

CHARACTERISATION OF A PURIFIED LOW MOLECULAR WEIGHT AND THERMO-ALKALINE TOLERANT XYLANASE PRODUCED BY *Aspergillus brasiliensis* IN SUBMERGED FERMENTATION (SmF)

Hooi Ling Ho, Li Sha Soh and Soon Hwa Ong

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

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 glycosidase that catalyze the conversion of xylan to xylose through endohydrolysis of 1,4- β -D-xylosidic linkage in xylan 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 a 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 in submerged fermentation (SmF). Subsequently, two-step column chromatography was used to purify xylanase followed by step-wise manner of elucidation on the enzyme characterisation of 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 pH 6.42 was obtained at 48 h of SmF. Xylanase extracted from *A. brasiliensis* was then purified with DEAE Sepharose and Sephadex G-75 column chromatography. At the end of purification, the xylanase was purified up to 3.6-fold with its recovery yield of 1.68% and specific activity of 116.64 U/mg. The low molecular weight of 36 kDa of this purified xylanase was also detected from SDS-PAGE. The purified xylanase was then subjected to the step-wise characterisation study. The xylanase was found to be thermo-tolerant from 40°C to 60°C. In fact, the purified xylanase was detected to be most stable at 50°C whereby 98.3 3% of its activity was retained even after 3 h of incubation. Xylanase from *A. brasiliensis* was also found to be most active at 50°C where its relative activity increased from 95.24% at 45°C to the maximum activity of 20.51 U/mL at 50°C. On the other hand, the pH stability of the xylanase was appreciable from pH range of 4 to 8. The enzyme apparently showed the highest stability at pH 5 as 94.87% of activity was retained even after 3 h of incubation. Furthermore, the activity of the purified enzyme remained relatively high at pH buffer ranging from pH 3 to 9. Indeed, xylanase activity reached its maximum activity of 22.18 U/mL at pH 5. Additionally, xylanase activity was detected to be the highest, producing 24.46 U/mL when 1% xylan was used as the optimised substrate for the incubation period of 30 minutes. 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. Nonetheless, chelating agent of EDTA inhibited the xylanase marginally. The non-ionic detergent of Tween 80 acted as an enhancer whilst the ionic detergent of SDS was an inhibitor.

On the other hand, xylanase showed a better tolerance to glycerol with 51.2% of xylanase activity 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* revealed its enormous potential as thermo-alkaline tolerant enzyme in xylan degradation process that is applicable in the manufacturing of animal feeds, fruit juice and paper pulping.

Keywords: Xylanase, *Aspergillus brasiliensis*, submerged fermentation (SmF), purification, characterisation

1. INTRODUCTION

Agriculture sector has made significant contributions towards the economical growth of Malaysia. Wastes or by-products from agriculture industries would cast serious effect on environment if they are not managed in proper manner. Utilisation of the agro-industrial waste 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 at much lowest cost [1]. As a result, these wastes are said to be high-valued products and their recovery or re-utilisation would be economically attractive. In this study, wheat bran was therefore, used as a carbon source for fungi, *Aspergillus brasiliensis* in the production of the xylanase enzyme. Wheat bran consists about 650 mg/g of cell wall polysaccharides which are mainly of heteroxylans, cellulose, and lignin [2]. It is a cheap agro-industrial residue which is suitable to be used as carbon and nitrogen sources in the area of fermentation for enzyme production [3]. There are studies where wheat bran as the indigenous carbon source was utilised for xylanase production [4, 5, 6, 7, 8]. Xylanase has been used extensively for various industrial processes. According to Yang et al. [9], recent market trends revealed that xylanase take a major chunk of share up to 20% of the world enzyme market along with cellulases and pectinases. Apparently, it has been widely used in many industries including textile, pulp and paper, food processing, detergents and animal feeds [10]. The technology of utilising xylanase has reached the stage where this enzyme has become indispensable. Nonetheless, high production cost and low yields of the xylanase hinder its further production, as a result, they remain the paramount problems for industrial applications especially in huge industry scale. In order for commercial applications, xylanase should be produced rapidly in large quantity using simple and inexpensive substrates. Therefore, investigations on xylanase-producing ability of fungal strains by utilising inexpensive substrate have been extensively carried out. In fact, much effort has been done on the optimisation of fermentation processes to further reduce xylanase production cost. Besides that, the characterisation of xylanase is equally crucial where the understanding of the optimum parameters for the xylanase is essential for its applications in different industries. As a result, step-wise manner was used for the elucidation of the xylanase characterisation. Hence, the objectives of this study were to produce extracellular xylanase from *A. brasiliensis* under the optimum conditions using wheat bran as the carbon source and to purify and characterise the purified xylanase using different parameters including temperature and pH stability, effects of temperature, pH, substrate specificity, substrate concentration, incubation time, metal ion, chelating agent, detergent and organic solvent on the

xylanase enzyme activity. In conclusion, this study summarised the economical utilisation of wheat bran in the production of xylanase by *A. brasiliensis* under submerged fermentation (SmF) followed by purification and enzyme characterisation in step-wise manner.

2. MATERIAL AND METHODS

2.1 *Aspergillus brasiliensis* ATCC 16404

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

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 before the pre-treatment started. 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 because 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°C 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, Culture Medium Formulation and Growth Conditions

Spore suspension was prepared from *A. brasiliensis* on the PDA. Approximately 10 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, the volume of inoculum used to inoculate into the culture medium was calculated. Standard inoculum of 3×10^6 spores was used in this study. 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; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 and KCl, 0.52 according to Ho and Heng, [11] where wheat bran and yeast extract were applied as the carbon and nitrogen source. The medium was adjusted to pH 6.5 before autoclaved at 121°C for 15 min. The inoculum with the standard size of 3×10^6 spores was added into 150 mL working volume in 250 mL Erlenmeyer flask. Subsequently, the culture was incubated in orbital rotary shaker at 30°C and agitated at 150 rpm. Sample was harvested at 24 h interval and analysis was conducted accordingly.

2.4 Sampling and Analysis

10mL of sample was harvested at every 24 h interval from the flasks into 15mL Falcon tubes. The samples were collected for analysis including protein assay, xylanase activity, medium pH and spore

concentration. The samples were centrifuged at 8500 rpm for 15 min prior to the analysis. The cell pellet was discarded and supernatant was used for further analysis.

2.5 Protein Assay

The total amount 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.

2.6 Xylanase Activity Assay

Xylanase activity assay was performed using 3, 5-dinitrosalicylic (DNS) method by measuring the reducing sugar of xylose according to Bailey et al. [13]. 1% of beechwood xylan was used as the xylanase substrate in the activity assay. 0.1 mL of supernatant was added into 0.9 mL of 1% xylan and incubated in water bath at 50°C for 30 min. Subsequently, 1.5 mL of DNS reagent was added into the mixture and incubated at 90°C for 5 min. Then, 0.5 mL of 40% potassium sodium tartrate (Rochelle salt) was added and cooled to room temperature before measuring the absorbance at 575 nm using spectrophotometer. The amount of xylose obtained 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 min under assay condition.

2.7 Measurement of Medium pH

The pH of samples was measured using a pH meter. Prior to the pH measurement, the pH meter was calibrated with buffer pH 4, 7 and 10.

2.8 Quantification of Spores of *A. brasiliensis*

Spores count of *A. brasiliensis* was carried out using haemocytometer.

2.9 Purification of Xylanase

The purification of xylanase by *A. brasiliensis* has been optimised by Ho and Ilyia [14]. All purification steps were carried out at 4°C unless otherwise specified. The crude supernatant containing xylanase was obtained after centrifugation at 8500 rpm for 15 min at 4°C. Then, ammonium sulphate was added slowly into the supernatant until it reached 70% salt saturation. It was swirled overnight for protein precipitation to take place at 4°C. The protein precipitate was then collected after centrifugation at 8500 rpm for 15 min at 4°C. The precipitate was suspended into 0.05 M sodium phosphate buffer, pH 5.3 before transferred into a dialysis tube. The tube containing protein precipitate was then suspended in the same buffer. Dialysis process was carried out overnight at 4°C. After that, the enzyme sample was ready for a two-step chromatographic procedure. To begin, resins of DEAE Sepharose and Sephadex G-75 were swelled before packed and mounted upright into two separate columns using retort stands. The enzyme sample was loaded into anion exchange column packed with DEAE Sepharose that was equilibrated with the same buffer. Then, the enzyme sample was eluted with increasing concentration of NaCl from 0 to 1.0 M. The eluates were collected in

fractions of 0.5 mL. The fractions were assayed to determine the xylanase activity and protein concentration. Then, the fractions with relatively high xylanase activity were pooled and loaded into a gel filtration column packed with Sephadex G-75. Subsequently, the enzyme sample was eluted with cold 0.05 M sodium phosphate buffer (pH 5.3). Eluates were collected in fractions. The fraction with the highest xylanase activity was loaded into SDS-PAGE to visualise the molecular weight of the purified xylanase. The specific activity of xylanase was calculated by division of total 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.

2.10 Xylanase Activity and Protein Concentration Assays for Purified Xylanase

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

2.11 Characterisation of Purified Xylanase

The biochemical characterisation of the purified xylanase was performed to compare its characterisation with that of the other reported xylanases. Biochemical characterisation study including temperature and pH stability of xylanase, effects of temperature, pH, substrate specificity, substrate concentration, incubation period, metal ion, organic solvent and detergent on xylanase activity was also 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 of parameters in order to ensure the results obtained through the characterisation study was reliable.

2.11.1 Temperature stability of xylanase

The long-term stability of xylanase at different temperatures was elucidated. The purified sample was incubated without substrate up to 3 h at 40°C, 50°C, 60°C, 70°C and 80°C, respectively. Sample was withdrawn at every hour interval and the xylanase stability was assayed under standard assay condition.

2.11.2 pH stability of xylanase

The long-term stability for xylanase at different pH was also analysed in this study. The purified sample was incubated without substrate up to 3 h in different 0.05 M buffer consisted of 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 the xylanase stability was assayed under standard condition.

2.11.3 Effect of temperature on xylanase activity

The determination of the optimum temperature for xylanase is important because temperature may cause the conformation changes which leads to the decrease in enzyme activity and to prevent the

loss of profit in industry. To elucidate the effect of incubation temperature on xylanase activity, 0.9 mL of 1% xylan was added to 0.1 mL of purified sample and incubated at different temperatures ranging from 45°C, 50°C, 55°C, 60°C, 65°C and 70°C. The xylanase activity of purified samples was determined under standard condition as mentioned earlier.

2.11.4 Effect of pH on xylanase activity

In order to determine the effect of pH on xylanase activity via a step-wise manner, using the optimised temperature obtained from the previous characterisation study, 0.9 mL of 1% 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. The xylanase activity was then being determined under the standard assay condition.

2.11.5 Effect of substrate specificity on xylanase activity

After the optimum temperature and pH were obtained, xylanase activity was accessed for the specificity towards its substrates. The substrates used in this study were consisted of 1%: xylan, psyllium husk, rice bran, sawdust, wood chip, wheat bran, dehulled soya and palm kernel, respectively. All of the mentioned substrate was purchased from Country Farm Organics, Malaysia except for xylan which was obtained from Sigma-Aldrich. The xylanase activity was tested using the DNS method with different substrates under standard assay condition, respectively.

2.11.6 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 xylanase worked the best. The analysis was carried out by substituting the optimised 1% substrate with different concentrations consisted of 0%, 0.25%, 0.50%, 0.75%, 1.00%, 1.25% and 1.50%, respectively. The xylanase activity was then quantified under the standard assay condition.

2.11.7 Effect of incubation period on xylanase activity

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

2.11.8 Effect of metal ions on xylanase activity

To determine the effect of metal ions on xylanase activity, the purified sample was incubated in the presence of 5 mM of various metal ions including Mg^{2+} , Ca^{2+} , Mn^{2+} , Al^{3+} , K^+ , Cu^{2+} and Zn^{2+} at 50°C for 30 min, respectively. The xylanase activity was quantified under the standard assay condition.

2.11.9 Effect of chelating agent and detergents on xylanase activity

To elucidate the effect of chelating agent and detergents on xylanase activity, the purified sample was incubated in the presence of 5 mM of chelating agent of EDTA and 5% (w/v) of detergents including SDS and Tween 80 at 50°C for 30 min, respectively. The xylanase activity was then quantified under standard assay condition.

2.11.10 Effect of organic solvents on xylanase activity

To investigate the effect of organic solvents on xylanase activity, the purified sample was incubated in the presence of 50% (v/v) of various organic solvents consisted of glycerol, ethanol and acetone at 50°C for 30 min, respectively. The xylanase activity was then quantified under 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, 10 days of SmF was carried out in this study. Sample was withdrawn at every 24 h interval and analysis such as xylanase activity, protein assay, spore count and pH measurement of fermentation medium were elucidated. The correlation between xylanase production and spore count is illustrated in Figure 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. Meanwhile, 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, large amount of xylanase was required to hydrolyse wheat bran which was the sole carbon source found in the medium that provided energy for the fungal growth. As a result, xylanase was synthesised at the higher rate during the first 48 h of SmF. Indeed, a large amount of xylan from wheat bran in the medium was hydrolysed by xylanase to yield sufficient amount of simplified nutrient to support the fungal growth. Subsequently, *A. brasiliensis* began to reproduce rapidly using the reducing sugar of xylose available in the medium before 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 the rate of death during the stationary phase of fungal growth. The depletion of the essential nutrients of xylan and the build-up of 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 fact, the choice of using wheat bran for the xylanase production is a brilliant decision as it is cheap and it contributes higher xylanase production.

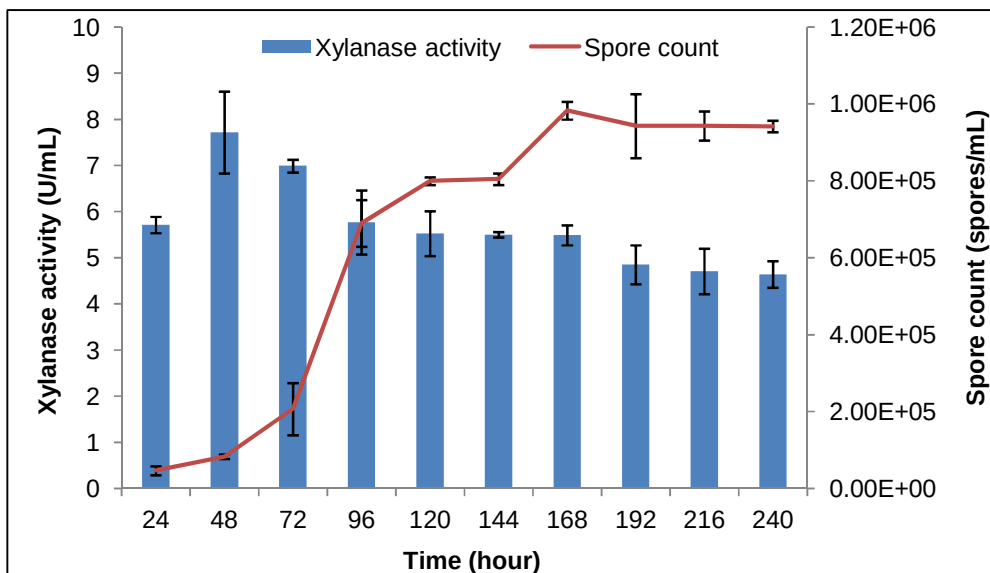


Fig.1. Xylanase production by *A. brasiliensis* in SmF. The correlation of xylanase activity with the number of spores over fermentation time using wheat bran as the sole carbon source.

Figure 2 illustrates the correlation between the protein concentration and xylanase activity. The trends of protein concentration and xylanase activity possessed high resemblance. As the fermentation time increased, the 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.

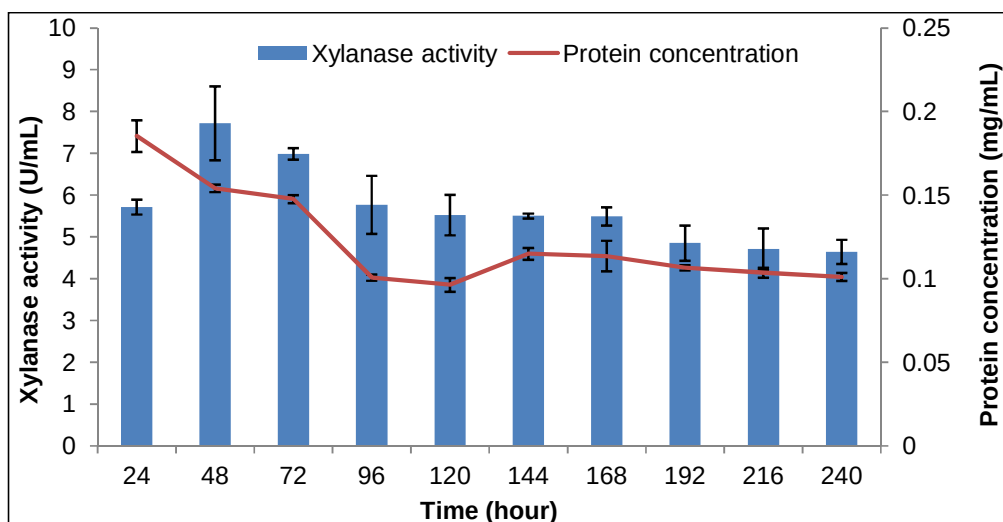


Fig. 2. Xylanase production by *A. brasiliensis* in SmF. The correlation of xylanase activity with protein concentration over fermentation time using wheat bran as the sole carbon source.

Based on Figure 3, the pH of medium increased as fermentation time prolonged. The initial pH of medium was adjusted to pH 6.5 prior to fermentation. However, 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 short, 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 xylanase production 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 range from pH 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 with the initial medium pH of 7.0 [19].

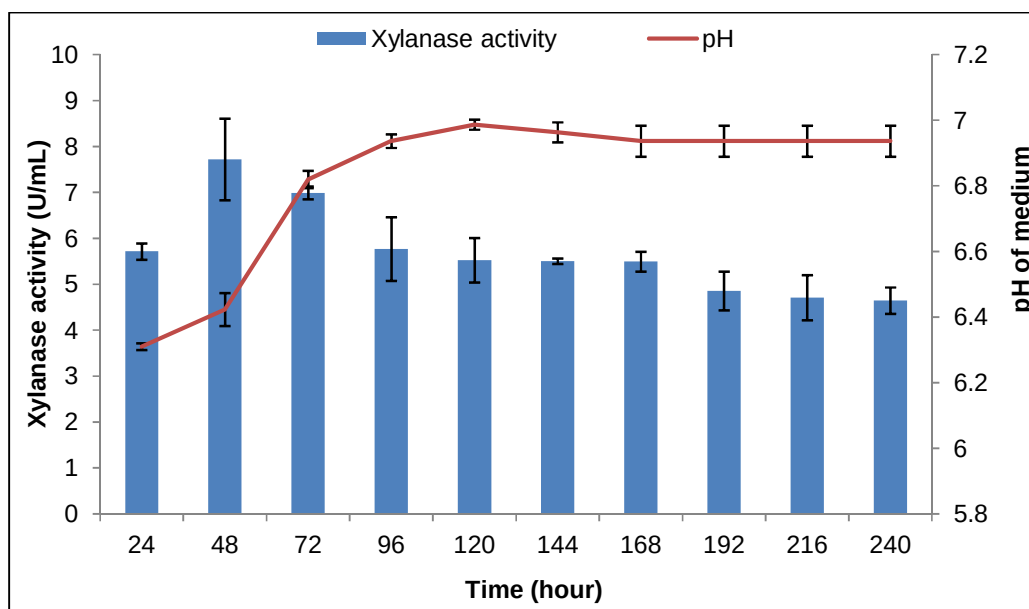


Fig. 3. Xylanase production by *A. brasiliensis* in SmF. The correlation of xylanase activity with the pH of medium over fermentation time using wheat bran as the sole carbon source.

3.2 Purification of Xylanase

A. brasiliensis was cultivated under the optimum condition to produce the maximum production of xylanase. The purification of xylanase in this study was carried out according to Ho and Ilyia [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% 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 this optimised salt saturation percentage. According to the result of Milala et al. [21], 70% saturation of ammonium sulphate was found to be the optimum concentration for most of the xylanase precipitation of *Aspergillus* spp, achieving the specific xylanase activity of 16.06 U/mg. A study was conducted by Pal and Khanum [22] using 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% ammonium sulphate saturation which was the first step of protein isolation and extraction was added to 300 ml of crude supernatant at 4°C [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°C, the

supernatant was discarded while the pellet was dissolved in fresh 0.05 M sodium phosphate buffer, pH 5.3 before preceded 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 precipitate obtained from the previous 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 resuspended in the same buffer. The dialysis process was carried out overnight at 4°C. At the end of this step, the specific activity was assayed to be 47.53 U/mg. 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 purified by a two-step chromatographic procedure. DEAE Sepharose column chromatography was used as the first step of chromatographic procedure. It is an anion exchange chromatography which separates samples based on the difference in charges. The elution of sample was carried out using NaCl solution of different concentrations ranging from 0 to 1.0 M. The eluates were collected in small fractions and assayed for xylanase activity and protein concentration. The result was plotted into a chromatogram as shown in Figure 4. Since fraction 1 to 46 has xylanase activity of less than 0.01 U/mL which were insignificant, thus, Figure 4 shows the chromatogram of DEAE Sepharose starting from fraction 47 to 80. There is single peak of chromatogram that showed the highest xylanase activity together with its maximum protein concentration. 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 together with specific xylanase activity of 46.37 U/mg. About 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. Purification fold increased by around 1.44-fold from 1.43 to 2.87-fold.

According to Okafor et al. [23], xylanase produced from *A. niger* ANL 301 was purified using DEAE Sephadex A-50 and specific activity of 90.82 U/mg was obtained. The specific activity obtained in this study was 92.79 U/mg which was relatively higher than the result of Okafor et al. [23]. Based on the results, different strains of *Aspergillus* produced different amount of xylanase activity and the variation in medium formulation used for growth of the microorganism strains was also affected the activity of xylanase. In addition, resin used for the chromatography also affects the result of purification. This was due to that different resins elute different ranges of protein which were based on their ionic charge as well as pI value [24]. Another study conducted by Yang et al. [25], who used *A. niger* C 3486 for the production of xylanase at 72 h of fermentation. A specific activity of 123.68 U/mg and 14.79 of purification fold were achieved. About 40.25% of xylanase was recovered from its crude

supernatant. Additionally, Lu et al. [26] showed the specific xylanase activity of 288.7 U/ mg and approximately 82.3% was recovered from its crude filtrate.

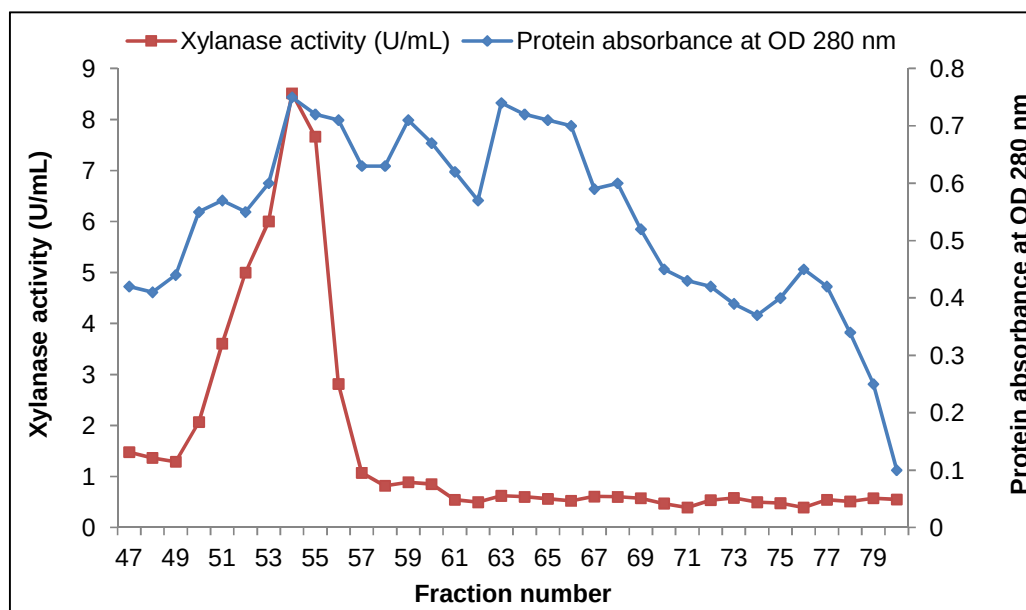


Fig. 4. Chromatogram of xylanase from *A. brasiliensis* using DEAE Sepharose chromatography.

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 into a chromatogram as shown in Figure 5. According to Figure 5, both xylanase activity and protein concentration showed single peak of the chromatogram. Fractions with the number from 10 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 1.68% of recovery at the end of the purification process. According to the result obtained in this study, a specific activity of 0.73-fold increase was observed compared to specific activity of 92.79 U/mg after DEAE Sepharose chromatography. At the end of the purification, a specific enzyme activity of 116.64 U/mg with the total protein of 0.894 mg was obtained.

On the other hand, xylanase produced by *A. terreus* achieved a specific activity of 6.92 U/mg. This study was performed by Sorgatto et al. [15] using a CM-cellulose column for purification of xylanase. The specific activity of the mentioned study was significantly lower than specific activity of xylanase from this study. However, the purification fold was increased majorly from 1 to 45-fold at the end of purification [15]. In contrast, the study from Yang et al. [25] achieved the specific activity of 123.68

U/mg from xylanase produced by *A. niger* which was slightly higher than specific activity of this study. Hence, it is suggested that besides column chromatography, the medium and cultivation conditions might also play an important role in purification of enzymes. In conclusion, xylanase produced in this study had achieved a specific activity of 116.64 U/mg at the end of the purification. The purification fold had increased from 1 to 3.6-fold. Subsequently, low molecular weight of 36 kDa of purified xylanase was detected from SDS-PAGE as shown in Figure 6 [14]. Then, the purified xylanase was subjected to various characterisation studies conducted in this study. Results from all of the purification steps in this study are summarised in Table 1.

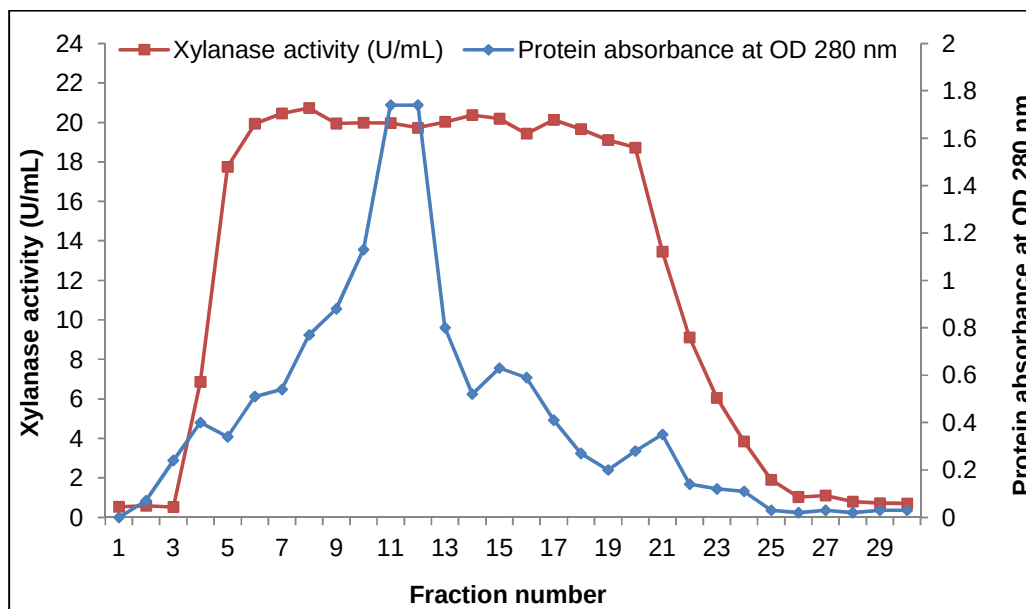


Fig. 5. Chromatogram of xylanase from *A. brasiliensis* using Sephadex G-75 chromatography.

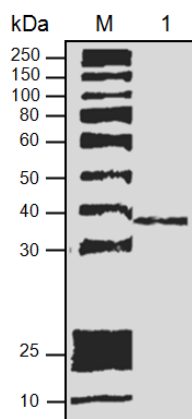


Fig. 6. Pictograph of SDS-PAGE of purified xylanase by *A. brasiliensis*. Lane M: protein marker. Lane 1: Purified xylanase with an estimated molecular weight of 36 kDa.

Table 1. Purification steps of xylanase by *A. brasiliensis*.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification Yield (%)	Purification fold
Crude extract	192.00	6215.4	32.37	100	1
Ammonium sulphate	125.40	9696.5	46.37	93.56	1.43
Dialysis	8.06	383.08	47.53	6.16	1.47
DEAE Sepharose	1.971	182.88	92.79	2.94	2.87
Sephadex G-75	0.894	104.28	116.64	1.68	3.60

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 industrial processes. Thus, temperature stability of an enzyme is one of the most important factors in studying its characterisation. The result on temperature stability of xylanase by *A. brasiliensis* is displayed in Figure 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°C to 60 °C with its maximum temperature stability maintained at 50°C. At 40°C, xylanase retained almost 95.62% of its stability after 3 h of incubation. In fact, xylanase showed the maximum stability at 50°C where it maintained the highest, 98.33% of its stability even after incubated for 3 h. At 60°C, the xylanase stability was gradually declined as the incubation time increased. In fact, at 60°C, the xylanase stability was found to be 66.03% after 1 h, 40.21% after 2 h and 34.4% retained after 3 h of incubation. At much higher temperatures of 70°C and 80°C, the xylanase stability was declined drastically with only 1% to 2% of activity after 3 h of incubation.

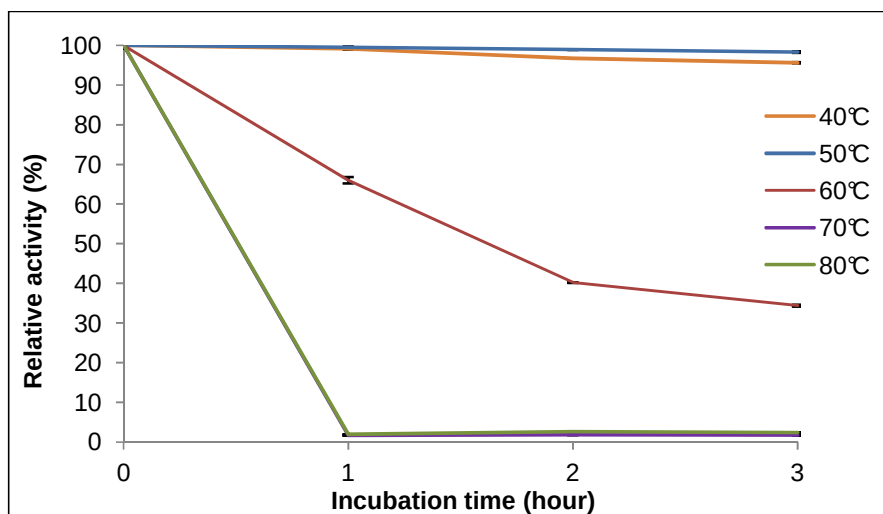


Fig. 7. Temperature stability of the purified xylanase by *A. brasiliensis*.

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°C with half-life of 36 min followed by 45°C. In a study by Fialho and Carmona [27], two types of xylanases produced by *A. giganteus* exhibited the highest thermo-stability at 50°C with half-life of 22.5 min and 17.5 min, respectively. While xylanases by *A. caespitosus* were at the most stable activity at 55°C with its half-life of 27.3 min and 90 min, respectively [28]. Besides that, the xylanase by *A. niger* C3486 showed thermo-stability at 55°C to 60°C and retained more than 80% of its activity after 90 min of incubation [25]. Generally, xylanase from most of *Aspergillus* spp. are stable at the temperature range of 40°C to 60°C. Nonetheless, some *Aspergillus* spp. such as *A. niger* produced more thermo-tolerant xylanase which remained stable up to 100°C with approximately lost of 50% to 52% of its activity [20, 29]. Based on the result, xylanase produced in this study is suggested to be a good candidate for 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 obtained is displayed in Figure 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 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. Xylanase was also active at slight alkaline pH in lesser extent. It retained 57.22% and 33.59% of xylanase stability at pH 7 and 8 after incubated for 3 h, respectively. In contrary, xylanase was less stable at pH 3 and 9. At pH 3, around 4% of 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 stability was retained after 1 h of incubation and declined drastically to 5.49% and 1.4% after 2 and 3 h of incubation, respectively. The considerable stability of xylanase by *A. brasiliensis* occurred at wider range of pH from pH 4 to 8 which entitled it to be potentially effective for utilisation in various industries including detergent, paper pulping and food industries. The result in this study was also in accordance with the results isolated from other studies. In general, xylanase 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 high stability from pH 4 to 8, whereby around 80% of the xylanase stability was retained after 1 h of incubation. Xylanase produced by *A. niger* maintained 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 had a broader pH stability from pH 5 to 10.

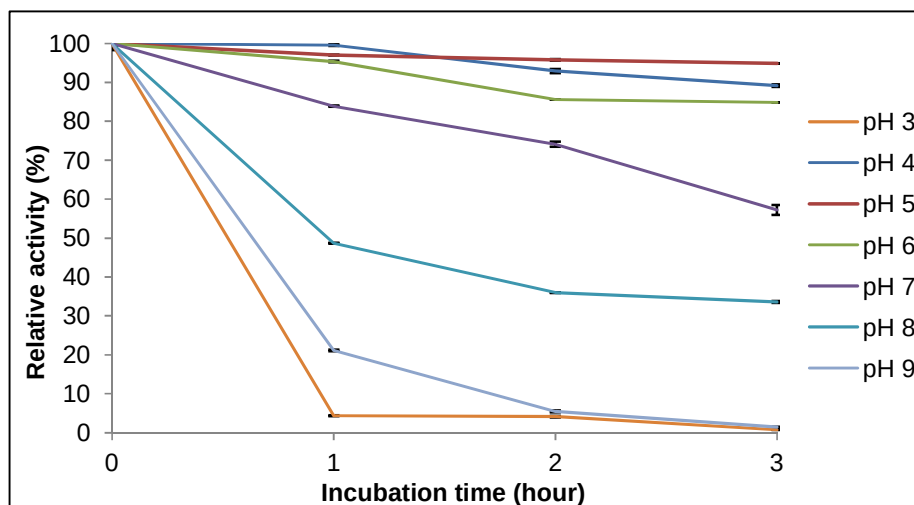


Fig. 8.pH stability of the purified xylanase by *A. brasiliensis*.

3.2.5.3 Effect of temperature on xylanase activity

Due to the wide variety of xylanase usage, a lot of studies have been carried out on the elucidation of the xylanase characterisation. According to Sharma and Chand [34], the optimum temperature for xylanase produced from *Pseudomonas* sp. XPB-6 was occurred at 60°C with its specific activity of around 80 U/mg. The result of Abirami et al. [35] showed that the optimum temperature for xylanases produced from *Penicillium janthinellum* and *Neurospora crassa* were detected to be at 50°C and 60°C with their enzyme activity of around 50 U/mL and 40 U/mL, respectively. This proved that xylanase produced from different strains may have different optimum parameters. Hence, it is important for experimental research to investigate and elucidate the characterisation of xylanase, including the effect of temperature on the activity of xylanase produced by *A. brasiliensis*. Indeed, xylanases which perform efficiently at relatively higher temperatures would be favourable in the paper pulping (from 55°C to 70°C) and animal feeds industries (from 70° C to 95°C) [36]. The result of the effect of temperature on xylanase activity is plotted in a graph shown in Figure 9. The relative xylanase activity was expressed as a percentage of the optimal activity at 50°C where 100% xylanase activity was quantified to be 20.51 U/mL. Based Figure 9, xylanase from *A. brasiliensis* was found to be active at the temperature ranging from 45°C to 70°C. The xylanase relative activity increased from 95.24% with its activity of 19.53 U/mL at 45°C to the maximum 100% with its activity of 20.51 U/mL at 50°C. Thereafter, the xylanase activity decreased gradually as the temperature increased to 17.9 U/mL with 87.27% of relative activity at 70°C.

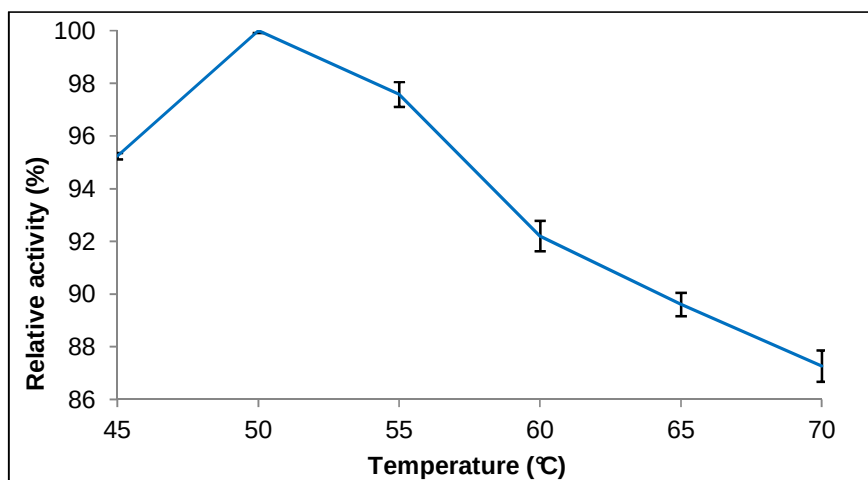


Fig. 9. Optimum temperature of the purified xylanase by *A. brasiliensis*.

In general, the optimum temperature for xylanase produced by *Aspergillus* spp. is usually ranging from 45°C to 60°C. Xylanase by *A. awamori* VTCC-F312 was active at a much larger temperature ranging from 40°C to 80°C, while its optimum temperature was 50°C to 55°C 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°C to 60°C 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°C [15, 38]. Similarly, Fialho and Carmano [26] reported that xylanase produced by *A. giganteus* possessed the optimum temperature of 50°C which matched with the result of this present study. On the other hand, xylanase produced by *A. fumigatus* MKU1 showed relatively high xylanase activity over 70°C to 90°C, while 70°C was its optimum temperature detected [39].

3.2.5.4 Effect of pH on xylanase activity

pH is capable of greatly affecting the activity of an enzyme because the binding of substrate with its enzyme is usually relied on the charge distribution of both substrate and enzyme molecules. Besides that, pH is also one of the important criteria of an enzyme 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 result obtained is shown in Figure 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 Figure 10, the 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% 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 activity retained at pH 10.

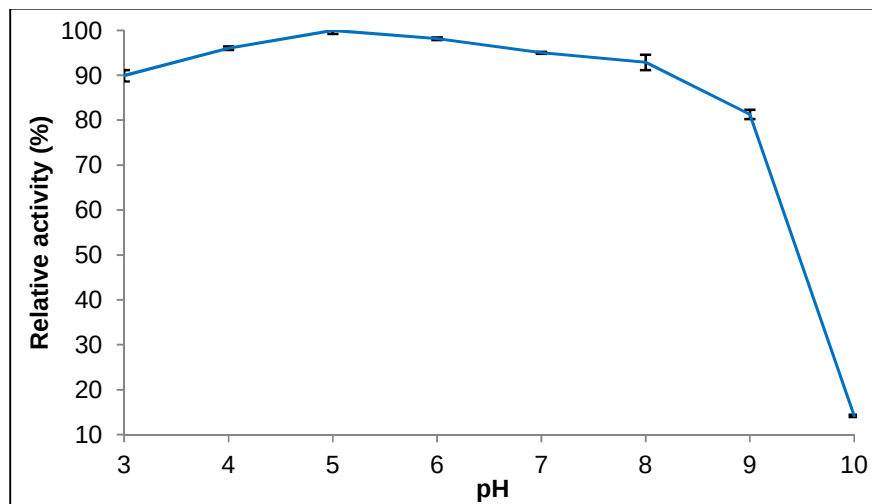


Fig. 10. Optimum pH of the purified xylanase by *A. brasiliensis*.

These results were also agreed by other studies. Do et al. [32] reported that xylanase by *A. awamori* VTCC-F312 was active from pH 3 to 8 and achieved its maximum activity 556.2 U/mg at pH 5. While 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 is other fungal strains possessed 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]. Apparently, xylanases which work optimally at acidic condition are potentially applicable in feeds and food industries while xylanases which active at alkaline condition are suitable for paper pulping and bleaching industries [35]. As a result, xylanase by *A. brasiliensis* which was active from pH 3 to 9 in the present study possessed greater suitability and larger potential in industrial applications especially in animal feeds production.

3.2.5.5 Effect of substrate specificity on xylanase activity

Among all the substrates used for the present study, the action of purified xylanase with xylan exhibited the highest xylanase activity by giving out the xylanase 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 xylanase activity of 6.01 U/mL. Subsequently, sawdust and wheat bran gave the same value of 4.5 U/mL while wood chip which showed the activity of 3.5 U/mL followed by psyllium husk with 3.4 U/mL while soya produced the lowest activity reading of 2.23 U/mL.

According to Do et al. [32], the 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. Moreover, the results from Mander et al. [43] also showed that xylan was the substrate specifically suitable to xylanase by giving out relatively high xylanase activity. Based on their results, xylanase activity of 39,996 U/mg and 37,831 U/mg were obtained after using beechwood and birchwood xylan as the substrate, respectively. On the other hand, substrate specificity of purified xylanase from *A. niger* was also investigated with substrates such as carboxymethyl cellulose, oat spelt, birchwood and beechwood xylan by Dobrev and Zhekova [44]. 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 was detected on carboxymethyl cellulose. Similar result also reported by Kamble and Jadhav [42] where the purified xylanase from a new species of *Bacillus* was specific to birchwood xylan while showing no activity towards carboxymethyl cellulose. In fact, the xylanase activity towards birchwood xylan was detected as 291.9 U/mg. Additionally, according to Krisana et al. [33], the result showed that the purified xylanase from *A. cf. niger* was specific to xylan, producing 5870 U/mg while showing no activity to carboxymethyl cellulose. The conclusion drawn from the results of the present study proved that xylan was a specific substrate for xylanase as the biochemical reaction of purified xylanase towards xylan produced the highest xylanase activity as shown in Figure 11.

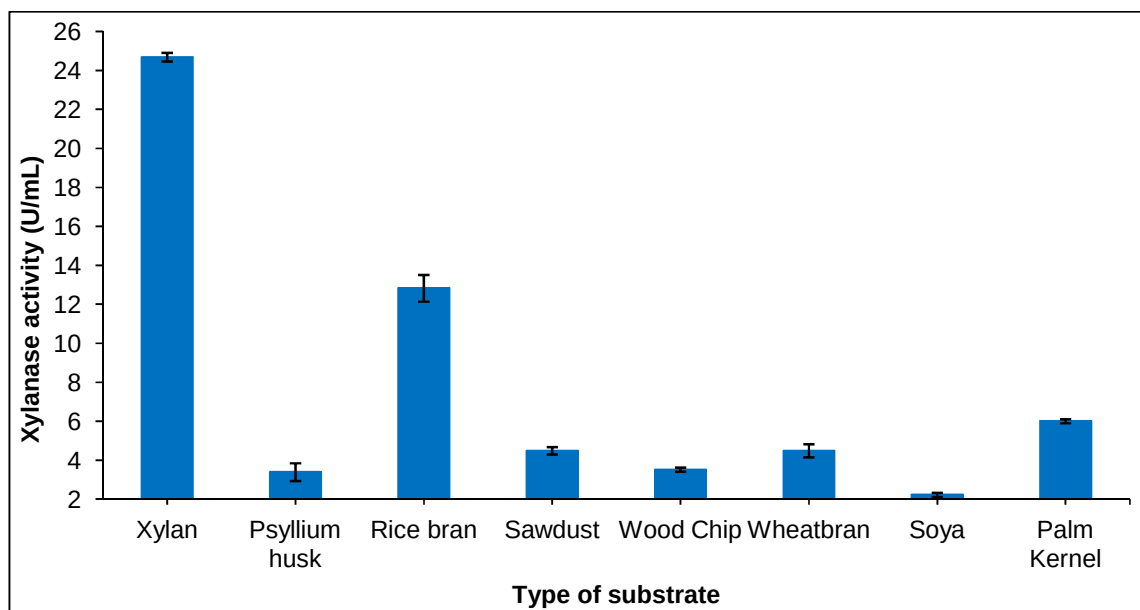


Fig. 11. Effect of substrate specificity on xylanase activity.

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 giving excess amount or concentration of substrate that would hinder the production rate that eventually leads 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. The increase in xylanase activity happened from 0.25% to 1.0% xylan concentration. After that, the xylanase activity remained almost constant until 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% 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 gave the enzyme activity of about 500 U/mL. Similar trend of xylanase activity was observed whereby the xylanase activity increased as the xylan concentration increased. 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 enzyme produced by *A. brasiliensis* in this study as shown in Figure 12.

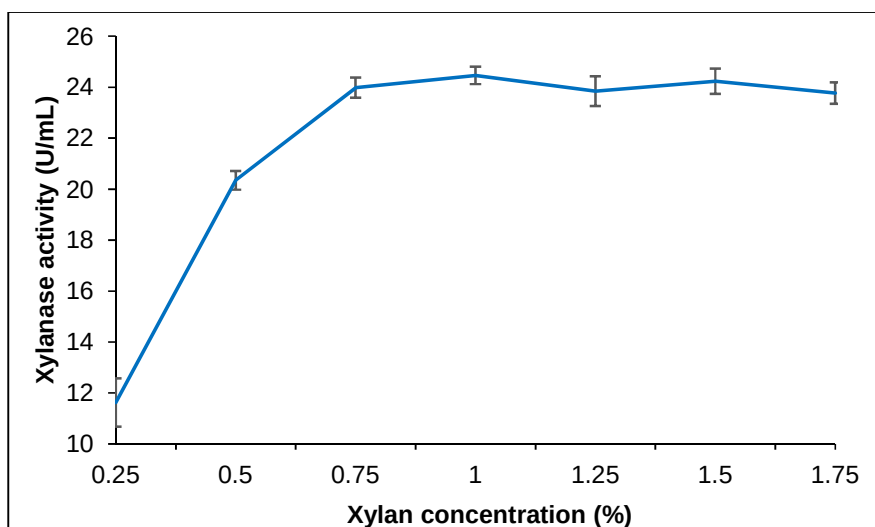


Fig. 12. Effect of xylan concentration on xylanase activity.

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 leads to possible lower production rate and increase loss of profits. From 10 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 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 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 from 40 min to 80 min as the purified xylanase activity remained 100%, respectively. According to Isil and Nilufer [45], the optimum incubation time for the purified xylanase produced from *T. harzianum* was 50 min with its xylanase activity of about 520 U/mL. Nonetheless, the result from Sharma and Chand [34] showed that 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. The results from different journals mentioned above proved that the purified xylanase produced by different strains possessed 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* that are drawn based on the result in the present study shown in Figure 13.

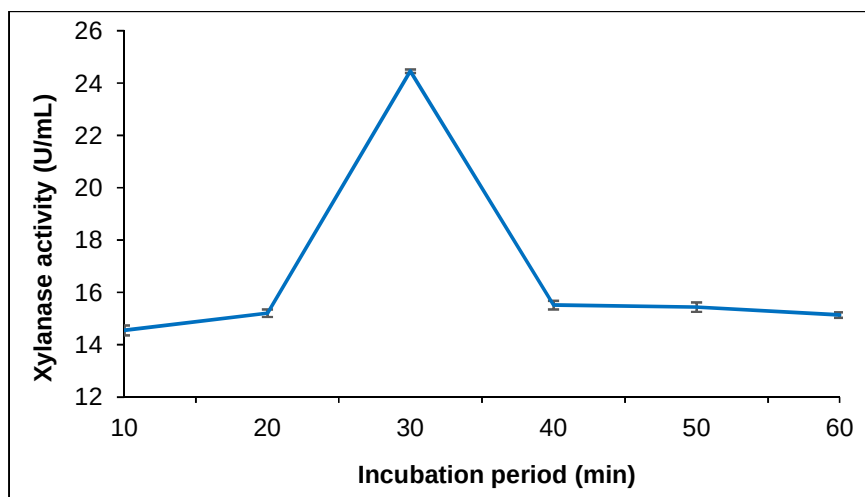


Fig. 13. Effect of incubation time on xylanase activity.

3.2.5.8 Effect of metal ions 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 protein. Thus, it indirectly changes the properties of solvent water, mainly the water activity [40]. Nevertheless, metal ions which exist in raw pulp as impurities might inhibit the activity of xylanase. As a result, the influence of various metal ions on xylanase activity was investigated to elucidate its suitability for paper pulping application specifically and also to reveal which of the ions should be precluded or included in this industrial process.

The xylanase activity was assayed in the presence of several metal solutions in 5 mM buffer is showed in Figure 14. Based on the results, 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 result, all metal ions were proven to act as marginal effectors on xylanase activity. Apparently, Cu^{2+} , Mn^{2+} and Zn^{2+} slightly increased the xylanase relative activity by 1.3%, 0.9% and 0.4% in 5 mM buffer 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 among the metal ions on xylanase activity in this study.

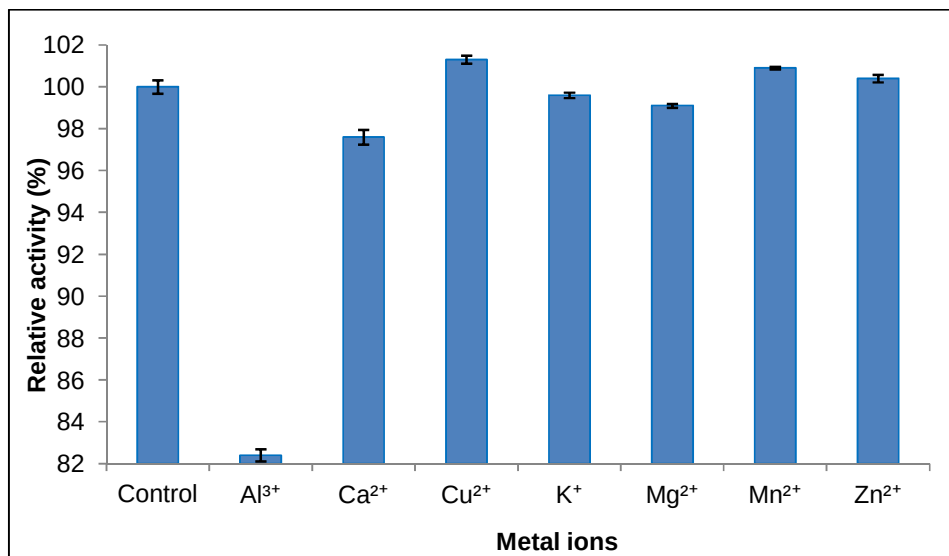


Fig. 14. Effect of metal ions on xylanase activity produced by *A. brasiliensis*.

Similar studies were also conducted in determining the effect of metal ions on xylanase activity. Sorgatto et al. [15] 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+} , 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 from *A. ficuum* AF-98 was activated by Cu^{2+} up to 16% of activity, however, xylanase from *A. niger* C3486 was inhibited by the same ion [25, 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 study, 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, 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 from *A. brasiliensis* (this study) and *A. ficuum* AF-98 but acted as an inhibitor for xylanase 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.

3.2.5.9 Effect of chelating agent and detergents on xylanase activity

Some additives such as detergents and chelating agents are potentially inhibited enzyme activity during production and industrial applications. Thus, their effects on xylanase activity were investigated in the present study and the results obtained are showed in Figure 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 is responsible to remove metal ions from the enzymes [49]. In this study, EDTA did not drastically affect the xylanase activity as it reduced its activity by 2.7%. This suggested that metal ions were not required as cofactors 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 from other *Aspergillus* spp. including *A. giganteus*, *A. niger* BCC14405 and *A. terreus* experienced 22%, 40% and 60% 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 were also investigated on the xylanase activity in this study. Based on the result, SDS was a strong inhibitor where it reduced the xylanase activity by 36.1% in comparison to the control reaction without the additive. 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 this study was still able to retain its activity at 63.9%.

In general, SDS 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 result of the interaction occurred between SDS and the hydrophobic group of amino acids [50]. On the other hand, Tween 80 did not change the xylanase activity drastically in this study but increase its activity of 0.5% compared to the control.

Some researchers also reported similar experimental findings. 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. Likewise, 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 effects on xylanase produced by *Termitomyces* sp., termite *Macrotermes subhyalinus* worker and *A. oryzae* DSM1863 by 3%, 4% and 14% reduction in xylanase activity, respectively [51, 52, 53]. Interestingly, Bandivadekar and Deshpande [54] reported that Tween 80 enhanced the thermal stability of xylanase produced by *Chainia* sp. at 60°C.

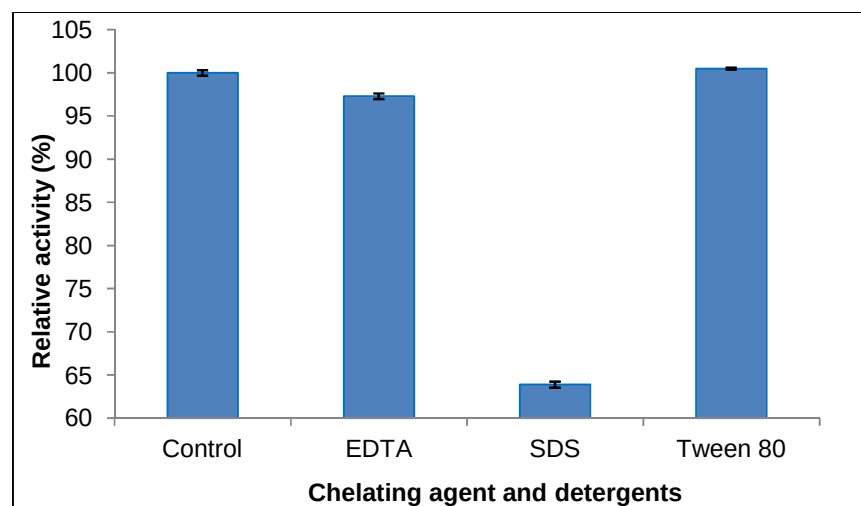


Fig. 15. Effect of chelating agent and detergents on xylanase activity produced by *A. brasiliensis*.

3.2.5.10 Effect of organic solvents 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 Figure 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 application of purified xylanase reaction in an organic phase. Based on the result, xylanase revealed the highest tolerance to glycerol, in which 51.2% of xylanase activity was maintained although high concentration of 50% (v/v) glycerol was added. Similar results were 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 [25]. Interestingly, it was found that glycerol possessed a positive effect against thermal denaturation. Likewise, in a study by Lemos et al. [56], the stability of *A. awamori* xylanase 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. Ethanol also showed inhibitory effects on xylanase activity by *A. niger* C3486 which was around 90% of its activity retained at the concentration of 2% (v/v) while 40% of the activity retained at 30% (v/v), respectively [25]. In contrast, the activation of xylanase activity by ethanol was 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, xylanase from *A. oryzae* DSM1863 and *Providencia* sp. X1 showed 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 from *A. niger* DSM1957 with the enhancement of xylanase activity by 1.5 to 2 times after incubation for 1 to 2 h [59]. Interestingly, xylanase activity from *Bacillus altitudinis* DHN8 was also stimulated in the presence of acetone by 2.96% increment of its activity [57].

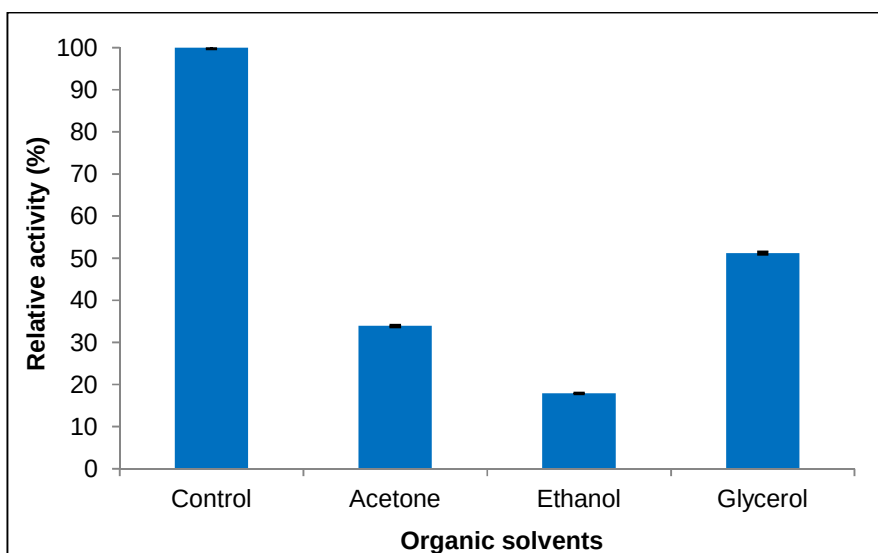


Fig. 16. Effect of various organic solvents on xylanase activity produced by *A. brasiliensis*.

3.2.5.11 Summary

Different strains of *Aspergillus* produce xylanase with their distinctive characteristics and properties which are applicable in various industries. According to the characterisation study of xylanase in this research, xylanase produced by *A. brasiliensis* is suggested to use in the involvement of animal feeds, fruit juice and paper pulping industries due to its greater stability and activity at elevated temperatures, high performance in broad range of pH buffer and huge tolerance to 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°C
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 chosen to grow and produce xylanase using wheat bran as the sole carbon source under SmF. The maximum xylanase and protein production were found to occur at 48 h of fermentation with its activity of 7.72 U/mL and protein concentration of 0.19 mg, respectively. As the fermentation proceeded, an increment of pH of the culture medium was observed. The medium pH stayed constant around 6.94 at the end of fermentation. Then, xylanase was purified from the culture of *A. brasiliensis* to 3.6-fold after anion chromatography of DEAE Sepharose and Sephadex G-75 gel filtration chromatography. At the end of purification, the purified xylanase possessed a specific activity of 116.64 U/mg with its low molecular weight of 36 kDa detected from SDS-PAGE. Subsequently, the purified xylanase by *A. brasiliensis* was characterised to uncover and identify its potential in industrial applications.

The purified xylanase by *A. brasiliensis* was thermostable as it displayed relatively high stability at the temperature ranging from 40°C to 60°C with its optimum temperature of stability at 50°C. Furthermore, the purified enzyme showed the highest stability at 50°C 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°C where its relative activity was increased from 95.24% of 19.53 U/mL at 45°C to the maximum 100% with activity of 20.51 U/mL at 50°C. 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 the most stable at pH 5 as it retained 94.87% of relative activity even after 3 h of incubation. Furthermore, the activity of the enzyme remained relatively high at pH buffer ranging from pH 3 to 9 where the xylanase activity 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 thermo-alkaline stable enzyme in xylan destruction and degradation process. Additionally, this purified xylanase from *A. brasiliensis* also possessed its optimum activity when 1% xylan was utilised as the optimised substrate in the sodium acetate buffer at pH 5 at 50°C for 30 min of incubation time. On the other hand, all metal ions investigated in this study were found to be marginal effectors on 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 while Al^{3+} was the toughest inhibitor compared to other metal ions on xylanase activity. 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 to glycerol, in which 51.2% of xylanase activity was still maintained even though relatively high concentration of 50% (v/v) glycerol was added. With the addition of 50% (v/v) acetone, the xylanase maintained 33.9% of its activity. Conversely, the purified xylanase showed the poorest tolerance towards ethanol where 17.9% of activity was retained after addition of 50% (v/v) ethanol.

In a nutshell, in the present study, *A. brasiliensis* ATCC 16404 utilised the agro-industrial waste of wheat bran to produce highly substantial amount of xylanase which capable of articulating its high stability and activity over the broad range of pH and elevated temperatures in the presence of various additives such as metal ions, organic solvents and detergents. With the application of low cost agro-industrial waste as the carbon source for the production of xylanase from *A. brasiliensis* ATCC 16404 in fermentation couple with the uniqueness of these enzyme characteristics, both would enhance and strengthen the competitive power of this enzyme in global enzyme market. As a result, xylanase is anticipated to be commercially high value bioproduct to use effectively in xylan biodegradation process in variety of industrial applications especially in the manufacturing of animal feeds, pulp bleaching and paper industries.

REFERENCES

1. Singh R, Kapoor V, Kumar V. Utilisation of agro-industrial wastes for the simultaneous production of amylase and xylanase by thermophilic *actinomycetes*. *Brazilian Journal of Microbiology*. 2012;43(4): 1545-1552.
2. Hossain K, Ulven C, Glover K, Ghavami F, Simsek S, Alamri MS, Kumar A, Mergoum M. Interdependence of cultivar and environment on fiber composition in wheat bran. *Australian Journal of Crop Science*. 2013;7(4): 525-531.
3. Zhang WB, He XL, Liu HN, Guo HY, Ren FZ, Wen PC. Statistical optimisation of culture conditions for milk-clotting enzyme production by *Bacillus amyloliquefaciens* using wheat bran, an agro-industry waste. *Indian Journal of Microbiology*. 2013;53(4): 492-495.
4. Nair SG, Sindhu R, Shashidhar S. Fungal xylanase production under solid state and submerged fermentation conditions. *African Journal of Microbiology*. 2008;2: 82-86.
5. Ahmad Z, Butt MS, Anjum FM, Asgher M. Effect of wheat bran concentration on xylanase biosynthesis by *Aspergillus niger*. *International Journal of Agriculture and Biology*. 2009;11: 571-576.
6. Kavya V, Padmavathi T. Optimisation of growth conditions for xylanase production by *Aspergillus niger* in solid state fermentation. *Polish Journal of Microbiology*. 2009;58(2): 125-300.
7. Venegas MI, Fuentes-Hernández J, Garcia-Rivero M, Mertinez-Trujillo A. Characteristics of *Aspergillus niger* xylanases produced on rice husk and wheat bran in submerged culture and solid-state fermentation for an applicability proposal. *International Journal of Food Science & Technology*. 2013;48: 1798-1807.
8. El-Shishtawy RM, Mohamed SA, Asiri AM, Gomaa AM, Ibrahim IH, Al-Talhi AH. Solid fermentation of wheat bran for hydrolytic enzymes production and saccharification content by a local isolate *Bacillus megatherium*. *Biomedcentral Biotechnology*. 2014;14(29).DOI: 10.1186/1472-6750-14-29.
9. Yang ST, El-Enshasy HA, Thongchul N. Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals and polymers. 1st ed. John Wiley & Sons. Inc. Hoboken. NJ. USA. 1-26. DOI: 10.1002/9781118642047.

10. Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. Xylanases from fungi: properties and industrial applications. *Applied Microbiology Biotechnology*. 2005;67: 577-591.
11. Ho HL, Heng KL. Xylanase production by *Bacillus subtilis* in cost-effective medium using soybean hull as part of medium composition under submerged fermentation (SmF) and solid state fermentation (SsF). *Journal of Biodiversity Bioprospecting and Development*. 2015;2:1. DOI: 10.4172/2376-0214.1000143.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. *J of Biological Chemistry* 1951;193: 265-275.
13. Bailey MJ, Biely P, Poutanen K. Laboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*. 1992;23: 257-270.
14. Ho. HL, Ilyia Z. Optimised production of xylanase by *Aspergillus brasiliensis* under submerged fermentation (SmF) and its purification using a two-step column chromatography. *Journal of Advances in Biology and Biotechnology*. 2015;4(3): 1-22.
15. Sorgatto M, Guimaraes NCA, Zanoelo FF, Marques MR, Peixoto-Nogueira SC, Gianne GG. Purification and characterization of an extracellular xylanase produced by the endophytic fungus, *Aspergillus terreus*, grown in submerged fermentation. *African Journal of Biotechnology*. 2012;11(32): 8076-8084.
16. Kanimozhi K, Nagalakshmi PK. Xylanase production from *Aspergillus niger* by solid state fermentation using agricultural waste as substrate. *International Journal of Current Microbiology and Applied Science*. 2014;3(3): 437-446.
17. Kurniati E, Arfarita N, Imai T. Potential use of *Aspergillus flavus* strain KRP1 in utilization of mercury contaminant. *Procedia Environmental Sciences*. 2014;20: 254-260.
18. Azad K, Halim MA, Hossain F. Optimization of culture conditions for the production of xylanase by two thermophilic fungi under solid state fermentation. *Journal of Asiatic Society of Bangladesh*. 2013;39(1): 43-51.
19. Ghoshal G, Banerjee UC, Chisti Y, Shivhare US. Optimization of xylanase production from *Penicillium citrinum* in solid-state fermentation. *Chemical Biochemical Engineering Quarterly*. 2012;26(1): 61-69.
20. Ahmad Z, Butt MS, Riaz M. Partial purification and characterisation of xylanase produced from *Aspergillus niger* using wheat bran. *Pakistan Journal of Agricultural Sciences*. 2013;50(3): 433-437.
21. Milala MA, Nok AJ, Inuwa HM, Ibrahim S. Isolation, purification and characterisation of a xylanase from *Aspergillus flavus* for possible use in paper industry. *ARPJ Journal of Science and Technology*. 2013;3(11): 1090-1104.
22. Pal A, Khanum F. Identification and optimization of critical medium components using statistical experimental designs for enhanced production of xylanase from *Aspergillus flavus* DFR-6. *Food Technology and Biotechnology*. 2011;49(2): 228-236.
23. Okafor UA, Okochi VI, Onyegeme-Okerenta BM, Nwodo-Chinedu S. Xylanase production by *Aspergillus niger* ANL 301 using agro-wastes. *African Journal of Biotechnology*. 2007;6(14): 1710-1714.

24. Fritz J, Gjerde DT. Ion chromatography. Weinheim: Wiley-VCH, 2009.
25. Yang YL, Zhang W, Huang JD, Lin L, Lian HX, Lu YP, Wu JD, Wang SH. Purification and characterization of an extracellular xylanase from *Aspergillus niger* C3486. African Journal of Microbiology Research. 2010;4(21): 2249-2256.
26. Lu F, Lu M, Bie X, Zhao H, Wang Y. Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. Bioresource Technology. 1999;13: 5938-5941.
27. Fialho MB, Carmona EC. Purification and characterisation of xylanases from *Aspergillus giganteus*. Folia Microbiologica. 2004;49(1): 13-18.
28. Sandrim VC, Rizzatti ACS, Terenzi HF, Jorge JA, Milagres AMF, Polizeli MLTM. Purification and biochemical characterisation of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. Process Biochemistry. 2005;40: 1823-1828.
29. Coral G, Arikan B, Unaldi MN, Guvenmez HK. Some properties of thermostable xylanase from an *Aspergillus niger* strain. Annals of Microbiology. 2002;52: 299-306.
30. Dai XJ, Liu MQ, Jin HX, Jing MY. Optimisation of solid-state fermentation of *Aspergillus niger* JL-15 for xylanase production and xylo-oligosaccharides preparation. Czech Journal of Food Sciences. 2011;29(5), 557-567.
31. Izidoro SC, Knob A. Production of xylanases by an *Aspergillus niger* strain in wastes grain. Acta Scientiarum. Biological Sciences. 2014;36(3): 313-319.
32. Do TT, Quyen DT, Dam TH. Purification and characterisation of an acid-stable and organic solvent-tolerant xylanase from *Aspergillus awamori* VTCC-F312. Science Asia. 2012;38: 157-165.
33. Krisana A, Rutchadaporn S, Jarupan G, Lily E, Sutipa T, Kanyawim K. Endo-1,4-xylanase B from *Aspergillus cf. niger* BCC14405 isolated in Thailand: purification, characterisation and gene isolation. Journal of Biochemistry and Molecular Biology. 2005;38(1): 17-23.
34. Sharma PK, Chand D. Production of cellulase-free thermostable xylanase from *Pseudomonas* sp. XPB-6. International Research Journal of Biological Sciences. 2012;1(5): 31-41.
35. Abirami V, Meenakshi SA, Kanthymathy K, Bharathidasan R, Mahalingam R, Panneerselvam A. Partial purification and characterization of an extracellular xylanase from *Penicillium janthinellum* and *Neurospora crassa*. Journal of Natural Product and Plant Resource. 2011;1(4): 117-125.
36. Collins T, Gerday C, Feller G. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiology Reviews. 2005;29: 3-23.
37. Hmida-Sayari A, Taktek S, Elgharbi F, Bejar S. 2012. Biochemical characterization, cloning and molecular modelling of a detergent and organic solvent-stable family 11 xylanase from the newly isolated *Aspergillus niger* US368 strain. Process Biochemistry. 2012;47(12): 1839-1847.
38. Taneja K, Gupta S, Kuhad RC. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresource Technology. 2002;85: 39-42.
39. Thiagarajan S, Jeya M, Gunasekaran P. Purification and characterization of an endoxylanase from solid-state culture of alkali tolerant *Aspergillus fumigatus* MKU1. Indian Journal of Biotechnology. 2006;5: 351-356.
40. Shah A, Madamwar D. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterisation. Process Biochemistry. 2005;40: 1763-1771.

41. Nair SG, Sindhu R, Shashidhar S. Purification and biochemical characterization of two xylanases from *Aspergillus sydowii* SBS 45. *Applied Biochemical and Biotechnology*. 2008;149: 229-243.
42. Kamble RD, Jadhav AR. Isolation, purification and characterization of xylanase produced by a new species of *Bacillus* in solid state fermentation. *International Journal of Microbiology*. 2012. DOI:10.1155/2012/683193.
43. Mander P, Choi YH, Pradeep GC, Choi YS, Hong JHC, Cho SS, Yoo JC. Biochemical characterization of xylanase produced from *Streptomyces* sp. CS624 using an agro-residue substrate. *Biocatalysis and Agricultural Biotechnology*. 2014;49(3): 451-456.
44. Dobrev G, Zhekova B. Purification and characterization of endoxylanase Xln-2 from *Aspergillus niger* B03. *Turkish Journal of Biology*. 2012;36: 7-13.
45. Isil S, Nilufer A. Xylanase production *Trichoderma harzianum* 1073 D3 with alternative carbon and nitrogen sources. *Food Technology and Biotechnology*. 2005;43(1): 37-40.
46. Tabosa-Vaz S, Kumar A, Permaul K, Singh S. Purification and biochemical characterization of xylanase purified from a crude enzyme extract for the determination of active site residues. *African Journal of Biochemistry Research*. 2011;5(2): 43-56.
47. Dobrev G, Zhekova B, Delcheva G, Koleva L, Tziporkov N, Pishtiyski I. Purification and characterization of endoxylanase Xln-1 from *Aspergillus niger* B03. *World Journal of Microbiology and Biotechnology*. 2009;25: 2095-2102.
48. Lu F, Lu M, Lu Z, Bie X, Zhao H, Wang Y. Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. *Bioresource Technology*. 2008;99: 5938-5941.
49. Juturu V, Wu JC. Microbial xylanases: engineering, production and industrial applications. *Biotechnology Advances*. 2012;30: 1219-1227.
50. Yasemin CAF, Nihan A, Burhan A. Isolation and characterization of thermostable, alkaline, detergent and H₂O₂ resistant cellulase (CMCase) from a novel strain *Bacillus* sp. CY8 isolate. *Journal of Applied Biological Sciences*. 2014;8(2): 50-54.
51. Faulet BM, Niamke S, Gonnety JT, Kouame LP. Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus, *Termitomyces* sp. *African Journal of Biotechnology*. 2006;5: 273-82.
52. Faulet BM, Niamke S, Gonnety JT, Kouame LP. Purification and characterization of two thermostable cellulase-free xylanases from workers of the termite *Macrotermes subhyalinus* (Isoptera: Termitidae). *International Journal of Tropical Insect Science*. 2006;26: 108-120.
53. Do TT, Quyen DT. Purification and properties of a thermoactive xylanase from *Aspergillus oryzae* DSM1863. *Middle-East Journal of Scientific Research*. 2012;6(4): 392-397.
54. Bandivadekar KR, Deshpande VV. Enhanced stability of cellulase-free xylanase from *Chainia* sp. (NCL 82.5.1). *Biotechnology Letter*. 1994;16: 179-182.
55. Prakash B, Vidyasagar M, Jayalakshmi SK, Sreeramulu K. Purification and some properties of low-molecular-weight extreme halophilic xylanase from *Chromohalobacter* sp. TPSV 101. *Journal of Molecular Catalysis B: Enzymatic*. 2012;74: 192-198.
56. Lemos JLS, Bon EPS, Santana MFE, Junior NP. Thermal stability of xylanases produced by *Aspergillus awamori*. *Brazilian Journal of Microbiology*. 2000;31: 206-211.

57. Adhyaru DN, Bhatt NS, Modi HA. Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. *Biocatalysis and Agricultural Biotechnology*. 2014;3: 182-190.
58. Raj A, Kumar S, Singh SK, Kumar M. Characterisation of a new *Providencia* sp. strain X1-producing multiple xylanases on wheat bran. *The Scientific World Journal*. 2013; 1-10.
59. Do TT, Dam TH, Quyen DT. Purification and biophysical characterization of xylanase from *Aspergillus niger* DSM 1957. *Journal of Agricultural and Rural Development*. 2009;6: 16-21.