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ORIGINAL ARTICLE

Evaluation of co-culture of cellulolytic fungi for enhanced cellulase and xylanase activity and saccharification of untreated lignocellulosic material

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Abstract

Bioethanol production from lignocellulosic materials is hindered by the high costs of pretreatment and the enzymes. The present study aimed to evaluate whether co-cultivation of four selected cellulolytic fungi yields higher cellulase and xylanase activities compared to the monocultures and to investigate whether the enzymes from the co-cultures yield higher saccharification on selected plant materials without thermo-chemical pretreatment. The fungal isolates, *Trichoderma reesei* F118, *Penicillium javanicum* FS7, *Talaromyces* sp. F113, and *Talaromyces pinophilus* FM9, were grown as monocultures and binary co-cultures under submerged conditions for 7 days. The cellulase and xylanase activities of the culture filtrates were measured, and the culture filtrates were employed for the saccharification of sugarcane leaves, Guinea grass leaves, and water hyacinth stems and leaves. Total reducing sugars and individual sugars released from each plant material were quantified. The co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 and of *T. reesei* F118 with *T. pinophilus* FM9 produced significantly higher cellulase activities compared to the corresponding monocultures whereas no effect was observed on xylanase activities. Overall, the highest amounts of total reducing sugars and individual sugars were obtained from Guinea grass leaves saccharified with the co-culture of *T. reesei* F118 with *T. pinophilus* FM9, yielding 63.5% saccharification. Guinea grass leaves were found to be the most susceptible to enzymatic saccharification without pre-treatment, while water hyacinth stems and leaves were the least. Accordingly, the study suggests that fungal co-cultivation could be a promising approach for the saccharification of lignocellulosic materials for bioethanol production.

Keywords Bioethanol production · Lignocellulosic materials · Cellulases · Xylanases · Fungal co-cultures · Saccharification

Introduction

Fossil fuel resources are limited, and their consumption results in global warming (Soeder 2021). Hence, it is necessary to utilize renewable and eco-friendly alternatives. Even though ethanol produced from lignocellulosic materials such as invasive plants and crop wastes is an attractive alternative, its commercial production is hampered by lack of economic viability (Zhang 2019). The process of producing ethanol from lignocellulosic materials involves pretreatment, enzymatic hydrolysis, fermentation, and purification (Margeot et al. 2009). The costs of pretreatment and enzymes contribute significantly to the overall cost of production. Thus, reducing the cost of the enzymes by enhancing the enzyme activities and minimizing the need for pre-treatment could help improve the economic viability of the process.

Cellulases and xylanases are the major enzymes needed for the hydrolysis of lignocellulosic materials (Gupta and Bisaria 2018). Enzymes degrading cellulose are collectively known as cellulases (Wilson 2011). Based on their mode of action, they are classified into three types: exoglucanases, endoglucanases, and β-D-glucosidases. Exoglucanases act on the reducing or non-reducing terminals of cellulose, releasing either cellobiose (cellobiohydrolases, EC 3.2.1.91), or glucose (cellodextrinases, EC 3.2.1.74). Endoglucanases cleave randomly within amorphous regions,

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but not crystalline regions, of cellulose (EC 3.2.1.4). β-D-Glucosidases (EC 3.2.1.21) act on non-reducing ends of β-D-glucosyl residues, releasing beta-D-glucose (Lynd et al. 2002). Based on the mode of action of xylanases, endoxylanases and β-xylosidases are the two main enzymes which involve in xylan hydrolysis. Endoxylanases act on the homopolymeric backbone of 1,4-linked ß-D-xylopyranose and produce xylooligomers while β-xylosidases release xylose by acting on those xylooligomers (Ahmed et al. 2009).

Even though numerous fungi and bacteria are known to produce these enzymes (Bajaj and Mahajan 2019), only few fungal species produce high levels of cellulases capable of extensive saccharification of lignocellulosic material (Mandels 1975). The fungus *Trichoderma reesei* QM6 was identified as one of the best sources of cellulases for saccharification. Mutants of *T. reesei* QM6 with higher enzyme productivity such as *T. reesei* QM9414 and *T. reesei* RutC30, have been well-studied for industrial production of cellulases and xylanases (Peterson and Nevalainen 2012; Silva et al. 2020).

Different classes of cellulases, such as endoglucanases, exoglucanases and β-D-glucosidases, act synergistically during the hydrolysis of cellulose (Bajaj and Mahajan 2019). Similarly, different classes of xylanases act in synergy during the hydrolysis of xylan (Shallom and Shoham 2003). A single microbial isolate may not produce all the different types of cellulases and xylanases in adequate amounts. Hence, mixed cultures of fungi may produce higher cellulase and/or xylanase activities compared to the monocultures. For instance, culture filtrates of *Trichoderma reesei* lack secreted β-glucosidases (Messner et al. 1990), whereas mixed culture of *T. reesei* and *Aspergillus niger* produce adequate amounts of secreted β-glucosidases, resulting in higher cellulase activity (Ahamed and Vermette 2008). If such efficient microbial enzyme cocktails can be used in the process, together with minimal need of pretreatment, it would be beneficial in making the bioethanol production economically viable.

Therefore, the present study aimed to determine whether the binary co-cultivation of the selected cellulolytic fungal isolates, namely, *Trichoderma reesei* F118, *Penicillium javanicum* FS7, *Talaromyces* sp. F113, and *Talaromyces pinophilus* FM9, results in higher cellulase (FPU/mL) and/or xylanase (U/mL) activities compared to the corresponding monocultures and whether the culture filtrates from the co-cultures yield higher saccharification on three selected plant materials, namely sugarcane (*Saccharum officinarum*) leaves, Guinea grass (*Megathyrsus maximus*) leaves and water hyacinth (*Pontederia crassipes*) stems and leaves, without thermo-chemical pretreatment, compared to those from the corresponding monocultures. Through preliminary experiments, these fungal strains were screened and selected for binary co-cultivation based on their high cellulase and xylanase activities. Guinea grass and water hyacinth are highly invasive plants with a rapid growth (Kariyawasam et al. 2021; Amarasinghe 2021). Sugar cane leaf is an agricultural waste with potential use as substrate for biofuel production. Thus, they were selected as the plant materials for saccharification, with the aim of utilizing them as low cost substrates in bioethanol production.

Materials and methods

Microbial isolates, media, and culture conditions

The cellulolytic fungal isolates, *Trichoderma reesei* F118, *Penicillium javanicum* FS7, *Talaromyces* sp. F113, and *Talaromyces pinophilus* FM9 (Genbank accession numbers OP295120.1, OP295121.1, OP295119.1, and OP295406.1, respectively) (Fig. 1), were obtained from the culture collection of the National Institute of Fundamental Studies, Kandy, Sri Lanka.

The four selected filamentous fungi were grown as monocultures and as co-cultures in an enzyme production medium. With the aim of forming fungal-fungal co-cultures, all possible pairs of the four fungi were used except the pair of *Talaromyces* sp. (F113) and *Talaromyces pinophilus* (FM9) as they belonged to the same genus. The fungal strains were initially inoculated on potato dextrose agar

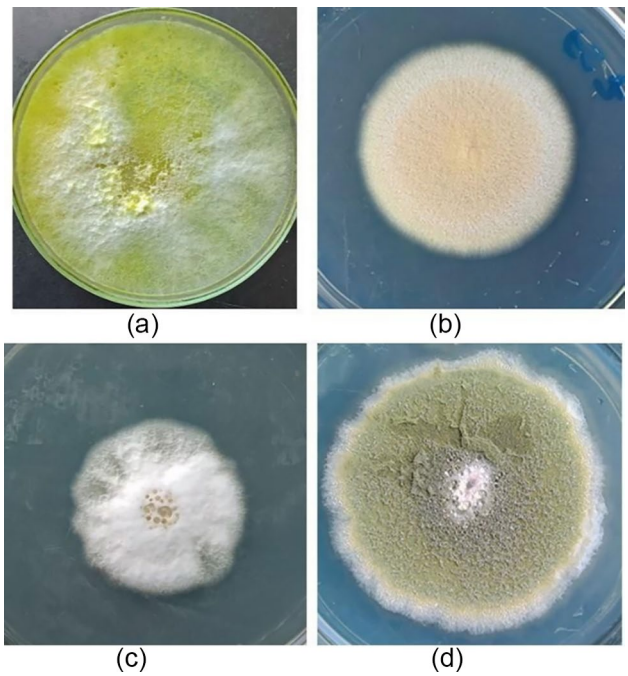

Fig. 1 Colonies of the selected cellulolytic fungal isolates grown on potato dextrose agar: **a** *Trichoderma reesei* F118, **b** *Penicillium javanicum* FS7, **c** *Talaromyces* sp. F113, and **d** *Talaromyces pinophilus* FM9

slopes in glass tubes and incubated at room temperature (~25 °C) for 10 days and the spores were harvested by adding a sterile 0.1% (v/v) tween 80 (aq) solution. The spore concentrations were estimated by using an improved Neubauer counting chamber and were adjusted to 10^7/mL. For fungal monocultures of each strain, 100 µL of fungal spore suspensions was inoculated whereas for fungal-fungal co-cultures consisting of two fungi, 50 µL of spore suspension from each component fungus was inoculated into 20 mL of the enzyme production medium (Mandels and Weber 1969) in 100 mL Erlenmeyer flasks. The medium contained (per liter): 2 g of KH_2PO_4, 1.4 g of $(NH_4)_2SO_4$, 0.3 g of urea, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of yeast extract, 10 mL of trace elements solution, 0.5 g of glycerol, and 10 g of cellulose powder (pH = 5.5). The trace elements solution contained per liter: 500 mg of $FeSO_4 \cdot 7H_2O$, 200 mg of $CoCl_2 \cdot 6H_2O$, and 160 mg of $MnSO_4 \cdot H_2O$. The cultures were incubated at 28 °C with orbital shaking at 100 rpm in a shaking incubator (JeioTech Lab Companion ISF-7100R) for 7 days. The cultures were then filtered with a Whatman No. 42 filter paper, and the filtrates were used for enzyme assays. The culture filtrates were filter-sterilized by using syringe filters with Nylon membrane (0.22 µm) before being used for the saccharification of the plant materials.

Enzyme assays

The cellulase activities of monocultures and co-cultures were determined by using a Whatman No. 1 filter paper as the substrate according to Mandels et al. (1976) as described by Ghose (1987). In brief, to 50 mg of a Whatman No. 1 filter paper (1 cm × 6 cm) fully immersed in 1 mL of 50 mM citrate buffer (pH 4.8), 0.5 mL of culture filtrate was added and incubated at 50 °C for 60 min. Then, 300 µL of the reaction mixture was transferred to a separate test tube containing 600 µL of 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959), and the tube was kept in a boiling water bath for 5 min. The tube was quickly cooled to ambient temperature; 4 mL of distilled water was added and mixed thoroughly. Absorbance was measured at 540 nm. The amount of sugars released from filter paper was determined by using a calibration curve plotted with glucose standards. Total cellulase activity was expressed in filter paper units/mL (FPU/mL), which is the amount of reducing sugar released, in micromoles, by 1 mL of culture supernatant per minute.

Xylanase assay was carried out by using beechwood xylan (Sigma, USA) as the substrate according to Gottschalk et al. (2010). In brief, to 100 µL of 10 mg/mL xylan solution in 50 mM citrate buffer (pH 4.8), 50 µL of culture filtrate was added and incubated at 50 °C for 5 min. Then 300 µL of 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959) was added, and the tube was kept in a boiling water bath for 5 min. The tube was quickly cooled to ambient temperature, 4 mL of distilled water was added and mixed thoroughly. Absorbance was measured at 540 nm. In order to determine the amount of reducing sugar released from xylan due to boiling, a xylan blank was also included by adding 50 µL of distilled water instead of culture filtrate. Xylose standards were used to plot the calibration curve which was used to determine the amounts of xylose released. The xylanase activity was expressed as units/mL (U/mL), which is the amount of xylose released due to enzyme activity, in micromoles, by 1 mL of culture supernatant per minute.

Preparation of the plant materials

Sugar cane leaves, Guinea grass leaves, and water hyacinth stems and leaves were oven-dried at 50 °C to constant weight, ground by using a plant grinder and sieved (pore size: 80 µm). The resultant powder was used for compositional analysis and for saccharification. An amount of 500 mg of the resulted dry powder was initially used to remove extractives from the plant material. Then the extractive-free plant material was used for compositional analysis.

Compositional analysis of the plant materials

Extractive contents of the plant materials were determined according to Foster et al. (2010). The plant materials remaining after the removal of the extractives were used for the determination of polysaccharide and lignin contents. The total insoluble polysaccharide contents and acid insoluble lignin contents were determined according to Sluiter et al. (2008), using 100 mg of the extractive-free plant material. The non-cellulosic insoluble polysaccharide contents were determined using 10 mg of extractive-free plant material as described by Foster et al. (2010). The ash contents were measured using 300 mg of extractive-free plant material according to Sluiter et al. (2005).

Saccharification of the plant materials by the culture filtrates

To 50 mL screw-capped culture bottles, 250 mg of each powdered plant material was added followed by 5 mL of 100 mM acetate buffer (pH 4.8). The bottles were sterilized by autoclaving at 121 °C for 20 min with the aim of killing any microbial contaminants. To each bottle, 5 mL of sterile culture filtrate was added and incubated for 7 days at 40 °C with rotary shaking at 70 rpm.

For controls, 5 mL of sterile distilled water was added instead of culture filtrates. At the end of the incubation, the hydrolysates were sampled and were checked for microbial contamination by sub-culturing on nutrient agar and potato dextrose agar. Any replicate found to be contaminated was repeated. The hydrolysates were centrifuged at 10000 g for

5 min, and the supernatants were used for the measurement of total reducing sugars and individual sugars released.

Measurement of the total reducing sugars and individual sugars released

The amounts of total reducing sugars present in the enzymatic hydrolysates were determined by using DNS reagent against glucose standards (Miller 1959). The individual sugars present in the enzymatic hydrolysates were quantified by HPLC according to Ball et al. (2011), under the following conditions: Column: Agilent Hi-plex H (7.7 × 300 mm, 8 µm, part number-PL1170-6830), mobile phase: 0.005 M H_2SO_4, flow rate: 0.7 mL per minute, column temperature: 60 °C, detector type: refractive index, detector temperature: 55 °C.

Statistical analysis

Statistical comparisons were made by ANOVA followed by Fisher's pairwise comparison, using Minitab Software. The cultures and saccharification were carried out in triplicates ($p < 0.05$). Compositional analysis was carried out in duplicates.

Results

Enzyme activities of the culture filtrates

The cellulase and xylanase activities of the fungal co-cultures and the corresponding monocultures are shown in Figs. 2 and 3, respectively. The co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 and the co-culture of *T. reesei* F118 with *T. pinophilus* FM9 produced significantly higher cellulase activities compared to both corresponding monocultures. The highest mean cellulase activity of 0.29 FPU/mL was produced by the co-culture of *Talaromyces* sp. F113 and *Penicillium javanicum* FS7 (Fig. 2). Even though the xylanase activities of the co-cultures containing *T. reesei* F118 were significantly higher compared to the monoculture of *T. reesei* F118, they were not significantly higher than the xylanase activities of the remaining corresponding monocultures. Similarly, none of the other co-cultures produced xylanase activities that were significantly higher than those produced by both corresponding monocultures (Fig. 3). The highest mean xylanase activity was produced by the co-culture of *T. reesei* F118 and *T. pinophilus* FM9 (7.9 U/mL).

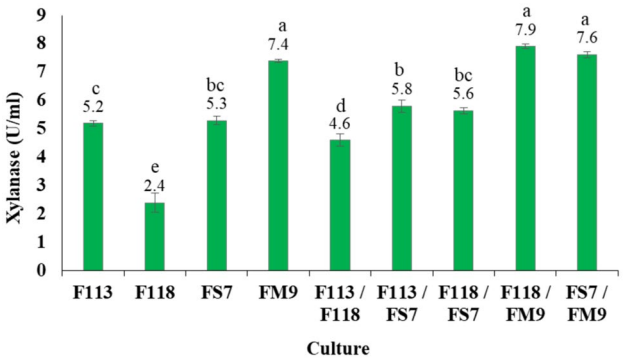

Fig. 3 Xylanase activities of the culture filtrates from the fungal monocultures and co-cultures. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9. Error bars indicate standard errors of the means. Means that do not share a letter are significantly different

Composition of the plant materials

The compositions of the three plant materials selected for saccharification are shown in Table 1. Sugarcane leaves contained the highest insoluble polysaccharide content (65.7% w/w) followed by Guinea grass leaves (54.7% w/w) and

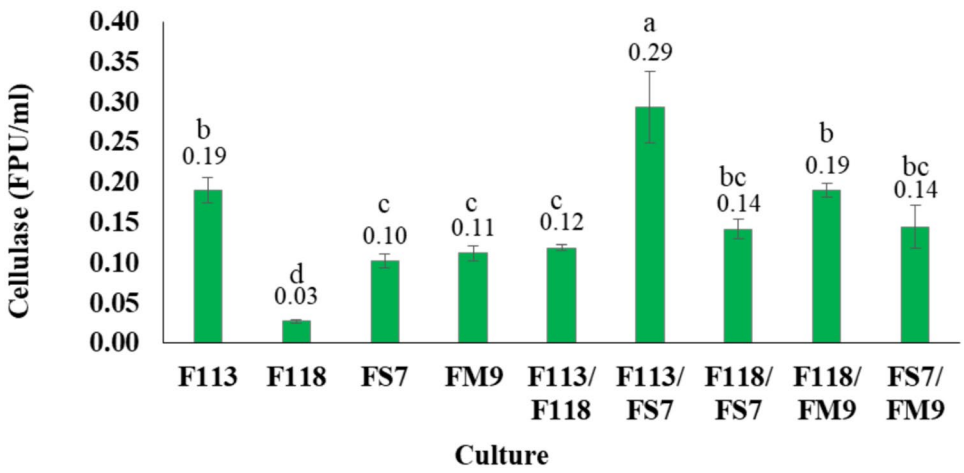

Fig. 2 Cellulase activities of the culture filtrates from the fungal monocultures and co-cultures. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9. Error bars indicate standard errors of the means. Means that do not share a letter are significantly different

Table 1 Compositions of the three plant materials selected for the study

Composition (% w/w)[a]					
Plant material	Extractives	Total insoluble polysaccharides	Non cellulosic insoluble polysaccharides	Acid insoluble lignin	Ash
SC	10	65.7	24.4	18.8	10.3
GG	13.7	54.7	22.8	19.4	10.1
WH	17.4	44.1	17.9	10.1	15.5

SC sugarcane leaves, *GG* Guinea grass leaves, *WH* water hyacinth stems and leaves

[a]Percentages based on dry weights obtained by oven drying at 50 °C

water hyacinth stems and leaves (44.1% w/w). Thus, based on the polysaccharide content per unit mass, sugarcane leaves could be considered as the most suitable for biofuel production. Water hyacinth leaves contained the lowest acid-insoluble lignin content (10.1% w/w), as can be expected from an aquatic plant. They also contained the highest ash content (15.5% w/w). Guinea grass leaves and sugarcane leaves contained similar acid-insoluble lignin and ash contents (Table 1). The results of the compositional analysis of the three selected plant materials were found to be similar to previously published data (Hodgson-Kratky et al. 2019; Premaratne et al. 1993; Guna et al. 2017).

Saccharification of the plant materials

Total reducing sugars released from the plant materials

The amounts of total reducing sugars released from 250 mg of the plant materials are shown in Fig. 4. The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 released significantly higher amounts of total reducing sugars from sugarcane leaves, compared to both corresponding monocultures. This is in correlation with the observed cellulase activities (Fig. 2). The highest amount of total reducing sugars released from sugarcane leaves was 60.4 mg, released by the co-culture of *T. reesei* F118 with *T. pinophilus* FM9.

Three of the co-cultures, namely, *T. reesei* F118 with *T. pinophilus* FM9, *Penicillium javanicum* FS7 with *T. pinophilus* FM9, and *T. reesei* F118 with *Penicillium javanicum* FS7, released significantly higher amounts of total reducing sugars from Guinea grass leaves compared to the corresponding monocultures. The highest amount of total reducing sugars released from Guinea grass leaves was 86.8 mg, released by the co-culture of *T. reesei* F118 with *T. pinophilus* FM9.

The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 released significantly higher amount of total reducing sugars from water hyacinth stems and leaves compared to both corresponding monocultures. However, the highest amount of total reducing sugars released from water hyacinth stems and leaves (29 mg) was released by the co- culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 (Fig. 4).

Table 2 shows the percentages of saccharification of the three plant materials achieved with the cultures. Overall, the highest amount of total reducing sugars released from 250 mg of the three plant materials was 86.8 mg, released from Guinea grass leaves by the co-culture of *T. reesei* F118 and *T. pinophilus* FM9, which amounts to 63.5% saccharification. Thus, among the three plant materials, Guinea grass leaves could be considered as the most suitable for biofuel production without thermochemical pre-treatment.

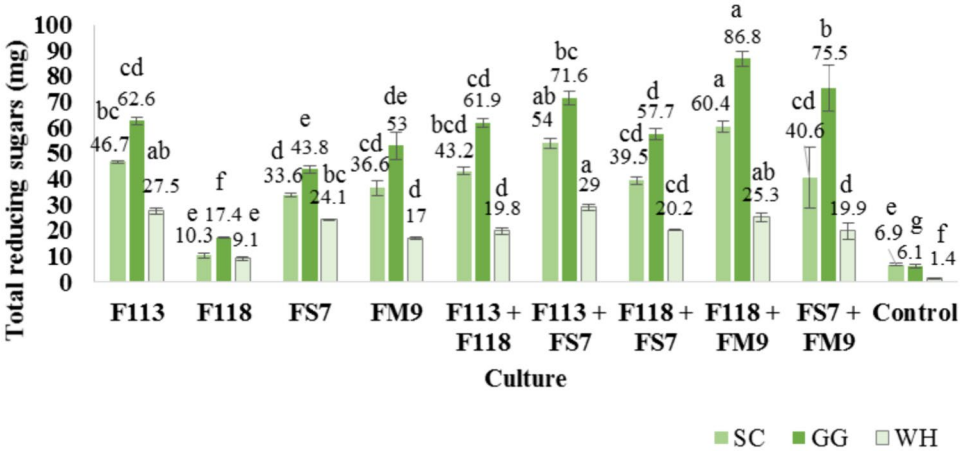

Fig. 4 Amounts of total reducing sugars released from the plant materials by the culture filtrates. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9, SC: sugarcane leaves, GG: Guinea grass leaves, WH: water hyacinth stems and leaves. Error bars indicate standard errors of the means. For each plant material, means that do not share a letter are significantly different

Table 2 Percentages of saccharification of the three plant materials achieved with the culture filtrates

Culture	% Saccharification of SC	% Saccharification of GG	% Saccharification of WH
F113	28.4	45.8	24.9
F118	6.3	12.7	8.3
FS7	20.5	32	21.9
FM9	22.3	38.8	15.4
F113 + F118	26.3	45.3	18
F113 + FS7	32.9	52.4	26.3
F118 + FS7	24	42.2	18.3
F118 + FM9	36.8	63.5	22.9
FS7 + FM9	24.7	55.2	18
Control	4.2	4.5	1.3

Codes: *SC* sugarcane leaves, *GG* Guinea grass leaves, *WH* water hyacinth stems and leaves. *F113 Talaromyces* sp. F113, *F118 Trichoderma reesei* F118, *FS7 Penicillium javanicum* FS7, *FM9 Talaromyces pinophilus* FM9

Individual sugars released from the plant materials

Among the different types of sugars released by saccharification, glucose can be fermented to ethanol by most fermenting micro-organisms while xylose can be fermented to ethanol by some micro-organisms (Margeot et al. 2009). Cellobiose can be converted to glucose by fermenting micro-organisms possessing β-glucosidase activity.

The amounts of glucose + cellobiose (the amount of glucose plus the amount of glucose equivalent of cellobiose) released from the three plant materials are shown in Fig. 5. The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 released significantly higher amounts of glucose + cellobiose from all three plant materials compared to the corresponding monocultures. The co-cultures of *T. reesei* F118 with *Penicillium javanicum* FS7 and *Penicillium javanicum* FS7 with *T. pinophilus* FM9 released significantly higher amounts of glucose + cellobiose from Guinea grass leaves whereas the co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 released significantly higher amounts of glucose + cellobiose from water hyacinth stems and leaves, compared to the corresponding monocultures. These findings are similar to those made with the amounts of total reducing sugars released.

The amounts of xylose released from the three plant materials are shown in Fig. 6. All the co-cultures released significantly higher amounts of xylose from Guinea grass leaves compared to the corresponding monocultures. The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 released significantly higher amounts of xylose from sugarcane leaves compared to the corresponding monocultures. None of the co-cultures released significantly higher amounts of xylose from water hyacinth stems and leaves compared to the corresponding monocultures. However, it was noted that the monocultures of the fungi *Talaromyces* sp. F113 and *Penicillium javanicum* FS7 and their co-culture released significantly higher amounts of xylose from water hyacinth stems and leaves compared to the other cultures.

The amounts of arabinose released from the three plant materials are shown in Fig. 7. The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 released significantly higher amounts of arabinose from sugarcane leaves and Guinea grass leaves, compared to the corresponding monocultures, whereas the co-culture of *Penicillium javanicum* FS7 with *T. pinophilus* FM9 released significantly higher amounts arabinose from Guinea grass leaves compared to the corresponding monocultures. It was noted that only the monocultures of the two fungi *Talaromyces* sp. F113 and *Penicillium javanicum* FS7 and their co-culture released detectable

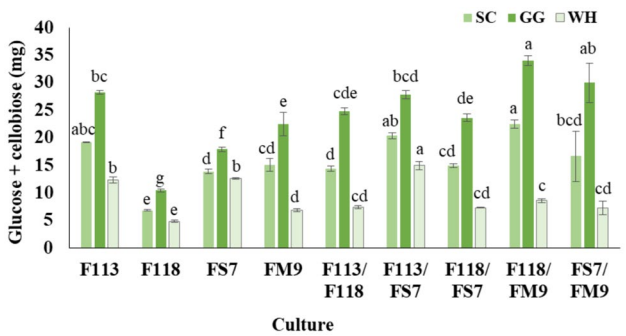

Fig. 5 Amounts of glucose + cellobiose released from 250 mg of the plant materials by the culture filtrates. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9, SC: sugarcane leaves, GG: Guinea grass leaves, WH: water hyacinth stems and leaves. Error bars indicate standard errors of the means. For each plant material, means that do not share a letter are significantly different

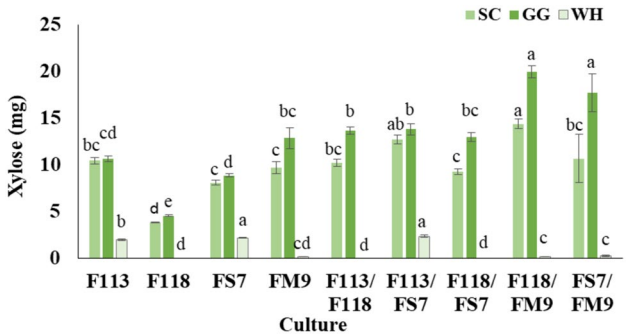

Fig. 6 Amounts of xylose released from 250 mg of the plant materials by the culture filtrates. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9, SC: sugarcane leaves, GG: Guinea grass leaves, WH: water hyacinth stems and leaves. Error bars indicate standard errors of the means. For each plant material, means that do not share a letter are significantly different

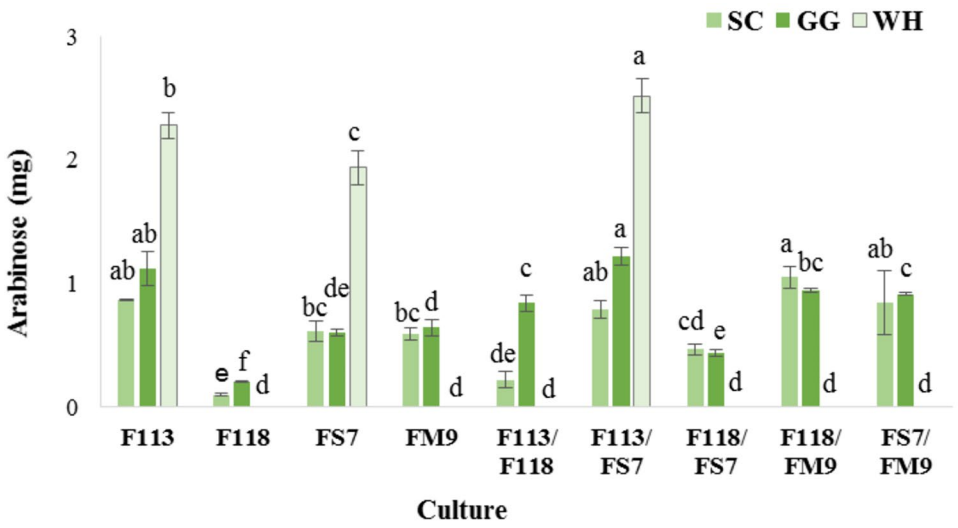

Fig. 7 Amounts of arabinose released from 250 mg of the plant materials by the culture filtrates. Codes: F113: *Talaromyces* sp. F113, F118: *Trichoderma reesei* F118, FS7: *Penicillium javanicum* FS7, FM9: *Talaromyces pinophilus* FM9, SC: sugarcane leaves, GG: Guinea grass leaves, WH: water hyacinth stems and leaves. Error bars indicate standard errors of the means. For each plant material, means that do not share a letter are significantly different

amounts of arabinose from water hyacinth stems and leaves. Interestingly, the amounts of arabinose released by them from water hyacinth stems and leaves were significantly higher than the amounts released by them from the other two plant materials. Also, the release of arabinose from water hyacinth stems correlates with the release of higher amounts of glucose + cellobiose and xylose.

Discussion

Several previous studies have found that co-culture of different species of cellulolytic fungi under submerged or solid-state conditions could lead to enhanced cellulase activity (Ahamed and Vermette 2008; Deshpande et al. 2008; Fatma et al. 2021; Peláez et al. 2022; Morilla et al. 2023). In the present study, significantly higher cellulase activity was observed from two of the co-cultures without concurrent effect on xylanase activity. This suggests that the effect is probably not caused by better growth of the fungi in co-culture. In the case of the co-culture of *T. reesei* F118 and *T. pinophilus* FM9, synergy between the cellulases of the two fungi could be the cause for enhanced cellulase activity, as *T. pinophilus* is known to produce high amounts of secreted β-glucosidase (Trollope et al. 2018), which is lacked by *T. reesei* (Ahamed and Vermette 2008).

Another possible reason for enhanced cellulase activity in fungal co-cultures is better induction of cellulases in co-culture. The cellulases and xylanases of most fungi are induced by the degradation products of cellulose and xylan as well as their transglycosylation products (Amore et al. 2013). Gentiobiose, a transglycosylation product of cellobiose, induces cellulases and xylanases in species of *Pencillium* (Kurasawa et al. 1992). The genus *Talaromyces* was initially considered as a teleomorph of *Penicillium* and was later classified as a closely related but separate genus (Yilmaz et al. 2014). Hence, it is quite likely that the cellulases and xylanases of the isolates *Talaromyces pinophilus* (F113) and *Penicillium javanicum* (FS7) are induced by the same inducer molecules such as gentiobiose. Thus, the production of inducer molecule by any one of the two fungi through transglycosylation may result in increased induction of the enzymes by the other, which may explain the significantly higher production of cellulases by their co-cultures. *Trichoderma* and *Penicillium* are somewhat distantly related (Gusakov and Sinitsyn 2012). The cellulases and xylanases of *Trichoderma* species, but not *Pencillium* species, are induced by the transglycosylation product sophorose. Similarly, gentiobiose is not an inducer of cellulases from *Trichoderma* (Gusakov and Sinitsyn 2012). Hence enhanced induction of cellulases by the co-culture of *T. reesei* F118 and *T. pinophilus* FM9 is less likely.

The co-culture of *T. reesei* F118 with *T. pinophilus* FM9 produced significantly lower cellulase activity compared to the co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 (Fig. 2). However, it released significantly higher amounts of total reducing sugars from sugarcane leaves and Guinea grass leaves compared to the latter (Fig. 4). This could be due to its higher xylanase activity (Fig. 3), as the action of xylanase would remove the xylans and expose the cellulose fibrils embedded in them to the action of cellulases. In contrast, the highest saccharification of water hyacinth stems and leaves was caused by the co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 (Fig. 2). This could be due to structural differences between the cell walls of water hyacinth stems and leaves and those of sugarcane leaves and Guinea grass leaves. Sugar cane and Guinea grass belong to the family Poaceae whereas water hyacinth belongs to the family Pontederiaceae (Global

Biodiversity Information Facility 2019). The primary cell walls of the family Poaceae are rich in glucoronoarabinoxylan and contain less than 10% of pectin whereas those of the other families of monocotyledons contain xyloglucan and/or glucomannan and 20–35% pectin (Carpita and Gibeaut 1993; O'Neill and York 2003).

Overall, the highest amounts of the fermentable sugars, namely, glucose + cellobiose (33.9 mg) and xylose (20.0 mg) released in the present study, were from Guinea grass leaves by the co-culture of *T. reesei* F118 and *T. pinophilus* FM9. Hence, the highest concentration of glucose + cellobiose and xylose obtained in the present study were 3.39 g/L and 2.0 g/L, respectively. This could be further increased by increasing the substrate and/or enzyme loadings.

Saccharification of sugarcane leaves following microwave assisted (MA) inorganic salt pretreatment by Moodley and Kana (2017) resulted in a reducing sugar yield of 0.406 g/g. Odorico et al. (2018) reported 54.2% saccharification of Guinea grass pre-treated with an ionic liquid. In the present study, even though 63.5% saccharification was achieved from Guinea grass, it was only from the leaves of the plant. In a study reported by Santana et al. (2020), water hyacinth was hydrolyzed without chemical pre-treatment resulting in about 9% saccharification. In the present study, a maximum saccharification of 26.3% was achieved with the co-culture of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7. Since switchgrass (*Panicum virgatum*) is a plant species which is phylogenetically closely related to Guinea grass (Burke et al. 2016), it would be interesting to study the enzymatic saccharification of switchgrass with no or minimal thermochemical pretreatment. Zhang et al. (2007) achieved about 13% of saccharification of untreated switchgrass (*Panicum virgatum*) in 3 days, using a commercial cellulase and a β-glucosidase. Extrapolation of the saccharification curve from their study suggests less than 20% saccharification after 7 days. The lack of xylanase activity in the enzyme mixture used could have been the reason for the lower percentage of saccharification achieved in their study.

Conclusions

Co-cultivation of *Talaromyces* sp. F113 with *Penicillium javanicum* FS7 and *T. reesei* F118 with *T. pinophilus* FM9 results in significantly higher cellulase activity compared to the corresponding monocultures. Among the fungal cultures made in the present study, the co-culture of *T. reesei* F118 and *T. pinophilus* FM9 was found to be the most suitable for the saccharification of sugarcane leaves and Guinea grass leaves without pretreatment whereas the co-culture of *Talaromyces* sp. F113 and *Penicillium javanicum* FS7 was found to be the most suitable for the saccharification of water hyacinth stems and leaves. Among the three plant materials studied, Guinea grass leaves were found to be the most susceptible to enzymatic saccharification without thermochemical pretreatment whereas water hyacinth stems and leaves were found to be the least susceptible. The highest percentage of saccharification achieved in the present study was 63.5% from Guinea grass leaves by the co-culture of *T. reesei* F118 and *T. pinophilus* FM9. Further increase in the percentage of saccharification achieved and a reduction in the time taken for the saccharification are needed for potential commercial bioethanol production.

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Declarations

Conflict of interest The authors declare no competing interests.

References

Ahamed A, Vermette P (2008) Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. Biochem Eng J 42(1):41–46. https://doi.org/10.1016/j.bej.2008.05.007

Ahmed S, Riaz S, Jamil A (2009) Molecular cloning of fungal xylanases: an overview. Appl Microbiol Biotechnol 84:19–35. https://doi.org/10.1007/s00253-009-2079-4

Amarasinghe SR (2021) Use of invasive water hyacinth for composting of ordinary leaf litter. Sri Lankan J Agric Ecosyst 3(1):5–16

Amore A, Giacobbe S, Faraco V (2013) Regulation of cellulase and hemicellulase gene expression in fungi. Curr Genom 14(4):230–249

Bajaj P, Mahajan R (2019) Cellulase and xylanase synergism in industrial biotechnology. Appl Microbiol Biotechnol 103:8711–8724

Ball S, Bullock S, Lloyd L, Mapp KP, Ewen A (2011) Analysis of carbohydrates, alcohols, and organic acids by ion-exchange chromatography. Agilent Hi-plex Columns Applications Compendium. Agilent Technologies Inc., Santa Clara, California. https://www.agilent.com/cs/library/applications/5990-8801EN%20Hi-Plex%20Compendium.pdf

Burke SV, Wysocki WP, Zuloaga FO, Craine JM, Pires JC, Edger PP, Mayfield-Jones D, Clark LG, Kelchner SA, Duvall MR (2016) Evolutionary relationships in panicoid grasses based on plastome phylogenomics (Panicoideae; Poaceae). BMC Plant Biol 16(1):1–11. https://doi.org/10.1186/s12870-016-0823-3

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3(1):1–30

Deshpande SK, Bhotmange MG, Chakrabarti T, Shastri PN (2008) Production of cellulase and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state

fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). Ind J Chem Tech 15:449–456
Fatma S, Saleem A, Tabassum R (2021) Wheat straw hydrolysis by using co-cultures of *Trichoderma reesei* and *Monascus purpureus* toward enhanced biodegradation of the lignocellulosic biomass in bioethanol biorefinery. Biomass Convers Biorefin 11:743–754
Foster CE, Martin TM, Pauly M (2010) Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part II: carbohydrates. J vis Exp 37:1837. https://doi.org/10.3791/1837
Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59(2):257–268
Global biodiversity information facility (2019) GBIF. Gbif.org. https://www.gbif.org/. Accessed 24 February 2024
Gottschalk LMF, Oliveira RA, Bon EPS (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 51:72–78. https://doi.org/10.1016/j.bej.2010.05.003
Guna V, Ilangovan M, Anantha Prasad MG, Reddy N (2017) Water hyacinth: a unique source for sustainable materials and products. ACS Sustain Chem Eng 5(6):4478–4490. https://doi.org/10.1021/acssuschemeng.7b00051
Gupta MN, Bisaria VS (2018) Stable cellulolytic enzymes and their application in hydrolysis of lignocellulosic biomass. Biotechnol J. https://doi.org/10.1002/biot.201700633
Gusakov AV, Sinitsyn AP (2012) Cellulases from *Penicillium* species for producing fuels from biomass. Biofuels 3(4):463–477
Hodgson-Kratky K, Papa G, Rodriguez A, Stavila V, Simmons B, Botha F, Furtado A, Henry R (2019) Relationship between sugarcane culm and leaf biomass composition and saccharification efficiency. Biotechnol Biofuels. https://doi.org/10.1186/s13068-019-1588-3
Kariyawasam CS, Kumar L, Ratnayake SS (2021) Potential risks of invasive alien plant species on agriculture under climate change scenarios in Sri Lanka. Curr Res Environ Sustain 3:100051
Kurasawa T, Yachi M, Suto M, Kamagata Y, Takao S, Tomita F (1992) Induction of cellulase by gentiobiose and its sulfur-containing analog in *Penicillium purpurogenum*. Appl Environ Microbiol 58(1):106–110
Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506–577. https://doi.org/10.1128/MMBR.66.3.506-577.2002
Mandels M (1975) Microbial sources of cellulase. Biotechnol Bioeng Symp 5:81–105
Mandels M, Weber J (1969) The production of cellulases. Adv Chem 95:391–414
Mandels M, Andreotti R, Roche C (1976) Measurement of saccharifying cellulase. Biotechnol Bioeng Symp 6:21–33
Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009) New improvements for lignocellulosic ethanol. Curr Opin Biotechnol 20(3):372–380. https://doi.org/10.1016/j.copbio.2009.05.009
Messner R, Hagspiel K, Kubicek CP (1990) Isolation of a β-glucosidase binding and activating polysaccharide from cell walls of *Trichoderma reesei*. Arch Microbiol 154(2):150–155. https://doi.org/10.1007/BF00423325
Miller GL (1959) Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428. https://doi.org/10.1021/ac60147a030
Moodley P, Kana EBG (2017) Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste: Effect on physiochemical structure and enzymatic saccharification. Bioresour Technol 235:35–42. https://doi.org/10.1016/j.biortech.2017.03.031
Morilla EA, Taddia A, Sortino M, Tubio G (2023) Mixed cultures of *Aspergillus niger* and *Rhizopus oryzae* using lignocellulosic substrates to improve hydrolytic enzyme production. BioEnergy Res 16:2285–2296
O' Neill MA, York WS, (2003) The composition and structure of plant primary cell walls. Annual plant reviews, 8th edn. Rose JKC Blackwell publishing Ltd, Oxford, pp 1–54
Odorico FH, Morandim-Giannetti ADA, Lucarini AC, Torres RB (2018) Pretreatment of guinea grass (*Panicum maximum*) with the ionic liquid 1-ethyl-3-methyl imidazolium acetate for efficient hydrolysis and bioethanol production. Cellulose 25:2997–3009. https://doi.org/10.1007/s10570-018-1753-z
Peláez RDR, Wischral D, Cunha JRB, Mendes TD, Pacheco TF, Siqueira FGD, Almeida JRMD (2022) Production of enzymatic extract with high cellulolytic and oxidative activities by co-culture of *Trichoderma reesei* and *Panus lecomtei*. Fermentation 8(10):522
Peterson R, Nevalainen H (2012) *Trichoderma reesei* RUT-C30 – thirty years of strain improvement. Microbiol 158:58–68. https://doi.org/10.1099/mic.0.054031-0
Premaratne S, Ibrahim MNM, Perera HGD (1993) Effect of stage of growth and additives on digestibility and palatability of Guinea (*Panicum maximum*, Jacq) grass silage. J Natl Sci Found Sri Lanka 21(2):175–182
Santana JC, Souza Abud AK, Wisniewski A, Navickiene S, Romão LPC (2020) Optimization of an organosolv method using glycerol with iron catalysts for the pretreatment of water hyacinth. Biomass Bioenergy 133:105454. https://doi.org/10.1016/j.biombioe.2019.105454
Shallom D, Shoham Y (2003) Microbial hemicellulases. Curr Opin Microbiol 6(3):219–228. https://doi.org/10.1016/S1369-5274(03)00056-0
Silva JCR, Salgado JCS, Vici AC et al (2020) A novel *Trichoderma reesei* mutant RP698 with enhanced cellulase production. Braz J Microbiol 51:537–545. https://doi.org/10.1007/s42770-019-00167-2
Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D (2005) Determination of ash in biomass. National Renewable Energy Laboratory, USA. http://www.nrel.gov/docs/gen/fy08/42622.pdf
Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory, USA. http://www.nrel.gov/docs/gen/fy13/42618.pdf
Soeder DJ (2021) Fossil fuels and climate change. In: Fracking and the Environment. Springer, Cham. https://doi.org/10.1007/978-3-030-59121-2_9
Trollope K, Nel DW, Volschenk H (2018) The heterologous expression potential of an acid-tolerant *Talaromyces pinophilus* β-glucosidase in *Saccharomyces cerevisiae*. Folia Microbiol 63:725–734
Wilson DB (2011) Microbial diversity of cellulose hydrolysis. Curr Opin Microbiol 14(3):259–263
Yilmaz N, Visagie CM, Houbraken J, Frisvad JC, Samson RA (2014) Polyphasic taxonomy of the genus *Talaromyces*. Stud Mycol 78:175–341
Zhang YHP, Ding SY, Mielenz JR, Cui JB, Elander RT, Laser M, Himmel ME, McMillan JR, Lynd LR (2007) Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnol Bioeng 97(2):214–223. https://doi.org/10.1002/bit.21386
Zhang C (2019) Lignocellulosic ethanol: technology and economics. In: Yun Y (ed) Alcohol fuels-current technologies and future prospect, IntechOpen