Journal of Advances in Biology & Biotechnology
4(3): 1-22, 2015; Article no.JABB.18129
ISSN: 2394-1081

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Optimised Production of Xylanase by *Aspergillus brasiliensis* under Submerged Fermentation (SmF) and Its Purification Using a Two-step Column Chromatography

Hooi Ling Ho[1*] and Zaki Iylia[1]

[1]Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, Cheras, 56000 Kuala Lumpur, Malaysia.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2015/18129
Editor(s):
(1) Anil Kumar, Professor & Head (Chair), School of Biotechnology, Devi Ahilya University, Madhya Pradesh, India.
Reviewers:
(1) Ponchang Apollos Wuyep, Plant Science and Technology, University of Jos, Nigeria.
(2) Anonymous, Universiti Sains Malaysia, Malaysia.
(3) Anonymous, Federal University of MatoGrosso of Sul, Brazil.
(4) Anonymous, Pondicherry University, India.
Complete Peer review History: http://sciencedomain.org/review-history/10261

Original Research Article

Received 6th April 2015
Accepted 11th July 2015
Published 21st July 2015

ABSTRACT

Aims: Carbon and nitrogen source play a significant role in microorganisms growth especially for the production of enzymes. Expensive medium composition creates huge setbacks especially in the production of commercial enzymes such as xylanase. Hence, in this study, wheat bran as the agricultural residue supplemented with yeast extract was used as the sole carbon and nitrogen source to culture *Aspergillus brasiliensis* ATCC 16404. This cheap alternative carbon source was used comprehensively to replace the expensive medium especially xylan in the production of xylanase. In fact, one of the main factors of using wheat bran is due to its cost effectiveness and availability in current market. Therefore, the main objectives of this study were to elucidate the production of xylanase from *A. brasiliensis* under submerged fermentation (SmF) using wheat bran as the sole carbon source followed by purification of the xylanase to its homogeneity. SmF was chosen primarily as the mode of fermentation for the production of xylanase since it was easily

**Corresponding author: E-mail: hohooiling@gmail.com;*

operated in terms of controlling its parameters including the supply of oxygen and nutrients in a large culture volume for the growth of fungi using the optimised medium composition proposed. Subsequently, the purification of xylanase was performed in an easy two-step column chromatography after the precipitation with ammonium sulphate and dialysis, respectively.

Methodology: In this study, the production of xylanase by *A. brasiliensis* was conducted under SmF using wheat bran as the prime carbon source followed by a two-step column chromatography of diethylaminoethanol (DEAE) Sepharose and Sephadex G-75 for the purification of xylanase before subjected to zymography analysis to detect the biological activity of the purified xylanase based on its substrates hydrolysation.

Results: From the results, high production of xylanase was exerted, producing 11.49 U/mL with protein concentration of 2.33 mg/mL at 72-hour of fermentation. The maximum spore count of 7.91 $\times 10^5$ cells/mL with final pH of 6.9 was able to produce the optimum xylanase activity of 11.49 U/mL. Xylanase secreted from *A. brasiliensis* was precipitated using ammonium sulphate and purified via DEAE Sepharose and Sephadex G-75 column chromatography. The purified xylanase was illustrated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of 36 kDa. Approximately a two and the half purification fold with a surprisingly high recovery of 38.15% was obtained. From our study, the purified extracellular xylanase was subjected to the zymography analysis using xylan supplemented potato dextrose agar (PDA) to analyze the biological application of xylanase in hydrolyzing its substrate of xylan. Since xylanase showed its activity only on xylan, therefore, the formation of visible halo ring in the zymography analysis was managed to indicate that this enzyme was an active xylanase after the two-step column chromatography.

Conclusion: The purified xylanase that obtained in this study possessed much greater potential in industrial applications since it had successfully demonstrated a visible convincing halo ring as a result of its active functional activity against its substrate of xylan.

Keywords: Xylan; xylanase; Aspergillus brasiliensis; wheat bran; two-step column chromatography; DEAE sepharose and sephadex G-75; zymography analysis.

1. INTRODUCTION

Xylanase has been found to be the most well known enzyme used practically in industrial applications especially in textile, baking and paper industry [1]. Xylanase is hydrolase which depolymerises plant cell wall component of xylan, the second most abundant polysaccharide. Xylanase are sourced variously from plants, marine algae, insects, snails and microorganisms. However, microbial xylanases are preferred as great producers of xylanase. Specifically, xylanases are glycosidases which catalyze the endohydrolysis of glycosidic bonds that hold the xylan structure [2]. Xylanase is assigned in enzyme code of E.C. 3.2.1.8 and its official name is endo-1,4-β-D-xylanase [3]. However, this name has alternatively preferred to be used in synonyms that include xylanase, endoxylanase, β-1,4-xylanase, 4-xylanase and many more [2]. In addition to this, xylanases are commonly sourced from two different families which are glycanase Family 10 or formerly known as Family 'F' and glycanase Family 11, formerly known as Family 'G'. They are basically categorized into these two families following the basis of the glycanases classifications on hydrophobic cluster and sequence similarities [4]. The crucial factors of microbial xylanases classifications are based on their molecular mass and isoelectric point (pI) [5]. Family 10 xylanases comprise of those with high molecular mass but possess low pI values whereas Family 11 xylanases consist of those with low molecular mass but exert high pI values [5]. Recently, this production of hydrolyzing enzyme, xylanase has crucially created a specific demand in several industries. According to Kanwar and Sunita [2], due to the vital ability of microbial xylanase degrading xylan, xylanase has caught up more attention due to its potential role in pulp and paper, food and feed production, textile processing and organic waste treatment [6]. As a result, obtaining a thermostable xylanase has greatly increased the biotechnological importance especially in various industries nowadays, many species and genera of microorganisms have been therefore screened for their potentials to meet the commercial requirements [2]. Indeed, thermophilic and hyperthemophilic species of microorganisms which include both bacteria and fungi have been carefully selected prior to the production of xylanase in large scale. Bacteria such as

Bacillus, Streptomyces, *Thermomyces* and *Pyrococcus* are commonly known to be great producers of thermophilic xylanase [7]. Some thermophilic fungi which have been identified to be able of producing thermostable xylanase are those from *Aspergillus, Penicillum* and *Trichoderma spp* [5]. In conjunction to this, purification of xylanase is also crucial to enhance enzyme efficiency and performance in their conventional industrial applications. The first person to discover the optimization of several parameters in production and purification of fungal enzymes was Dr Takamine in the year of 1894 and subsequently in 1914. Medium formulations prior to the incubation of fungi used for the production of fungal enzymes have been optimized. Indeed, wheat bran was optimised as the most satisfying substrate in producing great amount of fungal enzymes [8]. Generally, production and purification of microbial enzymes involved tremendous practical steps. Many circumstances are needed to be optimized especially in determining the right medium and other growth parameters prior to enzymes purification. In this study, *A. brasiliensis* has been chosen due to its potential in producing great amount of xylanase. Subramaniyam and Prema [9] proposed that filamentous fungi is one of the most crucial producers of enzymes especially xylanase that has important commercial applications compared to those from bacteria. As such, various measures have to be well managed and understood prior to production and purification of xylanase from microbial sources. The objectives of the study were to determine the production of xylanase from *A. brasiliensis* using wheat bran as the prime carbon source under SmF and to purify the xylanase using a two-step column chromatography.

2. MATERIALS AND METHODS

2.1 Microorganism

The fungus that used for xylanase production and purification in this study was *A. brasiliensis* ATCC 16404. Potato Dextrose Agar (PDA) was prepared and autoclaved at 121°C for 15 min before it was poured on the sterile Petri dishes using aseptic technique. Then, *A. brasiliensis* was subcultured at 30°C until the formation of colonies appeared after 5-day of incubation.

2.2 Plate Hydrolysis Assay

Plate hydrolysis assay is an important step to determine the possibility of a culture in producing desired enzymes. Hence, this method is crucial prior to production of desired enzymes. In this research project, an agar known as xylan-PDA agar that supplemented with 1% xylan and essential nutrients were prepared. The medium composition for plate hydrolysis assay was consisted of (g/L): PDA, 39; beechwood xylan, 10; peptone, 5; yeast extract, 5; $MgSO_4.7H_2O$, 0.2 and K_2HPO_4, 1. Beechwood xylan was weighed and mixed to 1 L of distilled water to prepare the 1% xylan solution. Upon that, PDA and the other medium compositions which consisted of yeast extract, peptone, $MgSO_4.7H_2O$ and K_2HPO_4 were also added before sterilization at 121°C for 15 min. Subsequently, the medium solution was poured onto Petri dishes to form the xylan-PDA agar. The plates were left to solidify before stored at 4°C. Upon usage, the stock culture of *A. brasiliensis* was taken into the laminar flow. Sterile distilled water was added to the fungal culture. Then, it was slowly scrubbed with a sterile L-hockey stick to form a fungal spore suspension. After that, 50 μl of the spore suspension was added onto the xylan-PDA agar and spread evenly using sterile L-rod. Then, the agar was incubated at 30°C overnight. On the next day, 1% Congo red dye used as the staining reagent was dripped onto the fungal growing colonies that grown on the surface of the agar. The excess staining reagent on the plate was discarded before the agar was dried for a few minutes. In order to perform the destaining, addition of 1 M NaCl was used to drain off the excess dye from the agar. The appearance of clear zone on the xylan-PDA agar as a result of decoloration indicates the presence of xylanase activity whereby xylan substrate was hydrolyzed by xylanase secreted by *A. brasiliensis.*

2.3 Optimisation of Culture Medium and Growth Conditions for the Maximum Production of Xylanase by *A. brasiliensis* under Submerged Fermentation (SmF)

2.3.1 Pre-treatment of wheat bran

First and foremost, many researches claimed that wheat bran is one of the great substrates for fungi especially *A. brasiliensis* to grow and to produce the optimum amount of xylanase [10]. Wheat bran was first being homogenized using a food grinder. This is to ensure the rough and large structure of wheat bran was being removed. As a result, a finer, smoother and

easily handled wheat bran structure was anticipated. Then, it was dried in oven at 65°C until the constant weight was achieved.

2.3.2 Inoculation of spores of *A. brasiliensis* for the production of xylanase

Sterile distilled water was added into the stock culture of *A. brasiliensis* before the spore suspension was harvested using a sterile L-hockey stick. Subsequently, the dilution was performed to obtain the standard inoculum size of 3 × 10^6 spores before transferred into 150 mL culture medium in a conical flask. The fermentation of *A. brasiliensis* for the production of xylanase was performed up to 144 h using wheat bran as the sole carbon source at the initial medium pH 6.5 at 30°C.

2.3.3 Medium formulation and growth conditions

A. brasiliensis was grown in the optimised medium formulation containing (g/L): yeast extract, 2.0; KH_2PO_4, 1.52; $MgSO_4.7H_2O$, 0.52; KCl, 0.52 and pre-treated wheat bran, 10.0 according to Ho and Heng, [11]. The fermentation of the fungi was carried out under optimised growth conditions in a medium working volume of 150 mL in 250 mL Erlenmeyer flasks at 30°C in a rotary shaker at 150 rpm for 144 h of SmF [12]. Then, 10 mL of culture sample was harvested at 24 h interval for sampling analysis. The samples were centrifuged at 8500 rpm at 4°C. The supernatants contained the crude extracellular xylanase used for further analysis steps. The production of xylanase by *A. brasiliensis* under SmF was conducted in triplicate.

2.4 Sampling Analysis and Extraction of Xylanase

At every 24 h interval, sample from culture medium was withdrawn from the culture flask incubated at 30°C. Sampling of the culture medium was conducted in a laminar flow to avoid from the contamination of microorganisms invading into the flask culture. After that, 15 mL culture medium was pipette at every 24 h up to 144 h of SmF. Then, 5 mL from the supernatant was used for the quantification of biomass concentration using spore count and determination of medium pH in order to study the pH profile of culture medium. On the other hand, 10 mL of the medium was then centrifuged at 8500 rpm at 4ºC to extract the crude enzyme from the supernatant for further analysis which included the quantification of xylanase activity and protein concentration assay.

2.4.1 Xylanase activity assay

Xylanase activity was performed according to Bailey et al. [13] by using 3,5-dinitrosalicyclic (DNS) method in order to detect the reducing sugar of xylose. In this assay, 1% beechwood xylan was used as the substrate in this assay. First, xylan was dissolved in 0.05 M sodium phosphate buffer (pH 5.3) until there were no clumps observed in the mixture. After that, 0.1 mL of crude enzyme was transferred into 0.9 mL of xylan mixture before incubated in water bath at 50°C for 30 min. Then, 1.5 mL of DNS reagent was added into the mixture and subjected to incubation at 90°C for 5 min. Subsequently, 0.5 mL of 40% Rochelle salt was added and the mixture was cooled down at room temperature. Absorbance reading was recorded using optical density (OD) at 575 nm. The activity of xylanase was measured according to the xylose standard curve of the absorbance reading at 575 nm against its concentrations. One unit of xylanase activity (U) is defined as the amount of xylanase required to release one µmole of xylose per mL of enzyme extract per min under assay condition. On the other hand, in the purification study of xylanase by *A. brasiliensis,* the xylanase activity of the fractions that collected from anion and gel filtration column chromatography were measured using the same method according to Bailey et al. [13].

2.4.2 Protein concentration assay

The total protein concentration obtained from the production of xylanase was assayed according to Lowry method [14]. The absorbance reading of protein was measured at OD of 750 nm using spectrophotometer before quantified using the bovine serum albumin (BSA) standard curve. The protein concentration was calculated as according to BSA standard curve of the absorbance reading at 750 nm against its concentrations from 0.0 to 0.1 mg/mL. On the other hand, in purification study of xylanase by *A. brasiliensis,* the protein concentration of the purified fractions collected from column chromatography were quantified using OD at 280 nm.

2.4.3 Quantification of biomass concentration and medium pH

The spore count of *A. brasiliensis* was performed using haemocytometer. The pH of the culture

medium was measured using pH meter to study the profile of pH during the growth and production of xylanase by *A. brasiliensis* under SmF using wheat bran as the prime carbon source.

2.5 Purification of Xylanase Using a Two-step Column Chromatography of Diethylaminoethanol (DEAE) Sepharose and Sephadex G-75

In this purification study, 70% saturation of ammonium sulphate was conducted according to Zulfiqar et al. [15] and Milala et al. [16]. To precipitate the xylanase, 252.88 g ammonium sulphate was added into 580 mL supernatant in order to achieve 70% saturation. After stirring overnight at 4°C, the enzyme sample was subjected to centrifugation at 8500 rpm for 15 min at 4°C. Then, the precipitate was resuspended in 0.05 M sodium phosphate buffer (pH 5.3) before desalted by dialysis. The dialysis tubing was loaded with dialyzed sample and left overnight at 4°C. The concentrated enzyme sample was collected and loaded into an anion exchange chromatography using diethylamino-ethanol (DEAE) Sepharose as the positively charged resin. At the beginning, the column was equilibrated with 0.05 M sodium phosphate buffer (pH 5.3) to prepare the system for chromatographic run. Subsequently, 15 mL of enzyme sample was gently loaded into the column. Then, the elutions were collected as flow-through. After that, 6 mL sterile distilled water was first subjected into the column. Thereafter, the desired proteins were eluted with a linear gradient of 0.1 to 0.5 M NaCl. Then, the fractions were collected to analyse xylanase activity and protein assay at OD 280 nm, respectively. When the column was not in use, the column was filled to the top with 0.05 M sodium phosphate buffer (pH 5.3) to avoid resin dehydration and cracking. The collected fractions with relatively high xylanase activity and protein concentration were pooled together and subjected to a Sephadex G-75 gel filtration column chromatography. The column was first equilibrated with 0.05 M sodium phosphate buffer (pH 5.3). Subsequently, the flow-through was collected. Fractions with relatively high xylanase activity and protein concentration were then loaded into the gel filtration column chromatography. Then, the fractions were eluted using 0.05 M sodium phosphate buffer (pH 5.3) before they were subjected to analysis including xylanase activity and protein assay at OD of 280 nm, respectively.

2.6 Determination of Molecular Weight of Purified Xylanase Using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

One of the practical methods in the determination of the molecular weight of purified xylanase was conducted using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was run according to the method mentioned by Laemmli [17] using 4% stacking gel and 12% resolving gel. The molecular weight of xylanase was compared to a standard protein marker with the range of 10 to 250 kDa (Bio-labs, New England). In order to determine the protein size of xylanase, SDS-PAGE gel was performed at a voltage of 90 V for approximately 2 h.

2.7 Zymography Analysis

Zymography analysis was illustrated as a simple yet sensitive technique in the detection of the biological activity of the purified xylanase based on its substrate hydrolysation [18]. 1% xylan agar was prepared to detect the activity of the purified xylanase activity. The xylan-PDA agar plates were left to solidify under a sterile environment in a horizontal laminar flow. A sterilized filter paper was dipped into the purified xylanase obtained after the two-step column chromatography. Subsequently, the filter paper was transferred onto xylan supplemented PDA agar. Then, it was left to air-dry for a few minutes. After that, a sufficient amount of 5% Congo red dye was dropped onto the filter paper and left to air-dried. Then, the agar plate was incubated at 50°C for 3 days to detect the formation of a clear halo ring. The control of the experiment was conducted by replacing the purified xylanase with sterile distilled water before incubation.

2.8 Data Analysis

In this study, the mean value of the sampling analysis including xylanase activity, protein concentration, spore count and medium pH was used to elucidate the production of xylanase by *A. brasiliensis* under SmF. The standard error was calculated and represented on all the Figures in this study.

3. RESULTS AND DISCUSSION

3.1 *Aspergillus brasiliensis* ATCC 16404

There are evidences by some scientific findings stated that *A. niger* especially strain ATCC 16404

has been reclassified as *A. brasiliensis* ATCC 16404. This statement was also mentioned by Acton [19] which explained that *A. brasiliensis* was formally known as *A. niger*. According to a scientific article in 2010 from Antimicrobial Testing Services (ATS) labs in United States of America, *A. niger* strain ATCC 16404 actually belongs to *A. brasiliensis* species based on the recent molecular testing. Even though, this culture is still belonged to the *Aspergillus* species, however its cell morphology differs in terms of colonies color. Initially, the colonies are in white or yellowish, but after a rapid growth of mycelium, it eventually turns into dark brown or black. The optimum incubation temperature of *Aspergillus spp* is from 25 to 30°C for about 3 to 7 days. This culture is also categorized as Biosafety Level 1 [19] in which practices and facilities are appropriate for undergraduates and high school education system [20].

Why do the researchers and many manufacturers prefer to use microorganisms as their ultimate source of enzymes instead of other sources? Frankly, microbial enzymes production is crucial due to its ability in economic production, consistency, ease of modification, purification and optimization [21]. Besides that, microbial enzymes are much stable compared to plants and animals enzymes besides it is more stable and convenient to employ in large scale production [22]. As a result, filamentous fungi have been chosen to be the main producer of xylanase. According to Pal and Khanum [23], filamentous fungi are useful producers of xylanase due to its capability of producing high amount of extracellular enzymes under submerged and solid state cultivation. The most well known xylanase producers are *Aspergillus spp* as well as *Trichoderma spp.* [23]. *A. brasiliensis* is a type of fungi which capable of producing xylanases including endo-1,4-β-xylanase. Related to this matter, there are also many other types of xylanases which are capable of being produced by fungi especially *Aspergillus spp.* Most of them are extracellular xylanase. However, xylanase produced from fungal are mostly being grouped into Family 11 as proven by Wang et al. [24]. Microbial xylanases have been ultimately used in various industrial applications that include pulp and paper industry [25]. Indeed, xylanase is used in the enzymatic treatment of pulp prior to bleaching in the pulp and paper industry. It is applied to replace the usage of harmful chemicals such as chlorine and chlorine oxide in producing a better quality of pulp for paper production industry [1]. Basically, enzymatic treatment is much environmental friendly as it is less harmful as compared to the chemicals treatment.

3.2 Plate Hydrolysis Analysis

As previously mentioned, xylanase is one of the most important enzymes used in recent commercial applications, especially in textile, pulp and paper industry as well as in baking process [1]. Determination of fungal ability of producing desired enzymes are crucial to ensure that the culture is continuously able of producing the desired enzymes before proceeding to other experimental steps. Hence, plate hydrolysis assay was conducted to verify the fungal ability in producing xylanase. Previous research has been done by several researches [26,27,28] and mostly Congo red was used to observe the decolorisation of clear zone formation. Congo red dye is a type of diazo dye which basically consisted of an aromatic amine structure of benzidine that binds to the carbohydare polymers [29]. Congo red dye was used extensively in the study of decolorizing zone of enzymes which applied onto a substrate supplemented agar with the presence of fungal culture. Besides that, it was claimed by Quratulain et al. [27] that fungal enzymes such as from *A. niger* was able to distinguish based on plate hydrolysis test. Similarly, cellulases are also able to hyrdrolyze carboxymethyl cellulose and therefore producing a clear zone around the ring structure. The appearance of the clear zone indicates there was presence of cellulase activity which was particularly able to hydrolyze its substrate [27, 30]. As for xylanase, it was also proven by Nair et al. [26] that the clear zones would form as a result of xylan hydrolysation. Therefore, any hydrolyzing enzymes that are capable to degrade its substrates have been further confirmed through plate hydrolysis analysis method. In fact, identical study was also performed by Ribeiro and his co-workers [31], using various filamentous fungi to screen for xylanase activity on a xylan-enriched agar. After 72 h of incubation, clear zone was evidently formed surrounding the fungi culture and Congo red dye was in fact, added for better observation of the clear zone [31]. More examples on plate hydrolysis assay in the screening of enzymatic activity from various filamentous fungi and other microbes are listed in Table 1.

Fig. 1 illustrates the results of plate hydrolysis assay by *A. brasiliensis* in this study. It therefore could be clearly seen that the clear zone was

formed on the surrounding of *A. brasiliensis* culture. 1% of Congo red dye was added onto the agar for better observation. After a few minutes, the decolorisation of Congo red dye was appeared as the result of the formation of different tones of red color with the distinct appearance of clear zone at the centre of the xylan-PDA agar as shown in the Fig. 1. The mycelium of *A. brasiliensis* was actively penetrated into the agar in order to secrete xylanase for the hydrolysis of its substrate of xylan that entrapped in the xylan-PDA agar. As a result, a clear zone of xylanase was evident and fully distributed around the growing fungal colonies on the surface of the agar. The decolorisation of the clear zone was large enough where it was easily observed and detectable with the naked eyes. In fact, it is also convinced by Jo et al. [30] in which the formation of the clear zones occurred as a result of the enzymatic reactions with its substrates. The enzymatic reactions may be varied and it is proven based on their different tones of colors. Some of fungal species may produce darker zone color compared to those from *Aspergillus spp*. As such, a recent study by Jo et al. [30] has shown that the differences in color formation of clear zones by different microorganisms could be explained by the differences in pH of Congo red dye used. In fact, this study was proven identical with the study by Jo et al. [30]. They clearly proposed that Congo red dye with pH ranging from 4.5 to 8.0 would give different tones of color in the process of the substrates hydrolysation by enzymes secreted by microorganisms. Therefore, lighter and even darker zones of decolorisation would anticipate to be appeared depending on the types of microorganisms used and its corresponding reactions towards Congo red and pH. On the other hand, there are several other dyes available used as an indicator in the hydrolysis assay such as phenol red and methylene blue. However, in this study, Congo red dye has been used efficiently as an indicator for the clear zone formation in the qualitative study of xylanase. This application is satisfied because Congo red dye is also well known to execute its interaction with β-1,3 and β-1,4 glucans backbone linkages which are found primarily in the hemicelluloses especially present in xylan [37]. Xylan is consisted of β-1,4 glucan linkages at its backbone in order to hold and manage the structure of xylan. Xylan has a complex structure, besides consisted of β-1,4-linked xylose residues at the backbone, residues such as O-acetyl, arabinofuranosyl, glucoronic and phenolic acid are attached together at the side [38]. As mentioned by Collins et al*.* [39], xylan is easily available from agricultural residues especially during wood processing. That is why in this study, xylan has been therefore, chosen as the primary substrate for the analysis of the xylanase activity. With the application of Congo red in this study, the clear zone formation due to the hydrolysis of xylan from xylanase was anticipated on the xylan-PDA agar as shown in the Fig. 1. In contrast, the control of the xylan-PDA agar (left) without the fungal inoculum shows no clear zone of decolorisation in the Fig. 1.

Table 1. Plate hydrolysis assay in screening of various enzymes based on the formation of decolorisation zone on substrate-enriched agar

Microorganism	Enzyme	Dye	Substrate	Reference
Aspergillus spp	Xylanase	Congo red	Birchwood xylan	Nair *et al.* [26]
Chaetomium thermophile	Xylanase	Congo red	Oat spelt xylan	Saleem *et al.* [32]
Trichoderma spp	Xylanase	Congo red	Birchwood xylan	Charitha Devi *et al.* [33]
Aspergillus spp	Cellulase	Congo red	Carboxymethyl Cellulose	Charitha Devi *et al.* [33]
Aspergillus niger	Cellulase	Congo red	Carboxymethyl Cellulose	Quratulain *et al.* [27]
Aspergillus niger, Aspergillus tereus	Cellulase	Congo red	Mandel’s media with sawdust	Jahangeer *et al.* [34]
Trichoderma spp	Cellulase	Congo red	Avicel	Florencia *et al.* [35]
Lentinus polychrous	Laccase	Congo red, Rhodamine B	Potato Dextrose Agar	Suwannawong *et al.* [36]

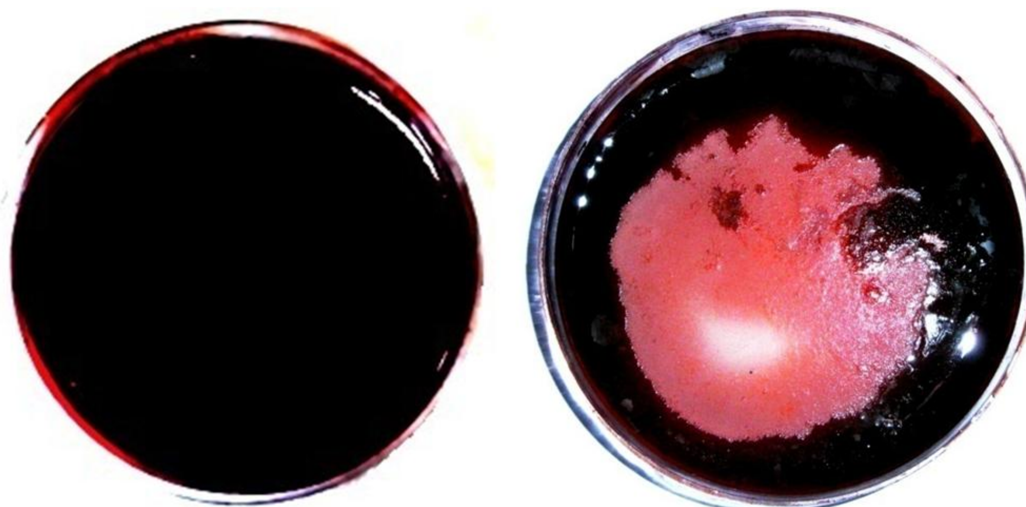

Fig. 1. Decolorisation of Congo red dye occurred after overnight incubation of *A. brasiliensis* on xylan-PDA agar

The appearance of the clear zone as a result of decolorisation of Congo red dye was observed on the xylan-PDA agar after the inoculation of A. brasiliensis overnight (right). Control of xylan-PDA agar without the fungal shows no clear zone of decolorisation (left)

3.3 Maximum Production of Xylanase by *A. brasiliensis* using the Optimised Medium Composition and Growth Conditions under Submerged Fermentation (SmF)

3.3.1 Optimised medium formulation and growth conditions

Prior to the production of xylanase under SmF, culture medium and various growth conditions such as pH, rpm as well as temperature have been regulated continuously. In fact, it was proven by many scientists that these factors are crucially important to optimize the production of enzymes. SmF is the most popular method for the production of fungal xylanase especially endo-1,4-β-xylanase. This is because SmF is easily operated and able to generate high sporulation rate for the fungi to grow optimally after being provided with the optimum medium and high oxygen supply [40]. Besides that, carbon and nitrogen sources are also important for the medium formulation in fermentation process. Without carbon and nitrogen sources, the cultivated microorganisms would not have a hospitable environment to grow for the production of desired enzymes under a fermented phase. As illustrated by Garg and Johri [41], carbon sources commonly used for the production of fungal xylanase are consisted of natural agricultural residues, purified xylan, sugars as well other enhancers or inhibitors. Usually, most scientists used undefined medium such as wheat bran as the primary carbon source in the production of xylanase [42,43]. Unlike defined medium, undefined medium is different in terms of its chemicals composition which is not clearly stated and is usually composed of natural materials [44]. This medium is much cheaper because of its availability in the market and its composition that consists of complex natural materials. For example, wheat bran, rice husks and corn cobs are examples of undefined medium used in the cultivation of microbes and also production of enzymes.

In fact, in this study, culture medium which has been optimised by Ho and Heng, [11] was applied for enzymes production by *A. brasiliensis*. In a scientific study by Muhammad Irfan et al. [45] proposed that in the industrial production of enzymes, production cost is absolutely critical especially when 30% to 40% of production cost is affected by the cost of growth substrate. Therefore, this liability cost should be reduced by implementing the utilization of low cost substrates from various agricultural residues. Some of the examples are wheat bran, sugarcane bagasse, rice husk, soybean meal and corn cobs. Tallapragada et al. [46] mentioned the hemicelluloses and celluloses of agricultural residues such as rice husk and wheat bran were suitable substrates for the xylanase

production. In addition, Tallapragada et al. [46] also mentioned oat spelt xylan and birchwood xylan were efficient inducers for xylanase activity. Likewise, another study conducted by Haltrich et al. [47] discussed the A. niger derived xylanase produced using wheat bran was able to excel out xylanase activity of 27.1 U. In conclusion, the best way of producing great amounts of xylanase is by supplementing wheat bran to further enhance the enzyme secretion. In fact, Archana and Satyanarayan [48] have reported that xylanase production was found to be the optimum by using wheat bran as its primary carbon source. In general, medium composition and growth conditions play an important role in the production of xylanolytic enzymes. Table 2 shows some examples on microbial xylanase production by various fungi and bacteria using wheat bran as the carbon source.

Nonetheless, undefined medium of wheat bran could lead to several setbacks such as inconsistency in the production of xylanase from batch to batch depending on the nutritional value and healthy stage of the undefined medium. Furthermore, the emergence of impurities found in natural materials of the undefined medium would cause contamination in production and difficulty in purification. In addition to this, production and recovery of xylanase would be a lengthy process compared to the usage of defined medium. As a result, the cost of production would eventually be interfered [40]. In a nutshell, the production of xylanase using undefined medium of agro-industrial materials is always anticipated to be relatively differ from using defined medium.

On the other hand, nitrogen source is also important in maintaining growth of microorganisms. Nitrogen source is either classified as inorganic or organic. Organic nitrogen has been used extensively in various fermentation processes. Satyanarayan et al. [58] proven nitrogen source is essential in influencing biomass production as well as metabolites build-ups. Bakri et al. [59] also reported that the best nitrogen source for the maximum xylanase production was involved the use of yeast extract. As a result, the optimum medium formulation containing 2.0 g/L yeast extract with the pre-treated wheat bran of 10.0 g/L as optimised by Ho and Heng, [11] was used in the present study for the maximum production of xylanase by *A. brasiliensis* prior to its purification and recovery process.

3.3.2 Correlation of biomass and xylanase production by *A. brasiliensis* using wheat bran as the carbon source under submerged fermentation (SmF)

As previously discussed, *A. brasiliensis* could be cultivated under both SmF and SsF. However, in our study, SmF has been chosen as a mode of fermentation by the filamentous fungi to grow and sporulate. Hence, to provide ample inoculum amount of *A. brasiliensis* for xylanase production under SmF, subculturing of *A. brasiliensis* was carried out weekly to ensure that the inoculum would be enough before subjecting to fermentation. Furthermore, the use of inexpensive agricultural waste as an alternative replacement of commercially produced xylan creates a big turnover in industrial biotechnology as a result of huge increment of biomass production. As such, wheat bran was selected as the most effective and efficient carbon source in producing greater amounts of multiple enzymes including xylanase. In conjunction, spore count was carried out and the results are shown in Fig. 2. Fig. 2 illustrates the growth profile of *A. brasiliensis* in response to the activity of xylanase using wheat bran as the prime carbon source. Based on the Fig. 2, spore count was increased tremendously from 24 to 72 h with the increment of 53.6% from 3.67×10^5 to 7.91×10^5 spores per mL at 72 h of SmF. The fungi reached its maximum logarithm phase at 72 h of fermentation based on the number of spores observed. In fact, the logarithm phase of *A. brasiliensis* with the highest xylanase was also observed, producing 11.49 U/mL at 72 h. Xylanase produced by *A. brasiliensis* was actively metabolised its hemicelluloses of xylan substrate from wheat bran, hence, releasing xylose as its simplest sugar or monomer. Due to the presence of nutrients which favour the fermentation environment, it resulted in a crucial growth of *A. brasiliensis*, promoting an increase of 0.73 fold of xylanase activity from 24 to 72 h. In fact, carbon, nitrogen source and minerals also play a significant role in the growth of microorganisms including *A. brasiliensis* to ensure an ample amount of xylanase is produced for industrial applications. Nonetheless, in the present study, the number of spores was reduced gradually to around 4.2×10^5 spores/mL at 96 h and it thereafter maintained stationary until the end of fermentation. This explains the profile of xylanase activity that correlated with the number of spores in the fermented culture in this study where the xylanase activity also remained

constant, producing 10.2 U/mL after 96 h of SmF.

3.3.3 Correlation of protein and xylanase production by *A. brasiliensis* using wheat bran as the carbon source under submerged fermentation (SmF)

In this study, xylanolytic enzymes were produced by *A. brasiliensis* in SmF using controlled optimised medium composition with adjusted medium pH 6.5 under controlled growth parameters at agitation at 150 rpm at 30°C for 144 h. Based on the result findings, the maximum production of xylanase was observed at 72 h of SmF. Fig. 3 shows the profile of xylanase activity and protein concentration secreted by *A. brasiliensis* in SmF. Based on Fig. 3, the maximum production of xylanase occurred at 72 h, producing 11.49 U/mL with its maximum concentration of total protein of 2.33 mg/mL. As the time of fermentation prolonged, more enzymes were secreted with the activity of enzymes increased gradually. As enzymes secretion increased, the increment of enzymes amount was reflected on the total protein production since enzymes are categorised as a type of globular protein. However, it was observed that the trends of xylanase activity and protein concentration reduced at 96 h and it thereafter remained constant from 96 h to the

Table 2. Microbial xylanase production using wheat bran as the carbon source

Microorganism	Xylanase activity	Reference
Aspergillus niger	12. 76 U	Guimaraes *et al.* [49]
Aspergillus niger	9.87 U/mL	Kavya and Padmavathi, [43]
Aspergillus niger	1.48 U/mL	Kanimozhi and Nagalakshmi, [50]
Aspergillus niger	6. 47 U/mL	Okafor *et al.* [51]
Aspergillus terricola	7.5 U/mL	Michelin *et al*. [52]
Bacillus arseniciselenatis	910.49 U	Kamble and Jadhav, [53]
Bacillus megatherium	350 U/g	El-Shishtawy *et al.* [54]
Bacillus pumilus AB-1	489.4 U/g	Banu and Ingale, [55]
Bacillus spp	2.0 U/mL	Saleem *et al.* [56]
Bacillus spp	0.45 U/mL	Dholpuria *et al.* [57]

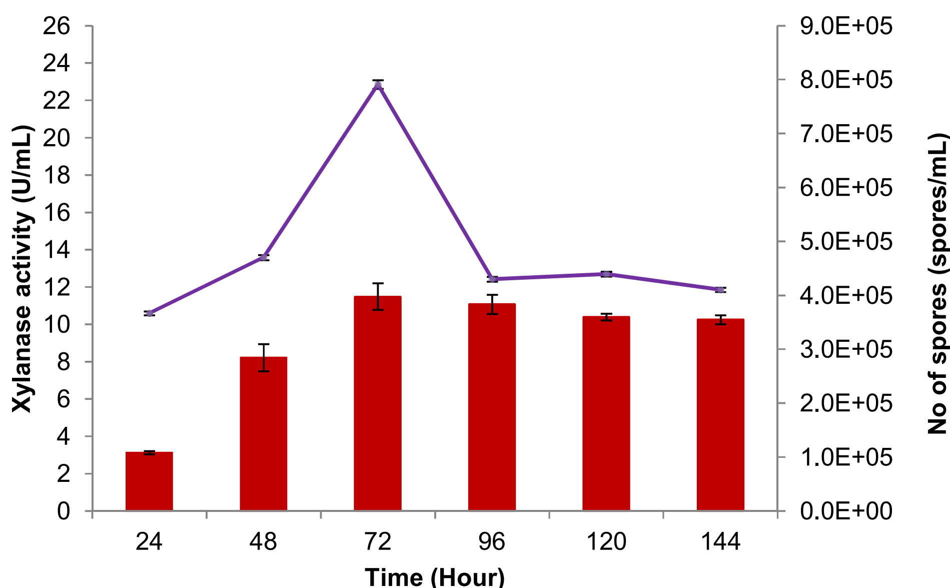

Fig. 2. Profile of xylanase activity and number of spores in culture medium against time of fermentation

A total of 144 h of fermentation was conducted and samples were analyzed at every 24 h. The experiments were performed in triplicate. Line graph illustrates number of spores per mL and bar chart represents xylanase activity in U/mL.

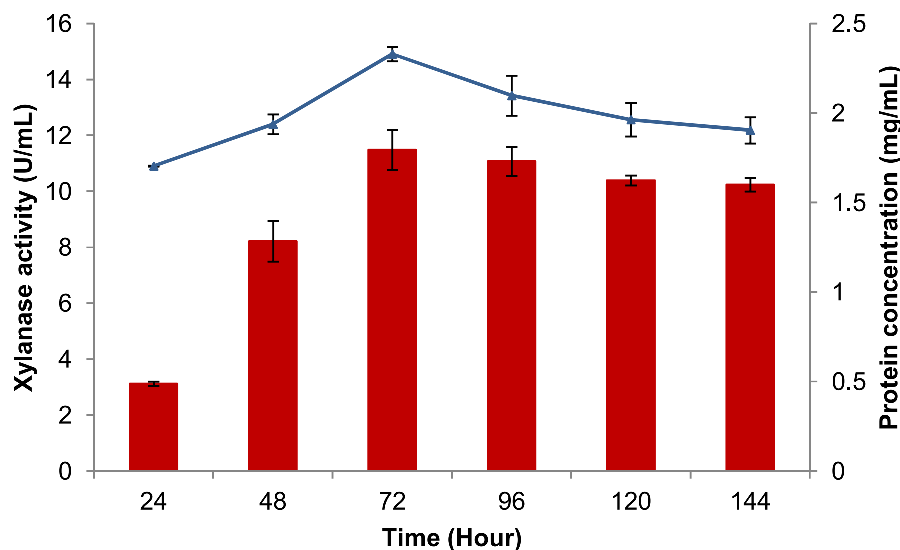

Fig. 3. Typical time course of xylanase production by *A. brasiliensis* in SmF

This figure demonstrates the correlation between the xylanase activity with its total protein concentration by A. brasiliensis. A total of 144 h of SmF was conducted and samples were analyzed at every 24 h. The maximum production of xylanase and protein were observed at 72 h, producing 11.49 U/mL and 2.33 mg/mL, respectively. Line graph illustrates the protein concentration in mg/mL while bar chart represents the xylanase activity by A. brasiliensis in U/mL.

end of fermentation process. This could be explained that the maximum production of xylanolytic enzymes and its protein was occurred at 72 h using wheat bran as the primary carbon source and production of enzymes after its optimum hour of incubation was therefore became constant. The fungi could not sustain the optimised amount of xylanase and proteins after the peak of logarithm phase at 72 h might due to nutrients restriction and struggling of its limited living conditions as fermentation prolonged. In fact, the pattern and profile of microbial metabolism, growth and enzymes production are differ depending on the environment of the fermentation provided [60]. In this study, the profile of xylanase activity was observed at the maximum, producing 11.49 U/mL at the 72 h of SmF. Xylanase from *A. flavus* favoured its optimum specific activity of 1.47 x 10^{-3} U/mg at the same fermentation time of 72 h [16]. Different microorganisms exhibit different chemical and kinetic properties which influence the enzymatic secretion and reaction [5,46].

3.3.4 Profile of medium pH during the optimum production of xylanase by *A. brasiliensis*

According to Satyanarayan et al. [58], medium pH is well known to be able of exerting a profound effect on the production of enzymes. Besides that, medium pH also plays a critical role in maintaining the stability and activity of extracellular enzymes. The optimum pH prior to the production of xylanase is therefore crucial. In fact, different microorganisms exert different optimal medium pH to maintain the enzyme activity and stability throughout the fermentation process. Thus, the optimum medium pH used for the maximum production of xylanase by *A. brasiliensis* in this study was adjusted to the optimised pH 6.5 as proposed by Ho and Jamila, [12]. Based on Fig. 4, in the present study, the medium pH exerted consistency between pH 6.82 to 7.13 from 24 h to the end of SmF. Since the optimum production of xylanase was occurred at 72 h, the optimum pH of culture medium was therefore detected at pH 6.97.

3.3.5 Summary of optimum production of xylanase by *A. brasiliensis*

The optimum time for the maximum production of enzyme is varied from organism to organism [61]. According to Antoine et al. [62], *Penicillium canescens* excelled the maximum xylanase activity after fermentation of 192 h. However, *Fusarium solani* F7 took about 6 days for the maximum xylanase activity to occur [63]. In another study performed by Nair et al. [10], *Aspergillus sydowii* SBS 45 needed about 9 days of fermentation for its maximum xylanase

production of 543 U/g. In a nutshell, it is clearly anticipated that the optimum time for the maximum production of xylanase activity depends on the types of microorganisms, environmental and cultural conditions and also genetic make-up of the organism [64]. Hence, this study showed that *A. brasiliensis* took 72 h of fermentation to produce the optimum amount of xylanase. Based on the present study, when the cells released xylanase, the enzyme was being used up to hydrolyze xylan of wheat bran during fermentation process. In fact, it exerted the highest xylanase activity at 72 h resulted in the highest spore count of 7.9 x 10^5 spores per mL at 72 h fermentation. Total of 72.8% increment of xylanase obtained from the beginning to 72 h of fermentation sufficient for the purification of xylanase in this study.

3.4 Purification of Xylanase by *A. brasiliensis* Using a Two-step Column Chromatography of Diethylaminoethanol (DEAE) Sepharose and Sephadex G-75

3.4.1 Precipitation with ammonium sulphate

The crude enzyme was subjected to protein precipitation phase or also known as salting out. This process is mainly used to precipitate out proteins from unwanted components including small ions. This step is one of the crucial steps in the early downstream process especially in purification means. Salting out is a process of precipitation of proteins through ammonium sulphate with the gradual increase of salt concentrations. At high saturation of salt, solubility of proteins is reduced and the desired proteins could therefore be precipitated out from other substances [65]. According to Rosenberg [66], the solubility of proteins is the result of polar interactions with aqueous solvents, ionic interactions with salts and repulsive electrostatic interactions between charged molecules. Rosenberg [66] also proposed that salting out with ammonium sulphate is achieved through dehydration process that occurs in the microenvironment of protein molecule. Therefore, in this purification scheme, protein precipitation was done using ammonium sulphate with gradual increase of salts concentration. The optimum concentration of ammonium sulphate used to precipitate most xylanases from *Aspergillus spp* was occurred at 70% according to Milala et al. [16]. Based on their results, the highest xylanase specific activity of 16.06 U/mg was obtained. In comparison with Zulfiqar and co-researchers [15], xylanase from *A. niger* possessed 6052 U of activity with its specific activity of 77.29 U/mg and total protein of 78.3 mg. In contrast, at 80% of salt saturation, there was no enzyme activity retained in the supernatant [67]. 70% of ammonium sulphate saturation was thus added to 580 mL of crude supernatant in this study. Another similar study performed by Pal and Khanum [68] using *A. niger,* the maximum xylanase activity of 182716 U with its specific activity of 142.30 U was observed using close to 70% salt saturation. From the similar study, *A. niger* xylanase has been successfully purified to the purification fold of 3.76 compared from its crude extract [68]. During this commencement step, this xylanase from *A. niger* has also been ultimately purified with the recovery of 81.92% [68]. In our study, the crude enzyme was subjected to protein precipitation by ammonium sulphate at 4ºC with the maximum saturation of 70%. At the 70% of salt saturation, the maximum xylanase activity obtained was 8.57 U/mL with the optimum specific activity of 16.06 U/mg. Xylanase from *A. brasiliensis* has been purified to a purification fold of 1.05, lesser than the study conducted by Pal and Khanum, [68] with its specific activity of xylanase at 3.76. However, due to different strains of *Aspergillus* used, thus, the xylanase activity and its specific activity were varied in the production and purification. In the present study, xylanase by *A. brasiliensis* was recovered to the maximum of 88.9% compared from its crude supernatant.

3.4.2 Diethylaminoethanol (DEAE) sepharose anion column chromatography

Ion exchange column chromatography is basically a type of protein separation based on protein charges and their affinity to the ion exchangers. Anion exchange column chromatography is basically used for negatively charged proteins which exert the high affinity binding towards the positively charged ions in the ion exchange resin. The anion chromatography used in this study was consisted of DEAE Sepharose equilibrated with 0.05 M sodium phosphate buffer (pH 5.3). The elution of protein is based on their charges and proteins that are bond to the resin. Then, the desired protein is eluted out with gradual increase of salt concentrations, for example, NaCl. In the present study, the elution of proteins was carried out using the linear gradient of salt concentrations from 0.0 M to 0.5 M of NaCl. Xylanase was successfully eluted out and screened for its

protein concentration using OD at 280 nm. The fraction with the highest protein concentration and xylanase activity was collected before subjected to the gel filtration column chromatography. Based on the chromatogram shown in Fig. 5, there was a distinctive peak observed from an eluted protein fraction with the highest xylanase activity and protein concentration. This xylanase fraction that possessed the highest xylanase activity was eluted with 0.3 M NaCl from the anion column chromatography. Total activity of 159.28 U with

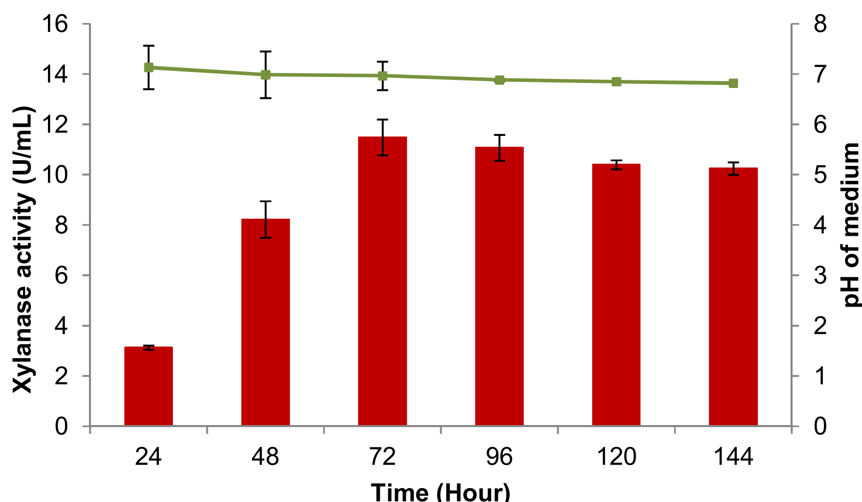

Fig. 4. Medium pH and xylanase activity against time of fermentation

Line graph represents pH of culture medium and bar chart represents xylanase activity in U/mL

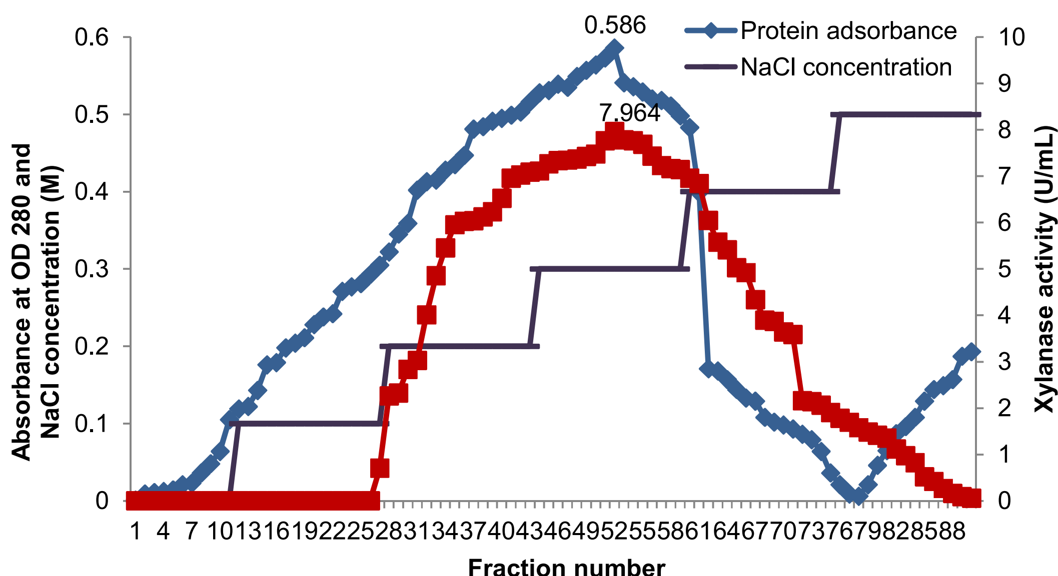

Fig. 5. Chromatogram of xylanase by *A. brasiliensis* in anion exchange column chromatography using DEAE Sepharose

Column was calibrated with 0.05 M sodium phosphate buffer (pH 5.3). The elution of samples was conducted using linear gradient of NaCl solution. Single protein and xylanase activity peak were obtained. Blue line indicates absorbance reading of protein at 280 nm. Black line shows the elution of desired protein using linear gradient of NaCl concentration and red line represents xylanase activity in U/mL

the total protein of 5.99 mg was observed from the fraction as shown in Table 3. The total xylanase activity and its total protein were reduced from 4968.15 U to 159.28 U and 309.36 mg to 5.99 mg after purification using anion column chromatography, respectively.

The total maximum xylanase activity was evident at 159.28 U/mL with its specific activity of 26.55 U/mg. There was about 39.5% increment of specific activity after the ion exchange column chromatography from 16.06 U/mg of protein precipitation using ammonium sulphate. Purification fold increased from 1.05 to 1.73. The desired enzyme was successfully purified based on the increment of purification fold. The washing steps were carried out after the collection of the last fraction prior to the next purification phase of gel filtration column chromatography. Comparing to scientific studies which have been done years ago, Okafor et al. [51] obtained the specific activity of xylanase as much as 90.82 U/mg after elution through DEAE Sephadex A-50 column chromatography which was higher than the specific activity obtained from this study. This could be explained through the variation in the medium formulations used. Different sources of agricultural residues and xylans would definitely exert different production of xylanase and therefore different activity of xylanase is then purified at the end of recovery [69]. Besides that, it also depends on the type of resin chosen prior to chromatography. Different types of resin would elute different range of proteins based on their ionic charges as well as protein pI values [70]. Based on the results of Okafor et al. [51], xylanase extracted from *A. niger* ANL301 was monitored at every 24 h intervals for a period of 168 h fermentation. This fungi was cultivated under various agro-wastes substrates including wheat bran, sugarcane pulp and sawdust. Xylanase production from agro-wastes was compared to those of commercial oat-spelt xylan. From the results, xylanase derived from *A. niger* was purified to its homogeneity with a purification fold of 2.38 and its specific activity of 16.36 U [51]. Another study conducted by Yang and co-workers [25], they discussed that xylanase from *A. niger* was purified up to 14.79 fold where 40.25% of this enzyme was recovered from its crude supernatant. Xylanase from *A. niger* C3486 possessed a specific activity of 123.68 U/mg at 72 h of fermentation. A purification study of xylanase from a different strain of *Aspergillus* was conducted by Lu and colleagues in 1999 [71]. Based on their study, *Aspergillus ficuum* was cultivated to produce xylanase. Based on the results, xylanase was purified to its homogeneity from the precipitation of 50-80% of ammonium sulphate. In their study, DEAE Sephadex A-50 was then used to separate proteins using anion column chromatography. Hence, the optimum specific xylanase activity obtained was achieved at 288.7 U/mg with a molecular mass of 35 kDa and 82.3% of xylanase was recovered from its crude filtrate [71].

In addition, the elution of proteins in our study was carried out using 0.05 M sodium phosphate buffer at pH 5.3 which were determined based on the pI value of xylanase. In a study conducted by Wong et al. [5], xylanases from *A. niger* possessed the pI value range from 4.0 to 4.2. It was also discussed in a scientific study that xylanase from various strains with their molecular masses ranged from 23 to 29 kDa have pI values between 3.7 and 4.1 [72]. The right pI value would affect the pH chosen for buffer of choice to be used in the calibration of resin upon elution of proteins. The right pH of buffer of choice would reduce the risk of resin cracking and bubbles formation that would greatly affect the elution of proteins. If the buffer is not compatible with the chosen resin, the packing bed would be able to destroy or crack upon elution of proteins [70]. Thus, elution buffer with the correct pH is very essential in this matter.

3.4.3 Gel filtration column chromatography using sephadex G-75

Gel filtration column chromatography or also known as size exclusion column chromatography is one of the simplest methods in separation of proteins which solely depends on the protein sizes. This separation technique plays as an important role in purification of proteins, enzymes, nucleic acids and polysaccharides [73]. In size exclusion column chromatography, samples are eluted isocratically using single buffer with no gradient involved. Therefore, buffer selectivity plays a crucial role in this separation basis. From the results obtained in Fig. 6, there is a single peak of protein elution obtained illustrating that xylanase had been successfully separated and purified based on its protein size in this study. It was previously mentioned by Sharma [74] where large proteins would elute faster since it could not pass through the pores of the beads whereas small proteins would take longer time to elute out. Small proteins also vary in its own sizes which would be separated at different course of time [75]. As according to the

results in Fig. 6, it could be clearly seen that the highest activity of xylanase with the highest peak was achieved from the fraction 11 out of 50 fractions in total. In fact, the gel filtration column chromatography had greatly purified xylanase from *A. brasiliensis* with a purification fold of 2.39 and 38.15% of recovery in the present study. About 28% of purification fold has increased from previous stage of anion exchange chromatography. As a result, the purified xylanase possessed a specific activity of 36.65 U/mg at the end of purification which increased about 1.38 fold compared to 26.55 U/mg from anion column chromatography. Besides that, by the end of gel filtration column chromatography, the total activity of xylanase obtained was 78.79 U with its total protein of 2.15 mg.

In recent study performed by Sorgatto et al. [76], it was discussed that xylanase from *A. terreus* has been purified using CM-Cellulose column chromatography with its specific activity retained at 6.92 U/mg, which is extensively lower than the specific xylanase activity from *A. brasiliensis* in this study. However, the purification fold has majorly increased from 1 to 45 fold at the end of purification [76]. There was also another scientific study conducted years ago and it had proven that xylanase from *A. niger* was successfully purified to its specific activity to achieve 123.68 U/mg [25]. The specific activity obtained was much higher compared to 36.65 U/mg in this study. Hence, it is greatly anticipated that medium compositions and cultivation conditions also play a big role in accessing a

Table 3. Purification of xylanase produced by *A. brasiliensis*

Purification step	Working volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	600	376.84	5775.52	15.33	100	1
$(NH_4)_2SO_4$ precipitation	580	309.36	4968.15	16.06	86.02	1.05
DEAE Sepharose	20	5.99	159.28	26.55	2.77	1.73
Sephadex G-75	10	2.15	78.79	36.65	1.36	2.39

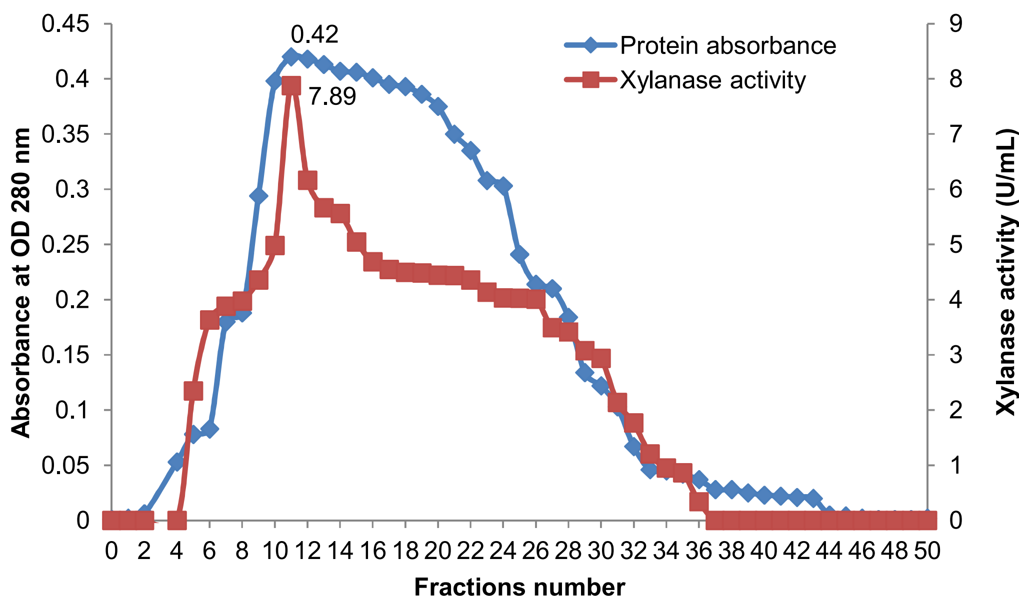

Fig. 6. Chromatogram of xylanase by *A. brasiliensis* in gel filtration column chromatography using Sephadex G-75

Column was calibrated with 0.05 M sodium phosphate buffer (pH 5.3). The elution of samples was carried out using 0.05 M sodium phosphate buffer (pH 5.3). Single protein and xylanase activity peak were observed. Blue line indicates the absorbance reading of protein at 280 nm while red line represents the xylanase activity in U/mL

better purification of proteins. Zulfiqar and colleagues [15] had discussed that *A. niger* derived cellulase-free xylanase was produced and purified to its homogeneity by a two-step purification which included the size exclusion chromatography of Sephadex G-75 that was equilibrated with 50 mM sodium phosphate buffer (pH 7.0). As a result, it was recorded that the maximum xylanase activity after subjection to Sephadex G-75 chromatography was 2943 U with its specific activity of 613.13 U/mg. It also confirmed that xylanase from *A. niger* was purified 7.93 fold to its homogeneity. In conclusion, xylanase from *A. brasiliensis* has been successfully purified to produce its specific activity of 36.65 U/mg and 38.15% of the final recovery at the end of purification stage. The increment in purification fold from 1 to 2.39 which was about 58% of its recovery was observed from beginning till the end of purification. However, in order to apply the end product commercially, xylanase by *A. brasiliensis* is promising to scale up to meet the corresponding market goals. The overall summary of the xylanase purification in this study is listed in Table 3.

3.5 Determination of the Molecular Weight of Purified Xylanase by *A. brasiliensis* Using SDS-PAGE Electrophoresis

SDS-PAGE is the most common method use to analyze proteins through gel electrophoresis. In this method, proteins are denatured and therefore no enzyme activity is involved in this process [77]. In SDS-PAGE, the homogeneity asses of purified enzymes should exhibit a single band on the gel to suggest it is a pure enzyme where it is a crucial confirmation step in purification [77]. The SDS-PAGE procedures are conducted based on the method described by Laemmli [17]. There are three different ways of protein staining in SDS-PAGE. They are Coomasive, silver and copper staining. The most commonly used for practical and routine work is Coomasive staining using Coomasive Brilliant Blue R-250 which is recommended to analyze protein in this study. As for silver staining, it is a relatively sensitive detection method compared to Coomasive staining [78]. On the other hand, copper staining is a newly developed and improved staining for sensitive proteins that could be done in a short period of time [78].

Xylanase is a type of hydrolyzing enzyme which capable of hydrolyzing xylan into its simple sugar which is xylose. Xylanases (endo-1,4-β-xylanase) are classified according to its molecular structure and genetic information. Therefore, endo-1,4-β-xylanases are further classified into the group of glycoside hydrolases [79]. In details, xylanases with EC.3.2.1.8 are classified into three branches which firstly according to their molecular weight and pI value with either having high or low molecular weight or acidic or basic pI value [5]. The structure of enzymes plays an important role in the enzyme classification. Molecular weight of an enzyme could also result in classifying enzymes into its own pI value. Basically, endo-1,4-β-xylanase is classified into a few families, however majority of these enzymes are belonged to Family 10 and 11 [79]. Generally, xylanases that belong to Family 10 usually possess higher molecular weight, large and more complex in structure compared to Family 11. It was also proven by Whitehurst and Van Oort, [79] and Kumar et al. [80], xylanases which fall under Family 11 are actually true xylanases. They exert consistent in low molecular weight and do not possess any cellulolytic activity. Nonetheless, pI value may vary either acidic or basic. As reported by Wong et al. [5], many xylanases have low molecular weight ranging from 16-22 kDa which normally exert basic pI points. On the other hand, those with high molecular weight xylanases with 43-50 kDa are usually having acidic pI value [5]. Simultaneously, the classification of enzymes is also determined based on their kinetic properties such as pH, temperatures, pressures, substrate specificity and many more.

SDS-PAGE of purified xylanase by *A. brasiliensis* from the present study is shown in Fig. 7. It revealed a single band with an estimated molecular weight of 36 kDa after confirmed by Coomasive staining using Coomasive Brilliant Blue G-250. Hence, xylanase from this protein band was categorized under Family 11 as discussed by Kumar et al. [80]. It was proposed that Family 11 of xylanase is considered as a true xylanase with commonly low molecular weight, cellulase-free with acidic or basic pI value [80]. Previously, Zulfiqar et al. [15] has described that a partial purified xylanase from *A. niger* was found to have a molecular mass of 30 kDa. The partial purified xylanase was optimally produced using wheat bran before undergone with one-step gel filtration column chromatography. The enzyme was successfully purified to its homogeneity producing only a single band on the gel [15]. Another comparative study conducted in 2010 proposed that a fully purified extracellular

xylanase from *A. niger* C3486 was purified homogenously with an apparent molecular weight of 25 kDa as illustrated from SDS-PAGE gel electrophoresis [25].

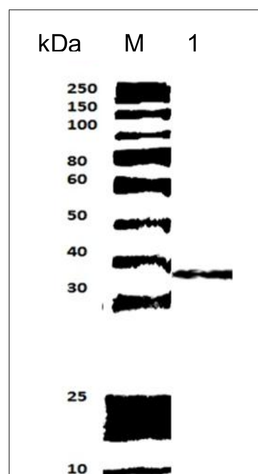

Fig. 7. Pictograph of SDS-PAGE of purified xylanase by *A. brasiliensis*
Lane M: protein marker. Lane 1: Purified xylanase with an estimated molecular weight of 36 kDa.

Furthermore, according to Sunna et al. [81], most of xylanases have a molecular weight ranging from 11-80 kDa depending on its specific types and species of organisms. Some research studies have been carried out years ago. Yang and colleagues [25] proposed that the molecular weight of purified extracellular xylanase extracted from *A. niger* C3486 was determined to be 25 kDa. On the other hand, Pal and Khanum [23] mentioned that the purified extracellular xylanase extracted from *A. niger* DFR-5 possessed a molecular weight of 32 kDa. In addition to this, a research investigated by Asad et al. [82] claimed that the molecular weight of purified xylanase by *A. fumigatus* isolated from soil was determined to be 43 kDa. Hence, these results have proven that different strains and species of organisms exert bizarre yet unique characteristics that differ from one another. In addition to this, Milala and co-workers [13] discussed the xylanase from *A. flavus* was purified to its absolute homogeneity which exerted an estimated molecular weight of 28 kDa. Hence, the purified xylanase isolated from *A. flavus* was reported to be potentially categorized under a Family 11 xylanase that is commonly applied in pulp and paper industry [83, 84]. In conclusion, based on this study, xylanase from *A. brasiliensis* was fully purified to its homogeneity and was evidently revealed a single band with the estimated molecular weight of 36 kDa as shown in Fig. 7. Therefore, the final purification step of xylanase was successfully conducted in the present study. Subsequently, the purified xylanase was subjected to in-situ zymography analysis.

3.6 Zymography Analysis

As mentioned previously, zymography possesses a simple yet sensitive technique in detecting purified enzymes based on the hydrolysis reaction on their substrates [85]. There are several types of zymogram which are either performed in-vivo or in-situ decolorisation. In this study, the zymography analysis was conducted via color decolorisation using xylan-PDA agar. From the results obtained in Fig. 8, it shows that xylanase was able to hydrolyze its

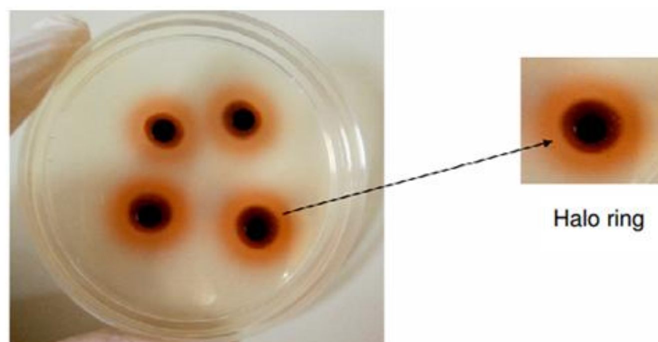

Fig. 8. Positive result of hydrolysation of xylan by purified xylanase from *A. brasiliensis*
Decolorisation of Congo red dye and a halo ring were formed at the outermost layer surrounding the filter paper which was dipped with purified xylanase and Congo red

substrate of beechwood xylan, hence a clear halo ring was observed on the agar. A positive result from the study of zymography gave an evident of halo ring on the surrounding of filter paper that was dipped with purified xylanase and Congo red. From the result shown in Fig. 8, it can be clearly seen that after 3 days of incubation, the halo ring structure was clearly formed, in fact, bigger decolorisation of Congo red dye was much visible. The decolorisation of Congo red dye was the result obtained from the hydrolysis of purified xylanase with its substrate of xylan.

4. CONCLUSION

Xylan is formally derived from plant cell wall component. It is the second most abundant polysaccharides after cellulose. Xylan is a type of hemicelluloses which is depolymerised through its hydrolyzing enzyme of xylanase. Hence, xylanases have created a specific demand in global market due to its significant role in various applications in the manufacturing of textile, pulp and paper, baking, animal feeds and many more. As previously described, xylanase is derived from various sources which include plants, snails, insects, crustaceans and microorganisms. However, microbial origin has found to be the greatest producer of xylanase which is well employed in industrial scale. Therefore, in this study, *A. brasiliensis* was chosen to be the producer of xylanase prior to purification study. As mentioned before, microorganisms play an important role in the production of xylanase. Again, *Aspergillus spp* has been found to be the greatest xylanase producers. Therefore, producing a pure and highly suitable xylanase in industrial applications has become a vital priority nowadays. Commercial enzymes must exert a significant effect through various operating conditions before applying the enzymes commercially. Therefore, many analysis are needed to be carried out to monitor the enzymes specificity, efficiency and stability before any further processes take place. From the study conducted, xylanase was optimally produced from *A. brasiliensis* at 72 h of SmF using wheat bran as the primary carbon source. Generally, lignoecellulosic materials especially those from agricultural crops are deprived to apply in fermentation of xylanase. They are great sources of xylan which are practically derived from corn cobs, wheat bran, barley husk and straw. Wheat bran has been chosen to be the primary carbon source in xylanase production from *A. brasiliensis.* One of the main factors is because of its cost effectiveness and its availability in current market. Furthermore, SmF was chosen primarily as the mode of fermentation for production of xylanase since it is easily operated in controlling its parameters including high oxygen and nutrients supply. Besides that, wider area with higher culture volume is provided to the fungi to grow with proper optimised medium composition under optimum growth control. Due to these advantages, SmF was chosen above soli state cultivation. Consecutively, *A. brasiliensis* that yielded ample amount of xylanases was subjected to purification.

Since the public concerns are more towards developing an alternative method which is less harmful to the environment and living things, various industrial applications such as chemical bleaching of paper using chlorine and the traditional usage of pumic stones in stonewashing of garments in the textile industry have been greatly improved by using biotechnology means. Hence, the ultimate vital goal in this research is to produce a cheap xylanase enzyme as biobleaching agent in the pulp and paper industry. Therefore, from the research project studied, the purification steps have been shorten and greatly monitored and improvised for a better efficiency of the desired xylanase. Purification methods involved in this research has successfully purified the desired xylanase. The screening of the xylanase activity and its efficiency of enzymatic reaction were also conducted. Hence, it has clearly shown that pure xylanase from *A. brasiliensis* might has a profound yet promising effect in hydrolyzing its substrate. Thus, this enzyme is anticipated to have a significant role and effect in industrial scales. In conclusion, there are still tremendous measures to be considered upon this research study. From the results obtained, it is clearly indicated that the maximum xylanase extracted from *A. brasiliensis* was occurred at 72 h of fermentation with the maximum activity of 11.49 U/mL. A purified xylanase was successfully obtained with an estimated molecular weight of 36 kDa. It also possesses great ability in industrial applications since it has successfully demonstrated a visible yet convincing halo ring as a result of its active substrate hydrolysation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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