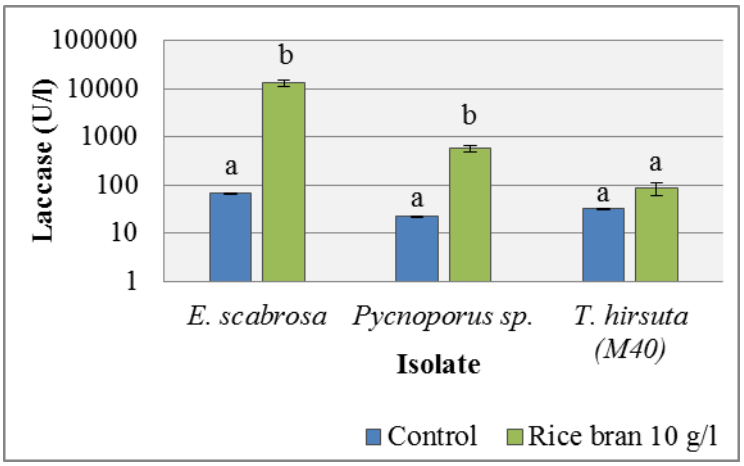

Figure 5: Production of laccase by the three selected basidiomycetes with and without rice bran. Note that the y axis is on a logarithmic scale. Error bars indicate standard errors of the means of triplicates. For individual isolates, values not sharing the same letter are significantly different.

CONCLUSIONS

Most of the basidiomycetes that were isolated produced cellulase and xylanase activities while several of them produced laccase and MnP activities (Table 1). *Pycnoporus* sp. produced the highest cellulase activity along with high xylanase activity. It also produced laccase and MnP activities and degraded alkali lignin in culture. Thus, *Pycnoporus* sp. may potentially be used as a single source of enzymes for saccharification as well as delignification. Alkali lignin significantly increases laccase production by *Pycnoporus* sp. and *T. hirsuta* (M40) at 2 g/l whereas Cu^{2+} causes significantly higher laccase production from *E. scabrosa* and *T. hirsuta* at 200 μM. Rice bran (10 g/l) causes higher laccase production from *E. scabrosa* and *Pycnoporus* sp. Whether these effects are due to induction needs further study by quantification of the mRNAs coding for the laccases. *E. scabrosa* produced very high laccase activity (79600 U/l) when cultured in 50 g/l rice bran. Thus, it can potentially be used for industrial production of laccase. Rice bran could be used as a cheap carbon source for high laccase production from *E. scabrosa*.

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