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Production, Purification and Characterisation of a Purified Low Molecular Weight and Thermo-alkaline Tolerance Xylanase by *Aspergillus brasiliensis* In Submerged Fermentation

Hooi Ling Ho[1*], Li Sha Soh[1] and Soon Hwa Ong[1]

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

Authors' contributions

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

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ABSTRACT

Aims: Xylanase is an enzyme which has been used extensively in many different industrial processes. Xylanase also known as endo-1,4-β-xylanase is a glycosidase that catalyses the conversion of xylan to xylose through endohydrolysis of 1,4-β-D-xylosidic linkage which is commonly found in various agro-industrial wastes such as wheat bran. Thus, the objectives of the present study were to produce, purify and characterise cost-effective xylanase from *Aspergillus brasiliensis* ATCC 16404.
Methodology: Wheat bran as the major sustainable low cost agro-industrial residual was utilised as the sole carbon source for the production of xylanase by *A. brasiliensis* in submerged fermentation (SmF). Subsequently, a two-step column chromatography was used to purify xylanase followed by step-wise manner of characterisation study on the purified xylanase.
Results: Based on the results, the maximum xylanase production of 7.72 U/mL with spore count of 8.33×10^4 spores/mL at medium pH 6.42 was obtained at 48 h of SmF. Xylanase extracted from *A.*

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

brasiliensis was then purified with DEAE Sepharose and Sephadex G-75 column chromatography. At the end of purification, xylanase was purified up to 3.6-fold with its recovery yield of 1.68% and specific activity of 116.64 U/mg. Additionally, the purified xylanase was detected to be a low molecular weight protein. Indeed, molecular weight of 36 kDa of the purified xylanase was visualized on SDS-PAGE. The purified xylanase was then subjected to the step-wise manner of characterisation study. Based on the results obtained, xylanase was found to be thermo-tolerance from 40℃ to 60℃. In fact, the purified xylanase wa s detected to be most stable at 50℃ whereby 98.33% of its activity was retained even after 3 h of incubation. Furthermore, xylanase from *A. brasiliensis* was also found to be most active at 50℃ where its re lative activity increased from 95.24% at 45℃ to the maximum activity of 20.51 U/mL at 50℃. Besides that, the pH stability of xylanase was appreciable from pH range of pH 4 to 8. Notably, the purified xylanase showed the highest stability at pH 5 as 94.87% of its activity was retained after 3 h of incubation. Additionally, the activity of the purified xylanase remained relatively higher in pH buffer ranging from pH 3 to 9. Indeed, the purified xylanase reached its maximum activity of 22.18 U/mL at pH 5. In addition, xylanase activity was detected to be the highest, producing 24.46 U/mL when 1% beechwood xylan was used as the optimised substrate for the incubation period of 30 min at 50℃. Besides that, metal ions such as Cu^{2+}, Mn^{2+} and Zn^{2+} were identified to enhance xylanase activity, whereas Al^{3+}, Ca^{2+}, K^{+} and Mg^{2+} performed otherwise. In particular, Cu^{2+} was identified as the strongest activator while Al^{3+} was found to be the toughest inhibitor of xylanase activity. Nonetheless, chelating agent of EDTA inhibited the xylanase activity marginally. Furthermore, the non-ionic detergent of Tween 80 was detected to be a weak enhancer whereas the ionic detergent of SDS was a strong inhibitor of xylanase activity. On the other hand, xylanase activity showed a better tolerance towards glycerol with 51.2% and acetone with 33.9% compared to ethanol with 17.9%.

Conclusion: In a nutshell, some of the characteristics of the purified xylanase by *A. brasiliensis* in this study revealed its enormous potential as a thermo-alkaline tolerance enzyme in xylan degradation process that is applicable and useful in the manufacturing of animal feeds, fruit juice and paper pulping.

Keywords: Xylanase; wheat bran; Aspergillus brasiliensis; submerged fermentation (SmF); production; purification; characterization.

1. INTRODUCTION

Agricultural sector has made significant contributions towards the economical growth of Malaysia. Wastes or by-products from agriculture industry would cast serious effects on the environment if they are not managed in proper manner. Reutilisation and recycling of the agro-industrial wastes is one of the solutions of resolving pollution problems. Agro-industrial wastes such as sugarcane bagasse, wheat bran, rice bran, corn cob and wheat straw are copiously available as natural carbon source for fermentation at much lower cost [1]. As a result, these wastes are anticipated to be high valued by-products and their recovery or reutilisation would be economically attractive. In this study, wheat bran was therefore, used as the carbon source for fungi, *Aspergillus brasiliensis* in the production of xylanase enzyme. Wheat bran consists about 650 mg/g of cell wall polysaccharides which are mainly comprised of heteroxylans, cellulose and lignin [2]. It is a cheap agro-industrial residue which is suitable to use as carbon and nitrogen source in fermentation for enzymes production [3]. There are studies where wheat bran as the indigenous carbon source was utilised for xylanase production [4-8]. Xylanase has been used extensively for various industrial processes. According to Yang et al. [9], recent market trends revealed that xylanase took a major chunk of share up to 20% of the world enzyme market along with cellulase and pectinase. Apparently, xylanase has been widely used in many industries including textile, pulp and paper, food, detergents and animal feeds [10]. The technology of utilising xylanase has reached the stage where this enzyme has become indispensable. Nonetheless, high production costs and low yields of xylanase hinder its further potentials. As a result, they remain as the paramount problems for industrial applications especially in large industrial scale. In order for commercial applications, xylanase should be produced rapidly in huge quantity using simple and inexpensive substrate. Therefore, investigations on the ability of xylanase-producing fungal strains utilising inexpensive substrate have been extensively carried out in

our previous studies. In fact, much effort has been done on the optimisation of fermentation to further reduce the production costs of xylanase. Besides that, the characterisation study of xylanase is equally important where the understanding of its optimum parameters is necessary for its applications in various industries. As a result, step-wise manner was applied for the characterisation study of purified xylanase in this work. Characterisation study using different parameters including temperature and pH stability of xylanase, effects of temperature, pH, substrate specificity, substrate concentration, incubation time, metal ion, chelating agent, detergent and organic solvent on the xylanase activity was elucidated in this work. Hence, the objectives of this study were to produce extracellular xylanase from *A. brasiliensis* using wheat bran as the sole carbon source under the optimum growth conditions in submerged fermentation (SmF), to purify and lastly to characterise the purified xylanase using step-wise manner. In conclusion, this study summarised the economical utilisation and recycling of wheat bran in the production of xylanase by *A. brasiliensis* under SmF followed by purification using a two-step column chromatography and step-wise manner of characterisation study.

2 MATERIALS AND METHODS

2.1 *Aspergillus brasiliensis* ATCC 16404

Filamentous fungi known as *Aspergillus brasiliensis* ATCC 16404 was used in this study. The subculture of fungi was carried out using potato dextrose agar (PDA) in a sterile laminar flow cabinet. After the agar was solidified in the petri dish, a loopful of *A. brasiliensis* was streaked on the plate. Then, the agar was sealed with parafilm and incubated for 6 days at 30℃.

2.2 Pre-treatment of Wheat Bran

Wheat bran as the exclusive sole carbon source was used for the growth of *A. brasiliensis* in this study. Wheat bran was flaky prior to the pre-treatment. Hence, wheat bran was homogenised into finer particles using stainless steel blender before subjected to sieving to obtain the consistency in size. The process was carried out to ensure that *A. brasiliensis* possessed better accessibility to the carbon source of the agro-industrial waste. Much constant size of wheat bran was easier to disperse and dissolve in the liquid culture medium of SmF. After achieving the desired size of wheat bran, it was dried in an oven at 65℃ until constant weight was achieved. Extra wheat bran was then vacuum packed and kept under cold and dry condition for longer storage.

2.3 Inoculum Preparation

Spore suspension was prepared from *A. brasiliensis* grown on the PDA. Approximately 5 mL of sterile distilled water was poured onto the PDA. A sterile hockey stick was used to gently scrap the spores of *A. brasiliensis*. The spore suspension was then collected and transferred to a sterile Falcon tube. Then, the number of viable spore was counted under microscope using haemocytometer. Subsequently, serial dilution was performed to obtain the standard inoculum size of 3×10^6 spores before inoculated into 150 mL working volume of culture medium in a conical flask.

2.4 Culture Medium Formulation and Growth Conditions

The culture medium used for the xylanase production by *A. brasiliensis* under SmF in this study was consisted of (g/L): wheat bran, 10; yeast extract, 2; KH_2PO_4, 1.52; $MgSO_47H_2O$, 0.52 and KCl, 0.52 where wheat bran and yeast extract were applied as the carbon and nitrogen source. The medium pH was adjusted to 6.5 before autoclaved at 121℃ for 15 min [11]. Subsequently, the inoculum with the standard size of 3×10^6 spores was inoculated into 150 mL culture medium in a 250 mL Erlenmeyer flask. Then, the culture was incubated in an orbital rotary shaker at 30℃ and agitated at 150 rpm for 240 h under SmF [11]. Sample was harvested at regular 24 h interval and analysis was conducted accordingly.

2.5 Sampling and Analysis

10 mL sample was harvested from the culture flask into 15 mL Falcon tubes at every 24 h interval up to 240 h of SmF. The samples were collected for analysis of protein concentration, xylanase activity, medium pH and spore count. In order to perform the analysis, 5 mL sample was used for the quantification of spore count and determination of medium pH. On the other hand, the remaining 5 mL of the sample was then centrifuged at 8500 rpm at 4℃ for 15 min. The cell pellet was discarded and the supernatant was used to extract the crude enzyme for quantification of xylanase activity and protein concentration.

2.6 Quantification of Soluble Protein Concentration

The total concentration of soluble protein was measured according to Lowry et al. [12]. The absorbance of protein was measured at the optical density (OD) 750 nm using spectrophotometer. The protein concentration of samples was calculated using bovine serum albumin (BSA) as the standard.

2.7 Xylanase Activity Assay

Xylanase activity assay was performed using 3, 5-dinitrosalicyclic (DNS) method by measuring the reducing sugar of xylose according to Bailey et al. [13]. 1% of beechwood xylan in 0.05 M sodium phosphate buffer, pH 5.3 was used as the substrate in activity of xylanase. 0.1 mL of supernatant was added into 0.9 mL of 1% beechwood xylan and incubated in water bath at 50℃ for 30 min. Subsequently, 1.5 mL of DNS reagent was added into the mixture and incubated at 90℃ for 5 min. Then, 0.5 mL 40% potassium sodium tartrate (Rochelle salt) was added and cooled down at room temperature before measured the absorbance at 575 nm using spectrophotometer. The amount of xylose obtained from the activity was calculated using xylose standard curve. One unit (U) of xylanase activity was defined as the amount of xylanase required to release one µmol of xylose per mL of enzyme extract per min under assay condition.

2.8 Measurement of Medium pH

The pH of samples was measured using a pH meter after it was calibrated with buffer pH 4, 7 and 10.

2.9 Spore Count of *A. brasiliensis*

Spore count of *A. brasiliensis* was carried out under microscope using haemacytometer.

2.10 Purification of Xylanase

The purification of xylanase by *A. brasiliensis* has been optimised by Ho and Iylia [14]. All purification steps were carried out at 4ºC unless otherwise specified. The supernatant containing crude xylanase enzyme was obtained after centrifugation at 8500 rpm for 15 min at 4℃. Then, ammonium sulphate was added slowly into the supernatant until it reached 70% salt saturation. It was swirled overnight at 4℃ for protein precipitation to take place. The protein precipitate was then collected after centrifugation at 8500 rpm for 15 min at 4℃. Subsequently, the precipitate was suspended with 0.05 M sodium phosphate buffer, pH 5.3 before transferred into a dialysis tube. The tube containing protein precipitate was then immersed in the same buffer. Dialysis process was carried out overnight at 4℃. Then, the enzyme sample was ready for a two-step chromatographic purification. To begin, resins of DEAE Sepharose and Sephadex G-75 were swelled before packed into two separate columns and mounted upright to a retort stand. The enzyme sample was firstly loaded into an anion exchange column packed with DEAE Sepharose that was equilibrated with 0.05 M sodium phosphate buffer, pH 5.3. Then, the enzyme samples were eluted with increased concentration of NaCl from 0 M to 1.0 M. The eluates were collected as fraction of 0.5 mL. After that, the fractions were assayed to determine the xylanase activity and protein concentration. Based on the results, the fractions with relatively high xylanase activity were pooled and loaded into a gel filtration column packed with Sephadex G-75. Subsequently, the enzyme samples were eluted with 0.05 M sodium phosphate buffer, pH 5.3. Again, the eluates were collected as fraction of 0.5 mL. Lastly, the fraction that possessed the highest xylanase activity and protein concentration was loaded into SDS-PAGE to visualise the molecular weight of the purified xylanase. The specific activity of purified xylanase was calculated by division of total xylanase activity with its total protein concentration. On the other hand, the purification fold was quantified by division of specific activity with its initial specific activity. The purified xylanase sample was thereafter reserved for the characterisation study in step-wise manner.

2.11 Quantification of Protein Concentration and Xylanase Activity of Purified Xylanase

The protein concentration of the purified samples was measured using spectrophotometer at the absorbance reading of 280 nm. On the other hand, xylanase activity of the purified samples was determined according to Bailey et al. [13].

2.12 Characterisation of Purified Xylanase in Step-wise Manner

Characterisation study including temperature and pH stability, effects of temperature, pH, substrate

specificity, substrate concentration, incubation time, metal ion, chelating agent, detergent and organic solvent on xylanase activity was investigated in this study.

The elucidation of xylanase characterisation was conducted in step-wise manner, where the optimum parameter found in one characterisation was used over to the next characterisation in order to ensure the results obtained throughout the study were reliable. Subsequently, the characterisation of the purified xylanase was analysed by comparing its characterisation results with other reported studies.

2.12.1 Temperature stability of xylanase

The long-term stability of xylanase at different temperatures was elucidated in this study. The purified sample was incubated without substrate up to 3 h at 40℃, 50℃, 60℃, 70℃ and 80℃, respectively. Sample was withdrawn at every hour interval and xylanase activity was then performed and quantified under the standard assay condition.

2.12.2 pH stability of xylanase

The long-term stability of xylanase at different pH was also analysed in this study. The purified sample was incubated without substrate up to 3 h in 0.05 M of different pH buffers: Sodium citrate buffer (pH 3), sodium acetate buffer (pH 4 and 5), sodium phosphate buffer (pH 6, 7 and 8), sodium carbonate-bicarbonate buffer (pH 9) and sodium phosphate buffer (pH 10), respectively. Sample was withdrawn at every hour interval and xylanase activity was then performed and quantified under the standard assay condition.

2.12.3 Effect of temperature on xylanase activity

The determination of the optimum incubation temperature for xylanase activity is important because temperature might cause the conformational changes of enzyme which lead to the decrease in activity and eventually loss of profit in industry. To elucidate the effect of temperature on xylanase activity, 0.9 mL of 1% beechwood xylan was added to 0.1 mL of purified sample and incubated at different temperatures: 45℃, 50℃, 55℃, 60℃, 65℃ and 70℃, respectively. Xylanase activity of the purified samples was determined under the standard condition as mentioned earlier.

2.12.4 Effect of pH on xylanase activity

In order to determine the effect of pH on xylanase activity via step-wise manner, by using the optimised incubation temperature obtained from the previous characterisation study, 0.9 mL of 1% beechwood xylan in 0.05 M of different pH buffers: sodium citrate buffer (pH 3), sodium acetate buffer (pH 4 and 5), sodium phosphate buffer (pH 6, 7 and 8), sodium carbonate-bicarbonate buffer (pH 9) and sodium phosphate buffer (pH 10) was mixed with 0.1 mL of purified sample, respectively. Xylanase activity of the purified samples was then quantified under the standard assay condition.

2.12.5 Effect of substrate specificity on xylanase activity

After the optimum incubation temperature and pH buffer were determined, xylanase activity of the purified sample was accessed for specificity towards its substrates. Various substrates were tested in this study which consisted of 1%: beechwood xylan, psyllium husk, rice bran, sawdust, wood chip, wheat bran, soya and palm kernel.

All of the substrates were obtained from Country Farm Organics, Malaysia except for beechwood xylan which was purchased from Sigma-Aldrich. Xylanase activity of the purified samples with different substrates was then elucidated using DNS method under the standard assay condition, respectively.

2.12.6 Effect of substrate concentration on xylanase activity

After the determination of the optimised substrate specificity, analysis was carried out to find out its optimum substrate concentration where the purified xylanase worked the best.

The analysis was carried out by substituting the optimised substrate with different concentrations consisted of 0%, 0.25%, 0.50%, 0.75%, 1.00%, 1.25% and 1.50%, respectively. Xylanase activity of the purified samples was then quantified under the standard assay condition.

2.12.7 Effect of incubation time on xylanase activity

The effect of incubation time towards xylanase activity of the purified sample was elucidated

after the optimised substrate concentration was confirmed. The analysis was carried out by substituting the incubation time of xylanase activity with a set of different periods: 0 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, respectively. Subsequently, xylanase activity of the purified samples was quantified under the standard assay condition.

2.12.8 Effect of metal ion on xylanase activity

To determine the effect of metal ion on xylanase activity, the purified sample was incubated with its optimised substrate in the presence of 5 mM of different metal ions consisted of Mg^{2+}, Ca^{2+}, Mn^{2+}, Al^{3+}, K^{+}, Cu^{2+} and Zn^{2+}, respectively. Xylanase activity of the purified samples was quantified under the standard assay condition.

2.12.9 Effect of chelating agent and detergent on xylanase activity

To elucidate the effect of chelating agent and detergent on xylanase activity, the purified sample was incubated with its optimised substrate in the presence of 5 mM chelating agent of EDTA, 5% (w/v) of SDS and 5% (w/v) of Tween 80, respectively. Xylanase activity of the purified samples was then quantified under the standard assay condition.

2.12.10 Effect of organic solvent on xylanase activity

To investigate the effect of organic solvent on xylanase activity, the purified sample was incubated with its optimised substrate in the presence of 50% (v/v) of different organic solvents consisted of glycerol, ethanol and acetone, respectively. Xylanase activity of the purified samples was then quantified under the standard assay condition.

3. RESULTS AND DISCUSSION

3.1 Production of Xylanase by *A. brasiliensis* Using Wheat Bran as Carbon Source

To determine the fermentation time for the maximum xylanase production by *A. brasiliensis* using wheat bran as carbon source, 10 days of SmF was carried out in this study. Sample was withdrawn at every 24 h interval and analysis including xylanase activity, protein concentration, spore count and medium pH were elucidated.

The correlation between xylanase production and spore count is illustrated in Fig. 1. The maximum xylanase production was found to occur at 48 h of SmF. Based on the study, the xylanase activity increased by 1.35-fold from 5.715 U/mL at 24 h to 7.720 U/mL at 48 h of SmF. Thereafter, it started to decline to 5.501 U/mL at 144 h. On the other hand, there was an increase in spore count by 1.78-fold from 4.67×10^4 spores/mL at 24 h to 8.33×10^4 spores/mL at 48 h, followed by 3.89-fold from 2.07×10^5 spores/mL at 72 h to 8.05×10^5 spores/mL at 144 h. It was suggested that at the beginning of fermentation process, large amount of xylanase was secreted by *A. brasiliensis* to hydrolyse wheat bran which was the sole carbon source found in the medium in order to provide energy for the fungal growth. As a result, xylanase was synthesised at the higher rate during the first 48 h of SmF. Indeed, we anticipated that large amount of xylan from wheat bran in the medium was hydrolysed by xylanase to yield sufficient amount of simplified nutrient to support the growth of *A. brasiliensis*. Subsequently, the fungi began to reproduce rapidly using the reducing sugar of xylose available in the medium. Then, the xylanase synthesis was constantly produced from 120 h to 168 h. Thereafter, xylanase activity reduced gradually to almost constant from 168 h onwards. At 168 h, the xylanase activity obtained was 5.490 U/mL while spore count reached its maximum peak of 9.83×10^5 spores/mL. Consequently, a stationary phase was observed from 192 h onwards. It was suggested that the growth rate of *A. brasiliensis* was equivalent to its death rate during the stationary phase of the fungal growth. The depletion of the essential nutrients of xylan and the build-up of growth inhibitory by-products from *A. brasiliensis* were anticipated to occur after the stationary phase of *A. brasiliensis.* As a result, the fungal growth and xylanase production were limited after the stationary phase.

Likewise, Sorgatto et al. [15] induced the xylanase production from *A. terreus* utilising different lignocellulosic wastes as the main carbon source. Among all the lignocellulosic wastes, wheat bran was found to be the best inducer for xylanase production with approximately 65 U/mg of xylanase produced. Similar result finding was also observed in another study by Kanimozhi and Nagalakshmi [16] whereby wheat bran contributed to the highest xylanase production of 1.4 U/mL compared to the other substrates.

In a nutshell, the choice of using wheat bran for the xylanase production was a brilliant decision as it was more economical in producing higher amount of xylanase compared to other substrates.

Fig. 2 illustrates the correlation between the xylanase activity and protein concentration produced by *A. brasiliensis.* The trends of xylanase activity and protein concentration possessed high resemblance. As fermentation time increased, protein concentration decreased. The protein concentration was at the highest at 24 h, producing 0.19 mg/mL in the medium. Thereafter, it decreased gradually until 120 h to support the fungal growth during the log phase. The protein concentration remained nearly stationary from 144 h to 240 h. Meanwhile, the production of xylanase was remained constant from 120 to 168 h before it reduced gradually to remain unchanged from 168 h onwards.

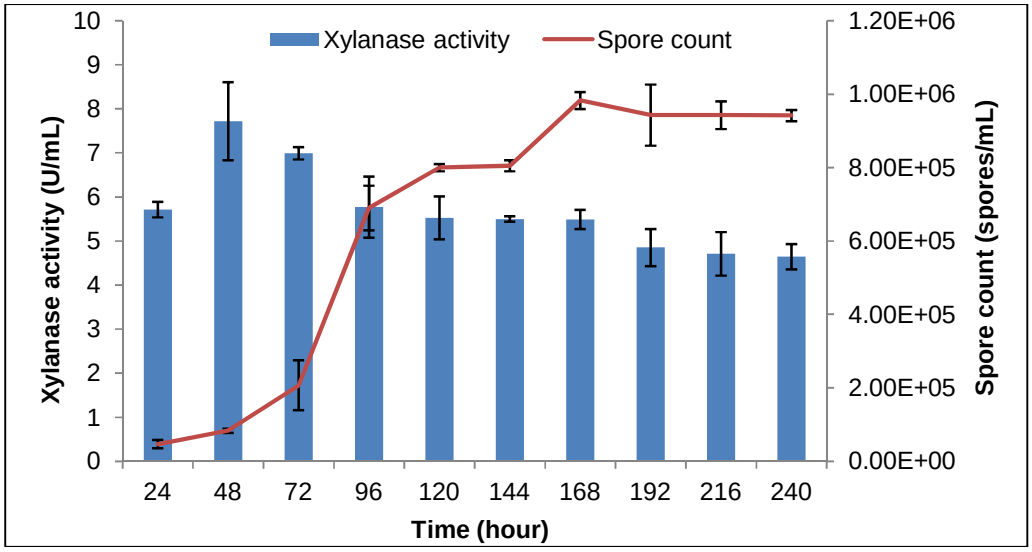

Fig. 1. The correlation of xylanase activity with the number of spores of *A. brasiliensis* using wheat bran as the sole carbon source in SmF

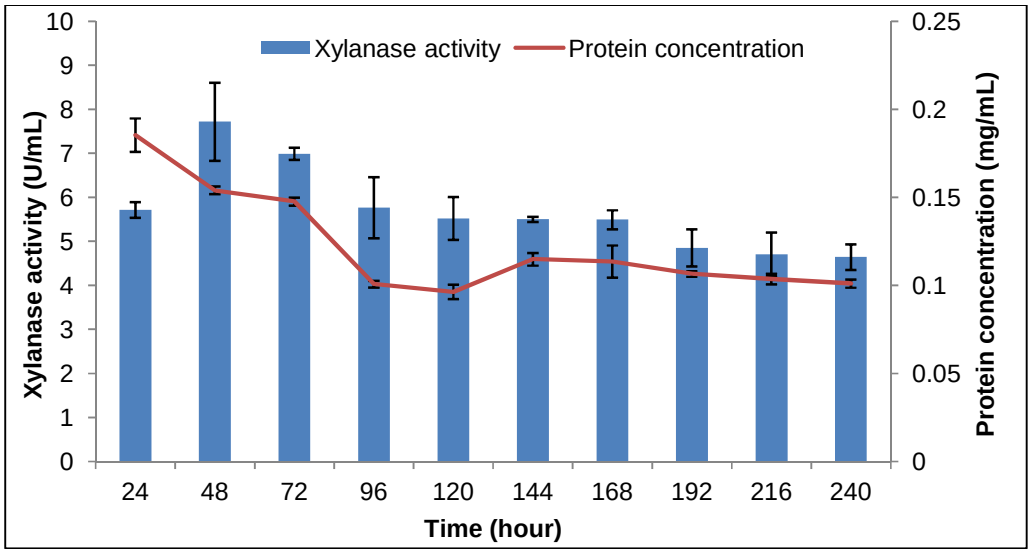

Fig. 2. The correlation of xylanase activity with the protein concentration produced by *A. brasiliensis* using wheat bran as the sole carbon source in SmF

Based on Fig. 3, the pH of medium increased as fermentation time prolonged. The initial pH of medium was adjusted to pH 6.5 prior to fermentation. Nonetheless, the medium pH changed after fermentation began. The pH of medium increased from pH 6.31 to 6.99 by 1.11-fold at fermentation time of 24 h to 120 h of SmF. After 144 h, the medium remained unchanged at around pH 6.94. In summary, the medium pH increased from the very beginning of fermentation before remained constant when fermentation proceeded. It was suggested that increment in medium pH to around pH 6.94 stimulated the constant production of xylanase from 120 h onwards. Based on some research findings, majority of the filamentous fungi synthesize xylanase optimally between pH 6 and 7. Preference pH for fungal growth of *A. flavus* strain KRP1 was detected to be at the pH range from 5 to 7 where the optimum growth occurred at pH 6 according to Kurniati et al. [17]. Apparently, *A. niger* possessed the highest xylanase production by optimising the pH of medium at 6 [16]. In addition, in a study conducted by Azad et al. [18], *Thermomyces lanuginosus* BPJ-10 was found to grow optimally at pH 7.0. Likewise, the xylanase activity produced by *Penicillium citrinum* MTCC 2553 was maximised at the initial medium pH of 7.0 [19].

3.2 Purification of Xylanase

The purification of xylanase in this study was carried out according to Ho and Iylia [14].

3.2.1 Ammonium sulphate precipitation

In this study, ammonium sulphate was used for the precipitation of protein. There are several studies which used 70% saturation of ammonium sulphate for precipitation of xylanase. Ahmad et al. [20] purified xylanase produced by *A. niger* using 70% saturation of ammonium sulphate. The xylanase activity was found to be 6052 U/mg while the protein concentration possessed 78.3 mg. The enzyme was purified by 1.85-fold using the optimised salt saturation of 70%. According to the result obtained from Milala et al. [21], 70% saturation of ammonium sulphate was found to be the optimum for most of the xylanase precipitation of *Aspergillus* spp, achieving the specific xylanase activity of 16.06 U/mg. Likewise, a study was conducted by Pal and Khanum [22], who used 65% ammonium sulphate saturation on xylanase from *A. niger.* Based on their results, the maximum xylanase activity of 182716 U and specific activity of 142.30 U were obtained. When compared to the crude enzyme, a purification fold of 3.76 was achieved.

Hence, in this study, 70% saturation of ammonium sulphate as the first step of protein precipitation was added into 300 mL of crude supernatant at 4℃ [14]. At the end of this step, the specific activity was assayed to be 46.37 U/mg. The sample was purified by 1.43-fold and achieved 93.56% of recovery from its crude supernatant. After centrifugation at 8500 rpm for 15 min at 4℃, the supernatant was discarded while the protein pellet was dissolved in fresh 0.05 M sodium phosphate buffer, pH 5.3 before subjected to the next step of purification.

3.2.2 Dialysis

Dialysis was the second step of protein isolation and extraction in the present study. The protein precipitate obtained from the precious step was resuspended into 0.05 M sodium phosphate buffer, pH 5.3 before transferred into a dialysis tube. The dialysis tube was tied securely and immersed in the same buffer. Then, the dialysis process was carried out overnight at 4℃. At the end of this step, the specific activity was assayed to be 47.53 U/mg. In fact, the sample was purified by 1.47-fold and achieved 6.16% of recovery.

3.2.3 DEAE Sepharose column chromatography

After being precipitated by ammonium sulphate and dialysed in the dialysis tube, the sample was then subjected to a two-step chromatographic purification. To begin, DEAE Sepharose column chromatography was used as the first step of chromatographic purification. It is an anion exchange chromatography which separates samples based on the differences in protein charge. The elution of sample was carried out using NaCl solution of different concentrations ranging from 0 M to 1.0 M. The eluates were collected in small fractions and assayed for xylanase activity and protein concentration. The result was plotted as chromatograms as shown in Fig. 4. Since the fraction 1 to 46 possessed xylanase activity of less than 0.01 U/mL which were insignificant, thus, Fig. 4 shows the chromatogram of DEAE Sepharose starting from the fraction 47 to 80 instead. There are two main peaks of the chromatograms that possessed the highest xylanase activity along with its maximum protein concentration obtained from the fraction

number 54 after eluted with 0.3 M NaCl. Hence, the fractions with number 53 to 55 eluted with NaCl gradient of 0.3 M were pooled together before proceeded to the next purification step. The maximum xylanase activity of 8.51 U/mL was obtained with its specific xylanase activity of 46.37 U/mg. Approximately 100% increment in specific xylanase activity was observed after anion exchange chromatography whereby the specific xylanase activity increased from 46.37 U/mg to 92.79 U/mg while protein concentration was elucidated to be 1.971 mg. Furthermore, purification fold increased by around 1.44-fold from 1.43 to 2.87-fold.

According to Okafor et al. [23], xylanase produced by *A. niger* ANL 301 was purified using DEAE Sephadex A-50 and specific activity of 90.82 U/mg was obtained. The specific activity attained in this study was 92.79 U/mg which was relatively higher than the result achieved by Okafor et al. [23]. Another study was conducted by Yang et al. [24] who used *A. niger* C 3486 for the production of xylanase at 72 h of fermentation. The specific activity of 123.68 U/mg and 14.79 of purification fold were achieved. 40.25% of xylanase was recovered from its crude supernatant. Additionally, Lu et al. [25] attained the specific xylanase activity of 288.7 U/mg and approximately 82.3% was recovered from its crude filtrate produced by *Aspergillus ficuum* AF-98. Based on the results, different strains of *Aspergillus* produced different amounts of xylanase activity and the variation in medium formulation used for growth of the microorganism strains was also affected the activity of xylanase. Besides that, resin used in chromatography is also playing crucial role in affecting the result of purification. Different resins of chromatography elute different ranges of protein which are based on their ionic charges as well as pI values [26].

3.2.4 Sephadex G-75 column chromatography

Sephadex G-75 column chromatography was used as the last step of purification in this study. It is a size exclusion chromatography which separates protein molecules based on their differences in size. Samples were eluted using 0.05 M sodium phosphate buffer, pH 5.3. The eluates were collected in small fractions and subjected to assays including xylanase activity and protein concentration. The result obtained was plotted as chromatograms as shown in Fig. 5. Apparently, the maximum xylanase activity and protein concentration possessed two main peaks of the chromatograms obtained from the fraction number 11 and 12. Thus, fractions with the number from 11 to 12 were pooled together. About 25% increment in purification fold was observed compared to its previous step. Xylanase was purified by 3.6-fold with its specific activity of 116.64 U/mg and recovery of 1.68% at the end of the purification process. According to the results obtained in this study, increase of specific activity of 0.73-fold was observed compared to specific activity of 92.79 U/mg after

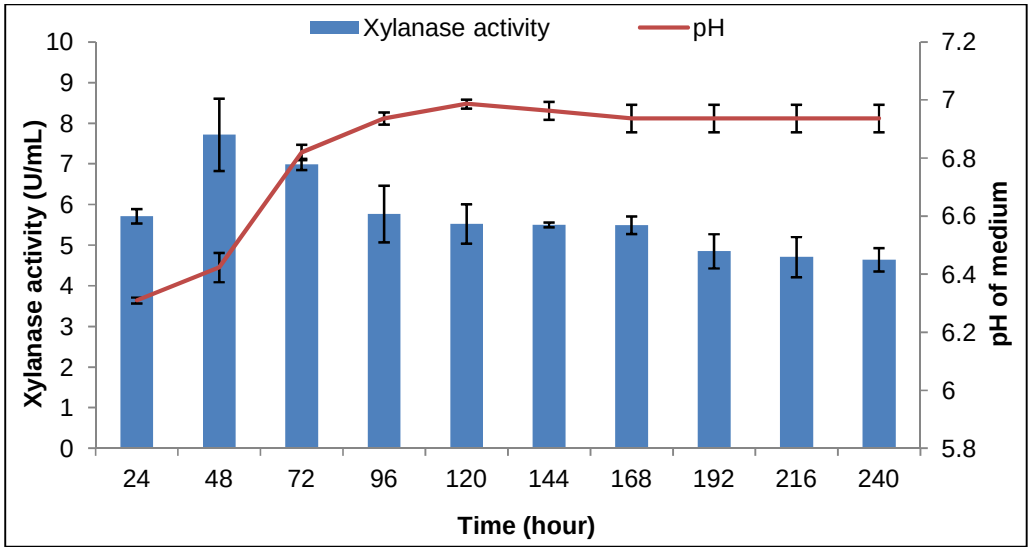

Fig. 3. The correlation of xylanase activity produced by *A. brasiliensis* with the profile of medium pH in SmF

DEAE Sepharose chromatography. At the end of the purification, specific enzyme activity of 116.64 U/mg with the total protein concentration of 0.894 mg was obtained. On the other hand, xylanase produced by *A. terreus* achieved the specific activity of 6.92 U/mg. This study was performed by Sorgatto et al. [15] using a CM-cellulose column chromatography for the purification of xylanase. The specific activity of the study was significantly lower than specific activity of xylanase obtained from this study. Nonetheless, the purification fold was increased majorly from 1 to 45-fold at the end of purification process [15]. In contrast, Yang et al. [24] achieved the specific xylanase activity of 123.68 U/mg produced by *A. niger* which was relatively higher than the specific activity of this study. Hence, it is suggested that besides column chromatography, medium composition and cultivation conditions might also play important role in production and purification of enzymes. In conclusion, xylanase produced in this study achieved the specific activity of 116.64 U/mg. In fact, the purification fold increased from 1 to 3.6-fold at the end of the purification. Additionally, the purified xylanase was detected to be a low molecular weight protein. 36 kDa of purified xylanase was detected from SDS-PAGE as shown in Fig. 6. The purified xylanase was thereafter subjected to the characterisation study in step-wise manner. The results obtained from the purification steps in this study are summarised in Table 1.

3.2.5 Characterisation of purified xylanase

3.2.5.1 Temperature stability of xylanase

Thermal inactivation of enzymes is a frequent encountered problem during the applications of enzymes in industry. Thus, temperature stability of an enzyme is one of the most important factors in the study of characterisation. The results of temperature stability of xylanase by *A. brasiliensis* are displayed in Fig. 7. The relative xylanase activity was expressed as the percentage of the control reaction at time zero. In this study, xylanase produced by *A. brasiliensis* was relatively stable at the range of temperatures from 40℃ to 60℃ with its maximum temperature activity (stability) maintained at 50℃. At 40℃, xylanase retained almost 95.62% of its activity after 3 h of incubation. In fact, xylanase showed the maximum activity at 50℃ where it maintained the highest, 98.33% of its activity even after incubated for 3 h. Nevertheless, at 60℃, the xylanase activity was gradually declined as the incubation time increased. Notably, at 60℃, the xylanase activity was found to be 66.03% after 1 h, 40.21% after 2 h and 34.4% retained after 3 h of incubation, respectively. In contrary, at much higher temperatures of 70℃ and 80℃, the xylanase activity declined drastically to 1% and 2% after 3 h of incubation, respectively.

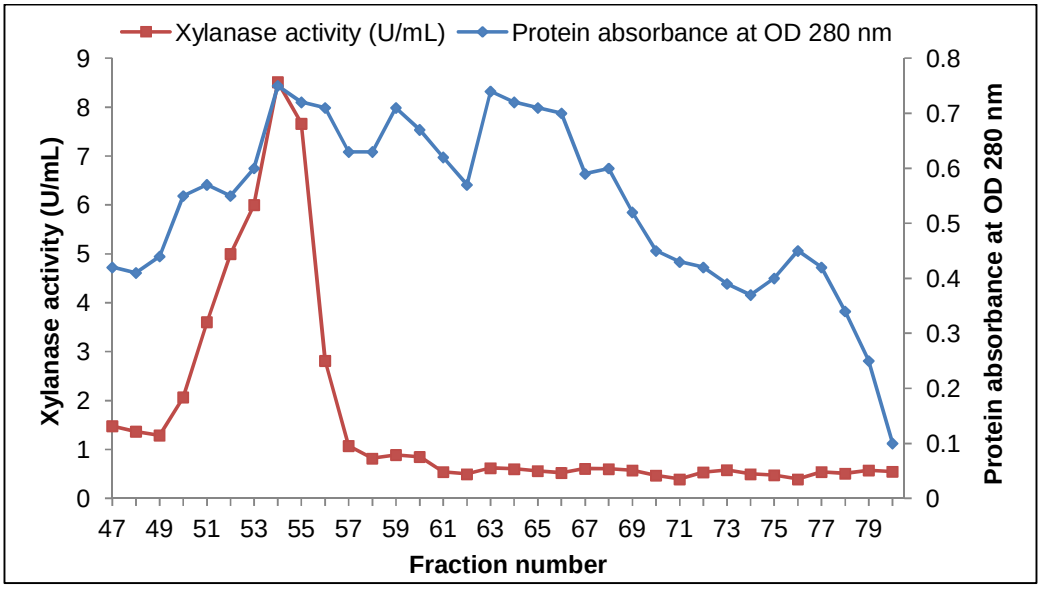

Fig. 4. Chromatograms of xylanase activity and protein absorbance reading of fraction 47 to 80 eluted from DEAE Sepharose chromatography

Table 1. Summary of purification of xylanase by *A. brasiliensis*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification yield (%)	Purification fold
Crude extract	192.00	6215.40	32.37	100.00	1.00
Ammonium sulphate	125.40	9696.50	46.37	93.56	1.43
Dialysis	8.06	383.08	47.53	6.16	1.47
DEAE Sepharose	1.97	182.88	92.79	2.94	2.87
Sephadex G-75	0.89	104.28	116.64	1.68	3.60

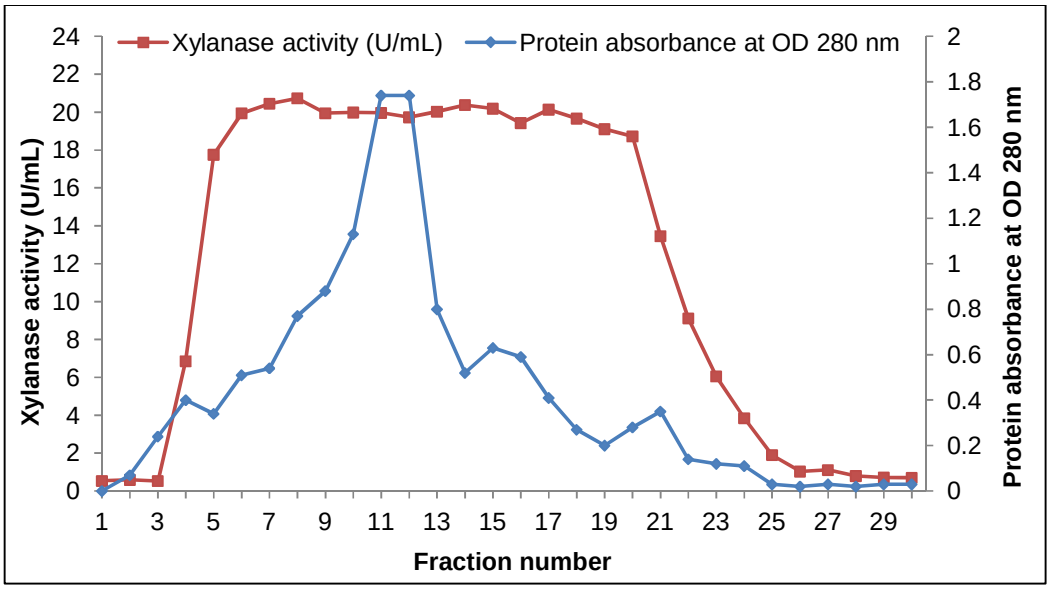

Fig. 5. Chromatograms of xylanase activity and protein absorbance reading of fraction 1 to 30 eluted from Sephadex G-75 chromatography

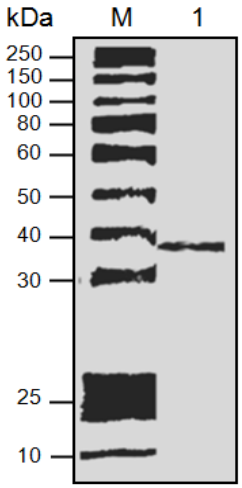

Fig. 6. Pictograph of purified xylanase produced by *A. brasiliensis* on SDS-PAGE
Lane M: protein marker. Lane 1: purified xylanase with the molecular weight of 36 kDa

The result of the present study was also in accordance with other studies. Sorgatto et al. [15] reported that xylanase stability by *A. terreus* was found to be the most stable at 50℃ with half-life of 36 min followed by 45℃. In a study by Fialho and Carmona [27], two types of xylanases produced by *A. giganteus* exhibited the highest thermo-stability at 50℃ with half-life of 22.5 min and 17.5 min, respectively. Similarly, xylanases produced by *A. caespitosus* were at the most stable activity at 55℃ with its half-life of 27.3 m in and 90 min, respectively [28]. Likewise, the xylanase produced by *A. niger* C3486 showed its thermo-stability at 55℃ to 60℃ with more than 80% of its activity retained after 90 min of incubation [24]. Generally, xylanases from most of *Aspergillus* spp. are stable at the temperature range of 40℃ to 60℃. In fact, some *Aspergillus* spp. such as *A. niger* produced more thermo-tolerance xylanase which remained stable up to 100℃ with approximately lost of 50% to 52% of its activity [20,29]. Based on the results, xylanase

produced in this study is suitable to use in various industrial applications which require high thermo-stability characteristic especially in paper pulping and food processing industry.

3.2.5.2 pH stability of xylanase

pH stability study is one of the important parameters in enzyme characterisation. Therefore, pH stability of xylanase by *A. brasiliensis* was investigated at pH ranging from pH 3 to 9. The result of the pH stability of xylanase is displayed in Fig. 8. The relative xylanase activity was expressed as a percentage of the control reaction at time zero. Xylanase produced by *A. brasiliensis* maintained its stability over a broad range of pH from pH 4 to 8. It possessed the highest activity (stability) at pH 5 followed by pH 4 and 6. As much as 94.87%, 89.17% and 84.81% of relative activity were retained at pH 5, 4 and 6 after 3 h of incubation. Furthermore, xylanase was also stable at slight alkaline pH in much lesser extent. The purified xylanase retained 57.22% and 33.59% of its activity at pH 7 and 8 after incubated for 3 h, respectively. In contrary, xylanase was less stable at pH 3 and 9. Notably, at pH 3, only 4% of xylanase activity retained at the first 2 h of incubation and less than 1% of activity retained after 3 h incubation. Whereas at pH 9, 21.1% of xylanase activity was retained after 1 h of incubation but declined drastically to 5.49% and 1.4% after 2 and 3 h of incubation, respectively. Based on the result findings, the considerable activity of xylanase by *A. brasiliensis* at wider range of pH from pH 4 to 8 in this study suggested it is potentially useful in variety of industries including detergent, paper pulping and food processing.

The result in this study was also in accordance with the results isolated from other studies. In general, xylanases from most of the *Aspergillus* spp. are stable at pH range of pH 4 to 8. Dai et al. [30] reported that xylanase by *A. niger* JL-15 displayed its high stability from pH 4 to 8, whereby around 80% of the activity was retained after 1 h of incubation. Xylanase produced by *A. niger* maintained its high stability over pH 4.5 to 7.0 with activity above 50% [31]. Besides that, xylanase from *A. awamori* VTCC-F312 showed relatively great stability from pH 4 to 8 without any reduction in enzyme activity even after 4 h of incubation [32]. Another study conducted by Krisana et al. [33] showed that xylanase from *A. niger* BCC14405 possessed a broader pH stability from pH 5 to 10.

3.2.5.3 Effect of temperature on xylanase activity

Due to the wide variety of xylanase applications, a lot of studies have been elucidated on the characterisation of xylanase. According to Sharma and Chand [34], the optimum temperature for xylanase produced by *Pseudomonas* sp. XPB-6 was occurred at 60℃ with its specific activity of around 80 U/mg.

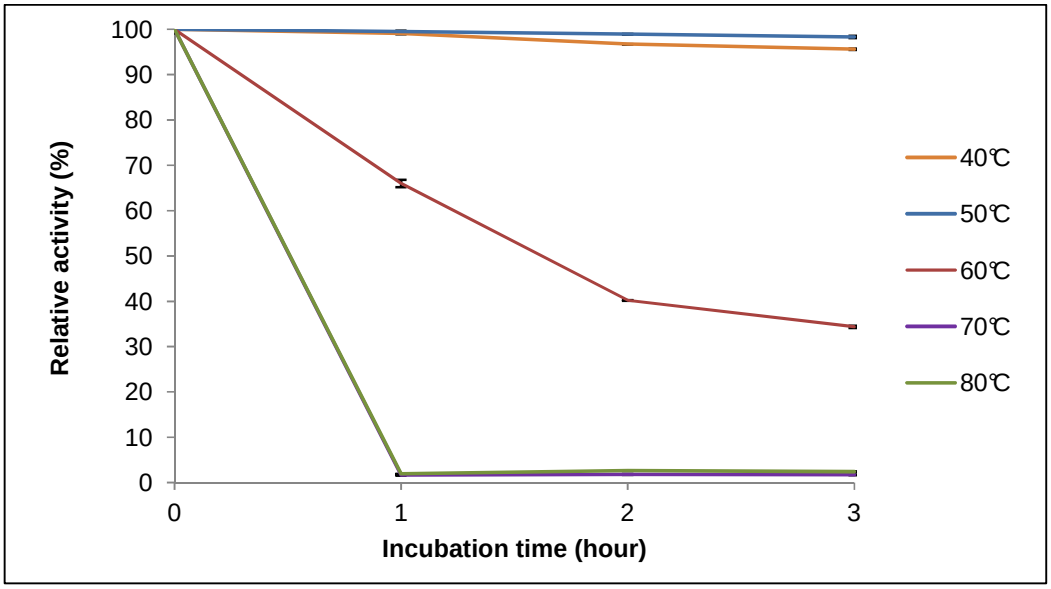

Fig. 7. Temperature stability of the purified xylanase by *A. brasiliensis*

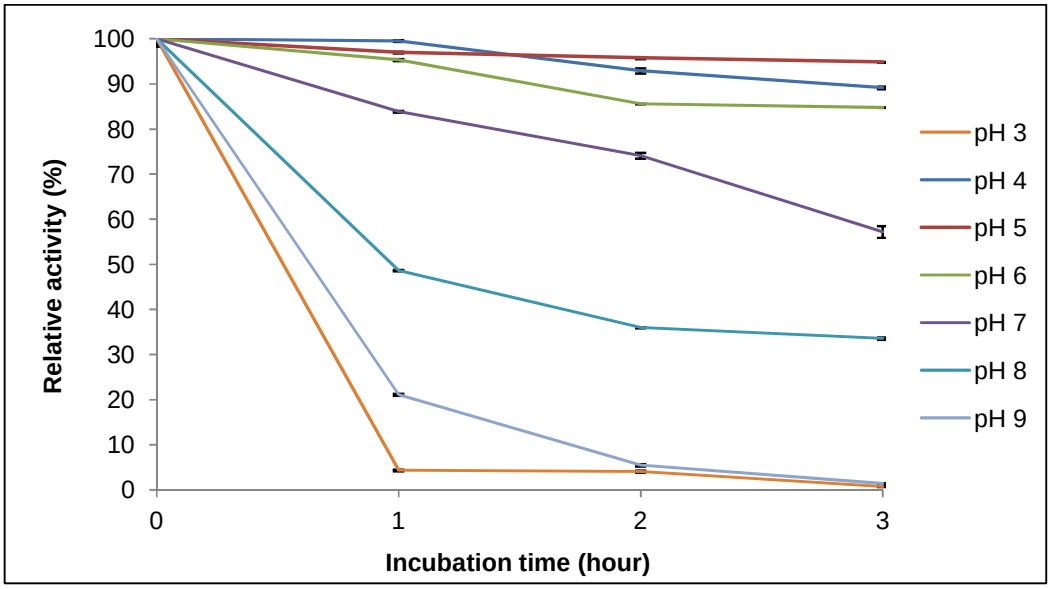

Fig. 8. pH stability of the purified xylanase by *A. brasiliensis*

Likewise, the result of Abirami et al. [35] showed that the optimum temperature for xylanases produced by *Penicillium janthinellum* and *Neurospora crassa* were detected to be at 50°C and 60℃ with their xylanase activity of around 50 U/mL and 40 U/mL, respectively. This proved that xylanases produced by different microorganism strains might have different optimum parameters in their characterisation study. Hence, it is important to investigate and elucidate the characterisation of xylanase including the effect of temperature on its activity. Indeed, xylanases which perform efficiently at relatively higher temperatures of 55℃ to 70℃ and 70℃ to 95℃ would be favourable in the processing and manufacturing industry of paper pulping and animal feeds, respectively [36]. The result of the effect of temperature on xylanase activity is plotted in a graph shown in Fig. 9. The relative xylanase activity was expressed as a percentage of the optimal activity at 50℃ where 100% xylanase activity was quantified to be 20.51 U/mL. Based Fig. 9, xylanase from *A. brasiliensis* was found to be active at the temperatures ranging from 45℃ to 70℃. Indeed, the xylanase relative activity increased from 95.24% with its activity of 19.53 U/mL at 45℃ to the maximum 100% with its activity of 20.51 U/mL at 50℃. Thereafter, the xylanase activity decreased gradually to 17.9 U/mL with 87.27% of relative activity as the temperature increased to 70℃.

In general, the optimum temperature for xylanase activity produced by *Aspergillus* spp. is usually at the range from 45℃ to 60℃. Xylanase by *A. awamori* VTCC-F312 was active at much higher temperatures ranging from 40℃ to 80℃, while its optimum temperature was between 50℃ and 55℃ with the maximum xylanase activity of 346.4 U/mg [32]. Besides that, the optimum temperature of xylanase by *A. niger* US368 was detected to be in the range from 50℃ to 60℃ with its activity found around 800 U/mg [37]. Likewise, xylanases by *A. nidulans* KK-99 and *A. terreus* were optimally found active at 55℃ [15, 38]. Similarly, Fialho and Carmano [27] reported that xylanase produced by *A. giganteus* possessed the optimum temperature of 50℃ which matched with the result of this present study. On the other hand, xylanase produced by *A. fumigatus* MKU1 showed relatively higher xylanase activity from 70℃ to 90℃ while 70℃ was its optimum temperature detected [39].

3.2.5.4 Effect of pH on xylanase activity

pH is greatly capable of affecting the activity of an enzyme because the binding of substrate with its enzyme is usually relied on the charge distribution of substrate and enzyme molecules. Besides that, pH is also one of the important criteria in enzyme characterisation to determine its suitability in industrial applications. As a result, the effect of pH on xylanase activity by *A. brasiliensis* was investigated in the present study. The results obtained are shown in Fig.10. The relative xylanase activity was expressed as a percentage of the optimal activity at pH 5 where the 100% xylanase activity was quantified to be

22.18 U/mL. As shown in Fig. 10, xylanase remained active at pH ranging from pH 3 to 9. The xylanase activity increased from 19.95 U/mL with its relative activity of 89.91% at pH 3 to 21.30 U/mL with 96.02% of relative activity at pH 4. In fact, the xylanase activity reached its maximum of 22.18 U/mL at pH 5. Consequently, the xylanase activity decreased gradually from 21.78 U/mL with 98.16% of relative activity at pH 6 compared to 18.04 U/mL with 81.33% at pH 9. Thereafter, the xylanase activity decreased drastically to 3.15 U/mL where only 14.22% of relative activity retained at pH 10.

The results of the present study were also agreed by other researchers. Do et al. [32] reported that xylanase by *A. awamori* VTCC-F312 was active from pH 3 to 8 and achieved its maximum activity of 556.2 U/mg at pH 5. Likewise, xylanase by *A. niger* US368 was active from pH 3 to 7 with its maximum activity at pH 5 [37]. On the other hand, xylanases by *A. niger* BCC14405 and *A. foetidus* possessed their maximum activity at pH 5 and 5.3, respectively [33,40]. In general, the optimum pH of xylanase activity by *Aspergillus* spp. is found to be around pH 5. However, there are other fungal strains possess xylanase activity at the extreme optimum pH. For instant, xylanase activity by *A. nidulans* KK-99 was at the most active at pH 8 while xylanase activity by *A. sydowii* SBS 45 was optimum at pH 10 [37,41]. Notably, xylanases

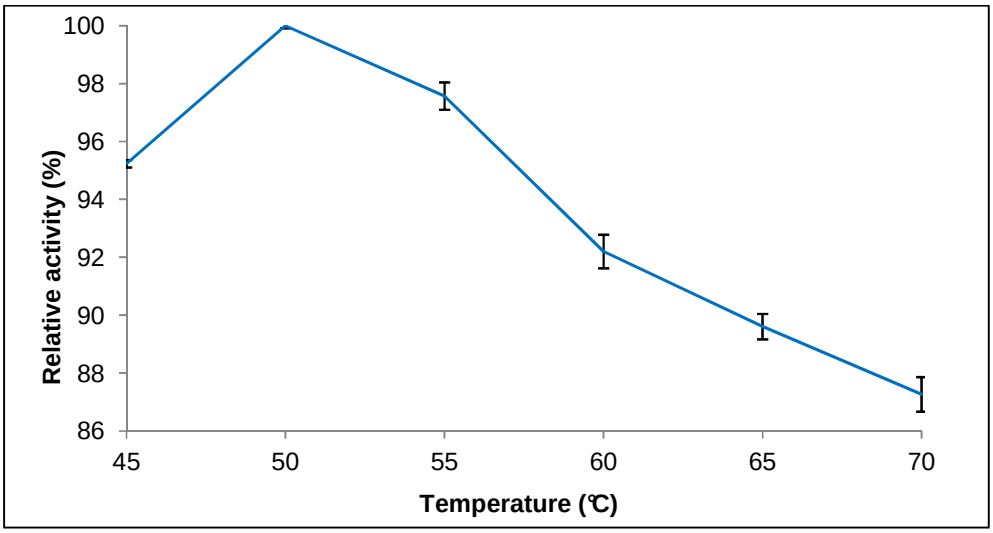

Fig. 9. Effect of temperature on the purified xylanase activity by *A. brasiliensis*

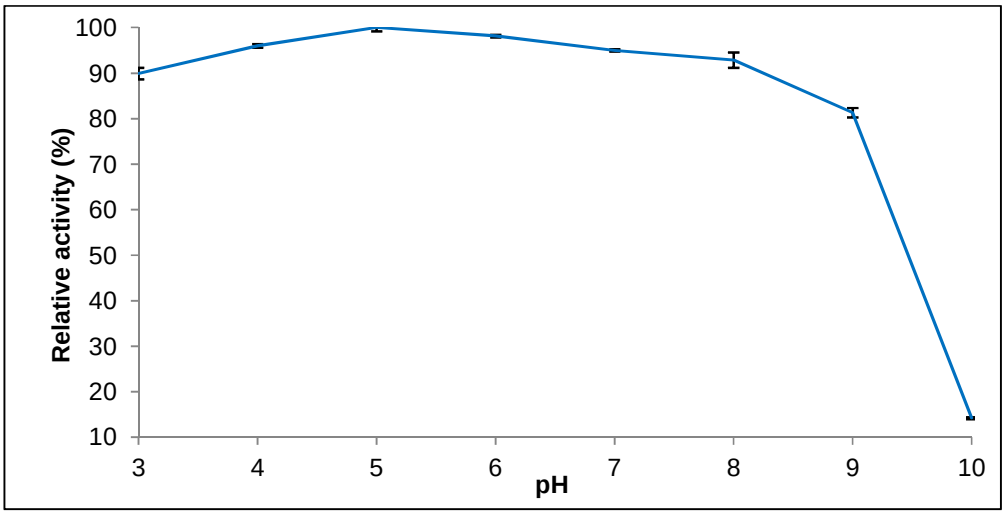

Fig. 10. Effect of pH on the purified xylanase activity by *A. brasiliensis*

which work optimally at acidic condition are potentially applicable in animal feeds and food processing industry while xylanases which active at alkaline condition are suitable for paper pulping and bleaching industry [35]. Therefore, xylanase produced by *A. brasiliensis* which active from pH 3 to 9 in the present study possessed greater suitability and larger potentials in industrial applications especially in animal feeds production and food processing industry.

3.2.5.5 Effect of substrate specificity on xylanase activity

Compared all the substrates elucidated in the present study, the enzymatic reaction of purified xylanase with beechwood xylan exhibited the highest activity of 24.7 U/mL. Substrate that possessed the second highest xylanase activity was detected to be rice bran with its xylanase activity of 12.8 U/mL followed by palm kernel with its xylanase activity of 6.01 U/mL. Subsequently, sawdust and wheat bran gave the same value of 4.5 U/mL while wood chip showed the activity of 3.5 U/mL followed by psyllium husk with 3.4 U/mL and soya with the lowest activity of 2.23 U/mL.

According to Do et al. [32], xylanase activity using the substrate of oat spelt xylan possessed the highest activity of 522 U/mg. Then in the same journal, xylanase activity with rice bran as the substrate gave a value of 194.18 U/mg with 37.2% of relative activity. In addition, the result of Kamble and Jadhav [42] also showed similar finding where birchwood xylan produced the highest xylanase activity of 291.9 U/mg. Likewise, the results from Mander et al. [43] also showed that xylan was the optimum substrate for xylanase activity by giving out relatively higher amount of activity. Based on their results, the highest xylanase activity of 39,996 U/mg was produced using beechwood xylan as the optimum substrate followed by 37,831 U/mg after using birchwood xylan as the substrate. On the other hand, substrate specificity of purified xylanase from *A. niger* was also investigated by Dobrev and Zhekova [44] with substrates such as carboxymethyl cellulose, oat spelt, birchwood and beechwood xylan. Their results showed that oat spelt xylan possessed the highest xylanase activity with the K_m value of 12.2 mg/mL compared to no enzyme activity detected on carboxymethyl cellulose. Similar result also reported by Kamble and Jadhav [42] where the purified xylanase from a new species of *Bacillus* was found to be specific to birchwood xylan but showing no activity towards carboxymethyl cellulose. In fact, higher xylanase activity towards birchwood xylan was detected as 291.9 U/mg. Additionally, according to Krisana et al. [33], the purified xylanase from *A. niger* was specific to xylan by producing 5870 U/mg while showing no activity towards carboxymethyl cellulose. Apparently, the conclusion that drawn from the results of the present study showed beechwood xylan was the optimum substrate for xylanase activity because it possessed the highest activity of 24.7 U/mL as shown in Fig. 11.

3.2.5.6 Effect of substrate concentration on xylanase activity

Substrate concentration is also one of the important parameters in the characterisation study especially for the industrial applications of xylanase. Large industries are tended to avoid using excess amount or concentration of substrate that would hinder the production rate that eventually lead to the loss of profit as far as the industries are concerned. Based on the result findings, as the concentration of xylan increased, xylanase activity was also increased. Apparently, the increase in xylanase activity occurred from 0.25% to 1.0% beechwood xylan. Thereafter, the xylanase activity remained almost constant until it reached 1.75% substrate concentration. The highest activity of 24.46 U/mL was obtained at 1.0% xylan concentration while the lowest activity of 11.62 U/mL was obtained at 0.25% of xylan concentration in this study.

According to Isil and Nilufer [45], 1% xylan concentration was found to be the optimum concentration for purified xylanase from *Trichoderma harzianum* 1073 D3. It produced the xylanase activity of about 500 U/mL. Similar trend of xylanase activity was also observed whereby the xylanase activity increased as the xylan concentration increased. Nevertheless, after the optimum concentration of 1% xylan, the xylanase activity started to fall down. According to Sharma and Chand [34], 1% xylan was found to be the optimum concentration for purified xylanase by *Psuedomonas* sp. XPB-6 with its specific xylanase activity of about 78 U/mg. Similarly, the trend of the result was identical with this study whereby when the substrate concentration increased, the xylanase activity also increased. In conclusion, 1% xylan concentration was the optimum concentration for purified xylanase produced by *A. brasiliensis* in this study as shown in Fig. 12.

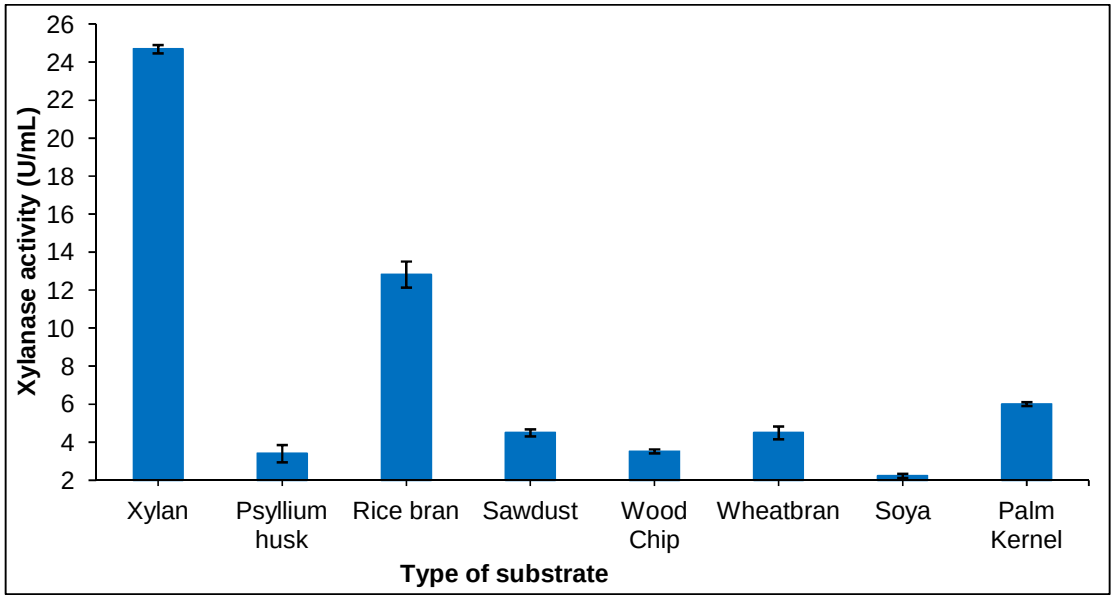

Fig. 11. Effect of substrate specificity on the purified xylanase activity by *A. brasiliensis*

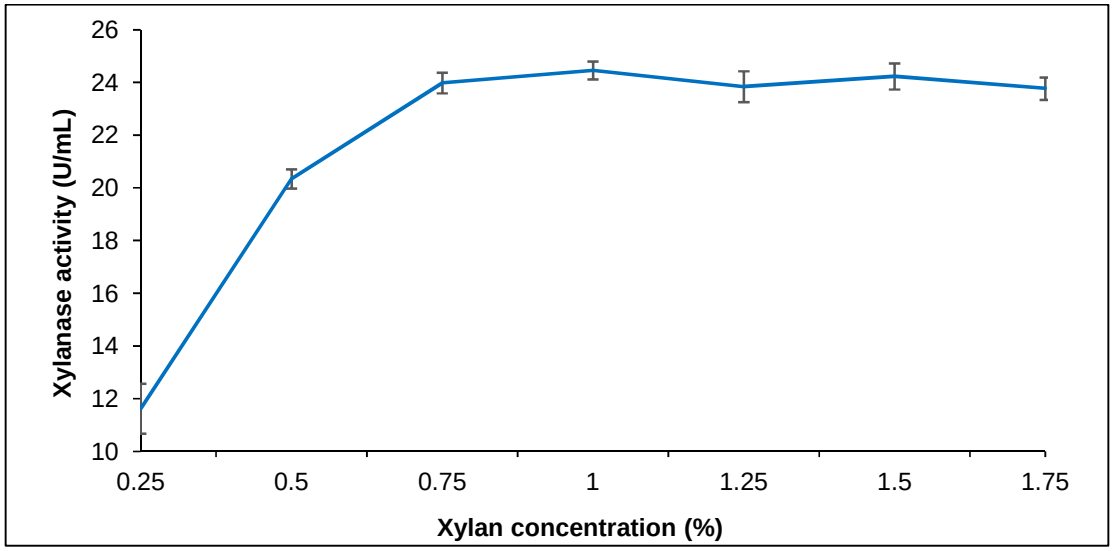

Fig. 12. Effect of xylan concentration on the purified xylanase activity by *A. brasiliensis*

3.2.5.7 Effect of incubation time on xylanase activity

Incubation time is yet another key parameter that needed to be characterised. The incubation time is important because xylanase is a temperature sensitive globular protein, if the enzyme is stored for long period of time at certain temperature, the temperature would cause the conformational changes which ultimately lead to possible lower production rate and loss of profits. Based on the result in this study, from 10 min to 30 min of incubation time, the increase in incubation time would result in the increase of xylanase activity. Nevertheless, after 30 min, the xylanase activity started to decrease drastically until it reached 60 min of incubation. Obviously, in the present study, the optimum incubation time for the purified xylanase produced by *A. brasiliensis* was found to be 30 min with its maximum xylanase activity of 24.46 U/mL. In contrast, 10 min of the incubation time gave the lowest xylanase activity of 14.55 U/mL.

According to the result from Tabosa-Vaz et al. [46], the optimum incubation time obtained was found in the range of 40 min to 80 min as the purified xylanase activity remained 100%. According to Isil and Nilufer [45], the optimum incubation time for the purified xylanase produced from *T. harzianum* was 50 min with its maximum xylanase activity of about 520 U/mL. Nonetheless, the result from Sharma and Chand [34] showed the optimum incubation time for the purified xylanase produced by *Psuedomonas* sp. was 5 min with its specific xylanase activity of about 76 U/mg. Apparently, purified xylanases produced by different strains possess different optimum incubation time of enzyme activity. In the present study, a conclusion of 30 min was the optimum incubation time for the activity of purified xylanase produced by *A. brasiliensis* in the present study as shown in Fig. 13.

3.2.5.8 Effect of metal ion on xylanase activity

Inorganic salts or metal ions are additives that influence stability and activity of enzyme directly by the preferential binding to the folded or unfolded proteins. As a result, it changes the properties of solvent water, especially the water activity [40]. Nevertheless, metal ions which exist in raw paper pulp as impurities might inhibit the activity of xylanase. Hence, the influence of various metal ions on xylanase activity was investigated to elucidate its suitability in application of paper pulping and to reveal which of the ions should be precluded or included in this industrial process.

In this study, the xylanase activity was assayed with the optimised 1% beechwood xylan in the presence of 5 mM of different metal ions, respectively. Based on the results shown in Fig. 14, the relative xylanase activity was expressed as a percentage of the control reaction without addition of any metal ions where the 100% xylanase activity was quantified to be 21.03 U/mL. According to the results, all metal ions were proven to act as the marginal effectors of xylanase activity. Indeed, Cu^{2+}, Mn^{2+} and Zn^{2+} were detected to slightly increase the xylanase relative activity by 1.3%, 0.9% and 0.4% compared to the control. In contrast, the inhibition on xylanase activity was found in the presence of Al^{3+}, Ca^{2+}, K^{+} and Mg^{2+} by reduction of relative activity of 17.6%, 2.4%, 0.4% and 0.9%, respectively. In particular, Cu^{2+} was the strongest enhancer whereas Al^{3+} was the strongest inhibitor compared to other metal ions on xylanase activity in this study.

Similar studies were also conducted in determining the effect of metal ions on xylanase activity. Based on Sorgatto et al. [15], they reported that the activity of xylanase from *A. terreus* reduced by 30%, 47% and 60% after the addition of 5 mM Mg^{2+}, Cu^{2+} and Zn^{2+}, respectively. Whereas, the xylanase activity increased by 19% and 34% after addition of $Mn^{2+}$and K^{+}, respectively. Besides that, xylanase produced by *A. niger* US368 was activated by Mg^{2+} and Zn^{2+} up to 10% and 4.3%, while inhibited by Ca^{2+}, Cu^{2+} and Mn^{2+} by 0.3%, 44.2%, and 80%, respectively [37]. Nair et al. [41] reported that Al^{3+}, Ca^{2+} and Zn^{2+} enhanced the activity of xylanase from *A. sydowii* SBS 45 at the concentration of 10 mM. According to study by Dobrev et al. [47], xylanase from *A. niger* B03 was totally inhibited by Cu^{2+} but activated by Mn^{2+} up to 64%. Likewise, the xylanase produced by *A. ficuum* AF-98 was activated by Cu^{2+} up to 16% of its activity, however, xylanase from *A. niger* C3486 was inhibited by the same ion [24, 48].

Thiagarajan et al. [39] showed that xylanase originated from *A. fumigatus* MKU1 experienced reduction in xylanase activity by 21% after addition of Mg^{2+} whereas activation by 38% was observed after addition of Zn^{2+}. In some other studies, the addition of Ca^{2+}, Mg^{2+} and Zn^{2+} reduced the activity of xylanase obtained from *A. brasiliensis* (this study), *A. sydowii* SBS 45, *A. fumigatus* MKU1 and *A. terreus,* respectively. On the other hand, the addition of Mn^{2+} was found to increase activity of xylanase from *A. brasiliensis* (this study), *A. niger* B03 and *A. terreus,* respectively.

In spite of that, there are some contradicting results obtained in comparison to other studies. For example, Cu^{2+} was found to be an activator for xylanase activity from *A. brasiliensis* (this study) and *A. ficuum* AF-98 but acted otherwise as an inhibitor for xylanase activity from *A. niger* C3486, *A. terreus* and *A. niger* US368, respectively. Besides that, Zn^{2+} increased activity of xylanase from *A. brasiliensis* (this study), *A. sydowii* SBS 45 and *A. niger* US368, but reduced its activity of xylanase from *A. fumigatus* MKU1 and *A. terreus,* respectively. Based on these result findings, xylanases produced by different species of *Aspergillus* behaved differently in the presence of metal ions. Therefore, further research would be necessary using more activators, inhibitors and their combinations to uncover the mode of action of xylanase from *Aspergillus* spp.

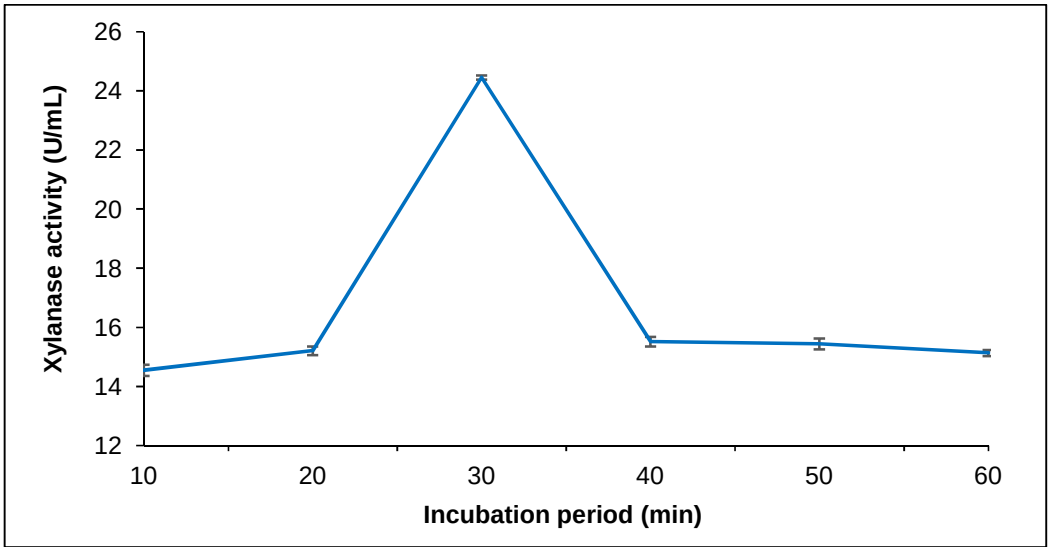

Fig. 13. Effect of incubation time on the purified xylanase activity by *A. brasiliensis*

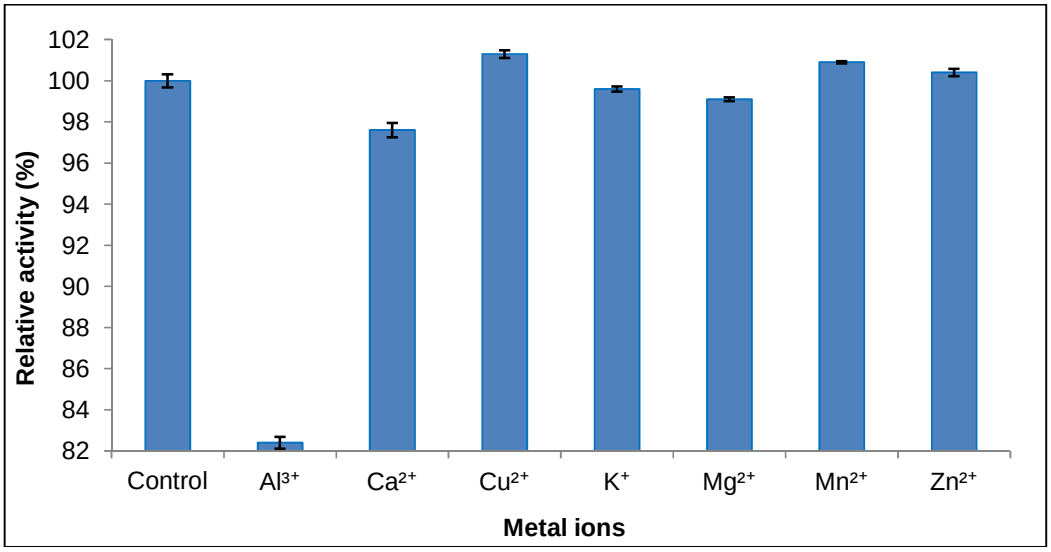

Fig. 14. Effect of metal ion on the purified xylanase activity by *A. brasiliensis*

3.2.5.9 Effect of chelating agent and detergent on xylanase activity

Some additives such as detergents and chelating agents might be potential inhibitors for enzyme activity especially during their applications in industry. Thus, their effects on xylanase activity were investigated in the present study and the results obtained are showed in Fig. 15. The relative xylanase activity was expressed as a percentage of the control reaction without addition of any chelating agents and detergents where the 100% xylanase activity was quantified to be 21.03 U/mL.

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent which commonly known to remove metal ions from enzyme molecules [49]. In this study, EDTA did not drastically affect the xylanase activity as it reduced its activity by only 2.7%. This result suggested that EDTA was not required as cofactor for xylanase activity. Similar study on xylanase activity by *A. caespitosus* also indicated that only 1% to 2% of activity was reduced by EDTA [28]. In fact, xylanase activity produced by other *Aspergillus* spp. including *A. giganteus, A. niger* BCC14405 and *A. terreus* experienced 22%, 40% and 60% of reduction

due to the presence of EDTA, respectively [15,27,33].

The effect of ionic detergent of SDS and non-ionic detergent of Tween 80 was also investigated on the xylanase activity in this study. Based on the results, SDS was a strong inhibitor of xylanase activity where it reduced the activity by 36.1% in comparison to the control reaction without any additives. It was suggested that xylanase obtained from *A. brasiliensis* exerted higher tolerance towards SDS as compared to *A. awamori* VTCC-F312. This is because when the same concentration of 5% (w/v) SDS was used in both studies, xylanase activity from *A. awamori* VTCC-F312 was completely inhibited while xylanase from *A. brasiliensis* in this study was still able to retain its activity at 63.9%. In general, SDS was greatly inhibited activity of xylanase from *Aspergillus* spp. including *A. giganteus*, *A. niger* US368 and *A. nidulans* KK-99, respectively [27,37,38]. The reduction in xylanase activity was elucidated to be the consequence from the interaction occurred between SDS and the hydrophobic group of amino acids [50].

On the other hand, even though, Tween 80 did not change the xylanase activity drastically in this study but it increased its activity by 0.5% compared to the control. Notably, Do et al. [32] reported that xylanase produced by *A. niger* was activated by 2% (w/v) Tween 80 to achieve 18% increment in activity. 33.32% of increment in the xylanase activity by *A. niger* US368 was observed after the addition of 1% Tween 80 [37]. In contrary, Tween 80 exerted slight inhibitory effect on xylanase activity from *Termitomyces* sp., termite *Macrotermes subhyalinus* worker and *A. oryzae* DSM1863 by 3%, 4% and 14% reduction in activity, respectively [51-53]. Interestingly, Bandivadekar and Deshpande [54] reported that Tween 80 enhanced the thermal stability of xylanase produced by *Chainia* sp. at 60℃.

3.2.5.10 Effect of organic solvent on xylanase activity

Organic solvents have been used for solubilising hydrophobic substrates in enzymatic reactions. Addition of organic solvent reduces the polarity of the medium surrounding the enzyme molecules, as a result, the enzymes are usually inactivated in response to the increase in hydrophobic environment [55]. The influence of different organic solvents on xylanase activity is shown in Fig. 16. Organic solvents such as acetone, ethanol and glycerol were used to evaluate xylanase activity in the present study. The relative xylanase activity was expressed as a percentage of the control reaction without addition of any organic solvent where the 100% xylanase activity was quantified to be 21.03 U/mL. These results would provide primarily essential data for the applications of purified xylanase in organic phase. Based on the result, xylanase revealed its highest tolerance towards glycerol, in which 51.2% of its activity was maintained although high concentration of 50% (v/v) glycerol was added. Similar result was reported by Krisana et al. [33] where the addition of 10% glycerol reduced 20% of xylanase activity by *A. niger* BCC14405. Besides, xylanase from *A. niger* C3486 experienced 40% reduction in activity by addition of 10% glycerol [24]. Interestingly, it was found that glycerol also possessed a positive effect against thermal denaturation. Likewise, in a study by Lemos et al. [56], the stability of xylanase by *A. awamori* was markedly improved by the addition of 50% glycerol.

Based on the result in the present study, xylanase retained 17.9% of activity after the addition of 50% (v/v) ethanol. Notably, ethanol also showed its inhibitory effect on xylanase activity by *A. niger* C3486 which was 90% of its activity retained at the concentration of 2% (v/v) while 40% of the activity retained at 30% (v/v), respectively [24]. In contrast, the activation of xylanase activity by ethanol was also observed in other studies. Xylanase activity from *A. niger* US368 and *Bacillus altitudinis* DHN8 were increased by 26% and 8.69% in the presence of ethanol, respectively [37,57].

On the other hand, xylanase in this study maintained 33.9% of activity after the addition of 50% (v/v) acetone. The result was agreed with other experimental findings. Apparently, xylanases from *A. oryzae* DSM1863 and *Providencia* sp. X1 possessed good solvent tolerance in 10% and 25% acetone where they retained around 70% and 78% of activity, respectively [53, 58]. Indeed, acetone was found to be a good activator for xylanase activity by *A. niger* DSM1957 with the enhancement of activity by 1.5 to 2-fold after incubation for 1 to 2 h, respectively [59]. Interestingly, xylanase activity by *Bacillus altitudinis* DHN8 was also stimulated in the presence of acetone by 2.96% increment of its activity [57].

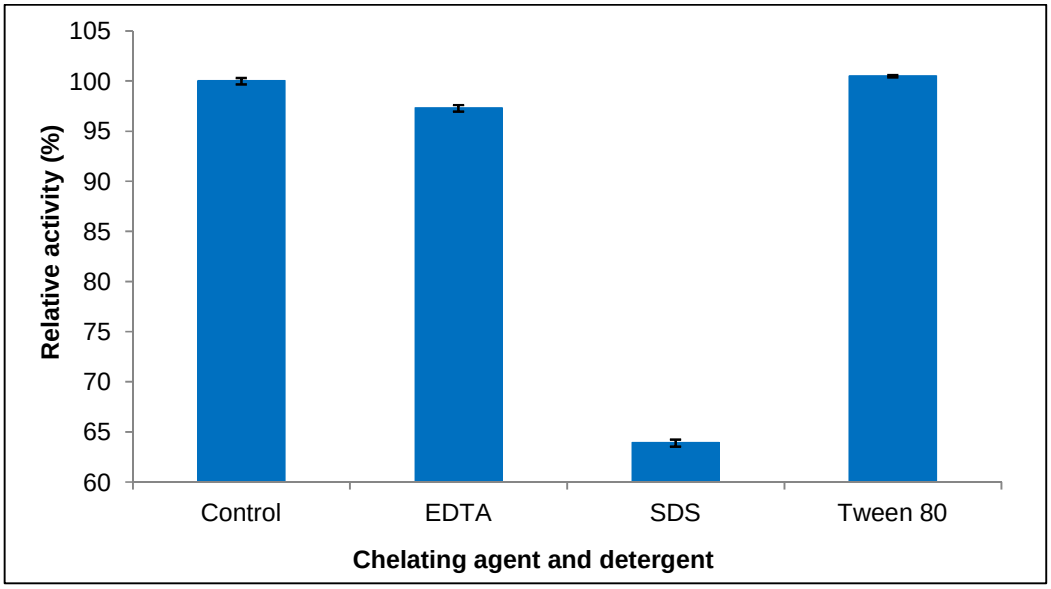

Fig. 15. Effect of chelating agent and detergent on the purified xylanase activity by *A. brasiliensis*

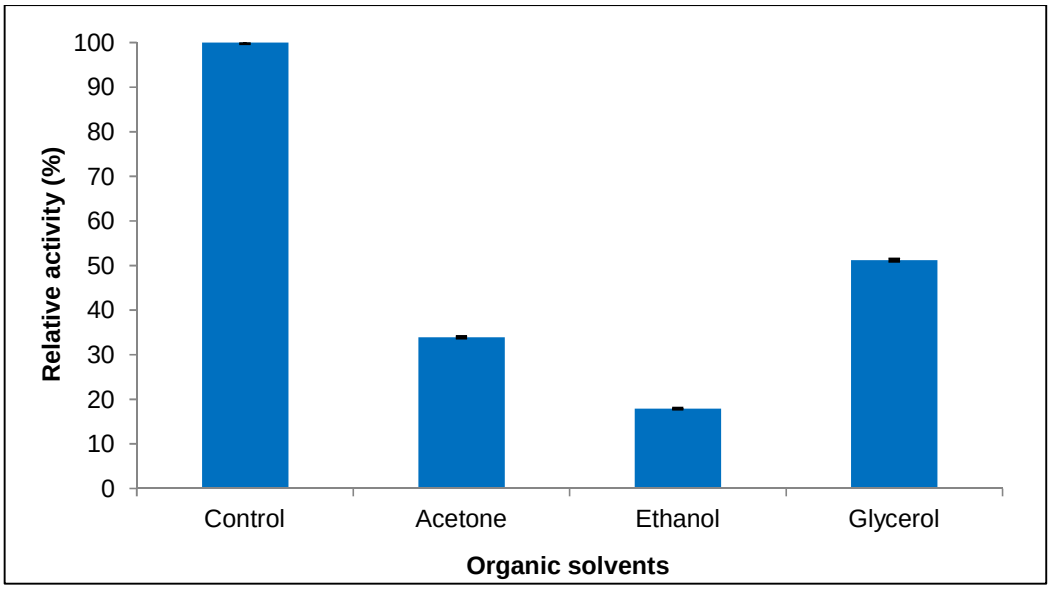

Fig. 16. Effect of various organic solvent on the purified xylanase activity by *A. brasiliensis*

3.2.5.11 Summary

Different strains of *Aspergillus* produce xylanases with their distinctive characteristics and properties which are applicable in various industries. According to the characterisation study of xylanase in this work, xylanase produced by *A. brasiliensis* is suggested to use in the manufacturing of animal feeds, fruit juice and paper pulping due to its greater activity and stability at elevated temperatures, higher performance in broad range of pH buffers and larger tolerance towards different metal ions, detergents and organic solvents. The results of the characterisation study of xylanase produced by *A. brasiliensis* in this study are summarised in Table 2.

Table 2. Summary of characterisation of purified xylanase by *A. brasiliensis*

Characterisation of purified xylanase		Optimum
Temperature of stability and activity		50℃
pH of stability and activity		5
Enhancer	(metal ion)	Cu^{2+}
	(non-ionic detergent)	Tween 80
Inhibitor	(metal ion)	Al^{3+}
	(ionic detergent)	SDS

4. CONCLUSION

In the present study, *A. brasiliensis* ATCC 16404 was selected to produce xylanase using wheat bran as the sole carbon source under SmF. The maximum xylanase activity and protein production were detected to occur at 48 h of fermentation with its activity of 7.72 U/mL and concentration of 0.19 mg attained, respectively. As the fermentation proceeded, an increment of pH of the culture medium was observed. Indeed, the medium pH stayed constant around 6.94 at the end of fermentation. Xylanase was then purified from the culture of *A. brasiliensis* to 3.6-fold after eluted from the anion chromatography of DEAE Sepharose and Sephadex G-75 gel filtration chromatography. At the end of purification, the purified xylanase possessed the specific activity of 116.64 U/mg with its low molecular weight of 36 kDa detected on SDS-PAGE. Subsequently, the purified xylanase by *A. brasiliensis* was characterised to uncover and to identify its potential in industrial applications.

The purified xylanase by *A. brasiliensis* was detected to be thermostable as it displayed relatively high stability at the wider range of temperatures from 40℃ to 60℃. Furthermore, the purified enzyme showed the highest stability at 50℃ because it maintained 98.33% of its relative activity even after incubated for 3 h. In fact, xylanase from *A. brasiliensis* was also found to be most active at 50℃ where its relative activity increased from 95.24% of 19.53 U/mL at 45℃ to the maximum 100% with activity of 20.51 U/mL at 50℃. In terms of pH stability, the purified enzyme remained stable in a wider range of pH from pH 4 to 8. In fact, it remained at the most stable condition at pH 5 with 94.87% of relative activity even after 3 h of incubation. Furthermore, the activity of the purified xylanase remained relatively higher at pH ranging from pH 3 to 9 where it reached its maximum of 22.18 U/mL in sodium acetate buffer at pH 5. Obviously, the purified xylanase by *A. brasiliensis* was successfully exerted its distinctive characteristic as a thermo-alkaline stable enzyme for degradation of xylan. Additionally, this purified xylanase from *A. brasiliensis* also possessed its optimum activity when 1% beechwood xylan was utilised as the optimised substrate in the sodium acetate buffer at pH 5 at 50^{o}C for 30 min of incubation time. On the other hand, all metal ions investigated in this study were found to be marginal effectors for xylanase activity. Cu^{2+}, Mn^{2+} and Zn^{2+} were vaguely increased the xylanase activity while Al^{3+}, Ca^{2+}, K^{+} and Mg^{2+} performed otherwise. In particular, Cu^{2+} was the strongest enhancer for xylanase activity while Al^{3+} was the toughest inhibitor compared to other metal ions. The chelating agent of EDTA was faintly reduced the xylanase activity by 2.7%. The ionic detergent of SDS was a strong inhibitor where it reduced the xylanase activity by 36.1%. In contrast, the addition of non-ionic detergent of Tween 80 increased the xylanase activity marginally by 0.5%. Besides that, organic solvents were used to evaluate their effects on xylanase activity. The purified xylanase showed the highest tolerance towards glycerol, in which 51.2% of xylanase activity was still maintained even though relatively high concentration of 50% (v/v) glycerol was added. On the other hand, with the addition of 50% (v/v) acetone, the purified xylanase maintained 33.9% of its activity. Conversely, the purified xylanase showed the poorest tolerance in activity towards ethanol where only 17.9% of activity was retained after addition of 50% (v/v) ethanol.

In the present study, we concluded that *A. brasiliensis* ATCC 16404 was managed to utilise the agro-industrial waste of wheat bran to produce highly substantial amount of xylanase which capable of its high activity and stability over the broad range of pH at the elevated temperatures in the presence of metal ions, organic solvents and detergents. With the utilisation and recycling of low cost agro-industrial waste of wheat bran as the carbon source for the production of xylanase by *A. brasiliensis* ATCC 16404 couple with its uniqueness of its enzyme characteristics, both would enhance and strengthen the competitive power of xylanase in global enzyme market. As a result, xylanase is anticipated to be a commercially high value bioproduct used in variety of industrial applications especially in the manufacturing of animal feeds and pulp bleaching.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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