Biocatalysis and Agricultural Biotechnology 11 (2017) 166–175

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

# Characterization of novel *Trichoderma* hemicellulase and its use to enhance downstream processing of lignocellulosic biomass to simple fermentable sugars

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ARTICLE INFO

*Keywords:*
*Trichoderma asperellum*
Co-hydrolysis
Oil palm empty fruit bunches
Hemicellulase
Enzyme characterization
Xylanase
Lignocellulosic bio-processing

ABSTRACT

Crude hemicellulase by novel mycoparasitic *Trichoderma asperellum* (GenBank accession nos. KU878976) was characterized and used in synergy with commercial cellulase to enhance the hydrolysis of Oil-palm-empty-fruit-bunches (OPEFB). Hemicellulase complex was cellulase-free, but was dominated by β-1,4-xylanase and a few accessory enzymes such as β-xylosidase, α-arabinofuranosidase, acetyl xylan esterase and β-glucosidase. Based on xylanase assay, optimum enzyme activity was shown at 50 °C and pH 5.3; while thermal and pH stability were respectively at 50 °C (1 h) and pH 4.8–10. Moreover, the crude hemicellulase from *T. asperellum* could withstand up to 50% selected hydrolytic and fermentation inhibitors. Supplement of hemicellulase crude to Celluclast 1.5 L at 1FPU:5U enhanced the hydrolysis of alkaline-treated OPEFB biomass achieving a 91.7% saccharification yield, higher than 77.9% yield or 12.2% yield when no hemicellulase was used or used without addition of cellulase respectively. The high saccharification yield, which has not been attained in any previous report on OPEFB, demonstrates the potential of *T. asperellum* for the development of efficient enzyme system for lignocellulosic bio-conversion. As far as we know, this is the first report on enhancement of lignocellulosic co-hydrolysis through synergism of hemicellulase crude by *T. asperellum* and commercial cellulase.

## 1. Introduction

Bio-energy from lignocellulosic sources is one of the renewable alternative energy sources currently being explored to replace the fast-depleting and environment-polluting fossil fuel sources. This is due to the abundance and renewability of lignocellulosic biomass all over the world (Jönsson and Martín, 2016). Conversely, due to its natural recalcitrance against enzymatic hydrolysis which is caused by the complexity in the arrangement of its major polymeric components (cellulose; hemicelluloses and lignin); the use of lignocellulosic biomass as secured energy source is being challenged. Hence, successful biomass hydrolysis is considered a major breakthrough for efficient lignocellulosic fuel production (Ding et al., 2008; Shin et al., 2011).

On the other hand, fermentation of the entire sugar complex (cellulose and hemicellulose) of lignocellulosic biomass determines the efficiency or otherwise of lignocellulosic ethanol system. However, in a development considered as primary factor for the prevailing cost-intensive lignocellulosic ethanol process (Kuhad et al., 2011), the conventional lignocellulosic technologies are routinely designed to remove both the lignin and hemicellulose fractions during acidic chemical pre-treatment (Rahman et al., 2007) causing xylose self-degeneration to non fermentation friendly products such as furfurals (Cheng et al., 2014). Beside that hemicellulose is the major structural component of plant's cell wall after cellulose which depending on source, accounts for about 25–35% of lignocellulosic biomass; an earlier study has identified that successful hemicellulose utilization will naturally enhance the resultant lignocellulosic ethanol yield at least by about 15% (Yano et al., 2009).

To enhance hemicellulose utilization, alkaline-treatment or other pre-treatment methods that retain the hemicelluloses fraction as part of the insoluble solids are much suitable (Sudiyani et al., 2013). Under this condition, co-hydrolysis of the cellulosic and hemicellulosic fractions through synergistic actions of cellulase and hemicellulase enzymes would be necessary. During co-hydrolysis of lignocellulosic biomass, cellulase enzyme catalyses the hydrolysis of the cellulosic component, while hemicellulases, made up usually of array of enzymes, enhance complete hydrolysis of the heteropolymeric hemicellulosic fraction. Several studies have been conducted (Banerjee et al., 2010; Gao et al., 2010; Shin et al., 2011); yet optimizing the effect of enzyme synergism to enhance hydrolysis of lignocellulosic biomass is an area of intense

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http://dx.doi.org/10.1016/j.bcab.2017.06.005
Received 26 October 2016; Received in revised form 2 June 2017; Accepted 7 June 2017
Available online 08 June 2017

research in the lignocellulosic ethanol industry. On the other hand, despite its rich cellulosic contents in comparison to other lignocellulosic biomass from agricultural wastes (Goh et al., 2010; Tye et al., 2016) and despite these advantages of co enzymatic hydrolysis, most available reports (Hamzah et al., 2011; Sudiyani et al., 2013; Ying et al., 2014) on the hydrolysis of OPEFB towards ethanol production concentrated only on the hydrolysis of its cellulosic fraction. This is due to the non-utilization of other important lignocellulosic hydrolytic enzymes during hydrolysis; and where co-enzymatic hydrolysis was done, cellulase enzyme is often supplemented by other enzymes such as β-glucosidase as reported by Hamzah et al. (2011) and Sudiyani et al. (2013) which are involved in the hydrolysis of only cellulose fraction of the biomass. There is scarcely a report which combined the use of cellulase enzyme and hemicellulase (xylanase) for the hydrolysis of the OPEFB fibre. Further, since the co-enzymatic hydrolysis is often designed to ensure maximum release of all the important structural sugars, glucose and xylose, selection of appropriate pretreatment is imperative. According to Han et al. (2011), alkaline-treatment is the suitable pre-treatment for agricultural residues and herbaceous crops; and when compared with other major pre-treatment methods like acidic and autohydrolysis, it is, according to Sudiyani et al. (2013), the suitable treatment which ensures large substrates availability for biomass saccharification, Hence this study was designed to employ mild alkaline pretreated OPEFB fibre as a substrate for co-enzymatic hydrolysis using a combination of commercial cellulase (Celluclast 1.5L) and crude hemicellulase sourced from *T. asperellum* USM SD4.

Generally, genus *Trichoderma* is considered a non-pathogenic symbiont of plants (Saba et al., 2012; Shahid et al., 2014) and *T. asperellum* is a known specie that have been well used to produce bio-control agents against plant pathogens (Asad et al., 2015; Shahid et al., 2014). Mycoparasitic properties of this species is, however, proportional to the presence of cell wall degrading enzymes among which xylanases is paramount component. Notwithstanding that xylanases is a very important industrial enzymes, most study on xylanase production from *T. asperellum* have focused on its bio-control ability at the expense of other benefits. Recently, Marx et al. (2013) reported a comparative composition analysis of secretomes of *T. asperellum* S4F8 and *T. reesie* Rut C-30 cultivated via solid state fermentation (SSF) of sugarcane bagasse. They proposed that in respect of its richer component enzymes, *T. asperellum* S4F8 stand a better chance to enhance lignocellulosic hydrolysis than the over exploited *T. reesie* Rut C-30. But since their work, there has not been new study on the potentials and applications of enzyme mix from *T. asperellum* for lignocellulosic bio-processing.

In this study, hemicellulase crude sourced from a novel strain of *T. asperellum* was characterized and its ability to enhance hydrolysis of lignocellulosic biomass was investigated using mild alkaline-treated OPEFB as a representative biomass. This work assessed the synergistic effect of the hemicellulase mix and commercial cellulase (Celluclast 1.5L) to enhance complete hydrolysis of lignocellulosic biomass. These were done with equal interest on the determination of optimum properties of the crude enzyme. More importantly, effects of selected non-conventional enzyme inhibitors which have not been reported in any earlier works on the activities of hemicellulase enzymes, were also assessed.

## 2. Methodology

### 2.1. Microorganism and isolation

*Trichoderma asperellum* was selected based on its high xylanolytic potential from among a consortium of xylanase-producing soil fungi isolated from Oil palm waste dumping site. It was identified as novel strain of *Trichoderma asperellum* following phylogenetic analysis of its rRNA (ITS-1 -5.8S- ITS-4) as earlier described (Ajijolakewu et al., 2016). Gene sequence of the organism has been deposited at the NCBI database as *Trichoderma asperellum* USM SD4 (GenBank accession number KU878976); while pure culture of the strain is being maintained at the culture collection centre of the School of Industrial Technology, Universiti Sains Malaysia as *T. asperellum* USM SD4 KU878976.

### 2.2. Enzyme production

Hemicellulase production was done at 27 °C in a 250 ml conical flask via SSF of untreated OPEFB in a production medium containing 12 ml modified Mandels and Rees medium (Mandels and Reese, 1960) and 3g (Oven dried bases) OPEFB. Other medium component at pH 7 includes (g/l): yeast extract (20), $K_2HPO_4$ (2), $CaCl_2$ (0.3), Tween-80 (2), $MgSO_4$ (0.3), $FeSO_4$ (0.005), $MnSO_4$ (0.002), $ZnSO_4{\cdot}7H_2O$ (0.0016), and $CoCl_2$ (0.0014). After sterilization at 121 °C for 15 min, fermentation (48 h and 27 °C) was initiated by the inoculation of 1 ml standardized fungal inoculum (2 x $10^6$ spores/ml) after which crude enzyme was filter-extracted (Whatman no. 1 filter paper) with 0.05 M citrate buffer pH 4.8 to obtain the crude enzyme. Filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used as the crude enzyme for enzyme characterization. Enzyme production was based on optimized conditions for hemicellulase production by *Trichoderma asperellum* USM SD4 as earlier reported (Ajijolakewu et al., 2016).

### 2.3. Enzyme characterization

#### 2.3.1. Proteome analysis

Hemicellulase crude filtrate was used for protein profiling and for the determination of physic-chemical properties. Modified Lowry et al. (1951) method for protein analysis was used to determine the concentration (mg protein/ml) of the crude enzyme. By this method, acetone was used in place of 10% Trichloroacetic acid while citrate buffer was used to dissolve pellets as against the use of $Na_2CO_3$ and 0.1 M NaOH solutions (Ghose, 1987). Corresponding protein concentration was extrapolated from Bovine Albumin standards (0.05–1.0 mg protein per ml). Crude hemicellulase enzyme profile was determined qualitatively via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using 10–250 kDa (Blue Plus™) as protein marker. Standard assay procedures were employed to determine corresponding concentration of respective enzymes in the crude.

#### 2.3.2. Enzyme assays

*2.3.2.1. Primary core enzymes*. Xylanase activity (IU/gds) was determined quantitatively by the DNSA method via the hydrolysis of 1% beech wood xylan (Sigma) in 50 mM citrate buffer pH 5.3 as described (Bailey et al., 1992). A 2.0% (w/v) sodium carboxymethyl cellulose (Sigma, MO, USA) was used to determine endo-β-1,4-glucanase (CMCase) activity (Zhang et al., 2009). In both cases, enzyme activities were determined after 5 min of incubation using the DNS method. Total cellulolytic activity (FPase) was measured using the filter paper assay (FPA) according to NREL protocol LAP00629 (Adney and Baker, 1996) based on an estimation of the released reducing sugars by DNS method. One unit of enzyme activity was defined as the amount of enzyme which liberate 1 μmol of reducing sugar as xylose or glucose per min under assay condition.

*2.3.2.2. Assay of accessory enzymes*. Activities of accessory enzymes including β-glucosidase; α-Arabinofuranosidase, acetyl-xylan esterase, and β-xylosi-dase activities were determined using p-nitrophenyl (pNP)- glucose, pNP -α-arabinofuranoside, pNP-acetate, and pNP-β-xylose as respective substrate, as described by Zhang et al. (2009) with slight modification of the enzyme-substrate ratio. Respective activity of each enzyme was measured by the release of pNP from each pNP substrate. Assay was done in a 5 ml total reaction mixture consisting of 100 μl of 20 mM of respective pNP- substrate, 4.4 ml 50 mM citrate buffer and 0.5 ml of crude enzyme. Mixtures were incubated at 50 °C