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Bioprocessing of Agricultural Wastes as Optimised Carbon Source and Optimisation of Growth Conditions for Xylanase Production by *Aspergillus brasiliensis* in Agitated Solid State Fermentation (Ssf)

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Abstract

Objective: Xylanase has been involved in many industrial applications especially in the pulp and paper, baking, detergent as well as food and beverage industries. This enzyme is produced by various microorganisms, mainly from fungal species. Therefore, in this study, *Aspergillus brasiliensis* ATCC 16404 was investigated for the optimum xylanase production under agitated solid state fermentation (SsF) using different agricultural wastes as alternative inexpensive carbon source.

Methods: *A. brasiliensis* was cultured in 10 g agricultural waste with addition of 2% yeast extract as the nitrogen source up to 120 h of SsF to elucidate xylanase production. Effect of various agricultural wastes such as wheat bran, rice bran, soybean hulls, barley husk, maize and palm kernel cake (PKC) on xylanase activity were enumerated in a stepwise manner, where one parameter was investigated at a time approach to obtain the optimum carbon source for the maximum xylanase production by *A. brasiliensis*. Thereafter, growth temperatures from 25 to 45°C, initial medium pH from 4 to 10 and agitation speeds of 50 to 200 rpm were also elucidated to determine the optimum growth conditions for the maximum production of xylanase by *A. brasiliensis*.

Results: Based on our results, the highest xylanase activity of 7.30 ± 1.93 U/mL was obtained using wheat bran as the prime carbon source with the initial medium pH 6.5 at 30°C at the agitation speed of 150 rpm.

Conclusion: Thus, in our study, the maximum xylanase production by *A. brasiliensis* using solid state bioprocessing of wheat bran as the carbon source was achieved with the addition of 2% yeast extract as the nitrogen source under the optimum growth conditions of 30°C with medium pH 6.5 at 150 rpm.

Keywords: Agitated solid state fermentation (SsF); *Aspergillus brasiliensis*; Xylanase activity; Xylan; Agricultural wastes; Wheat bran

Introduction

Xylanase is an inducible enzyme which is responsible for the complete hydrolysis of xylan into simpler compounds which are mainly consisted of xylose [1]. Over the past few years, worldwide market of xylanase is expanded rapidly due to its greater potential in industrial use, particularly in the biotechnological applications in the industry of baking, animals feed, food and beverages. In fact, the most critical application of xylanase is involved in pulp and paper industry. Xylanase acts as a pre-bleaching agent in improving the pre-bleaching process of kraft pulps by minimizing the use of hash toxic chemicals such as chlorine during the subsequent treatment stages [2]. Xylanase enhances rapid hydrolysis of xylan which is consisted of the cellulose and hemicellulose of the plant cell wall in order to improve the permeability of pulp fibers for better chlorine penetration [3]. As a result, xylanase reduces the treatment time of pulps and thus, produces greater final brightness and quality of papers [2,4]. Indeed, xylanase as a low cost bio-bleaching agent possesses tremendous benefit both environmentally and economically. Since the discovery of xylanase, ample experiments have been carried out to study the beneficial effects of xylanase and its roles in the industrial applications. Xylanase improves digestibility of animals feed and increases feed efficiency by enhancing the nutrients absorption in digestive system. In fact, the addition of xylanase in animals feed helps to degrade arabinoxylans which are the major components of the hemicellulosic complex found in plant cell wall. As a result, it fastens digestion of the animals feed to improve the nutrients absorption whereby the nutrients that trapped within the cell wall would easily release and absorb with the present of xylanase. Besides that, xylanase also plays an important role in bakery industry by improving the water absorption in dough. Xylanase increases dough volume and decreases its firmness through the changing of the water insoluble hemicelluloses into soluble form. Moreover, xylanase also improves the elasticity of gluten and structure of bread crumb and hence, increases the volume of bread. As a result, it enhances the overall quality of bread. Besides that, the shelf life of bread is prolonged by the addition of xylanase due to its pivotal role as an anti-staling action on bread storage [5]. In addition, xylanase is also commonly added together with pectinase and cellulase for the clarification and filtration of arabinoxylan and starch hazes in fruit juices [6]. Likewise, in brewing industry, the problem of viscous polysaccharides such as xylan in the final beer filtration process is overcome with the pre-treatment of using xylanase to break down the xylan and thus, increases the rates of filtration and ultimately prevents the accumulation of unwanted materials during brewing process [6].

Xylanase is produced by numerous numbers of different fungi. However, in the industrial scale, xylanase production is typically restricted to *Aspergillus spp*. and *Trichoderma spp*. [7]. Indeed, *Aspergillus spp*. are selected and optimised for xylanase production in SsF. *Aspergillus spp*. have many applications in SsF when compared to bacteria and yeast due to their physiological, biochemical and enzymological properties [8]. The hyphal of fungi penetrates into the solid substrates and survives under the controlled condition. In the absence of free-flowing water in solid substrates, it allows the fungi to become even more efficient and better growth than microflora bacteria [8]. Moreover, fungi produces higher xylanase activity compared to bacteria and yeasts [7,9]. In fact, Judith and Nei [10] demonstrated that *A. brasiliensis* was a better xylanase producer when compared to other *Aspergillus spp*. *A. brasiliensis* is a filamentous ascomycete fungi which possesses the ability to survive in the environment [11]. Apart from xylanase, *A. brasiliensis* also produces a huge variety of extracellular enzymes such as amylase, cellulase and protease [12]. They exert tremendous potentials in many industrial processes including textile, leather, detergent and baking [13].

In the production of fungal xylanase by *A. brasiliensis*, both solid state fermentation (SsF) and submerged fermentation (SmF) were used. Comparatively, the use of SsF for the production of xylanase has several advantages over SmF [14]. SsF is the growth of microorganisms under controlled condition without free-flowing water [15]. In fact, SsF is more economical compared to SmF. SsF is a simple approach uses little amount of water which in turn, it operates lesser expensive downstream processes that reduce the wastewater output. In addition, it requires cheaper capital and operational costs that demands lower energy supply. SsF is also suitable for using cheaper and abundant agricultural wastes as the carbon source in the fermentation process that often results in higher productivity of enzymes [16]. In fact, better exploitation of various agricultural wastes as the main substrate has always been the main investigation attempt in the study of SsF. Furthermore, SsF usually exerts lower risk of contamination. Most of the contaminants are not able to grow in the absence of free-flowing water [14]. Hence, SsF holds tremendous potentials in the production of xylanase. Therefore, SsF is an attractive and economical method especially for xylanase production.

Malaysia with its abundant natural resources like rainforest would be of great advantage in xylanase production by *A. brasiliensis* using SsF. Natural resources and agricultural wastes or lignocellulosic biomass which abundantly available in the environment are constituents of celluloses, hemicelluloses, lignin and xylan, respectively. They contain high concentration of about 15 to 30% of hemicelluloses which are the good inducer for xylanase production through SsF [17]. Wheat bran, rice bran, palm kernel cake (PKC), sugarcane bagasse, maize, barley husk, sawdust and soybean hulls could be used as the potential carbon sources in xylanase production through SsF [2]. This approach possesses economic value because abundance agro-industrial residuals could be used as alternative cheap raw materials for xylanase production. In fact, plant cell wall polysaccharides are mostly consisted of hemicelluloses and celluloses that manage to enhance the production of xylanase especially in SsF. All in all, xylanase is an extremely valuable enzyme which shows immense potential in biotechnological applications. In order to optimise the xylanase production by *A. brasiliensis* via SsF, the optimisation of medium formulation for xylanase production is pivotal using agricultural waste as the sustainable and economical carbon source. Besides that, the lack of precise data and information of the optimum growth conditions on xylanase production by *A. brasiliensis* in SsF has led to the objectives of this study. Therefore, the main objectives of this study are to determine the optimised carbon source from different agricultural wastes and thereafter, to enumerate the effect of different growth conditions on the xylanase production by *A. brasiliensis* via one parameter investigation at a time manner in SsF.

In this study, there were total of six different agricultural wastes consisted of wheat bran, rice bran, soybean hulls, barley husk, maize and PKC were selected to enumerate the effect of various carbon sources on the xylanase production by *A. brasiliensis* in SsF using the substrate to moisture content ratio of 1:1. Sterile water was absorbed into complex interior of the solid matrix of the agricultural wastes as the carbon source to create the optimum moisture content ratio of substrate in the present study. When the moisture content of the substrate was set at the optimum level of 1:1, it creates the swelling of the substrate particles and thus, facilitating the rapid absorption of nutrients from the substrate to the fungi. Therefore, SsF is more preferable choice of using agricultural waste as solid substrate to support the growth and xylanase synthesis by *A. brasiliensis* in this study.

Materials and Methods

Microorganism and inoculum preparation

Aspergillus brasiliensis ATCC 16404 was used for xylanase production in this study. The fungi was subcultured on PDA (pH 6.5) and incubated at 30°C for 5 days. Spores of *A. brasiliensis* were harvested and collected. Proper dilutions were performed to obtain the standard inoculum size of 1×10^6 spores for each experiment using haemocytometer under a microscope. All of the experiments were triplicate and the mean value was generated from the analysis.

Optimisation of carbon source using agricultural wastes on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF) with one parameter investigation at a time approach

Various agricultural wastes and growth conditions that influence the production of xylanolytic enzyme by *A. brasiliensis* during SsF were optimised in the stepwise manner where one parameter was elucidated at a time while keeping the other parameters at their original levels in SsF. Therefore, in order to determine the optimum carbon source for the maximum xylanase production by *A. brasiliensis* through SsF, total of six different agricultural wastes including barley husk, maize, rice bran, soybean hulls, wheat bran and PKC together with 2% yeast extract as the nitrogen source were used in the study, respectively. For the pre-treatment of agricultural wastes, these substrates were dried at 80°C in oven until their constant weights were achieved. Thereafter, they were grinded using an electric blender to homogenise into smaller residual particles before sieved to obtain the substrates with consistency in size and moisture content. Total of 10 g agricultural waste and 2% yeast extract were autoclaved separately at 121°C for 15 min to avoid the Millard reaction. 10 mL sterile water with pH 6.5 was added to the agricultural waste to acquire the substrate to moisture content ratio of 1:1 in SsF. After inoculated with the standard inoculum size of 1×10^6 spores, the culture flask was incubated at 30°C for 5 days. The rest of the experiments were repeated in the similar pattern except for replacing the carbon source with other agricultural wastes in order to elucidate the optimum

carbon source for the maximum xylanase production by *A. brasiliensis* in SsF.

Optimisation of initial medium pH on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF) with one parameter investigation at a time approach

The study was carried out at different initial medium pH from 4 to 10 in order to investigate the optimum medium pH for xylanase production using the optimised carbon source from agricultural waste with 2% yeast extract obtained from the earlier experiment.

Optimisation of growth temperature on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF) with one parameter investigation at a time approach

The effect of growth temperature on xylanase production was elucidated from 25 to 45°C in order to determine the optimum temperature for xylanase production using the optimised carbon source from agricultural waste with 2% yeast extract obtained from the earlier experiment.

Optimisation of agitation speed on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF) with one parameter investigation at a time approach

The optimum agitation speed for the maximum xylanase production was determined from 50 to 200 rpm using the optimised medium formulation and growth conditions obtained from the earlier experiments.

Sampling analysis and enzyme extraction

All of the samples were harvested every 24 h interval for analysis throughout the whole experiments. To extract the extracellular xylanase from the fermented mycelial substrate in SsF, 1 mL sterile distilled water was added and mixed homogenously with the substrate in the culture flask. Thereafter, the liquid sample was withdrawn, leaving the substrate in the culture flask for the rest of the fermentation. The liquid sample was used for the quantification of biomass concentration using spore count method. Then, the sample was centrifuged at 10,000 rpm for 15 min. The clear supernatant was used for xylanase activity and protein assays.

Xylanase activity assay

Xylanase activity assay was adopted from Bailey et al. [18] with slight modification. Xylanase activity was assayed using beechwood xylan as the standard enzyme substrate. 0.009 g of beechwood xylan was dissolved in 0.9 mL of 0.05 M sodium phosphate buffer, pH 5.3 at 50°C. 0.1 mL of supernatant was mixed with 0.9 mL of 1% xylan in sodium phosphate buffer. The mixture was incubated in a water bath at 50°C for 30 min. 1.5 mL of 3, 5-dinitrosalicylic acid (DNS) reagent was added and incubated in water bath at 90°C for 5 min. 0.5 mL of 40% Rochelle salt was added for better color stabilisation. The mixture was then allowed to cool down to room temperature and the developed color was measured using spectrophotometer at 575 nm. Xylose standard curve was used as the standard for xylanase activity. To quantify the xylanase activity, one unit of xylanase activity is defined as the amount of enzyme required to produce one micromole of xylose equivalent per minute of enzyme reaction per mL of enzyme solution under the assay condition.

Protein assay

Total soluble protein concentration was measured using Lowry assay with BSA (Bovine Serum Albumin) used as the standard [19]. The protein concentration of the supernatant was determined by absorbance reading using spectrophotometer at 750 nm.

Quantification of biomass concentration and measurement of medium pH

Spores of *A. brasiliensis* from the samples were estimated using haemocytometer and observed under microscope. In this respect, the sample was serially diluted before transferred to the haemocytometer for spore count. On the other hand, the medium pH of sample was determined using pH meter.

Results and Discussion

Optimisation of carbon source using agricultural wastes on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF)

Effect of different agricultural wastes on the production of xylanase: Based on our results, wheat bran showed the highest xylanase activity of 7.30 ± 1.93 U/mL at 24 h of SsF as shown in Figure 1. Similarly, a study conducted by Kavya and Padmavathi [20] which reported that wheat bran showed the highest xylanase activity of 9.87 U/mL in SsF by *A. brasiliensis* that was isolated from the mixed soil sample. In addition, another study conducted by Gawande and Kamat [21], they observed wheat bran produced the highest xylanase activity of 74.5 U/mL using *A. niger* strain 44. Indeed, according to Singh et al. [22], wheat bran as the agricultural by-product that contains sufficient nutrients of 40% xylans and 28% proteins supports better growth of *A. brasiliensis*. Wheat bran as the outer hard layer of grain is a highly nutritious agricultural by-product that abundantly available and usually used in the fermentation process of xylanase. Wheat bran is an economical by-product which highly abounds with xylan, and thus, it shows greater potential in enzymes production by many microorganisms [21]. Higher xylanase production of 74.5 U/mL was achieved using wheat bran by *A. niger* if compared with *A. terreus* [2]. In addition, Xu et al. [23] proved with a study that xylanase production by *A. niger* XY-1 reached as much as 14637 U/g dry substrate of wheat bran in shake flask culture under the optimised condition. Due to the natural present of arabinoxylan and hemicellulose in wheat bran, they possess the preferable particle size of this substrate in enhancing the xylanase production. The particle size of the substrate determines the amount of void space in the substrate. As a result, wheat bran increases the oxygen and nutrients transfer of *A. brasiliensis* for better growth of fungal and higher production of xylanase. In fact, wheat bran contains high concentration of protein, which is more than 14%, while other agricultural wastes such as corn cobs consists of only 2.0% [15]. In a nutshell, wheat bran of agricultural waste was found to be the most preferable optimised carbon source for the production of xylanase by *A. brasiliensis* in SsF.

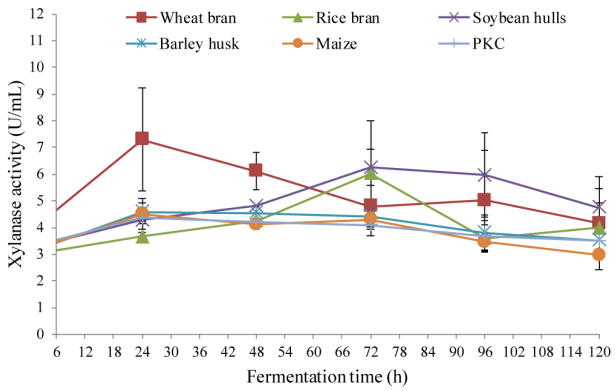

Figure 1: Time course of xylanase production by using different agricultural wastes as the carbon source in SsF.

In the present study, wheat bran as the prime carbon source produced the highest xylanase activity of 7.30 ± 1.93 U/mL compared to soybean hulls and rice bran that achieved their highest activity of 6.26 ± 0.69 and 6.02 ± 1.98 U/mL, respectively. Surprisingly, wheat bran as the main carbon source for xylanase production reached its highest activity at 24 h of SsF, the shortest fermentation period thus far. In fact, our results strongly indicated that wheat bran was the optimum carbon source for the maximum xylanase production by *A. brasiliensis* at 24 h of SsF. Comparatively, soybean hulls and rice bran achieved their highest xylanase production at much longer fermentation time of 72 h. Soybean hulls possess high cellulosic composition which contain 33.49% cellulose, 17.15% hemicellulose and 9.88% lignin, respectively [24]. However, due to the complex polymers structure of lignin that entraps cellulose and hemicellulose, soybean hulls are not easy hydrolysed in the shorter period of time. As a result, xylanase activity is anticipated to require much longer time to achieve its maximum activity but the production of xylanase is overcome once the hydrolysis is started. In fact, Kavya and Padmavathi [20] proved that soybean hulls possessed positive impact as the main substrate on xylanase production by *A. niger* in SsF.

On the other hand, rice bran is also anticipated to be a great potential substrate for xylanase production after wheat bran and soybean hulls in this study. Interestingly, Kavya and Padmavathi [20] reported that rice bran as the carbon source for xylanase production attained the second highest xylanase activity after wheat bran. Rice bran as agricultural by-product produced from milling process contains high amount of dietary fibers. Besides that, rice bran is also an inexpensive and abundantly available carbon source which typically used in most fermentation processes [20,25,26]. On a global basis, over 600 million tons of rice was harvested annually and rice bran was believed to have high amount of nutritional values [27]. In a study conducted by Fang et al. [28], rice bran contained high level of hemicelluloses which was approximately 7.55%. Additionally, according to Kuhad et al. [29], they reported that arabinoxylan of rice bran contained about 46% xylose and 44.9% arabinose, respectively. Hence, rice bran could be used as the alternative good substrate for the xylanase production by *Aspergillus spp* in SsF.

In the present study, barley husk, PKC and maize possessed relatively high xylanase production at 24 h of SsF from the range of 4.60 ± 0.35 to 4.37 ± 0.22 U/mL. Barley husk contains approximately 30% of hemicelluloses while xylan accounts for about 25.7g/100g of the hemicelluloses in barley husk [30]. In the latest study conducted by Soliman et al. [31], they remarked that barley husk as a substrate was able to show tremendous potential in xylanase production by *A. niger* and *T. viride* which they achieved their highest xylanase activity of 12.5 ± 0.13 and 11.0 ± 0.13 U/g substrate, respectively. In addition, an increase of xylanase activity by *A. niger* occurred from 12.5 ± 0.13 U/g substrate before optimisation to 42.0 ± 0.22 U/g substrate after optimisation using barley husk as the carbon source [31]. On the other hand, Pang and Omar [2] revealed that PKC contained high protein content was a good source for xylanase production, achieving 9.5 U/g production by *A. niger* AI 1 in SsF. Furthermore, in another study, xylanase activity of 192.50 U/g was produced by *A. niger* FTCC 5003 in a column bioreactor under the optimised condition using PKC as the main carbon source [32].

On the other hand, in a study conducted by Goyal et al. [33], maize straw was the prime carbon source for xylanase production compared to jowar and barseem straw because maize contained sufficient amount of hemicellulose. Doner and Hicks [34] also revealed that purified maize bran predominantly composed of 67.5% arabinoxylan, 22.5% cellulose and 2.4% protein was suitable for the xylanolytic enzymes production. Nonetheless, Archana and Satyanarayana [35] reported that there was no xylanase activity achieved when maize bran was used as the substrate for *Bacillus licheniformis* A99 in SsF. In order to induce the production of xylanase, the pre-treatment on the maize using acidic solution is suggested to destroy its complex structure. Consequently, it allows easier hydrolysis of maize by this xylanolytic enzyme, hence, higher xylanase activity is obtained from the fermentation process. In other words, the hydrolysis of arabinoxylan and cellulose of maize by xylanase is anticipated to be slower and achievable at much longer fermentation period of time. Therefore, maize is still suitable to be chosen as the carbon source for xylanase production in SsF after the pre-treatment of maize is carried out. Nevertheless, based on our results, we concluded that wheat bran as the low cost, time-saving and highly xylan-containing lignocellulosic material is suitable to use as the optimised carbon source for the maximum xylanase production by *A. brasiliensis* in SsF.

Effect of different agricultural wastes on the production of soluble protein: Lowry method was used to investigate the concentration of soluble protein produced by *A. brasiliensis* during xylanase production in this study. The soluble protein concentration of *A. brasiliensis* during 120 h of fermentation period using different agricultural wastes as the carbon source is shown in Figure 2. Apparently, wheat bran as the carbon source achieved the highest protein concentration of 0.018 ± 0.0048 g/mL at 24 h. According to Okafor et al. [36], they reported the highest protein concentration of 1.14 mg/mL by *A. niger* ANL 301 using wheat bran. On the contrary, the lowest protein concentration of 0.004 ± 0.0016 g/mL was obtained using barley husk at 120 h of SsF. Carbon sources such as rice bran, soybean hulls, barley husk, maize, and PKC showed their maximum protein concentrations of only 0.015 ± 0.002, 0.013 ± 0.00047, 0.013 ± 0.0008, 0.011 ± 0.00011 and 0.014 ± 0.0016 g/mL, respectively. According to Purwadaria et al. [37], with the presence of phytase and acid phosphatase in the fermentation processes of soybean, palm oil mill, rice bran and corn as the carbon sources, they hydrolysed the protein content of these agricultural wastes, as a result, the concentration of the soluble protein produced by *A. oryzae* GS-66 was increased. Even so, the production of soluble protein using these organic agricultural wastes was still remained low. In fact, soybean hulls were suggested to contain lower protein content of approximately 10% [24]. As a result, the production of soluble

protein by these agricultural wastes was anticipated to be lower compared to wheat bran.

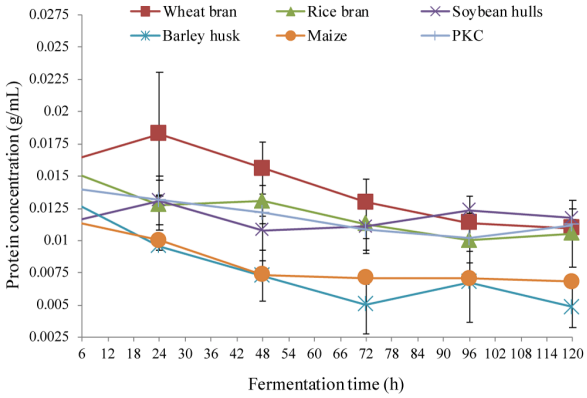

Figure 2: Time course of soluble protein production by using different agricultural wastes as the carbon source in SsF.

Effect of different agricultural wastes on the growth of *A. brasiliensis*: The maximum spore count of $60\times10^5 \pm 53\times10^5$ spores/mL was observed using soybean hulls as the carbon source at 72 h of fermentation as shown in Figure 3. This could be due to that the soybean hulls contained high cellulosic composition of 33.49% cellulose, 17.15% hemicellulose and 9.88% lignin which were greatly needed for the growth of *A. brasiliensis* through SsF. Meanwhile, rice bran and barley husk reached their maximum spore count at 72 h, producing $34\times10^5 \pm 8\times10^5$ and $6\times10^5 \pm 1\times10^5$ spores/mL, respectively. However, the maximum spore count of $17\times10^5 \pm 7\times10^5$ spores/mL at 96 h was obtained using wheat bran. On the other hand, the remaining carbon sources like maize and PKC produced only $4\times10^5 \pm 1\times10^4$ and $4\times10^5 \pm 5\times10^4$ spores/mL, respectively. This could be due to the low protein content in PKC. PKC has a poor amino acid profile that lacks of essential amino acids including lysine, methionine and tryptophan required for the growth and formation of enzymes synthesis process [38].

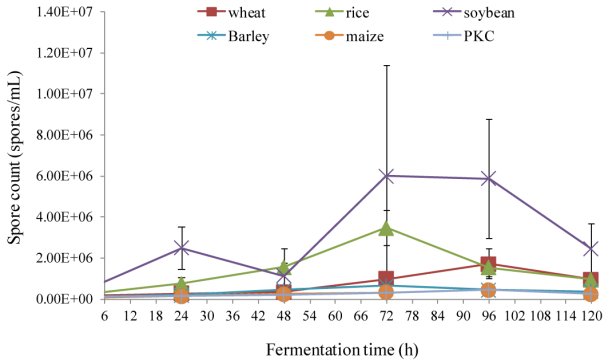

Figure 3: Growth profile of using different agricultural wastes as the carbon source in SsF.

Effect of different agricultural wastes on the pH of medium: The medium pH profile of *A. brasiliensis* during 120 h fermentation using different carbon sources is shown in Figure 4. In the present study, wheat bran as the prime carbon source produced the highest xylanase activity of 7.30 ± 1.93 U/mL at pH 6.5 at 24 h of SsF. Then, the medium pH increased to 7.91 ± 0.46 at 120 h, where the minimum xylanase activity of 4.16 ± 1.74 U/mL was observed. This could be the consequence of the depletion of nutrients after it reached its optimum activity. Besides that, the production of alkaline compounds as fermentation prolonged also prohibited the production of xylanase [39]. On the other hand, the remaining carbon sources like barley husk, maize and PKC decreased their medium pH from 6.50 to 5.10 ± 0.27, 4.97 ± 0.32 and 6.16 ± 0.01, respectively during their optimum xylanase activity at 24 h. Likewise, rice bran and soybean hulls also decreased their medium pH to 5.38 ± 0.24 and 5.34 ± 0.22 at 72 h, where their maximum production of xylanase was observed. Indeed, we suggested that the optimum xylanase production by *A. brasiliensis* occurred when the medium pH was reduced to slight acidic condition. Our results proposed that slight acidic xylanase was produced by *A. brasiliensis*. In fact, Rahman et al. [39] stated that the decrease of medium pH was due to the production of organic acids such as citric acid during the fermentation process. Based on the result findings, we suggested that the optimum synthesis of xylanase was liberated out during slight acidic condition but its activity was terminated at high alkaline conditions as the SsF prolonged was probably due to the changes in the configuration of xylanase that resulted in the denaturation of this enzyme.

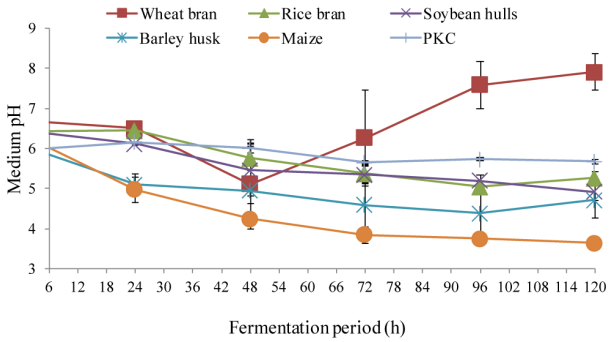

Figure 4: Medium pH profile of using different agricultural wastes as the carbon source in SsF.

Optimisation of initial medium pH on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF)

Effect of different initial medium pH on the production of xylanase: The optimisation of initial medium pH on xylanase production by *A. brasiliensis* was conducted in the stepwise manner where one parameter was elucidated at a time. The optimum growth conditions play a critical role in leading to the optimum cell growth and ultimately maximizing the production of xylanase. In other words, the optimum growth conditions of *A. brasiliensis* are the imperative key for the optimisation of xylanase production in SsF. In fact, Kavya and Padmavathi [20] reported that xylanase production was higher in the optimised conditions which produced 12.65 U/mL when compared to the unoptimised conditions which only generated about 9.38 U/mL. Using the optimised carbon source of wheat bran obtained from the earlier experiments, the initial medium pH of SsF from pH 4 to 10

were used to elucidate the optimum medium pH on xylanase production by *A. brasiliensis* in SsF. Initial medium pH is one of the crucial parameters that determines the success of xylanase production by *A. brasiliensis* in SsF in the present study. Majority of studies revealed their optimum xylanase activity occurred in slight acidic condition from the range of pH 5.0 to 6.0.

In the present study, xylanase production reached its optimum activity of 7.30 ± 1.93 U/mL at pH 6.5 at 24 h of SsF using wheat bran as the carbon source is shown in Figure 5. Thereafter, the enzyme activity decreased gradually until the end of fermentation. The initial medium pH of 4, 6, 8 and 10 exerted their maximum xylanase activity of 7.02 ± 1.13, 6.73 ± 1.35, 5.94 ± 0.60 and 6.94 ± 1.03 U/mL, respectively at 72 h of SsF. Meanwhile, 5.64 ± 0.11 U/mL of xylanase activity was produced by *A. brasiliensis* at pH 7 at 48 h of SsF. Based on the result findings, we concluded that the xylanase activity was attainable at its most productive level at the initial medium pH adjusted to 6.5 in SsF. According to Jayant et al. [40], they remarked that the growth medium with slight acidic pH was ideal for the growth of *A. niger* isolated from India in SsF. They further revealed that the medium with high acidic and high basic pH possessed negative effect on the growth of *A. niger* and thus, reduced its xylanase activity. In addition, according to Maciel et al. [41], *A. niger* LPB 326 achieved the optimum xylanase production of 3099 U/g at medium pH 6.0. They also concluded that the medium pH 6.0 was the optimum pH for the growth of fungi. Likewise, Ali et al. [42] also identified medium pH 6.0 was the optimum pH for the growth of *A. niger* GCBT7 using cane molasses as the main carbon source. Furthermore, Zulfiqar [26] concluded the optimum xylanase activity of 37.7 U/mL was achieved by *A. niger* at the medium pH 5.5 when wheat bran was used as the carbon source in his study. In general, *Aspergillus spp* are preferable to grow at the slight acidic condition. This characteristic is very important to the SsF because in this slight acidic condition, most of the bacteria responsible for contamination are prohibited. Based on our results, the positive effect of medium pH on xylanase activity occurred at the slight acidic pH 6.5.

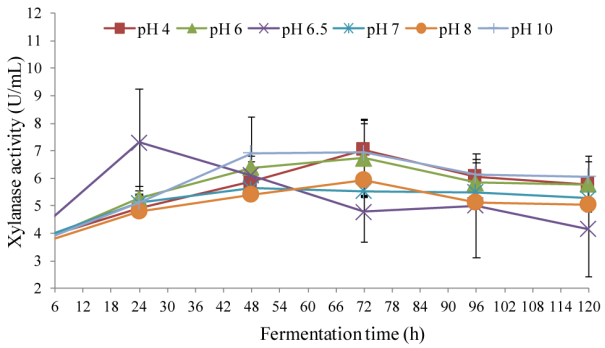

Figure 5: Time course of xylanase production by Aspergillus brasiliensis using different initial medium pH of wheat bran as the optimised carbon source in SsF.

Effect of different initial medium pH on the production of soluble protein: Figure 6 shows the effect of different initial medium pH on the soluble protein production using wheat bran as the carbon source. The initial medium pH 7 showed the highest protein concentration of 0.022 ± 0.003 g/mL at 24 h of SsF. Then, the protein concentration decreased gradually towards the end of fermentation. The initial pH 6, 6.5, 8 and 10 produced the protein concentration of 0.020 ± 0.0010, 0.018 ± 0.0048, 0.020 ± 0.0011 and 0.019 ± 0.0016 g/mL, respectively at 24 h. However, pH 4 showed it maximum protein concentration of 0.020 ± 0.00055 g/mL at 48 h before decreased gradually until the end of fermentation. Based on the result findings, there were not much differences of protein concentration achieved using different initial medium pH.

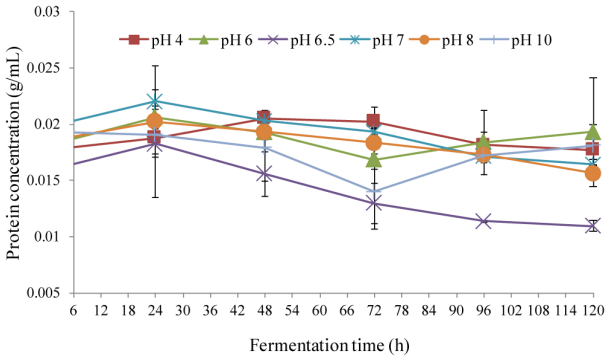

Figure 6: Time course of soluble protein production by using different initial medium pH of wheat bran as the optimised carbon source in SsF.

Effect of different initial medium pH on the growth of *A. brasiliensis*: Figure 7 shows the effect of different initial medium pH on spore production by *A. brasiliensis* using wheat bran as the carbon source. From the graph, it obviously showed that the initial medium pH 6.5 achieved the maximum spore count of $17\times10^5 \pm 7\times10^5$ spores/mL at 96 h of SsF. On the other hand, pH 6 showed the second highest spore count, producing $9\times10^5 \pm 7\times10^5$ spores/mL at 24 h. On the contrary, the initial medium pH of 4, 7, 8 and 10 showed relatively lower spore count of $6\times10^5 \pm 3\times10^5$, $4\times10^5 \pm 2\times10^5$, $5\times10^5 \pm 2\times10^5$ and $5\times10^5 \pm 3\times10^5$ spores/mL, respectively. These results strongly indicated that the protein concentration was at the most productive stage started from the initial medium pH 6.5. Jayant et al. [40] remarked that medium with slight acidic condition was ideal for the growth of *A. niger* in SsF. In fact, Donnell et al. [43] reported the growth inhibitory occurred on *A. niger* GEP when it grown at the medium pH outside of its optimum pH of cell growth. Based on our result findings, the optimal xylanase production was attainable at the slight acidic medium pH. Nonetheless, when the fermentation was prolonged, the medium pH from acidic to alkaline condition occurred as a result of gradual depletion of nutrients, limitation of oxygen transfer in the substrate particles and accumulation of the unwanted toxic wastes which were not preferable for the growth of fungi and synthesis of xylanase. Thus, this probably the reason why pH 4, 7, 8 and 10 showed relatively lower spore count and activity of xylanase by *A. brasiliensis* in SsF in this study.

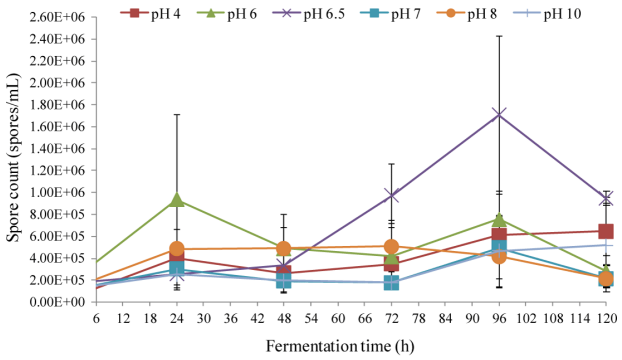

Figure 7: Growth profile of using different initial medium pH of wheat bran as the optimised carbon source in SsF.

Medium pH profile of *A. brasiliensis* from different initial pH: The medium pH profile of *A. brasiliensis* during 120 h of SsF from different initial medium pH is shown in Figure 8. Xylanase activity of 7.30 ± 1.93 U/mL was optimised at pH 6.5 at 24 h of SsF. Thereafter, the medium pH significantly increased to 7.91 ± 0.46 at 120 h of fermentation with its minimum xylanase activity observed. Likewise, the pH profile of the initial medium pH 6 showed a significant increase to alkaline condition of pH 8.44 ± 0.34 at 120 h of SsF where its minimum xylanase activity was detected. In fact, even with the initial medium pH 10 decreased to neutral condition of pH 7.22 ± 1.01 at 120 h, its lowest xylanase activity was also observed. These results indicated that the maximum production of xylanase by *A. brasiliensis* was preferably occurred at slight acidic condition. Therefore, we concluded that *A. brasiliensis* in our study possessed greater preference to grow and produce xylanase in slight acidic condition. This fungi possesses lower growth capacity in the extreme acidic and alkaline condition. This statement was in agreement with Rahman et al. [39] who reported that the depletion of nutrients occurred when the medium pH increased after it achieved its optimum xylanase activity at pH 6.5.

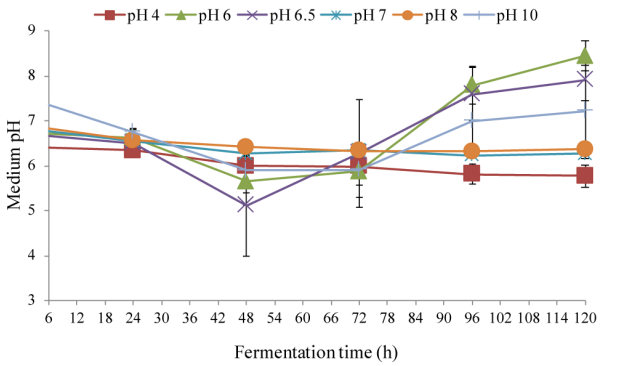

Figure 8: Medium pH profile of *Aspergillus brasiliensis* from different initial medium pH of wheat bran as the optimised carbon source in SsF.

Optimisation of growth temperature on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF)

Effect of different growth temperatures on the production of xylanase: Temperature is one of important parameters to determine the success of optimisation of growth conditions for xylanase production in SsF. In order to elucidate the effect of different growth temperatures on the xylanase production, there were total of five different temperatures ranging from 25 to 45°C elucidated as shown in Figure 9. The results indicated that the xylanase production was optimum at 30°C with the maximum activity of 7.30 ± 1.93 U/mL at 24 h of SsF. The xylanase production was significantly decreased as the temperature increased. Based on our results, relatively lower xylanase activity of 0.93 and 0.94 U/mL were attained at 40°C and 45°C, respectively. Notably, growth temperature has a huge impact on growth of microorganisms and level of xylanase production [7]. Likewise, Ali et al. [42] observed that the ideal growth temperature of *A. niger* GCBT7 was at 30°C which was the optimum temperature used for xylanase activity in a stirred fermentor. Similarly, the optimum growth temperature for *A. niger* USM A1I was at 28 ± 3°C which was similar to the optimum temperature for xylanase production in SsF [2]. In addition, Maciel et al. [41] observed that 30°C was the optimum temperature for xylanase activity of 3099 U/g by *A. niger* LPB 326 in SsF. This result was similar to the report by Shah and Madamwar (15) which stated that 30°C was the optimum temperature for xylanase production by *A. foetidus*. Xylanase production by *A. foetidus* reached its maximum production of 2701 U/g at 30°C, however, further increase of temperature to 42°C inhibited the xylanase production [15]. Furthermore, according to Kavya and Padmavathi [20], they reported *A. niger* that isolated from India grown well at 28°C with xylanase activity of 8.98 U/mL but an obvious decrease in xylanase activity to 2.64 U/mL when the temperature increased to 40°C. In another study conducted by Gawande and Kamat [21], they stated that both *A. niger* and *A. terreus* reached their maximum xylanase production at 35°C but decrease in the xylanase production occurred when further increase of the temperature from 35°C was implemented. Additionally, a study conducted by Fang et al. [28] observed that the optimum xylanase production of 22.2 U/mL by *A. carneus* was achieved at the temperature in the range of 32 to 37°C, however, further increase of temperatures caused the autolysis of fungi and hence, reducing the growth of fungi and xylanase production. Apparently, these results strongly suggested that the xylanase production in SsF is greatly influenced by the growth temperature of the microorganisms. Based on our results, 30°C was chosen as the most preferable growth temperature for the optimum production of xylanase due to a significantly shorter time of SsF needed to obtain its maximum activity. The fermentation period of SsF varies with the maximum xylanase production. Shorter fermentation period offers greater and inexpensive production of xylanase. In the nutshell, xylanase synthesis in SsF by *A. brasiliensis* was the most efficient at 30°C as compared to other temperatures in the present study.

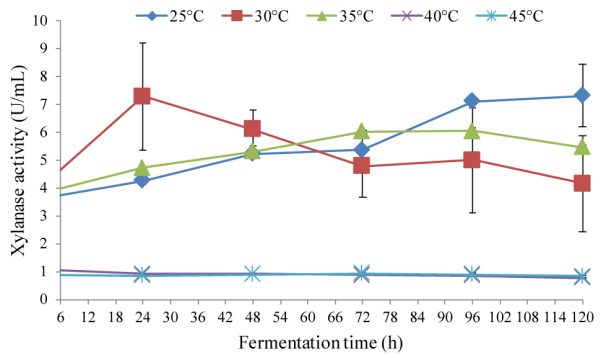

Figure 9: Time course of xylanase production by using wheat bran as the optimised carbon source under different growth temperatures in SsF.

Effect of different growth temperatures on the production of soluble protein: The response of *A. brasiliensis* to different growth temperatures for protein concentration using wheat bran as the optimised carbon source is shown in Figure 10. The maximum protein concentration of 0.021 ± 0.0029 g/mL was achieved at 35°C at 48 h of SsF. Then, the protein concentration decreased gradually until the end of fermentation. At 30°C, the highest protein concentration of 0.018 ± 0.0048 g/mL was observed at 24 h. The result was similar to Okafor et al. [36] who observed the maximum protein concentration of 1.14 mg/mL at 30°C by *A. niger* ANL 301 using wheat bran. On the other hand, 25°C and 40°C achieved their maximum protein concentration of 0.019 ± 0.00045 and 0.018 ± 0.0011 g/mL, respectively. Nonetheless, 45°C achieved the maximum protein concentration of only 0.015 ± 0.00045 g/mL. This might be due to the growth of *A. brasiliensis* was inhibited at much higher temperatures. Consequently, the soluble protein production was terminated. This statement was in agreement with Radley [44] who reported that higher temperatures than the optimum restricted the growth of microorganisms.

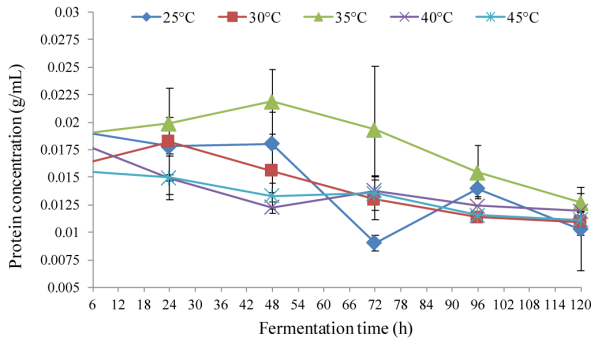

Figure 10: Time course of soluble protein production by using wheat bran as the optimised carbon source under different growth temperatures in SsF.

Effect of different growth temperatures on the growth of *A. brasiliensis*: Figure 11 illustrates the spore production of *A. brasiliensis* from different growth temperatures. Obviously, our result findings showed that the growth at 30°C produced the highest spore count of $17\times10^5 \pm 7\times10^5$ spores/mL at 96 h of SsF. On the other hand, growth at 35°C and 40°C produced their maximum spore count of only $1\times10^5 \pm 3\times10^4$ and $4\times10^5 \pm 1\times10^4$ spores/mL at 72 h of fermentation, respectively. Likewise, growth at 25°C and 45°C produced relatively lower maximum spore count of $1\times10^5 \pm 2\times10^4$ and $3\times10^4 \pm 4\times10^3$ spores/mL at 48 h. Nonetheless, it was apparent that when the growth temperatures were increased above or decreased below 30°C, *A. brasiliensis* showed lower biomass concentration. Pang and Omar [2] stated that the optimum temperature for xylanase production by *A. niger* was at the ambient temperature of 28 ± 3°C. They also proved that the highest xylanase activity was obtained at the temperature which was similar to the optimum temperature for the growth of fungi in SsF. Additionally, Fang et al. [28] also mentioned that both xylanase production and cell growth by *Aspergillus carneus* reached their optimisation at 30°C, respectively. Likewise, according Shah and Madamwar [15], the maximum xylanase production was attained at the temperature of 30°C, however, further increased of temperature up to 42°C resulted in poorer growth of fungi and lesser xylanase production. The growth of *A. brasiliensis* was inhibited at higher temperature than the optimum also reported by Radley [44]. This might be a reason why 45°C observed the lowest spore count in this study.

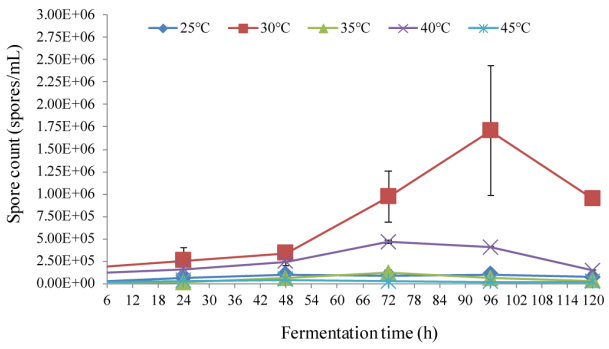

Figure 11: Growth profile of using wheat bran as the optimised carbon source under different growth temperatures in SsF.

Effect of different growth temperatures on the pH of medium: Figure 12 illustrates the impact of different growth temperatures of 25, 30, 35, 40 and 45°C on the medium pH profile by *A. brasiliensis* when the initial medium pH was adjusted to 6.5. Based on our result findings, the increase in pH of the medium was accompanied by decrease in xylanase production. The optimum xylanase activity was achieved, producing 7.30 ± 1.93 U/mL at the medium pH 6.5 at 30°C at 24 h of SsF. Nonetheless, when the pH increased to 7.91 ± 0.46 at 120 h of SsF, the minimum xylanase activity was observed in this study. In fact, at 40°C and 45°C, the medium pH increased from 6.50 at the beginning of fermentation to 7.73 ± 0.12 and 9.08 ± 0.14 at 120 h, where their lowest xylanase activities were also detected. The pH of the medium at the growth temperature of 25°C and 35°C increased gradually from pH 6.50 to 7.27 ± 0.05 and 6.62 ± 0.59 at 120 h, respectively. Therefore, we concluded that the optimum temperature for the maximum xylanase production by *A. brasiliensis* was achieved at 30°C, producing 7.30 ± 1.93 U/mL of xylanase activity at the medium pH 6.50 at 24 h of SsF. Likewise, this observation was similar to a study conducted by Ahmad et al. [45] whereby they studied the effect of growth temperature at 30°C on xylanase production by *A.*

niger that isolated in Pakistan using wheat bran as the main carbon source. Based on their study, the optimum xylanase activity of 78.03 U/mL was attained at 30°C. They also further explained that the isolated *A. niger* was mesophillic in nature, as a result, it performed its optimum xylanase activity at 30°C.

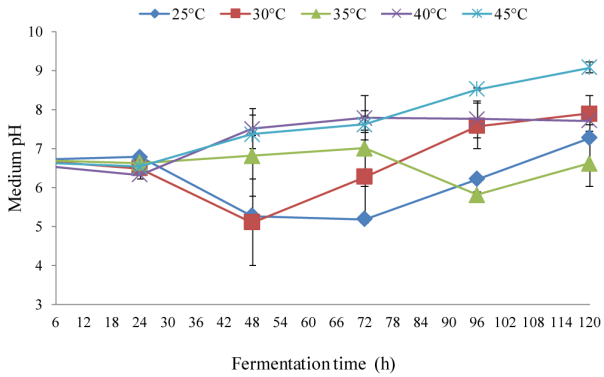

Figure 12: Medium pH profile of *Aspergillus brasiliensis* using wheat bran as the optimised carbon source under different growth temperatures in SsF.

Optimisation of agitation speed on xylanase production by *Aspergillus brasiliensis* in agitated solid state fermentation (SsF)

Effect of different agitation speeds on the production of xylanase: Agitation speed plays a very significant role in the fermentation process where it affects the dissolved oxygen and mass transfer of the microorganisms [7,46]. In the present study, there were four different agitation speeds from 50 to 200 rpm elucidated in order to study their effect on the optimisation of xylanase production. Obviously, the result showed the highest xylanase activity of 7.30 ± 1.93 U/mL was attained at 150 rpm at 24 h of fermentation as shown in Figure 13. While, the other agitation speeds such as 50, 100 and 200 rpm were resulted in relatively lower xylanase activity of only 1.58 ± 0.17, 1.54 ± 0.016 and 1.53 ± 0.04 U/mL, respectively. SsF at 200 rpm resulted in the lowest xylanase activity in the present study. This excessively high agitation speed of 200 rpm led to the shear stress occurred on *A. brasiliensis*. As a result, it causes damage to the mycelium of fungi. Likewise, same observation was also reported by Purwanto et al. [46], who suggested that higher agitation speed could result in shear force on the fungal cell and caused the disturbance that ultimately led to lower cell growth and thus, enzymes production. Similarly, relatively lower xylanase activity was also generated using 50 and 100 rpm, respectively. It might be the reason of the low inadequate supply of dissolved oxygen and mass transfer to the fungi in the culture flasks. Likewise, similar observation was in agreement with Dietmar et al. [7] who stated that low agitation speed of 130 rpm resulted in relatively lower xylanase activity by *Thermomyces lanuginosus* DSM 5826 as a result of lesser amount of dissolved oxygen in the growth medium. Therefore, the results obtained in our study obviously indicated that 150 rpm was the optimum agitation speed for the optimisation of xylanase production by *A. brasiliensis* under SsF.

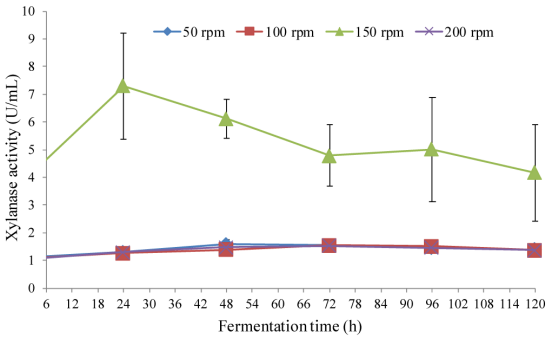

Figure 13: Time course of xylanase production by using wheat bran as the optimised carbon source under different agitation speeds in SsF.

Effect of different agitation speeds on the production of soluble protein: The profile of protein production by *A. brasiliensis* using wheat bran as the optimised carbon source at different agitation speeds under SsF is shown in Figure 14. Apparently, the graph shows that the agitation speed of 50 rpm achieved its maximum protein concentration of 0.018 ± 0.0021 g/mL at 48 h of SsF. On the contrary, other agitation speeds showed the negative effect on the protein production whereby their concentrations decreased from 48 h until the end of SsF. Again, the excessive high agitation speed than the optimum led to the higher shear stress on *A. brasiliensis* that damaged the fungal mycelium. According to Purwanto et al. [46], they reported that the exorbitant of agitation speed might result in higher shear force on *A. niger* that caused the fungal cell disturbance and eventually affected the growth of *A. niger* and thus xylanase production. In addition, Purwanto et al. [46] also suggested that when the higher agitation speed was applied, it possessed mechanical forces on the filamentous fungi such as *Aspergillus spp*, resulted in the formation of many smaller pellets in the culture of SsF. As the fermentation prolonged, oxygen deprivation was probably anticipated when the spores of *A. brasiliensis* and yeast extract were aggregated to form larger sticky pellets in the culture flasks. Consequently, oxygen, carbon dioxide, nutrients and toxic materials were not transferred as efficiently as they used to be. As a result, cell autolysis was triggered and the synthesis of enzyme was eventually terminated.

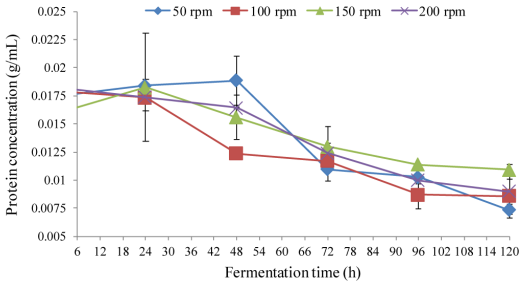

Figure 14: Time course of soluble protein production by using wheat bran as the optimised carbon source under different agitation speeds in SsF.

Effect of different agitation speeds on the growth of *A. brasiliensis*: Figure 15 shows the effects of different agitation speeds on the spore count of *A. brasiliensis* using wheat bran as the carbon source in SsF. The graphic illustration elaborates that when the biomass concentration was elucidated at different agitation speeds, the highest spore count of $17\times10^5 \pm 7\times10^5$ spores/mL was achieved at 150 rpm at 96 h of SsF. Meanwhile, the remaining agitation speeds such as 50, 100 and 200 rpm produced $5\times10^5 \pm 1\times10^5$, $9\times10^5 \pm 8\times10^4$ and $7\times10^5 \pm 2\times10^5$ spores/mL at 48 h of SsF, respectively. Nevertheless, it was apparent that the growth of *A. brasiliensis* was optimised at 150 rpm. On the other hand, higher or lower agitation speeds than the optimal resulted in relatively lesser growth of *A. brasiliensis*. The lower agitation speed leads to the inadequate supply of dissolved oxygen to *A. brasiliensis* while higher speed results in the damage of fungal cells due to the greater shear rate [7]. In short, both situations resulted in poorer growth of *A. brasiliensis* as shown in this study.

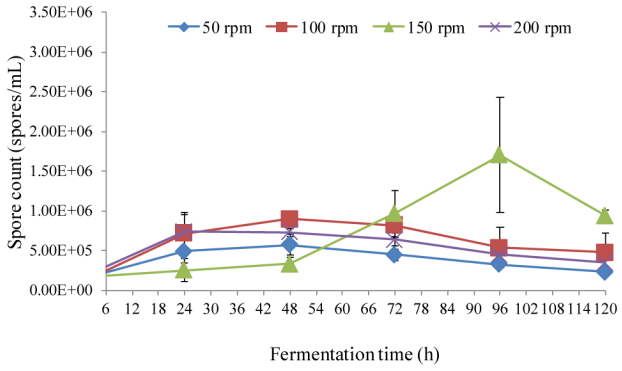

Figure 15: Growth profile of using wheat bran as the optimised carbon source under different agitation speeds in SsF.

Effect of different agitation speeds on the pH of medium: The medium pH profile of different agitation speeds using wheat bran as the prime carbon source by *A. brasiliensis* through SsF is shown in Figure 16. As mentioned earlier, 150 rpm achieved the highest xylanase of 7.30 ± 1.93 U/mL at 24 h of fermentation. Nonetheless, 50, 100 and 200 rpm apparently resulted in lower xylanase activity. At 150 rpm, the medium pH was remained at 6.50 ± 0.07 at 24 h, where the optimum xylanase activity was observed. Thereafter, the pH increased to 7.91 ± 0.46 at 120 h, where the minimum xylanase activity was attained. The pH of the remaining agitation speeds at 50 and 100 rpm increased slightly to 6.74 ± 0.06 and 6.8 ± 0.12 at 120 h, respectively. While, the pH at 200 rpm reduced slightly to 6.41 ± 0.26 at 120 h of fermentation. According to a study conducted by Jain et al. [47], they remarked that an increased in agitation speed caused the shear-dependent mechanism that was directly affected the growth and xylanase production.

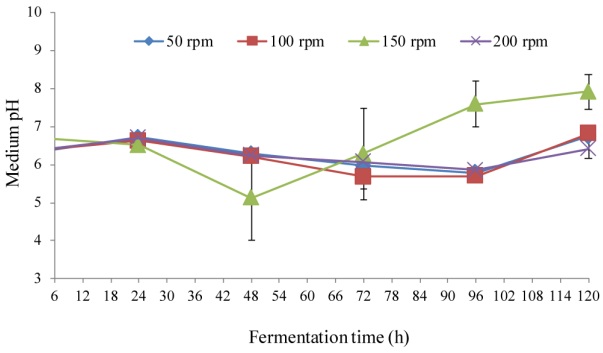

Figure 16: Medium pH profile of using wheat bran as the optimised carbon source under different agitation speeds in SsF.

Conclusion

The present study was conducted to produce the optimum xylanase activity by *A. brasiliensis* using various agricultural wastes as the prime carbon source in SsF. Besides that, a range of fermentation parameters were carried out in order to determine the optimal growth conditions for xylanase production by *A. brasiliensis* via SsF. In this study, SsF is preferred than SmF due to lesser use of water, higher capability of using cheap and abundant available agricultural wastes as carbon source, lower risk of contamination and thus, resulted in higher productivity of xylanase. Fungal xylanase produced by *A. brasiliensis* was found to be satisfactory in terms of its maximum activity which was determined by DNS method. Meanwhile, various agricultural wastes were analysed for the determination of the optimum carbon source on xylanase production. As a result, wheat bran was found to be the most productive and preferable carbon source on xylanase production by *A. brasiliensis* via SsF. Besides wheat bran, both soybean hulls and rice bran also showed relatively higher xylanase production. However, lower xylanase activity was observed in barley husk, maize and PKC, respectively. Fermentation parameters such as initial pH of the medium, growth temperature as well as agitation speed were significantly affected the xylanase production by *A. brasiliensis* at various levels. The maximum xylanase activity by *A. brasiliensis* was achieved when the medium pH was initially adjusted to pH 6.5. Furthermore, satisfactory of xylanase activity was attained at the growth temperature range of 25 to 35°C. More precisely, the maximum xylanase production by *A. brasiliensis* was produced preferably at 30°C. On the contrary, higher growth temperature above 40°C showed a negative impact on xylanase activity, most likely due to the inhibition and denaturation of xylanase. Apparently, *A. brasiliensis* exhibited the maximum xylanase activity at 150 rpm in SsF. Based on the current result, it obviously showed that with the further increase or decrease of the incubation agitation speed than 150 rpm, they resulted in a negative influence on xylanase activity. Notably, 150 rpm of SsF was attributed to the greater growth and thus, the maximum xylanase production in this study. Apart from that, according to the current findings, xylanase activity is also significantly affected by the fermentation period. The results explicated that the optimum xylanase activity was observed at 24 h of SsF. Therefore, the optimum fermentation period for optimising the xylanase production by *A. brasiliensis* via SsF occurred at 24 h. Using wheat bran as the prime carbon source, it satisfactorily achieved the highest xylanase yield

within much shorter fermentation period. In a nutshell, the objectives of this study involving the elucidation of the optimised carbon source and growth conditions on xylanase production by *A. brasiliensis* using SsF were successfully achieved in the present study. Based on our results, 10 g wheat bran as the prime carbon source with addition of 2% yeast extract as the nitrogen source were applied to culture *A. brasiliensis* for the maximum production of xylanase at the initial medium pH of 6.5 at 30°C with 150 rpm up to 24 h of SsF.

Potential establishment of xylanase

The development of industrial xylanase is emerging and growing rapidly in this recent decade. The world market of xylanase is expanding speedily due to its enormous pivotal roles in various industries, particularly in the biotechnology applications, including pulp and paper, baking, animals feed, detergent, food and beverage. The finding of this study using different agricultural wastes is still relatively new especially in Malaysia. Therefore, this study is needed to elucidate the optimum carbon source and growth conditions on xylanase production by *Aspergillus spp*. The ultimate goal of this study is to commercially produce xylanase in large industrial SsF system using alternative cheap and sustainable carbon source such as wheat bran under the optimal growth conditions. By using industrial SsF, growth temperature, medium pH, dissolved oxygen, nutrients supply and other factors are easily regulated to produce huge amount of xylanase by *Aspergillus* spp. Besides *A. brasiliensis*, other microorganisms are needed to explore and elucidate in order to determine their optimum growth conditions for xylanase production in SsF. As a result, it provides positive comparison among other microorganisms with *A. brasiliensis* in xylanase activity. Furthermore, better understanding and in-depth information regarding the production of this enzyme by different microorganisms via SsF possess greater insights in the industry point of view. In fact, several microorganisms have been reported as the suitable microorganisms on xylanase production, including *Aspergillus carneus* [28], *Aspergillus foetidus* [15], *Penicillium chrysogenum* [40] and *Paecilomyces themophila* [48], respectively.

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