Purification and Characterisation of Extracellular Cellulase Main Components from *Aspergillus terreus*

Mahdi Shahriarinour,[a] and Ramakrishnan Nagasundara Ramanan [b]

The filamentous fungus *Aspergillus terreus* was cultivated in a 2-L stirred tank bioreactor, and the resulting culture filtrate was used for protein purification. From the cultivation broth, seven crude extracts of glucanase and one of β-glucosidase were purified. A total of eight components were identified, including endoglucanases (Endo I, II, III, and IV), cellobiohydrolases (CBH I, II, and III), and β-glucosidase. The eight major components in the fermentation broth of *A. terreus*, which most likely constitute the essential enzymes for cellulose hydrolysis, were further purified by a series of column chromatography steps. Interestingly, the β-glucosidase from *A. terreus* displayed an extremely high activity on p-nitrophenyl-β-D-glucopyranoside (pNPG), which suggests that it is a good candidate enzyme for the conversion of cellobiose to glucose. The temperature and pH ranges for optimal activity of the purified enzyme were 46 to 62 °C and 5.0 to 6.0, respectively.

Keywords: Cellulase enzymes; Purification; Aspergillus terreus; Chromatography

*Contact information: a: Department of Microbiology, Rasht Branch, Islamic Azad University, Rasht, Iran; b: Chemical and Sustainable Process Engineering Research Group, School of Engineering, Monash University, Bandar Sunway 46150, Malaysia; *Corresponding author: mahdi.shahriari@iaurasht.ac.ir*

INTRODUCTION

Cellulases are enzymes that exist in multiple forms and catalyse reactions that degrade insoluble cellulose to soluble carbohydrates. In recent years, interest in cellulases has increased because of the many potential applications for these types of enzymes. For example, cellulases are involved in research and development and in the production of bio-energy and bio-fuel, as well as in the food, textile, laundry, pulp, paper, and agriculture industries (Wen *et al.* 2005; Ikeda *et al.* 2006; Tanaka *et al.* 2006). The growing shortage of fossil fuels, the emission of greenhouse gasses, and air pollution caused by incomplete combustion of fossil fuels have also resulted in an increased focus on the production of bioethanol from lignocellulosic biomass (Zaldivar *et al.* 2001), particularly using cellulases and hemicellulases to carry out enzymatic hydrolysis of the lignocellulosic material (Sun and Cheng 2002). However, in the production of bioethanol, the high costs of the enzymes used for the hydrolysis of the raw material must be reduced and their efficiency must be increased to make the process economically feasible (Hamelinck *et al.* 2005).

Enzyme production costs are closely related to the productivity of enzyme-producing microbial strains and the final protein yield and activity in the fermentation broth (Nieves *et al.* 1998). The production of cellulases is a key factor in the hydrolysis of cellulosic material, and this is essential to achieving the tremendous potential benefits of biomass utilisation by making the process economically feasible (Wen *et al.* 2005; Zhou *et al.* 2008). A number of fungi and bacteria capable of utilising cellulose as a

carbon source have been identified (Kim *et al.* 2003). Several researchers have extensively studied cellulases produced by fungi such as the *Aspergillus*, *Rhizopus*, and *Trichoderma* species (Murashima *et al.* 2002; Saito *et al.* 2003). Cellulases are inducible enzymes that are synthesised by microorganisms during their growth on cellulosic materials (Lee and Koo 2001).

A complete cellulase system consists of three classes of enzymes: endoglucanases (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4), cellobiohydrolases (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74), and β-glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21) (Zhou *et al.* 2008). The endoglucanases randomly hydrolyse the β-1,4 bonds in the cellulose molecule, and cellobiohydrolases attack from the non-reducing end of the cellulose with cellobiose as the primary structure. Lastly, β-glucosidases convert the cellobiose to glucose (Bhat and Bhat 1997). Enzymatic processes to hydrolyse cellulosic materials can be accomplished through a series of reactions with various enzymes. Reaction conditions and the production cost of the related enzyme systems significantly influence the application of the enzyme-based bioconversion technology. Therefore, much research has been devoted to obtaining new microorganisms to produce cellulolytic enzymes with higher specific activity and greater efficiency (Johnvesly *et al.* 2002; Zhou *et al.* 2008). The cellulolytic system of the filamentous fungus *A. terreus* has not previously been investigated in detail. Thus, the objective of the present study was to purify and characterise the main components of cellulases from *A. terreus* for use in the production of abundant cellulosic biomass.

EXPERIMENTAL

Materials

Strain and culture conditions

The fungus *A. terreus*, isolated from the compost of oil palm empty fruit bunch (OPEFB) waste at a local oil palm processing factory (Sri Ulu Langat Palm, Dengkil, Selangor, Malaysia) was used as the cellulase producer in this study. Details of the methods of isolation and identification of this fungus and pre-treatment OPEFB fibre as a substrate have been described previously (Shahriarinour *et al.* 2011a,b). After growing in the basal medium, as proposed by Mandels and Weber (1969) for 144 h, the mycelia were pelleted by centrifugation (Fixed-angle rotor model F-34-6-38 Eppendorf centrifuges 5810 R; Eppendorf AG, Hamburg, Germany) at 18,500×*g* for 15 min at 4 °C. The supernatant was filtered through a glass-fibre filter (GF/A grade; Whatman), and the clear supernatant was stored at -20 °C prior to purification.

Methods

Crude enzyme preparation

The sample supernatant was concentrated by first changing the media buffer to fresh Buffer A (20 mM Tris HCl, pH 7.5) using a Vivaspin concentrator with a 5 kD cut-off (Sartorius Stedim Biotech GmbH, Germany). At each purification step, the buffer was changed to fresh Buffer A (for hydrophobic column, 1 M $(NH_4)_2SO_4$, pH 5; for cation-exchange column, 50 mM NaAC, pH 4) using a Vivaspin concentrator.

Purification conditions

The purification of the enzymes produced by *A. terreus* was performed by anion exchange chromatography, hydrophobic interaction chromatography (HIC), cation-exchange chromatography, and gel filtration chromatography using an AKTA Explorer 100 Systems (GE Amersham Pharmacia) device. The following columns were used: 1 mL HiTrap pre-packed Q FF, 1 mL HiTrap pre-packed Phenyl FF (high sub), 1 mL HiTrap pre-packed SP FF, and HiLoad 16/60 Superdex 200 prep grade (Pharmacia Biotech, USA). An auto-fraction collector collected the purified samples, and each fraction was analysed for cellulase activity. Eluted fractions from the column were analysed for endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase activities, and the protein concentrations were detected at 280 nm. All solutions used for the chromatography runs were prepared by dissolving the reagents in water obtained from a Sartorius 611 Ultrapure Water Systems. All samples were filtered through a 0.2-μm low protein-binding filter before separation. The whole results of identification and characterizations are summarized in Table 1.

Table 1. Cellulase Complexes from *A. terreus* Identified by MALDI Mass Spectroscopy

Sample	Identified results	Coverage (%)	Mr (kDa)[a]	p*I*[b]
1	endo-glucanase I	15	42	4.81
2	endo-glucanase II	13	40	4.80
3	endo-glucanase III	21	43	5.22
4	endo-glucanase IV	31	66	4.84
5	Cellobiohydrolase I	31	51	5.25
6	Cellobiohydrolase II	23	87	5.34
7	Cellobiohydrolase III	24	79	4.77
8	β-glucosidase	23	95	5.08

[a]Determined by SDS-PAGE
[b]Determined by Isoelectric focusing (IEF)
Coverage (%): The percentage of protein covered by the matching peptides.
Mr (kDa): Molecular mass
pI: Isoelectric point

Enzymatic assays

Accumulated *A. terreus* biomass was removed by centrifugation, and the supernatant (crude enzyme) was assayed for cellulase activity, specifically for the activity of the individual enzyme components endoglucanase, cellobiohydrolase, and β-glucosidase. The cellulase activities of culture supernatants were determined using carboxymethylcellulose (CMC, 1%), p-nitrophenyl-β-D-cellobioside (pNPC), and p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrates, respectively. The cellulase activities were also determined by replacing CMC with 1% Avicel (insoluble Microcrystalline cellulose, Sigma).

To measure endoglucanase activity, a carboxymethylcellulose (CMC, 1%) solution was prepared in a 50 mM sodium acetate buffer (pH 5.0). A volume of 1 mL of CMC solution was incubated with 1 mL of the test enzyme solution at 50 °C for 30 min. Three millilitres of a 1% 3,5-dinitrosalicylic acid (DNS) reagent was added to terminate the reaction. The reaction was determined photometrically at 540 nm by using molar absorption coefficients (5901.1 $mol^{-1}.cm^{-1}$). One unit of endoglucanase activity was

defined as 1 µmol reducing sugar released/mL enzyme/min. The reducing sugar concentration produced from the enzymatic reaction was then measured and used to calculate the endoglucanase activity according to Eq. (1) (Afolabi 1997):

$$\text{Endoglucanase activity (U/mL)} = \text{Reducing Sugars Released} \times 0.66 \quad (1)$$

To measure the cellobiohydrolase activity, 1 mL of the test enzyme solution was added to 1 mL of 1% p-nitrophenyl-b-D-cellobioside (pNPC) suspension prepared in 50 mM sodium acetate buffer (pH 5.0). After incubating at 50 °C for 30 min, 3 mL of a 1% DNS reagent was added to end the reaction, and the resultant reducing sugar concentration was measured. The reaction enzyme was determined from absorbance measurements at 540 nm using molar absorption coefficients of 78000 $mol^{-1}.cm^{-1}$. One unit of cellobiohydrolase activity was defined as 1 µmol reducing sugar released/mL enzyme/min. The cellobiohydrolase activity was calculated according to Eq. (2) (Afolabi 1997):

$$\text{Cellobiohydrolase activity (U/mL)} = \text{Reducing Sugars Released (mg)} \times 0.19 \quad (2)$$

β-Glucosidase activity was estimated using p-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. The total assay mixture (5 mL) consisting of 4.5 mL of pNPG (1 mg/mL) and 0.5 mL of enzyme was incubated at 50 °C for 30 min. The liberated p-nitrophenol was measured at 410 nm after developing the colour with 2 mL of sodium carbonate (2%). Rates of pNPG hydrolysis were calculated by using the molar extinction coefficient for p-nitrophenol (10,718 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose in 1 min. The activity was calculated according to Eq. (3) (Afolabi 1997):

$$\text{β-glucosidase activity (U/mL)} = \text{glucose released (mg)} \times 0.09 \quad (3)$$

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12% (w/v) polyacrylamide gel. The proteins were stained using Coomassie Brilliant Blue R-250.

Protein identification

To identify the proteins that were expressed, secreted, and assembled into extracellular protein complexes, the bands of interest were excised from the gels. Eight gel samples stained with Coomassie blue were destained with acetonitrile and ammonium bicarbonate. The gels were digested with 1 µg trypsin at 37 °C overnight, then desalted and concentrated using a zip-tip (C18, Eppendorf). The samples were eluted directly onto AnchorChip sample plates (BrukerDaltonics, Germany) with 1 µL of matrix (α-cyano-4-hydroxy cinnamic acid, 1 mg/mL in 90% *v*/*v* acetonitrile, 0.1% trifluoroacetic acid (TFA) and allowed to air-dry. An additional 1 µL of matrix was spotted on top of the samples.

Matrix-assisted laser desorption ionisation (MALDI) mass spectroscopy was performed using an Applied Biosystems 4800 Proteomics Analyser. A Nd:YAG laser (355 nm) was used to irradiate the sample, and the spectra were acquired in reflection mode in the mass range of 700 to 3500 Da.

The instrument was then switched to MS/MS (TOF/TOF) mode, where the eight strongest peptides from the MS scan were isolated and fragmented by collision-induced dissociation using filtered air, then re-accelerated to measure their masses and intensities. A near-point calibration that gives a typical mass accuracy of 50 ppm or less was applied. The data were exported in a format suitable for submission to the database search program Mascot (Matrix Science Ltd, London, UK). Peak lists were searched against fungi that are tabulated in the NCBInr database (ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz). High scores in the database search indicate a likely match and were confirmed by operator inspection.

Protein determination

The concentration of protein was estimated using the dye-binding method of Bradford (1976) using Bio-Rad dye reagent (BioRad Protein Assay Dye Reagent; cat# 500-0006; kept at +4 °C) concentrate in microtiter plates. A standard curve was generated using solutions of 1 μg/μL bovine serum albumin (BSA). The absorbance was measured at 595 nm following 5 min of incubation at room temperature, and was performed in triplicate.

Temperature and pH Optimisation of Purified Cellulase Components

The optimum temperature of the purified cellulase components for hydrolysis of CMC, pNPC, and pNPG were determined by incubating each purified cellulase component (separately in 50 mM NaAc buffer, pH 5) with 1% (w/v) of their respective substrate for 60 min at temperatures ranging from 30 °C to 70 °C. After 60 min of incubation, the reaction was stopped by the addition of the DNS solution.

The optimum pH of the purified cellulase components was determined by incubating the different purified enzymes separately with 1% (w/v) of the appropriate buffers, to include a 50 mM citrate buffer (pH 3.0 to 6.0), a 50 mM sodium phosphate buffer (pH 6.0 to 8.0), a 50 mM Tris-HCl buffer (pH 8.0 to 9.0), and a 50 mM glycine-NaOH buffer (pH 9.0 to 11.0).

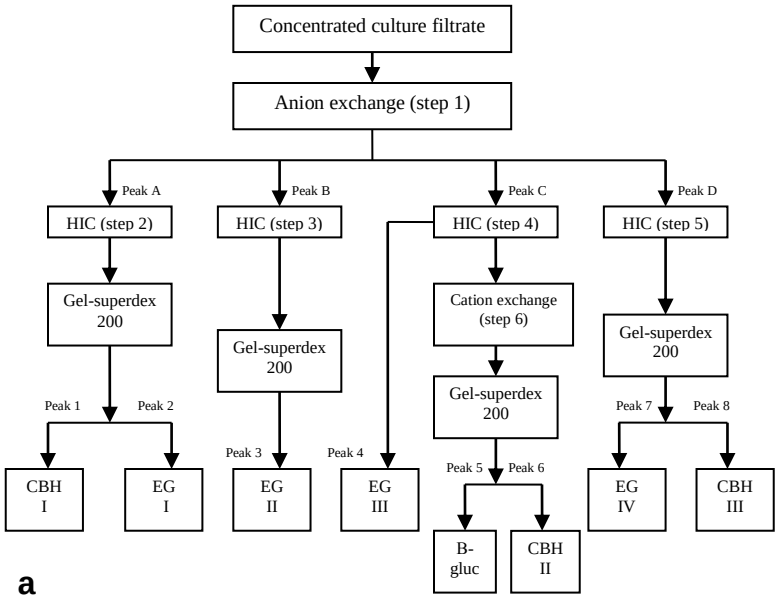
Each reaction mixture containing buffers of various pH values were incubated for 60 min at 50 °C, and the cellulase activity was assayed using the previously described DNS method.

RESULTS AND DISCUSSION

Purification of Cellulases

The entire purification scheme of the multi-enzyme complex produced by *A. terreus* is shown in Fig. 1. Enzyme purification using anion exchange chromatography produced four major peaks: A, B, C, and D. The enzymes present in the fractions corresponding to the four peaks showed activities toward the substrates CMC, Avicel, pNPC, and pNPG.

The collected fractions from peaks I, II, III, and IV were further purified, as outlined in Fig. 2. More detailed purification information is shown in Table 2.



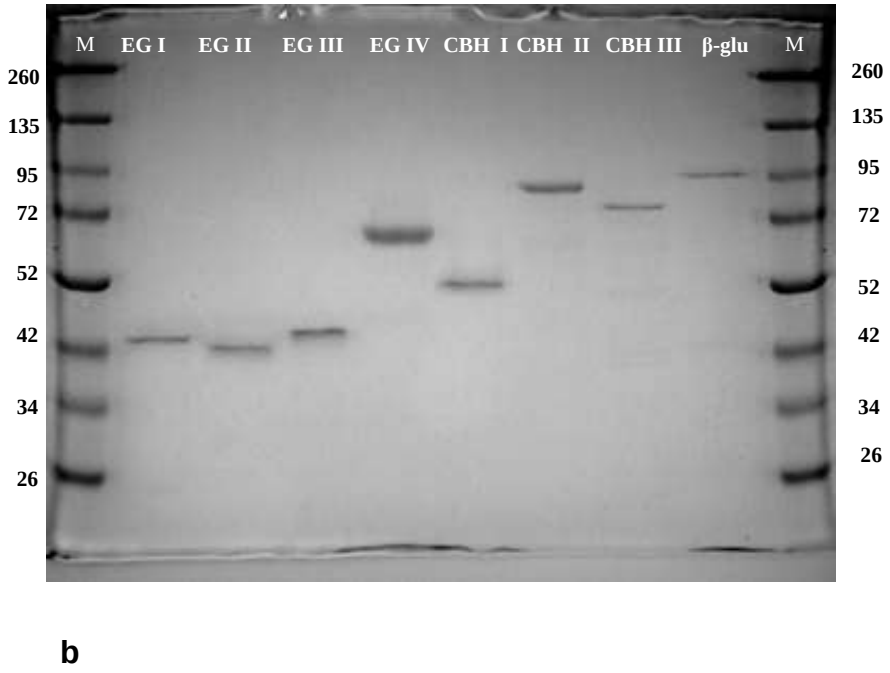


Fig. 1. Purification of cellulases from *A. terreus*. (a) The protein purification scheme from the culture filtrate. The parameters used in each purification step are described in Table 2. (b) SDS-PAGE (12% gel) of the purified enzymes (endoglucanases, cellobiohydrolase, and β-glucosidase)

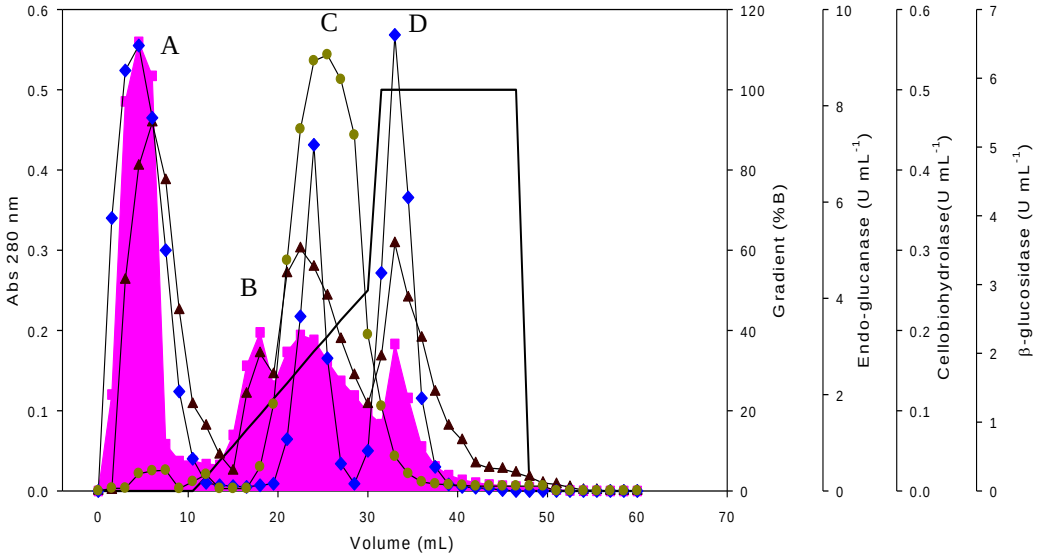


Fig. 2. Protein purification of the culture filtrate by anion exchange chromatography using a HiTrap QFF column. Distribution of the protein and elution profile of the proteins recorded at 280 nm (■); profile of the 1 M NaCl gradient (—) on endoglucanases (▲), cellobiohydrolase (♦), and β-glucosidase (●)

Table 2. Chromatographic Steps for Purification of Enzymes from *A. terreus*

Step	Sample	Column and buffer	Gradient	Flow (mL min^{-1})
1	Cultivation broth	1 mL HiTrap Q FF A: Tris-HCl 20 mM pH 7.5 B: A+ 1 M NaCl	10 mL A 20 mL 0-50% 20 mL B	1
2	Fractions in Peak A	1 mL HiTrap Phenyl FF A: $(NH_4)_2SO_4$ 1 M pH 5 B: NaAC 50 mM	10 mL A 20 mL 0-50% 20 mL B	1
3	Fractions in Peak B	1 mL HiTrap Phenyl FF A: $(NH_4)_2SO_4$ 1 M pH 5 B: NaAC 50 mM	10 mL A 20 mL 0-100% 20 mL B	1
4	Fractions in Peak C	1 mL HiTrap Phenyl FF A: $(NH_4)_2SO_4$ 1 M pH 5 B: NaAC 50 mM	10 mL A 20 mL 0-100% 20 mL B	1
5	Fractions in Peak D	1 mL HiTrap Phenyl FF A: $(NH_4)_2SO_4$ 1 M pH 5 B: NaAC 50 mM	10 mL A 20 mL 0-100% 20 mL B	1
6	Fractions from step 4	1 mL HiTrap SP FF A: NaAC 50 mM pH 4 B: NaAC 1 M pH 4	5 mL A 15 mL 0-20 % 10 mL 20-50 % 20 mL B	1
7	Gel filtration	Hiload 16/60 Superdex 200 prep grade A:NaAc 50 mM pH 5 B: NaCl 1 M	Liner Elute 85% A + 15% B	0.5

NaAC**:** sodium acetate

Purification of Enzymes from Peak A

The proteins eluting in peak A were further purified using a hydrophobic column (Table 2, step 2), leading to the separation of two major peaks: 1 and 2 (Fig. 3). These results show that the proteins in peak 1 did not bind to the HIC column and showed activity mainly toward pNPC, which indicates the presence of enzymes with cellobiohydrolase (CBH) activity. The proteins in peak 2 showed activity mainly towards CMC, with partial activity toward Avicel, suggesting that most of the proteins are enzymes with endoglucanase (EG) activity.

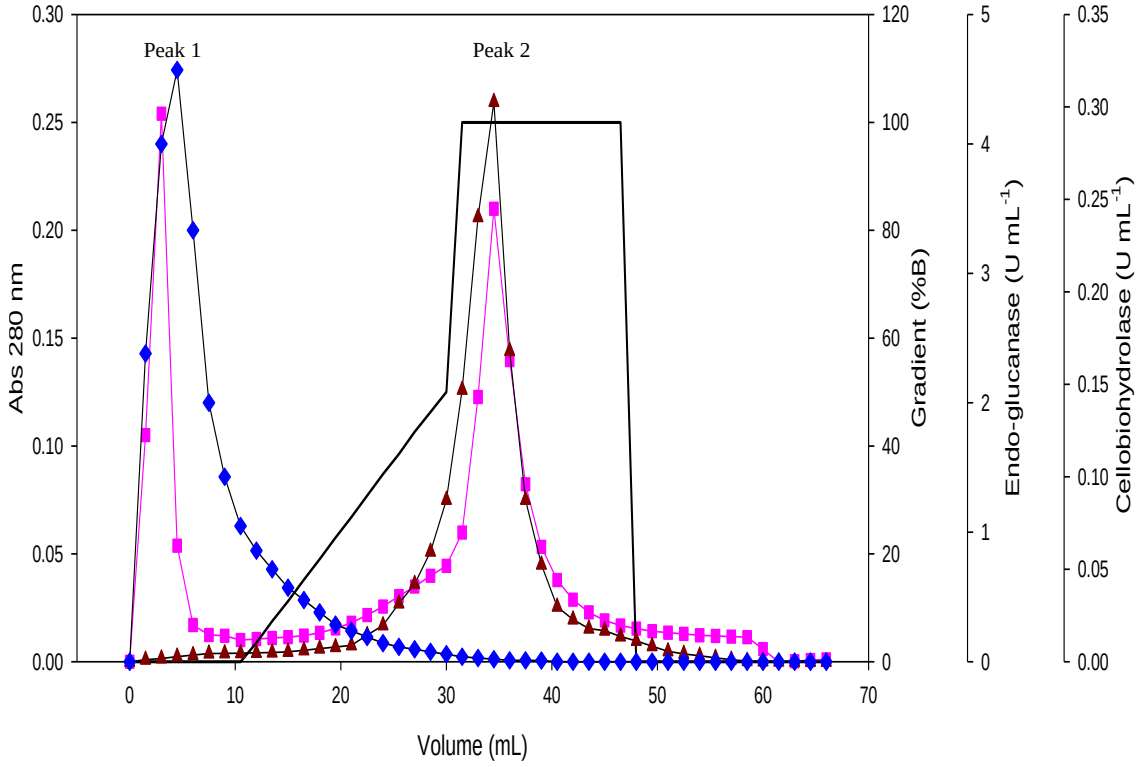


Fig. 3. Protein purification from the culture filtrate by hydrophobic chromatography using a HiTrap Phenyl FF column. (■) represents the distribution of the protein and elution profile of the proteins recorded at 280 nm; (—) gives profile of the 50 mM NaAc gradient on endoglucanases (▲) and cellobiohydrolase (♦)

Purification of Enzymes from Peak B

The proteins eluting in peak B were further purified using a hydrophobic column. These proteins showed activity mainly toward CMC and partial activity toward Avicel (Fig. 4), suggesting that most of the proteins are enzymes with endoglucanase activity. Analysis of the peak by SDS-PAGE following separation on a gel filtration column (Hiload 16/60 Superdex 200) revealed the presence of a single protein (EG II) in peak 3.

Purification of Enzymes from Peak C

The proteins eluting in peak C were further purified using a hydrophobic column, leading to the separation of three major peaks: 4, 5, and 6. The proteins in peak C showed enzyme activity toward Avicel, CMC, pNPC, and pNPG, suggesting that the proteins possessed characteristics of endoglucanases, cellobiohydrolase, and β-glucosidases.

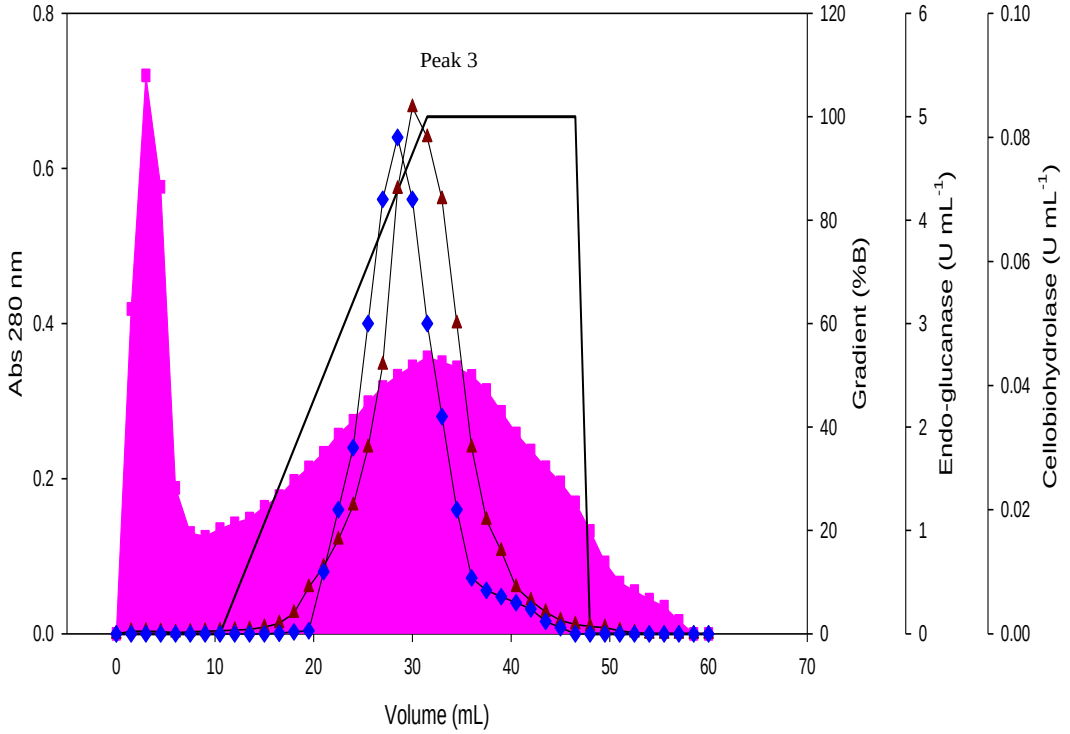


Fig. 4. Protein purification from the culture filtrate by hydrophobic chromatography using a HiTrap Phenyl FF column. Distribution of the protein and elution profile of the proteins recorded at 280 nm (■); profile of the 50 mM NaAc gradient (—) on endoglucanases (▲) and cellobiohydrolase (♦)

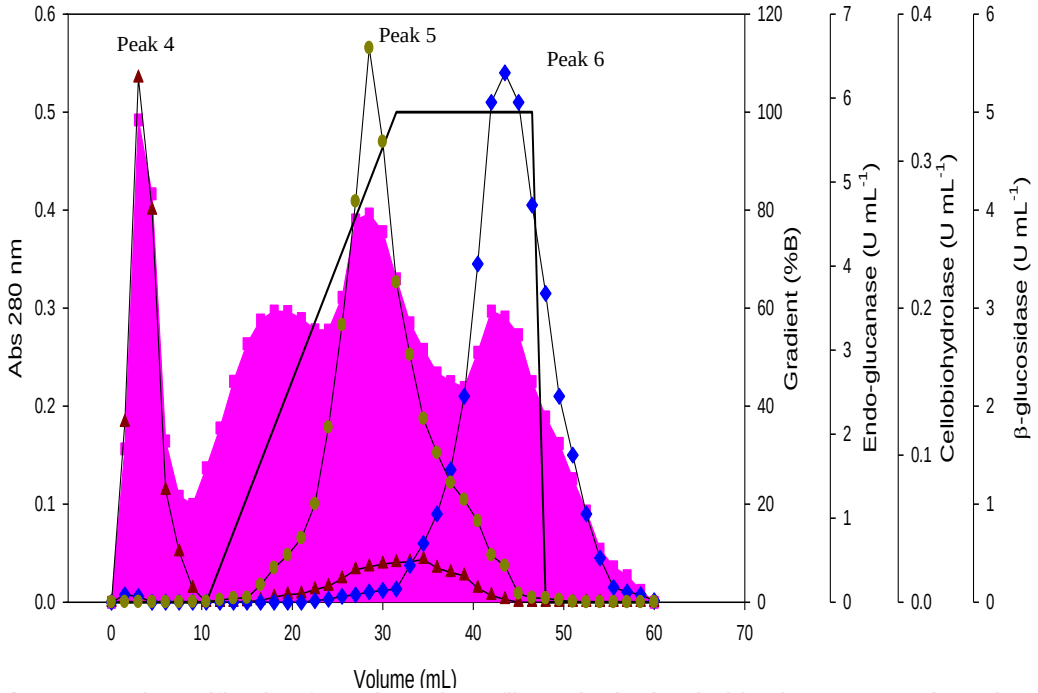


Fig. 5. Protein purification from the culture filtrate by hydrophobic chromatography using a HiTrap Phenyl FF column. Distribution of the protein and elution profile of the proteins recorded at 280 nm (■); profile of the 50 mM NaAc gradient (—) on endoglucanases (▲),cellobiohydrolase (♦), and β-glucosidase (●)

The proteins in peak 4 were active mainly toward CMC and showed partial activity toward Avicel, suggesting that most of the proteins were enzymes with endoglucanase activity. The proteins in peaks 5 and 6 were further purified using a cation-exchange column. Upon separation, the proteins in peak 5 showed active mainly toward pNPG, indicating β-glucosidase activity. The proteins in peak 6 were active mainly toward pNPC, indicating the presence of enzymes with cellobiohydrolase activity.

Analysis of SDS-PAGE revealed that the first part of the peak mainly contained enzymes with endoglucanase activity with a molecular mass of around 43 kDa. These proteins were further separated by cation-exchange (Table 2, step 7), resulting in two peaks: 5 and 6 (Fig. 5). Upon separation using an HIC column, the proteins in peak 3 showed activity mainly toward pNPG, which is an indication β-glucosidase activity. These proteins were classified as β-glucosidase (95 kDa) and cellobiohydrolase II (87 kDa).

Purification of β-Glucosidase and Cellobiohydrolase II

β-Glucosidase and cellobiohydrolase II purified from the previous step were further separated using cation-exchange chromatography at pH 4.0 (details are given in Table 2). Because this step assures complete separation of β-Glucosidase and CBH II from the contaminating peaks (Fig. 6), thorough separation in the first step is not entirely necessary. Alternatively, the entire breakthrough material from the first step could be treated together. In this case, the pools of β-Glucosidase and CBH II could be re-chromatographed using the same conditions to increase the sample purity. Ultra-filtration was then used to exchange the buffer and to concentrate the sample.

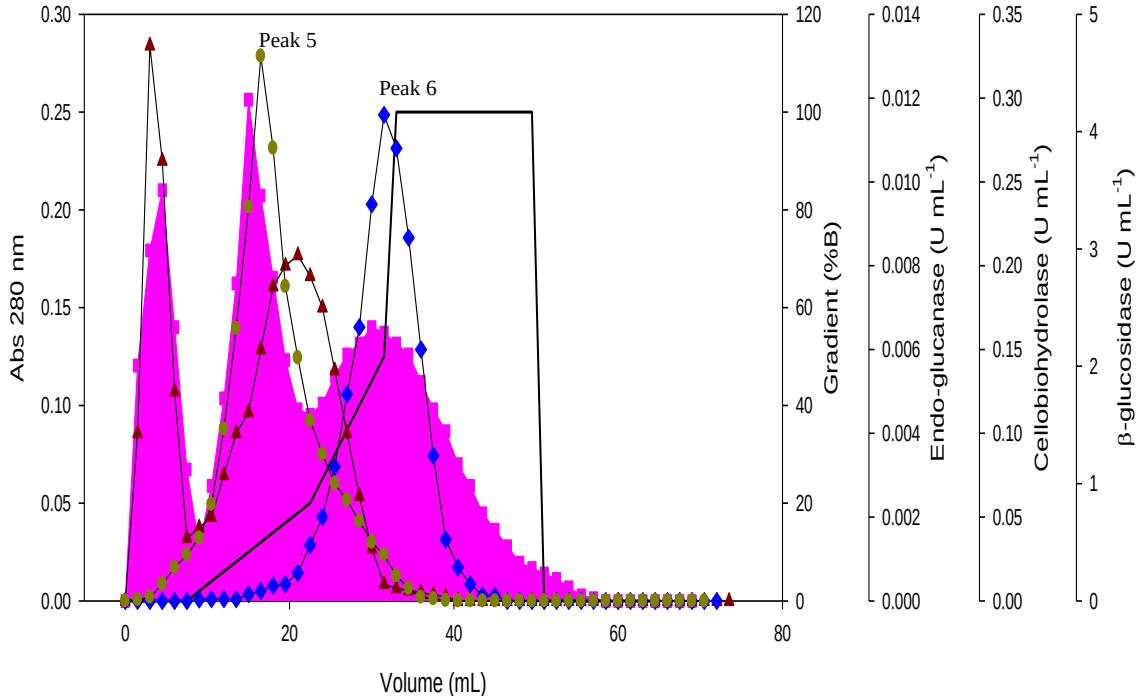


Fig. 6. Protein purification from the culture filtrate by cation exchange chromatography using a HiTrap SP FF column. Distribution of the protein and elution profile of the proteins recorded at 280 nm (■); profile of the 1 M NaAc gradient (—) on endoglucanases (▲), cellobiohydrolase (♦), and β-glucosidase (●).

Purification of Enzymes from Peak D

Protein eluting in peak D showed enzyme activity toward Avicel, CMC, and pNPC, suggesting that the proteins are enzymes containing characteristics of both cellobiohydrolase and endoglucanases. The next purification step of peak D (Table 2, step 6) resulted in a number of smaller peaks and one major peak that is slightly asymmetric. Analysis on SDS-PAGE revealed two peaks, which were further separated using a gel filtration column (Hiload 16/60 Superdex 200) (Table 2, step 7; Fig. 7). Peaks 6 and 7 were revealed to be Endo IV (66 kDa) and CBH III (79 kDa), respectively.

These results demonstrate that four peptides were present in sample 1 (Endo I, 42 kDa), sample 2 (Endo II, 40 kDa), sample 3 (Endo III, 43 kDa), and sample 4 (Endo IV, 66 kDa), and another three peptides were present in sample 5 (CBH I, 51 kDa), sample 6 (CBH II, 87 kDa), and sample 7 (CBH III, 79 kDa). A 110-kDa protein was identified as β-glucosidase (Table 1).

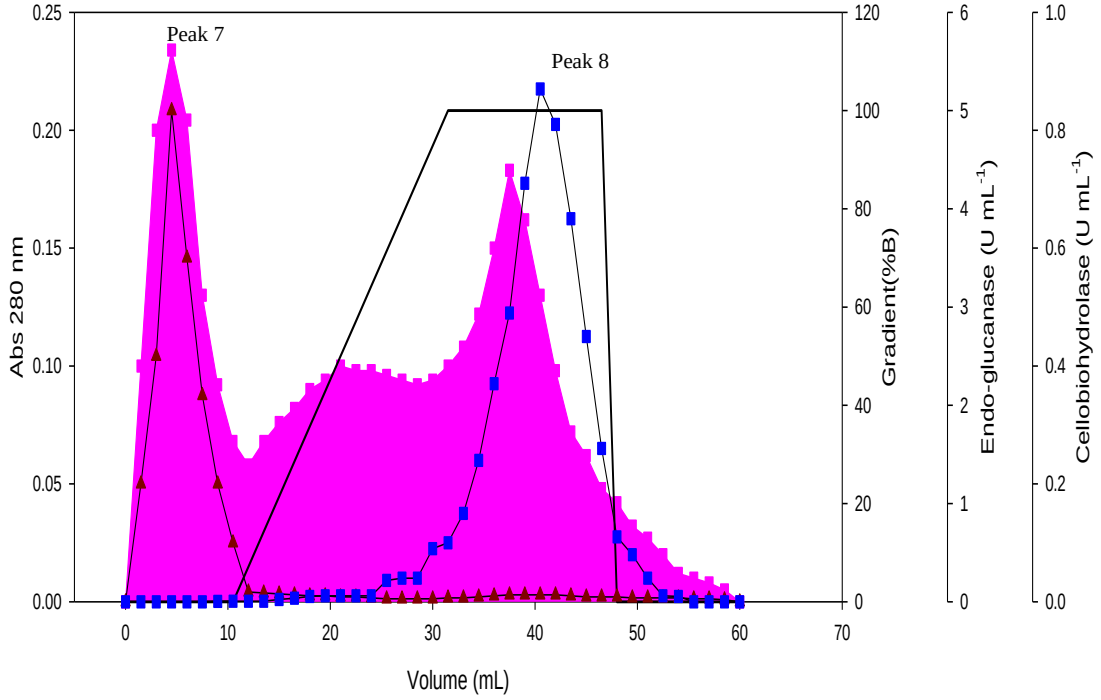


Fig. 7. Protein purification from the culture filtrate by hydrophobic chromatography using a HiTrap Phenyl FF column. Distribution of the protein and elution profile of the proteins recorded at 280 nm (■); profile of the 50 mM NaAc gradient (—) on endoglucanases (▲) and cellobiohydrolase (♦)

Chemical and Physical Properties of Purified Enzyme

The molecular masses and isoelectric points (p*I*s) of the purified endoglucanases, cellobiohydrolase, and β-glucosidase were estimated from the results of MS-MS. The purified enzymes had molecular masses in the range of 40 to 95 kDa, and p*I*s ranging from about 4.7 to 5.4, indicating that these are acidic enzymes. The purity, yield, and enzymatic activities of these enzymes are shown in Table 3.

Table 3. Cellulase Activities of Endoglucanases, Exoglucanases, and β-Glucosidase Purified from *A. terreus*

Enzyme	Yield (%)	Purity[a] (%)	Activity on cellulase substrates (µmol/min/mg protein)			
	Recovery		CMC	Avicel	pNPC	pNPG
EG I	1.2	90	3.6	0.2	0	0
EG II	1.3	97	4.2	0.3	0	0
EG III	2.3	98	5.7	0.7	0	0
EG IV	1.6	87	4.9	0.4	0	0
CBH I	0.8	91	0	0.3	0.26	0
CBH II	1.2	93	0	0.4	0.32	0
CBH III	3.3	96	0.1	0.6	0.79	0
β-glucosidase	7.1	82	0	0	0	5.15

[a] Determined by Total Lab software, version 1.11; Amersham

The Best Temperature Activity of Purified Cellulase Components

Cellobiohydrolase I and II achieved the best temperature cellulase activity at 50 °C, while CBH III achieved cellulase activity at 54 °C with very weak activity at lower temperatures. Endoglucanases I and II achieved the best activities at 58 and 62 °C, respectively, while endoglucanases III and IV achieved the best temperature activities at 54 °C. The β-glucosidase activity from *A. terreus* was the best at 46 °C (Table 4).

Table 4. Chemical and Physical Properties of Purified Cellulase Components from *A. terreus*

Cellulase component	best pH activity	best Temperature activity (°C)
Endoglucanases		
Endo-I	5.5	58
Endo-II	5.5	62
Endo-III	5.0	54
Endo-IV	6.0	54
Cellobiohydrolases		
CBH-I	5.0	50
CBH-II	5.5	50
CBH-III	5.5	54
β-glucosidase	6.0	46

The Best pH Activity of Purified Cellulases Components

Most of the best pH enzyme activities were determined to be between pH 5.0 and 6.0. Endoglucanases I and II were found to be most active at pH 5.5, while endoglucanases III and IV were most active at pH 5.0 and 6.0, respectively. Likewise, CBH I from *A. terreus* had maximal enzyme activity at pH 5.0. The best pH value for CBH II and III activities was pH 5.5, while β-glucosidase is most active at pH 6.0 (Table 4).

The filamentous fungus *A. terreus* isolated from OPEFB fibre in Malaysia has previously been optimised for batch fermentation in 2-L stirred tank bioreactors, and it was shown to be able to produce cellulases (Shahriarinour *et al.* 2011a). The bioconversion of renewable lignocellulosic biomass to ethanol as an alternative to liquid biofuels has attracted the attention of researchers since the beginning of the oil crisis. Cellulases provide a key opportunity for achieving the tremendous benefits of biomass utilisation (Wen *et al.* 2005). The enzymatic degradation of cellulosic materials by fungal enzyme systems has been suggested as a feasible alternative to produce fermentable sugars and ethanol biofuel from lignocellulosics (Oksanen *et al.* 2000; Shin *et al.* 2000). Therefore, cellulases produced by fungi, especially by *T. reesei* and *T. viride*, have been extensively studied, and much progress has been made thus far. In spite of present successes, the task of finding new, highly active cellulases or efficient producers of cellulases remains an unmet challenge. It should be noted that the mostly studied fungus *T. viride* has only two cellobiohydrolases, CBH I (Cel 7A) and CBH II (Cel 6A) (Teeri 1997; Schulein 2000; Foreman *et al*. 2003), and other fungi, such as *Humicola insolens*, also secrete only two cellobiohydrolases (Schulein 1997).

Seven purified glucanase fractions and one β-glucosidase were purified from the culture filtrate of *A. terreus* (Fig. 1). Activity of the purified enzymes toward different cellulose substrates, CMC, Avicel, pNPC, and pNPG, were detected. Substrate specificity is the traditional way of distinguishing endocellulolytic from exocellulolytic action. The relative specific activities of purified cellulase components are presented in Table 3. CBH I, II, and III are active mainly toward pNPC and are not active toward CMC. It is generally accepted that cellobiohydrolases are not able to hydrolyse CMC due to their carboxymethyl side groups, which prevents the cellulose chain from entering the narrow tunnel leading to the active site of the cellobiohydrolases (Teeri and Koivula 1995). Other hydrolysis studies with cellobiohydrolases from *T. reesei* have shown that cellobiohydrolases mainly produce cellobiose during hydrolysis of cellulose (Saloheimo *et al.* 1994). The results obtained with CBH I, II, and III in this study therefore strongly indicate that these three enzymes are three cellobiohydrolases. Comparing CBH I, II, and III to the cellobiohydrolases from *T. reesei* reveals some similarities. The enzymes CBHI, CBHII and CBHIII from *A. terreus* have shown that a molecular mass of 51, 87 and 79 respectively. According our result CBHI with 51 kDa molecular weight, is similar Cel6A (CBHII) from *T. reesei* has an estimated molecular weight 52 kDa on a SDS-PAGE. Cel6A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the inverting mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the non-reducing end (Saloheimo *et al.* 1994).

Endonucleases I, II, III, and IV have apparent activity toward CMC and partial activity to Avicel. Their ability to hydrolyse CMC clearly suggests that these four enzymes are endoglucanases, which can be further supported by the product pattern in the hydrolysis of Avicel (Medve *et al.* 2000). The enzymes EG I, II, III, and IV have

estimated molecular weights of 42, 40, 43, and 66 kDa, respectively. This could indicate some similarity between EGI from *A. terreus* and Cel5A (EGII) from *T. reesei*, with a molecular weight of 42 kDa (Saloheimo *et al.* 1994). β-glucosidase was shown to be mainly active towards pNPG, which is indicative of β-glucosidase activity. The high activity of β-glucosidase, which can alleviate the limitations of products and offset the small amounts of enzymes produced, advances the conversion of cellulose to glucose. The molecular weight of β-glucosidase was estimated to 95 kDa and the p*I* was 5.08. The enzymes Cel3A (BGLI) from *T. reesei* and *T. harzianum* was observed in molecular weight of 90.5 and 109 KDa, respectively. β-Glucosidase hydrolyzes the soluble oligosaccharides, produced by cellulases, to glucose. The addition of β-glucosidases into the *T. reesei* cellulases system achieved better saccharification than the system without β-glucosidases [Shahbazi *et al.* 2014]. β-Glucosidases hydrolyze the cellobiose which is an inhibitor of cellulase activity. β-Glucosidase produced by *A. terreus* can be considered an option for the future practical application in bio-ethanol yields.

Three cellobiohydrolases and four endoglucanases were found in this study based on activity stains in gels. MALDI mass spectroscopy and peptide mass fingerprinting were performed to clearly identify whether the active stained proteins were cellulase complexes. The eight bands stained were the previously known cellulolase components, EG I, II, III, and IV, CBH I, II, and III, and β-glucosidase.

Notably, the mostly studied fungus *A. terreus* has only one cellobiohydrolase (Araujo and D'Souza 1986), while some fungi such as *T. viride* secrete three cellobiohydrolases (Foreman *et al.* 2003; Beldman *et al.* 2005). Improved cellulase production by *A. terreus* has been previously reported, and the optimised conditions for both shake-flask and batch fermentation in 2-L stirred tank bioreactors were achieved through response surface methodology (Nour *et al.* 2010; Shahriarinour *et al.* 2011c; 2011d; 2011e). In this study, we observed that the level of β-glucosidase activity (5.15 U) was much higher than that produced by *A. terreus* (Hui *et al.* 2010) and *Trichoderma viride* (Jiang *et al.* 2011). The fact that the β-glucosidase, produced by *A. terreus*, has a relatively high specific enzymatic activity deserves much attention. The high activity of β-glucosidase, which can avoid inhibition of end-products and offset the small amounts of enzymes, brings advancement in the conversion of the cellulose to glucose. As a result, the β-glucosidase produced by *A. terreus* can be considered an option for future practical application in bio-ethanol yields. Because of the high activity compared with other purified β-glucosidases, the purified β-glucosidase of *A. terreus* shows potential as an industrial source of this important enzyme.

CONCLUSIONS

1. The cellulolytic system of the filamentous fungus *Aspergillus terreus* has not previously been investigated in detail. This study focused on the purification of some of the enzymes produced in the highest quantities. The purified enzymes were studied on different substrates to classify the enzymes. For the first time, in this study, four endoglucanases, three cellobiohydrolases, and one β-glucosidase were successfully purified from *A. terreus* with high purities and yields by column chromatography.

2. The molecular masses of purified cellulase components were found to be approximately 40 to 95 kDa by SDS-PAGE. The purified cellulase components degraded carboxymethyl-cellulose (CMC), p-nitrophenyl-β-D-cellobioside (pNPC), and p-nitrophenyl-β-D-glycopyranoside (pNPG) as well as Avicel, which is a microcrystalline cellulosic material.
3. The best temperature and pH ranges for maximal enzyme activity were 46 to 62 °C and 5.0 to 6.0, respectively.
4. *A. terreus* isolated and used in this study can utilise OPEFB fibres, one of the primary cellulosic waste-materials in Malaysia, as a substrate for growth, thus producing high levels of cellulases.

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