Journal of Physical Science, Vol. 28(Supp. 1), 171–184, 2017

Characterisation of Cellulases and Xylanase from *Trichoderma virens* UKM1 and its Potential in Oil Palm Empty Fruit Bunch (OPEFB) Saccharification

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Published online: 15 February 2017

To cite this article: Ngikoh, B. et al. (2017). Characterisation of cellulases and xylanase from *Trichoderma virens* UKM1 and its potential in oil palm empty fruit bunch (OPEFB) saccharification. *J. Phys. Sci*., 28(Supp. 1), 171–184, https://doi.org/10.21315/jps2017.28.s1.11

To link to this article: https://doi.org/10.21315/jps2017.28.s1.11

ABSTRACT: *A large amount of lignocellulosic waste biomass such as oil palm empty fruit bunch (OPEFB) is produced annually. Enzymes that are responsible for the degradation of lignocellulosic waste play a crucial role in converting the biomass into fermentable sugars and bio products. The aims of this work are to determine the ability of Trichoderma virens UKM1 to produce cellulases and xylanase using OPEFB as substrate in shake-flask fermentation system and to evaluate the potential of these enzymes for OPEFB saccharification. The highest enzyme activities recorded from T. virens UKM1 when 1% OPEFB used as the substrate were 0.79 U/mL for total cellulase (FPase), 53.22 U/mL for endoglucanase (CMCase), 6.5 U/mL for exoglucanase, 0.97 U/mL for β-glucosidase and 254.12 U/mL for xylanase. FPase, CMCase and exoglucanase exhibited their maximum activities at day two incubation, while xylanase and β-glucosidase activities were the highest at day six and seven, respectively. The enzymes obtained from this fungus were then used to hydrolyse OPEFB. The highest glucose production of 1.1 g/L was achieved when 5% OPEFB (w/v) was hydrolysed with 20 FPU enzyme units and with the addition of β-glucosidase (4 U/g OPEFB) for 24 h. These findings suggest that T. virens UKM1 is a potential cellulase producer for saccharification of OPEFB into simple sugars.*

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In addition, OPEFB can be used as a cheap substrate for the production of hydrolytic enzymes from T. virens UKM1.

Keywords: Cellulase, oil palm empty fruit bunch, Trichoderma virens, saccharification, fermentable sugars

1. INTRODUCTION

The lignocellulosic biomass feedstock is the most abundant renewable source in the world.[1,2] Lignocellulosic biomass is a renewable organic matter which includes energy crops, agricultural residues, aquatic plants, forest residues and wood residues as well as other waste materials.[3] Utilisation of these waste by-products could solve disposal problem and reduce the cost of waste treatment.[4] To date, lignocellulosic biomass has been explored extensively as the source of fermentable sugars for bio-ethanol and fine chemicals production. However, lignocellulosic biomass has a complicated composition and its structure makes it resistant to chemical and biological hydrolysis.[5] It consists of three major components which are cellulose, hemicellulose and lignin fraction. Cellulose, the most abundant carbohydrate exists in a lignocellulosic material, is a polymer of β-(1,4)-D-glucose.

Hemicellulose consists of multiple polymers such as pentose sugars (xylose and arabinose), hexose sugars (mannose and galactose), and sugar acid such as glucuronic acid and galacturonic acid.[6,7] Intermolecular interactions between the three major components in lignocellulosic biomass, determine the lignocellulosic biomass recalcitrant. Crystalline structure of cellulose microfibrils in the cell wall makes it resistant to chemical and biological hydrolysis. Besides that, the coverage of cellulose microfibrils by hemicelluloses and lignin also make it difficult for catalytic enzymes to access to cellulose microfibrils.[5,8] Therefore, conversion of lignocellulose to fermentable sugars requires two major steps: (1) physical or chemical pretreatment of lignocellulosic material to enhance its conversion by removing or reducing crystalline structure of cellulose and removing lignin; and (2) saccharification of lignocelluloses by means of enzymatic or chemical saccharification to produce sugars such as glucose and xylose.[9,10]

Oil palm empty fruit bunch (OPEFB) fibre is a lignocellulosic biomass, generated as a by-product of the oil palm industry. As one of the main oil palm exporters, Malaysia generates approximately 90 million tonnes of oil palm annually. Roughly 15–18 million tonnes of OPEFB were generated annually.[11,12] OPEFB is the solid residue obtained after stripping oil palm fruit from the oil palm bunch. Average

yield of OPEFB fibre is about 400 g per bunch.[13] This biomass is usually burned in incinerators by palm oil mills, which generate serious environmental concerns causing the authorities to formulate tight regulatory controls to curb air pollution from such activities.[14] OPEFB also has been used as compost material to nourish oil palm plantation soil.[15] Based on dry weight, OPEFB contains approximately 63% cellulose, 28% hemicellulose and 17.8% lignin.[16] This high cellulose content could be tapped to produce valuable products such as simple sugar as chemical feedstock and biofuel. The ideal lignocellulosic substrate for saccharification should be cheap, easy to handle and process, available in high amounts and its composition should be suited for both hydrolysis and production of cellulolytic enzyme.[17]

It is known that the cellulase complex secreted by filamentous fungi is formed by three major enzyme components: endoglucanases, exoglucanase (or cellobiohydrolases, CBH) and β-glucosidases. Cellulases are relatively slow-acting enzymes, primarily because of the complex, insoluble, and the semicrystalline nature of their substrate.[18] In addition, the maximal cellulase activity requires multiple, related enzymes such as endoglucanases, exoglucanases and β-glucosidases to act synergistically for complete conversion of cellulose into glucose.[19] Endoglucanases hydrolyse cellulose chains internally, providing new chain ends for exoglucanases. Exoglucanases hydrolyse the cellulose progressively from the ends to create mainly cellobiose. Fungal exoglucanase such as CBHII and CBHI split cellobiose from non-reducing ends and reducing ends of cellulose chains, respectively. The complementary activities of endoglucanases and exoglucanases lead to synergistic effects, whereby the enzymatic activity of two or several enzymes is higher than the sum of the activities of the individual enzymes.[20] β-glucosidase converts cellobiose and cellooligosaccharides to glucose.[21] Besides the cellulases, hemicellulases are also important enzyme in lignocellulose hydrolysis as the presence of hemicellulose can restrict the hydrolysis of the cellulose. The hemicellulases, such as xylanases, assist cellulose hydrolysis through removal of the non-cellulosic polysaccharides that coat the cellulose fibers.[7] Xylanase is responsible to hydrolyse the linear polysaccharide of xylan into xylose.[17]

Filamentous fungus *Trichoderma virens* (synonym *Gliocladium virens*) has been identified as a potential cellulase producer for bioconversion processes, but its cellulose degrading enzymes potential in saccharification have not been investigated in detail, as compared to other *Trichoderma* species such as *T. reesei*.[22] However *T. virens* is known to produce endoglucanase, exoglucanase and β-glucosidase extracellularly and has good xylanase and β-glucosidase activity.[23] The objectives of the present work are to investigate the possibility of using OPEFB fibres as

substrate for production of cellulase by *T. virens* UKM1 in shake-flask cultures, and to evaluate the potential of these enzymes in the saccharification of OPEFB fibres.

2. EXPERIMENTAL

2.1 Microorganism

Trichoderma virens UKM1 used in this study was obtained from the fungal stock collection of Molecular Mycology Laboratory, Universiti Kebangsaan Malaysia, Bangi, Malaysia. The fungus was cultured on Potato Dextrose Agar (PDA, Oxoid Ltd., UK) for 4–7 days at 30°C, and kept at 4°C refrigerator when not in use for up to 1 month. It was routinely maintained on the PDA on a monthly basis.

2.2 OPEFB Preparation

Raw OPEFB fibre was obtained from the Malaysian Palm Oil Board (MPOB). It was first pretreated by soaking in 5% (w/w) sodium hydroxide (NaOH) and later underwent thermomechanical pulping. Then, it was washed to eliminate any NaOH residue, and dried completely. Dried OPEFB were ground to 0.25 mm particles using a universal cutting mill, Pulverisette 19 (Fritsch, Germany).

2.3 Fungal Biomass Production

The medium used for biomass production of *T. virens* UKM1 was prepared as described by Domingues et al.[24] The medium contains 15 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 1.23 g/L $MgSO_4.7H_2O$, 0.8 g/L $CaCl_2.2H_2O$, 0.1% (v/v) Tween 80 and 30 g/L of glucose as carbon source. Trace elements were added according to the following concentration: 0.1% (v/v) trace element solution I ($MnSO_4$, 1.6 g/L; $ZnSO_4.7H_20$, 3.45 g/l; $CoC1_2.6H_20$, 2.0 g/l) and 0.1% (v/v) trace element solution II ($FeSO_4.7H_20$, 5 g/L). The media were sterilised by autoclaving at 121°C for 15 min. An approximately 10^6 spores/mL from 7 days old mycelium cultured on PDA plate was inoculated to 50 mL biomass production medium. The culture was incubated in an orbital shaking incubator at 28°C and shaken at 150 rpm for 48 h before transferring to the cellulase production medium.

2.4 Cellulase Production

The production of cellulase was carried out in 250 mL Erlenmeyer shake-flask, each having 45 mL induction medium containing 15 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$,

1.23 g/L $MgSO_4.7H_2O$, 0.8 g/L $CaCl_2.2H_2O$, 0.1% (v/v) and Tween 80 supplemented with a trace elements solutions: 0.1% (v/v) trace element solution I ($MnSO_4$, 1.6 g/L; $ZnSO_4.7H_20$, 3.45 g/L; $CoC1_2.6H_20$, 2.0 g/L) and 0.1% (v/v) trace element solution II ($FeSO_4.7H_20$, 5 g/L). OPEFB at a final concentration of 1% (w/v) was added to the medium as the main carbon source. The medium was inoculated with 5 mL mycelium suspension grown in biomass medium, incubated at 28°C and shaken at 150 rpm for 7 days. At periodical intervals (24 h), the culture medium was collected by centrifugation at 4,000 rpm at 4°C for 10 min and the supernatant was further concentrated using Vivaspin with 10 kDa molecular weight cut-off (Sartorius, Germany) until the volume reached 1 mL. The supernatant was tested for cellulase and xylanase activities. Results were presented as an average of two independents biological experiments, with each test were performed in triplicate.

2.5 Cellulase Characterisation

2.5.1 Filter paper assay

Filter paper assay (FPA) was performed as described by Xiao et al.[25] One unit of filter paper (FPU) is described as the amount of enzyme that forms 1 μmole of glucose per min during the hydrolysis reaction.

2.5.2 Endoglucanase assay

The assay was performed using 1% carboxymethyl cellulose (CMC) suspended in sodium acetate (NaOAc) buffer, pH 4.0. Reducing sugar liberated was measured using a 3,5-dinitrosalicylic acid (DNS) colorimetric assay method.[26] One unit of endoglucanase activity was defined as 1 μmole reducing sugar (as glucose equivalent) produced under specific assay condition.

2.5.3 Exoglucanase (Cellobiohydrolase) assay

Assay was performed using 1% (w/v) pure cellulose, Avicel (Sigma-Aldrich, USA) as substrate, suspended in 50 mM NaOAc buffer, pH 5.0. This was done at 50°C for 60 min. Reducing sugar liberated was measured using the DNS colorimetric assay method (Ramirez et al. 2004).[26] One unit of exoglucanase activity was described as 1 μmol reducing sugar (as glucose equivalent) produced under assay condition.

2.5.4 β-glucosidase assay

The assay was performed using 2 mM cellobiose as a substrate, suspended in 50 mM NaOAc buffer, pH 5.0. This assay was done at 50°C for 30 min. Glucose was

measured using GOD-PAP assay kit (Roche, Germany). One unit *β-glucosidase activity wa*s defined as 2 μmol glucose under specific assay condition.

2.5.5 Xylanase assay

The assay was performed using 1% (w/v) xylan from beechwood (Sigma, USA) as substrate, suspended in 50 mM NaOAc buffer, pH 5.0. This was done at 50°C for 30 min. Xylose liberated was measured using DNS colorimetric assay method.[26] One unit of xylanase activity was described as 1 μmol of xylose produced under assay condition.

2.6 Saccharification of OPEFB

OPEFB saccharification was performed using 5% (w/v) pretreated OPEFB as substrate with different *T. virens* UKM1 crude cellulase loadings with and without commercial β-glucosidase (Novozyme 188, Novozymes A/S, Denmark). The following cellulase formulations were used: 6.7 FPU/g OPEFB, 6.7 FPU/g OPEFB + 1 U/g OPEFB of β-glucosidase and 20 FPU/g OPEFB + 4 U/g OPEFB of β-glucosidase. As a comparison to *T. virens* UKM1 cellulase performance in OPEFB saccharification, commercial cellulase, Celluclast™ (Novozymes A/S, Denmark) was also used with the same enzyme loading. Saccharification was carried out for 24 h at 50°C in NaOAc buffer (50 mM, pH 5.0) with rotary speed of 150 rpm. Reducing sugar produced was measured with DNS reagent and glucose production was measured with GOD-PAP assay kit (Roche, Germany). The yield of enzymatic hydrolysis was calculated as follows:

$$\text{Yield (\%)} = \text{reducing sugar}\left(\frac{\text{mg}}{\text{mL}}\right) \Big/ \text{carbohydrate in substrate}\left(\frac{\text{mg}}{\text{mL}}\right) \times 100$$

3. RESULTS AND DISCUSSION

3.1 Characterisation of *T. virens* UKM1 Cellulases

Cellulase production of *T. virens* UKM1, which was isolated from soil, was characterised by cultivation in cellulase induction medium supplemented with 1% (w/v) pretreated OPEFB as the sole carbon source. The results of cellulase production by *T. virens* UKM1 was presented in Table 1. The highest activity of filter paper was recorded at day 2 with a value of 0.79 ± 0.16 FPU/mL. The highest activity for exoglucanase and endoglucanase was recorded at day 2, with values at 6.50 ± 0.11 U/mL and 53.22 ± 5.13 U/mL, respectively. Both exoglucanase and

endoglucanase activity profiles were correlated to FPA profile, as FPA measures total cellulase activity.[27]

Table 1: Cellulase and xylanase activities obtained at different incubation time from *T. virens* UKM1 grown in 50 mL minimal medium incubated at 28°C with shaking of 150 rpm. Data shown were determined in triplicate using two parallel cultures.

Incubation time (day)	Enzyme activity (U/mL)				
	FPU	CMcase	Cellobiohydrolase	β-glucosidase	Xylanase
1	0.51 ± 0.13	45.67 ± 9.83	5.21 ± 0.03	0.28 ± 0.06	250.17 ± 16.73
2	0.79 ± 0.16	53.22 ± 5.13	6.50 ± 0.11	0.46 ± 0.05	240.97 ± 34.87
3	0.50 ± 0.06	40.05 ± 5.35	4.32 ± 0.09	0.60 ± 0.08	221.08 ± 22.55
4	0.37 ± 0.13	39.11 ± 2.86	3.75 ± 0.17	0.78 ± 0.13	213.03 ± 02.79
5	0.41 ± 0.01	40.24 ± 6.53	3.34 ± 0.39	0.88 ± 0.04	194.62 ± 20.92
6	0.40 ± 0.08	39.40 ± 5.35	3.38 ± 0.05	0.80 ± 0.19	254.12 ± 22.32
7	0.39 ± 0.01	43.29 ± 1.30	3.09 ± 0.5	0.97 ± 0.05	230.62 ± 35.10

Endoglucanase activity of *T. virens* UKM1 was comparable to those of *Aspergillus terreus* (50.33 U/mL)[28] with the same carbon source, albeit under different pretreatment. High endoglucanase activity could be attributed to mixture of β-glucan enzymes present in the crude cellulase preparation; as substrate for endoglucanase activity, CMC can be degraded by these enzymes.[29] Decrease in cellulase activity over time might be due to the accumulative effect of oligosaccharides especially cellobiose, a dimer of glucose which is known to inhibit both endoglucanase and exoglucanase.

The highest level of β-glucosidase was 0.97 ± 0.05 U/mL at day 7. The activity showed an increased pattern, suggesting that cellobiose and cellooligosaccharides were presented or accumulated as OPEFB hydrolysis products of exoglucanase and endoglucanase activity in the cultivation medium. The activity is considerably low compared to other genera. Kim and Kim[30] reported that *Penicillum verruculosum* has an activity of 1.5 U/mL, while Umikalsom et al.[13] reported that *Chatemonium globosum* contains high level of β-glucosidase at 7.6 U/mL, and *A. terreus* reportedly has 6.1 U/mL.[28] However, *Trichoderma* sp. is generally not known to secrete high amount of β-glucosidase compared to *Aspergillus* sp. and *Penicillum* sp.[31]

Xylanase yield from *T. virens* UKM1 was considerably higher, with the highest activity recorded at 254.12 ± 22.32 U/mL on day six. OPEFB used in this experiment may contain high amount of xylan, as the higher amount of xylan present, the

higher xylanase activity will be recorded. However, even in the absence of xylan, low constitutive xylanase production could be observed.[17] Xylan is the main form of hemicelluloses present in plant cell walls. The structures vary from linear β-1,4-polyxylose main chains to highly branched polysaccharides containing acetyl, arabinosyl and glucuronosyl.[32]

T. virens UKM1 secreted a significant amount of cellulases into the culture medium containing pretreated OPEFB. Therefore, this fungus has potential to be used in OPEFB degradation. Cellulases of *T. virens* UKM1 were compared to cellulases of *G. virens* (synonym of *T. virens*) that was cultivated using wheat straw as sole carbon source. At 0.79 ± 0.16 FPU/mL, the filter paper activity of *T. virens* UKM1 in this study showed better activity compared to 0.33 FPU/mL by *G. virens*.[23] It proves that lignocellulose composition in OPEFB is more suitable to induce cellulase system in *T. virens*. The major factor governing cellulase productivity and yield is undoubtedly the type of lignocellulose used as a carbon source.[13,33] This could be influenced by structural and component complexity of lignocellulosic material itself.[13]

Cellulase production by *T. virens* UKM1 using pretreated OPEFB fibers as substrate may be improved through medium and cultural conditions optimisation, and use of different modes of fermenter operation. For example, using bioreactor could increase the production of cellulase, probably due to several related factors, for example, mixing and aeration effects which are absent in the shake-flask method.[14]

3.2 Saccharification of Pretreated OPEFB

To enhance the saccharification processes, OPEFB was first subjected to both physical and chemical treatments. Alkaline pretreatment with 5% NaOH solution was chosen as it leads to the irreversible mercerisation effect which increases the amount of amorphous cellulose at the expense of crystalline cellulose.[33] Alkaline pretreatment also tends to preserve hemicelluloses chain and is more effective for lignin removal.[6,34] High xylanase activity displayed by *T. virens* indicates that the alkaline treatment is mild enough, as hemicellulose is the first component to be disrupted in chemical treatment.[6] This is important as to increase the yield of fermentable hexose and pentose sugars by hydrolysis of both cellulose and hemicellulose chains. Physical treatment involving milling the OPEFB to a smaller size (0.25 mm) could increase the surface area for enzymatic attack. Increased cellulase production did not solely depend on the amount of cellulose in the medium, but might also depend on other factors such as pore size, surface area, and cellulose crystallinity.[35]

Table 2 shows the influence of the enzyme loading and the addition of β-glucosidase on the saccharification of pretreated OPEFB. To compare the performance of the *T. virens* enzymes, a commercial cellulase enzyme, the Celluclast™ was used to hydrolyse OFEFB. To further increase enzymatic hydrolysis yield, higher concentrations of cellulase and β-glucosidase were used and the production of reducing sugars was increased from 2.22 g/L to 3.45 g/L. Based on this result, increased cellulase loading leads to higher glucose recovery. But above a certain threshold, cellulase concentration might have lesser effects on cellulose hydrolysis compared to time effect.[36] The result in Table 2 also showed that addition of β-glucosidase (Novozyme 188) is necessary to relieve feedback inhibiton of cellobiose and other cellooligosaccharides in both *T. virens* cellulases and Celluclast™. This suggests that even though *T. virens* enzyme preparation already contains native β-glucosidase, it is not enough to relieve cellooligosaccharides inhibition. In addition, the result obtained in Table 2 also suggests the performance of *T. virens* cellulase is at par or slightly better than the commercial enzyme, Celluclast™.

Table 2: Concentration of reducing sugar at different enzyme loading on OPEFB saccharification.

Enzyme	6.7 FPU/g OPEFB	6.7 FPU/g OPEFB + 1 U/g OPEFB β-glucosidase	20 FPU/g OPEFB + 4 U/g OPEFB β-glucosidase
	Reducing sugar (g/L)		
T. virens UKM1	1.91 ± 0.40	2.22 ± 0.05	3.54 ± 0.05
Celluclast™	1.78 ± 0.02	1.67 ± 0.02	2.93 ± 1.08

Table 3 shows the glucose production from OPEFB saccharification by using the combination of 20 FPU *T. virens* cellulase and 4 U of β-glucosidase per gram of OPEFB. Cellulase from *T. virens* UKM1 gave the highest yield of glucose at 1.1 ± 0.06 g/L, corresponding to 2.2% of hydrolysis rate. The rate is slightly lower compared to commercial enzyme Celluclast™ (1.24 ± 0.01 g/L of glucose). Nevertheless, the reducing sugar production by *T. virens* UKM1 cellulase was higher than Celluclast™ (Table 2). Considering that, *T. virens* UKM1 cellulase was prepared from the crude supernatant; the preparation also contained hemicellulases such as xylanase and arabinofuranosidase, as cell wall degrading-enzyme. Accessory enzymes can give significant effects on enzymatic hydrolysis, presumably by improving cellulose accessibility.[37] The synergistic action of the said enzymes contributes towards efficient hydrolysis of EFB, which in turn contributes towards higher yield of reducing sugars.[38] Meanwhile, Celluclast™ has been reported to contain minor hemicellulase activity.[39]

Table 3: Different in saccharification yield of pretreated OPEFB by a cellulase produced by *T. virens* UKM1 and a commercial cellulase, Celluclast™. Enzymatic analysis was done using cellulase and β-glucosidase loading of 20 FPU/g OPEFB and 4 U/g OPEFB, respectively.

	Glucose (g/L)	Saccharification rate (%)	Glucose (mg/g of OPEFB)
T. virens UKM1	1.10 ± 0.06	2.2	22.0
Celluclast™	1.24 ± 0.01	2.5	25.0

Saccharification is an important step to produce lignocelluloses-based products. The aim for any lignocellulosic material saccharification is to obtain fermentable glucose as high as possible. There are two methods generally employed for biomass saccharification, acid hydrolysis and enzymatic hydrolysis, with varying efficiencies depending on treatment conditions, type of biomass and the properties of the hydrolytic agents.[36] The latter method which is under rapid development has immense potentials for improvement in cost and efficiency,[40] as it is more feasible, convenient, eco-friendly and gives appreciable sugar yields in optimised condition.[41] Recent demand for the production of fermentable sugars from lignocellulose leads to effort in finding novel and potent cellulase producing strains. Our result demonstrated that *T. virens* UKM1 may become a potential cellulase producer for saccharification of OPEFB.

4. CONCLUSION

T. virens UKM1 cellulases and xylanase has been successfully characterised. This fungus was found to be a potential cellulase producer with a good cellulase activity. Cellulase from this fungus shows the saccharification yield of OPEFB is comparable to the commercial cellulase, Celluclast™. Overall, this study has demonstrated that cellulase preparation from *T. virens* UKM1 has a potential in saccharification of lignocellulosic biomass resulting the fermentable sugar, glucose that could be further used for production of bio-based material.

5. ACKNOWLEDGEMENTS

The authors would like to extend their gratitude for the financial support generously provided by Malaysia's Ministry of Science, Technology and Innovation (MOSTI) under the project 07-05-MGI-GMB012 and Ministry of Higher Education under the project LRGS/2013/UKM-UKM/PT/01.

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