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Screening of lignocellulolytic fungi for hydrolyzation of lignocellulosic materials in paddy straw for bioethanol production

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ABSTRACT

Aims: Paddy straw is known to have lignocellulosic materials such as cellulose and hemicellulose which can be readily converted into fermentable sugar for production of bioethanol via simultaneous saccharification and fermentation (SSF). In order to produce ethanol competently, the degradation of biomass by cellulase and highly ethanol-producing microorganism in fermentation process are necessarily needed. However, there is lacking in cellulose degrading organism in producing adequate amount of lignocellulosic enzyme. Therefore, the screening and selection for the best fungi to hydrolyze the lignocellulosic materials as well as forming consortium between two species of fungi has become the main focus.
Methodology and results: Thirteen strains of fast-growing fungi were tested qualitatively for cellulase (congo red staining) and polyphenol oxidase (Bavendamm test). All tested strains displayed lignocellulolytic fungi characteristics. The selection was narrowed down by quantitative assay on endoglucanase, exoglucanase, β-glucosidase and xylanase and the highest cellulases enzyme producer were *Trichoderma asperellum* B1581 (3.93 U/mL endoglucanase; 2.37 U/mL exoglucanase; 3.00 IU/mL β-glucosidase; 54.87 U/mL xylanase), followed by *Aspergillus niger* B2484 (5.60 U/mL endoglucanase; 1.08 U/mL exoglucanase; 1.57 IU/mL β-glucosidase; 56.85 U/mL xylanase). In compatibility test, both *T. asperellum* B1581 and *A. niger* B2484 were inoculated on the same Petri dish for 4 days and the interaction showed by the two species was mutual intermingling.
Conclusions, significance and impact of study: Both *T. asperellum* B1581 and *A. niger* B2484 produced the highest cellulase enzyme. Since both strains can co-exist and produce enzymes that complete each other, a fungal consortium was suggested to increase the yield of sugars in saccharification process.

Keywords: Cellulose, hemicellulose, consortia, qualitative assay, quantitative assay

INTRODUCTION

In December 2017, Food and Agriculture Organization (FAO) has increased its estimation of paddy production across the globe in the year 2017 by 2.9 million tonnes to 759.6 million tonnes and China remains the largest paddy producer in the Asia region (FAO, 2018). The increased demand of rice production has led to an increase amount of world's paddy straw production, with an estimation of at least 731 million tons per annum (Wi *et al*., 2013). Paddy straw is the residual of rice production and has become one of the world's most commonly available lignocellulosic waste materials (Saini *et al*., 2015). Current disposal method by burning not only resulted in reducing the air quality (Rosmiza *et al*., 2014) but also wasting the potential of harvesting valuable carbohydrates for biofuel production as the paddy straw contains about 45-75% of complex carbohydrates (Fan *et al*., 2013). Bioethanol is a renewable energy source that have prospect in handling current world energy issues and the deteriorating quality of environment (Aditiya *et al*., 2016). Now, most technologies involve in biofuel production are focusing on turning lignocellulose biomass into transportation fuels (Bakker *et al*., 2013).

The first generations of biofuels are originated from crop plants, but they produced limited biofuel yields and threatening on food security (Aro, 2016). Alternative feedstock also known second-generation feedstock, are recovered from woody biomass which are more energy efficient, flexible and not competing with the human's food resources (Havlik *et al*., 2011). An estimation of 442 billion liters of bioethanol can be produced from lignocellulosic waste material, which the amount is 16 times higher than the real amount of bioethanol produced globally (Sarkar *et al*., 2012).

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The bioconversion of biomass into bioethanol involves several processes such as the pretreatment, saccharification and fermentation (Oberoi *et al*., 2012). Cellulolytic enzymes involve in hydrolysis of lignocellulosic materials (saccharification), which are commonly break down by cellulolytic bacteria and fungi (Belal, 2013). The by-products of the saccharification process are generally reducing sugars including glucose. According to Okamoto *et al.* (2011), several filamentous fungi has been explored for their capability in producing ethanol from biomass, such as the genera *Mucor*, *Rhizophus*, *Neurospora*, *Aspergillus*, *Fusarium*, *Monilia* and *Trichoderma*. Most of the *Trichoderma* sp. are fast-grower, produce large amount of conidia and variety of valuable enzymes such as cellulase (Khan *et al*., 2007). Cellulase is also known as cellulolytic enzymes, of which three classes are identified on the basis of the mode of the substrate specificities and enzymatic actions: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74 and EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Teeri, 1997; Kuhad *et al*., 2016). Only with the synergism of the above three enzymes makes the complete hydrolysis of cellulose into glucose (Gupta *et al*., 2012). An efficient and cost-effective enzyme system should contain balanced activities of cellulases (both endo- and exo-glucanase), β-glucosidase, and xylanase, and the system should also have high titer of enzyme activities to balance the cost of ethanol production (Brijwani *et al*., 2010).

The simultaneous saccharification and fermentation (SSF) processes involves enzymatic saccharification of cellulose with fermentation of glucose to ethanol (Kusmiyati *et al*., 2018). It has become favorable process as it improved the yield of ethanol via reducing the product inhibition excreted and also removes the need for separate reactors, which results in cost reduction and reduce the risk of contamination (Wang *et al*., 2013; Narra *et al*., 2015).

Regardless the fact that some of the fungal strains have the benefits of being lignocellulolytic and thermostable, most of these fungal strains failed to produce adequate amounts of lignocellulolytic enzymes, which are essentially required for efficient bioconversion process (Dashtban *et al*., 2009). According to Kausar *et al.* (2010), mixed cultures or consortium are able to stimulate colonization of the substrate by increasing the production of enzyme, withstand contamination by other microbes and results in high yield of sugars. However, the selection of suitable cellulase for hydrolyzation process of lignocellulolytic materials is very difficult because each biomass has structural and enzymatic activities differences (Takano and Hoshino, 2018). Hence, finding appropriate lignocellulolytic fungi for degradation of paddy straw is necessarily needed. In order to address the foregoing issues, this study was commenced to screen filamentous fungi with an ability to secrete hydrolytic enzyme for substrate degradation and to test the compatibility between two different species of fungi in an attempt to produce better yield of sugars in saccharification process.

MATERIALS AND METHODS

Collection of fungi

Fast growing and molecularly identified fungi strains (Table 1) were obtained from Mycology Laboratory, Faculty of Science, Universiti Putra Malaysia and School of Biological Sciences, Universiti Sains Malaysia. All strains were grown on Potato Dextrose Agar (PDA) incubated at 28 ± 2 °C for 7 days.

Table 1: The list of fungi strain used and the source of origin.

Fungi species	Strain	Source	References
T. asperellum	B1581	Soil	Sharifah Siti Maryam *et al*., 2016
T. asperellum	B1584	Soil	Sharifah Siti Maryam *et al*., 2016
A. niger	B2484	Vietnamese coriander	Nur Ain Izzati *et al*., 2017
A. niger	C2472	Winged bean	Nur Ain Izzati *et al*., 2017
F. oxysporum	B633T	Tomato	Nur Baiti *et al*., 2016
F. oxysporum	B645T	Tomato	Nur Baiti *et al*., 2016
F. fujikuroi	688	Rice	-
F. fujikuroi	4851	Rice	-
F. fujikuroi	4872	Rice	-
C. lunata	P1221	Rice	Nur Ain Izzati *et al*., 2019
C. lunata	P1244	Rice	Nur Ain Izzati *et al*., 2019
C. eragrostidis	P1262	Rice	Nur Ain Izzati *et al*., 2019
C. eragrostidis	K872	Rice	Nur Ain Izzati *et al*., 2019

Qualitative assays of lignocellulolytic activity

Cellulose degradation

A 5-mm mycelial disc was cut using cork borer from the edge of fungal culture (7 days old) and transferred to a prepared media (carboxymethylcellulose sodium salt 20.0 g/L; agar 15.0 g/L) before incubated at 28 ± 2 °C for 7 days as proposed by Mukhlis *et al*. (2013). The plate was stained with 1 mg/mL Congo red solution and formation of halo ring (clear zonation) around the fungal colony indicates the cellulase activity of the fungal (Kausar *et al*., 2010). The diameter of halo ring was measured and enzymatic index (EI) were calculated based on formula of Enzymatic Index = Diameter of Halo Ring / Diameter of Growth Colony (Florencio *et al*., 2012).

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Bavendamm test for laccase detection

Presence of polyphenol oxidase (PPO) indicates the existence of laccase. A disc of fungal hyphae was placed at the center of the prepared media (Tannic acid 5.0 g/L; Malt extract agar 15.0 g/L; Difco Agar 20.0 g/L) and incubated for 7 days a 28 ± 2 °C in dark condition to determine the presence of polyphenol oxidase (PPO) activity (Thormann *et al*., 2002). PPO was used to indicate laccase activity on lignin and the diameter of brown pigmentation formed around fungal colony was measured generally after five days.

Quantitative assays

The quantitative assays were also carried out to determine the activity of cellulase and hemicellulase enzymes in order to narrowing down to the best fungi producing hydrolytic enzyme. An amount of 1×10^6 spore/mL were cultured in basal medium [$(NH_4)_2SO_4$ 1.4 g/L; KH_2PO_4 2.0 g/L; $CaCl_2$ 0.3 g/L; $MgSO_4 \cdot 7H_2O$ 0.3 g/L; $CoCl_2$ 2.0 g/L] added with 1 mL of trace element ($MnSO_4 \cdot H_2O$ 1.56 g/L; $FeSO_4 \cdot 7H_2O$ 5.0 g/L; $ZnSO_4 \cdot 7H_2O$ 1.4 g/L) for 7 days at 150 rpm and 30 ± 1 °C (Ja'afaru, 2013) before crude culture was collected for quantitative assay.

Endoglucanase, exoglucanase and xylanase assays

The cellulase and hemicellulase enzymes were assayed using 3, 5-dinitrosalicyclic acid (DNS) method (Miller, 1959). Carboxymethyl-cellulose (CMC), Avicel® PH-101 and xylan from corn were used as substrate for endoglucanase, exoglucanase and xylanase assay, respectively. An amount of 0.09 mL substrate-buffer (1% (w/v) substrate; 0.05 M sodium acetate pH 4.8) was mixed with 0.01 mL of crude enzyme and incubated at 50 ± 1 °C for 30 min before 0.1 mL DNS were added to stop the reaction (Das *et al.*, 2012; Ja'afaru, 2013). The absorbance was read at 540 nm and the amounts of sugar were determined using glucose standard (for endoglucanase and exoglucanase) and xylose standard (for xylanase). One Unit (U) of enzyme activity was defined as the release of 1µmol of reducing sugar per min.

β-glucosidase assay

A mixture of 0.025 mL of substrate-buffer (1% (w/v) 4-nitrophenol β-D glucopyranoside; 0.05 M sodium acetate pH 4.8) and 0.025 mL of crude enzyme were incubated at 50 ± 1 °C for 15 min before been added with 0.05 mL 1 M Na_2CO_3 to stop the reaction (Jeya *et al*., 2010). The absorbance was read at 400 nm and the amount of *ρ*-nitrophenol (*ρ*NP) released was quantified based on pNP standard. One International Unit (IU) of β-glucosidase activity was expressed as the amount of enzyme required to release 1 µmole of *ρ*NP per min under the assay conditions.

Compatibility test

Seven days old fungal mycelial discs of selected fungi were place on the same PDA with a gap of 4 cm from each other (Kausar *et al.*, 2010). The plate was incubated at 28 ± 1 °C. Their growth rate and pattern were observed everyday till full grown; which the fungi mycelia fully occupied the surface of the agar (approximately 5 days). Mode of interaction between the filamentous fungi was observed and identified using a key reference by Mohammad *et al.* (2011) as shown in Table 2.

Table 2: Modes of interaction between filamentous fungi in compatibility test.

No.	Interaction	Definition
1	Mutual intermingling	Both fungi successfully grow into each other without any macroscopic indication of interconnections.
2	Partial intermingling	One of the funguses grows into the other fungus either above/below without forming any inhibition zone.
3	Invasion/ replacement	One mycelium propagates into the other and begins to ingest the weaker one before colonize it.
4	Inhibition at contact point	The fungi almost make contact with each other with a fine segregation line (1 to 2 mm) between the two colonies.
5	Inhibition at distance	Inhibition at a distance of more than 2 mm

RESULTS AND DISCUSSION

Microorganisms in which secretes cellulase have a major role in the environment, as they have decaying ability of biomass, and hence creating a crucial relation in the carbon cycle (Zhang *et al*., 2006; Damaso *et al*., 2012). Many studies have been highlighted on fungi producing cellulases as they are abundant, easy to extract and some of the fungal cellulases have been used widely used for commercials (Liang *et al*., 2014). All thirteen strains of molecularly identified fungi (Table 1) were selected based on their origin and ability as fast-growing fungi. However, their lignocellulolytic characteristics have yet to be tested.

In early screening, qualitative assays were performed to screen the quality of cellulase and laccase secreted by the fungi. In the qualitative screening, all carboxymethyl-cellulose (CMC) was used to raise the molecular disorder of the densely arranged cellulosic network and to uncover the cellulose chains buried within the microfibrils (Arantes and Saddler, 2010). Meanwhile the inefficiency of Congo red dye to bind with mono or disaccharides leading to a visible clear zone formation known as halo ring (Gohel *et al*., 2014) and relatively the diameter of halo ring produced is proportionate with the level of cellulase

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activity. An enzymatic index of ≥ than 0.5 is considered to be the best producers of cellulase enzyme. As shown in Table 2, out of 13 strains assayed, only 8 were found to have index more than 0.5. The largest enzymatic index (EI) was produced by *Trichoderma asperellum* B1584 at the value of 0.88. While the smallest EI value was produced by *Curvularia eragrostidis* P1262 with 0.25. However, there was no significant difference between all the samples tested.

Theoretically in Bavendamm test, utilization of phenolic compounds such as gallic acid or tannic acid in nutrient agar by the white rot fungi produce a deeply brown colored zone around the mycelium and this zone has become an indicator for the quality of the fungi polyphenol oxidase (PPO) activity (Mushimiyimana and Tallapragada, 2014). The ratio of brown pigmentation to growth colony was measured (Table 3) and the largest ratio produced was 1.60 by *C. eragrostidis* P1262 but it was not significantly different from other samples. All *Curvularia* sp. showed their ability to degrade lignin with the presence of polyphenol oxidase (PPO) but somehow failed to produce a good response in Congo red assay. The quality of cellulase has become the key of fungi selection as the successfulness of the whole bioconversion process mainly depends on the sources and production of cellulolytic enzyme as well as the optimal conditions for catalytic activity (Ram *et al.*, 2014). Therefore, both *C. lunata* and *C. eragrostidis* has been removed from list for the quantitative assays. To narrow down the selection and precisely measures the cellulase and hemicellulase activity, the quantitative assays were done as a secondary screening for final selection.

Trichoderma asperellum B1581 came out as the best producer of exoglucanase and β-glucosidase at 2.37 ± 0.34 U/mL and 3.00 ± 0.15 IU/mL, respectively (Table 4). On the other hand, *Aspergillus niger* B2484 had the highest endoglucanase (5.60 ± 0.43 U/mL) and xylanase (56.85 ± 2.75 U/mL) and considerably high amount of exoglucanase as well as β-glucosidase (1.08 ± 0.17 U/mL; 1.57 ± 0.02 IU/mL); thus making them as one of best fungi producing cellulase along with *T. asperellum* B1581.

The high production of exoglucanase; also known as cellobiohydrolase (CBH) is essential as they represent 60% of the enzyme cocktail population; primary exocellulase and responsible to degrades cellulose into cellobiose (Brady *et al*., 2015). According to Wang *et al*. (2012), the optimized ratios of cellulolytic enzymes for the best saccharification of crystalline cellulose is 60% CBH I: 20% CBH II: 20% endoglucanase. β-glucosidase plays a significant role in bioethanol production to eliminate cellobiose inhibition (Wang *et al*., 2012) and it has become a conundrum in producing bioethanol.

Table 3: Qualitative assessment on fungi strains for selection of the best fungi producing hydrolytic enzymes.

Fungi Strain	Qualitative Assay	
	Cellulase (EI)**	Laccase (ratio)
A. niger B2484	0.78^{ab}	1.01^{f}
A. niger C2472	0.64^{abc}	1.00^{f}
C. eragrostidis P1262	0.25^{abc}	1.60^{a}
C. eragrostidis K872	0.26^{abc}	1.55^{ab}
C. lunata P1221	0.38^{c}	1.43^{abcd}
C. lunata P1244	0.36^{bc}	1.45^{abc}
F. fujikuroi 688	0.49^{abc}	1.32^{abcde}
F. fujikuroi 4872	0.58^{abc}	1.31^{abcde}
F. fujikuroi 4851	0.58^{abc}	1.18^{cdef}
F. oxysporum B633T	0.61^{abc}	1.13^{def}
F. oxysporum B645T	0.62^{abc}	1.10^{ef}
T. asperellum B1581	0.68^{abc}	1.29^{bcdef}
T. asperellum B1584	0.88^{a}	1.35^{abcdef}

Values are means of three replicates with ±SD.
*Means in each column with same superscript letter are not significantly different amongst themselves when Tukey test were used at 5% significance level
** Enzymatic Index (EI) is a measurement for cellulase activity

Table 4: Qualitative and quantitative assessment on fungi strains for selection of the best fungi producing hydrolytic enzymes.

Fungi Strain	Quantitative Assay			
	Endoglucanase (U/mL)	Exoglucanase (U/mL)	β-glucosidase (IU/mL)	Xylanase (U/mL)
A. niger B2484	5.60 ± 0.43^{a}	1.08 ± 0.17^{cdef}	1.57 ± 0.02^{b}	56.85 ± 2.75^{a}
A. niger C2472	1.83 ± 0.15^{d}	1.75 ± 0.20^{abc}	0.05 ± 0.03^{c}	27.77 ± 2.69^{d}
F. fujikuroi 688	0.98 ± 0.01^{e}	1.01 ± 0.14^{cdef}	0.09 ± 0.04^{c}	56.00 ± 2.82^{a}
F. fujikuroi 4872	2.14 ± 0.09^{d}	0.25 ± 0.14^{f}	0.03 ± 0.02^{c}	37.69 ± 1.39^{cd}
F. fujikuroi 4851	0.95 ± 0.04^{e}	1.17 ± 0.16^{bcde}	0	32.04 ± 9.19^{cd}
F. oxysporum B633T	1.10 ± 0.09^{e}	0.95 ± 0.23^{cdef}	0	43.41 ± 6.92^{abc}
F. oxysporum B645T	0.93 ± 0.06^{e}	2.04 ± 0.53^{ab}	0	42.28 ± 2.06^{abcd}
T. asperellum B1581	3.93 ± 0.05^{b}	2.37 ± 0.34^{a}	3.00 ± 0.15^{a}	54.87 ± 8.23^{ab}
T. asperellum B1584	1.69 ± 0.10^{d}	2.00 ± 0.49^{ab}	0.03 ± 0.02^{c}	34.55 ± 4.69^{cd}

Values are means of three replicates with ±SD.
*Means in each column with same superscript letter are not significantly different amongst themselves when Tukey test were used at 5% significance level.

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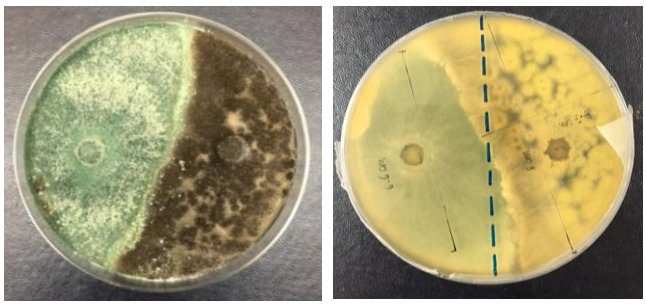
One of the major challenges in producing bioethanol is to search for a glucose tolerant β-glucosidase as they are the key component present in cellulase to complete the final step during cellulose hydrolysis and conversion process of cellobiose into glucose (Singhania *et al*., 2012). Currently, commercial cellulase was prepared using *T. reesei* due to its high activities of both exoglucanase and endoglucanase (Sohail *et al*., 2009). However, low levels of β-glucosidases in *T. reesei* have been complemented by mixing β-glucosidase from *A. niger* in *T. reesei* cellulolytic cocktail (Del Pozo *et al*., 2012; Rani *et al*., 2014). As in this study, combining these two species is expected to produce higher glucose yield for bioethanol production since *T. asperellum* B1581 obtained high exoglucanase and β-glucosidase activities while *A. niger* B2484 attained high endoglucanase and xylanase activities. In relation to qualitative assays, it was found that the diameter of clearance zone (in Congo red) exhibited almost a linear relationship with the enzyme activity.

Microbial interactions are usually referred as hostile, but they can also be supportive, related with a metabolic change of each partner and cause a modification in the arrangement of produced bioactive molecules (Benoit-Gelber *et al*., 2017). Consortia are a favorable method that could help to understand the synergistic effects between mixed enzymes for the biodegradation of lignocellulosic waste (Taha *et al*., 2015). The formation of consortia could be beneficial by eliminating problems such as incomplete synergistic enzymes and inhibition of end-product (Sheng *et al*., 2016). Through consortia, cellulase production was enhanced and the composition of cellulolytic enzymes was improved (Wen *et al*., 2005; Fang *et al*., 2013). Commercially, enzyme sets of *T. reesei* for cellulose degradation are often complemented with β-glucosidases from *A.niger* by combining *T. reesei*'s Celluclast® and *A. niger*'s β-glucosidase Novozym188® (Brink *et al*., 2014). A study by Liu *et al*. (2017) shows Napier grass could be utilized consortia of *T. reesei* and *A. niger* to produce glucose for bioethanol production by *Zygomonas mobilis.* However, according to Silva (2016), major obstacle is the compatibility since formation of consortia involves inter-relationship in managing the interaction, waste, space and resource to achieve optimum mutualism. Therefore prior to any fungi application as consortia for production, the compatibility test for fungal consortia is essential. In compatibility test, both *T. asperellum* B1581 and *A. niger* B2484 were grown on a same plate and *in-vitro* interaction between *T. asperellum* B1581 and *A. niger* B2484 after 5 days of incubation shown no negative interaction (Figure 1). The interaction was mutual intermingling growth where the fungus being observed is growing into the opposed fungus either above or below its colony. There was no visible boundary formed which indicates that both *T. asperellum* B1581 and *A. niger* B2484 can live together in the same culture. Since both of the strains can mutually co-exist, it was suggest mixing the cellulolytic cocktails of different fungi strains could enhance the end-product after saccharification process (Kalyani *et al*., 2013).



(a) (b)

Figure 1: shows the interaction between *T. asperellum* B1581 and *A. niger* B2484 after 5 days of incubation. (a) Front view (b) Rear view

CONCLUSION

Out of thirteen strains of filamentous fungi which were qualitatively and quantitatively assayed, the best two fungi strain were *T.asperellum* B1581 produces the highest exoglucanase and β-glucosidase (2.37 ± 0.34 U/mL; 3.00 ± 0.15 IU/mL respectively), while *A.niger* B2484 produces the highest endoglucanase and xylanase (5.60 ± 0.43 U/mL; 56.85 ± 2.75 U/mL respectively). Both of the fungi strain also showed their ability to formed mutual intermingling consortia in the *in-vitro* interaction of compatibility test. Since both of the strain can co-exist and produces enzymes that complete each other, the fungal consortium between *T.asperellum* B1581 and *A.niger* B2484 was suggested to increase the yield of sugars in saccharification process.

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