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Bioprocessing of Agricultural Residuals for the Optimum Production of Extracellular Xylanase by *Aspergillus brasiliensis* in Solid State Fermentation (SsF)

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Abstract

Objective: Xylanase production by *Aspergillus* species has become valuable and attractive due to its vast applications in pulp and paper, food and beverage, detergent and textiles industries. Xylanase is one of the hemicellulolytic enzymes that capable of hydrolysis of β-1,4 xylans present in the lignocellulosic materials. Therefore, *Aspergillus brasiliensis* ATCC 16404 was used to investigate the maximum production of xylanase using various agricultural residuals in solid state fermentation (SsF).

Methods: SsF is the fermentation process of culturing microorganisms using humid solid substrates without emerging in culture broth. SsF has always been an attractive substitute of liquid culture for xylanase production due to its higher productivity per reactor volume, lower capital investment and lesser energy demand. Hence, various parameters of medium formulation were investigated to obtain the maximum activity of xylanase by *A. brasiliensis* in SsF. Additionally, to reduce the costs of production, agricultural residuals were used instead of xylan. In this study, the optimisation of carbon source using agricultural residuals combined with nitrogen source was elucidated for the maximum xylanase production by *A. brasiliensis* in SsF.

Results: From our results, 10 g wheat bran as the optimum agricultural residual was able to produce xylanase activity of 6.7091 U/mL at 48 h of SsF. Subsequently, 6.7115 U/mL of xylanase was obtained using wheat bran as the optimised carbon source at the substrate to moisture content ratio of 1:1. Interestingly, when 2% yeast extract was added, further study reviewed the maximum xylanase activity increased to 28.75%.

Conclusion: In conclusion, the optimum medium formulation for the maximum production of xylanase by *A. brasiliensis* was achieved using 10 g wheat bran as the optimised carbon source at the substrate to moisture ratio of 1:1 combined with 2% yeast extract as the optimum nitrogen source cultured at optimum temperature of 30°C at 150 rpm up to 48 h of SsF.

Keywords: Solid state fermentation (SsF); *Aspergillus brasiliensis*; Xylanase activity; Xylan; Agricultural residuals

Introduction

The commercialised production of xylanase in the industrial scale creates great interest due to its advantages in producing greater catalytic efficiency, higher degree of specificity and better environmental friendliness compared to the chemical catalysts. Thus, many applications of xylanase are involved in bakery, food and beverage, animal feed and detergents industries. In the present day, the demand for xylanase is rising because of its prodigious utilisation in vast industries as one of the main ingredient components. The leading industrial application of xylanase is chlorine-free bleaching in pulp and paper, whereby xylanase is added into the pulp to degrade the xylan found in the lignin residuals during pre-bleaching process. This allows easy bleaching process of the pulp and hence, enhancing better brightness of paper [1,2]. Additionally, the initiative towards a greener environment is attained using xylanase where the usage of the costly bleaching chemicals that are potentially causing severe adverse effect to the environment was reduced [3]. There are several crucial fermentation parameters used to elucidate and optimise the production of xylanase including carbon and nitrogen source and their concentrations, initial medium pH, incubation temperature and agitation speed. These parameters are depended on the types of the microorganism that yield the xylanase in SsF. Apparently, carbon source from various agricultural residuals provides the prerequisite nutrients for the production of xylanase to promote the growth of microorganism. As a result, higher biomass concentration of microorganism was obtained from larger xylanase production. In order to further enhance the xylanase production, inexpensive but effective nitrogen source is generally supplemented as the additional nutrients during the fermentation. Thus, lower production costs but the maximum yield of xylanase could be easily achieved. Therefore, the optimisation of medium formulation would able to produce the desirable amount of xylanase at the maximum level through fermentation process. There are two types of fermentation used for xylanase production namely solid state fermentation (SsF) and submerged fermentation (SmF). Recently, SsF has become a well-known method to produce xylanase due to its economical process that does not involved high cost and expensive technology. SsF is the growth of microorganisms under controlled condition without free-

flowing water [4]. Viniegra-Gonzalez et al. [5] studied the comparison between SsF and SmF in terms of enzymes production. From the study, they found out that the higher biomass concentration and lower protein breakdown were the main factors in improving the production of enzymes in SsF. Many studies on the xylanase production have been conducted in SmF, yet only a few studies were performed on the production of xylanase using agricultural residuals in SsF.

Furthermore, the utilisation of inexpensive agricultural residuals in SsF would reduce the environmental pollutions as well as provide the sufficient nutrients as the alternative cheaper carbon source [1]. Agricultural residuals are lignocellulosic wastes, besides being cheap, abundant and readily available, have gained considerable interest as a result of their potential use in fermentation process as the main substrate source. Various agricultural residuals such as corn cob, wheat bran, rice husks, barley husks, sugarcane bagasse and sawdust used as the solid substrate for the abundant production of xylanase in SsF would be an economical approach to the extremely low operational cost for enzyme production rather than opting for the expensive pure xylan as the prime substrate. Little would be available for the production of xylanase using xylan as the optimum substrate especially in the industrial SsF. Thus, the bioprocessing of agricultural residuals using SsF, besides provides more economical solution, it exerts some engineering advantages including simplified downstream processing steps. In Malaysia, the agricultural residuals from palm oil, tapioca, pineapple, rice and sago wastes as well as the municipal solid waste are found bountiful, they are therefore, being utilised to produce valuable enzymes including xylanase via SsF [6]. Thus, continuous research efforts are constantly being given to SsF using agricultural residuals as the foremost carbon source to make it on the par with SmF to ensure SsF is more practicable using agricultural residuals. Additionally, filamentous fungi of *Aspergillus* species have always been the predilection choice as the producer for the extracellular production of xylanase due to their physiological, biochemical and enzymological properties [7]. They apparently produce higher activity of xylanase compared to the other sources, including bacteria, actinomycetes and yeast [8,9]. As a result, it is a notable research effort in SsF on the production of xylanase by *Aspergillus spp* using agricultural residuals as the carbon source. There are continuously endeavours to produce xylanase in a more profitable approach with much lower costs of production using agricultural residuals as the alternative cost-effective substrate for the production of xylanase by *Aspergillus spp*. Therefore, the main objective of this study is to develop the medium formulation for the optimum production of xylanase by *A. brasiliensis* in SsF. In this study, the elucidation of the maximum xylanase activity by *A. brasiliensis* was conducted in a stepwise manner where one parameter on medium formulation was investigated at a time while keeping the other parameters at their original level in SsF. In the present study, different agricultural residuals were used to determine the optimum carbon source followed by the optimisation of nitrogen source and its concentration in order to establish the optimum medium formulation on xylanase production by *A. brasiliensis* in SsF.

Materials and Methods

Microorganism and inoculum preparation

Aspergillus brasiliensis ATCC 16404 was used for xylanase production in this study. The strain was subcultured and maintained on PDA (pH 6.5) at 30°C. The inoculum of *A. brasiliensis* was obtained by pipetting sterile distilled water onto the PDA plate of *A. brasiliensis.* A sterile inoculation loop was used to scrap the surface of the agar and the spores were harvested and counted using a haemocytometer. Proper dilutions were performed to acquire the standard inoculum size of 1×10^6 spores for every experiment. All of the experiments were conducted twice and the mean value was generated from the analysis.

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

Various parameters of the medium formulation that influence the production of xylanolytic enzyme by *A. brasiliensis* during SsF were optimised over a wide range. The strategy to elucidate the optimum medium formulation using agricultural residuals combined with nitrogen source and its concentration on the maximum xylanase production was carried out to evaluate the effect of an individual parameter then incorporate it before standardising the next parameter in this study. The agricultural residuals such as barley husks, palm kernel cake (PKC), rice bran, sugarcane bagasse, soybean hulls, sawdust and wheat bran were elucidated as the carbon source for the optimum production of xylanase by *A. brasiliensis* in culture flasks under SsF. For the pre-treatment of agricultural residuals, 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. 10g agricultural residual of barley husks was autoclaved at 121°C for 15 minutes before 10 mL sterile water with optimum pH 6.5 was added in the culture flask to achieve the substrate to moisture content ratio of 1:1 in SsF. The culture flask was then covered with a non-absorbent cotton wool to prevent evaporation and contamination. After inoculated with 1×10^6 spores, the culture flask of SsF was carried out using the optimum growth conditions at 30°C at 150 rpm for 120 h. The rest of the experiments were prepared following the similar fashion except for replacing with other agricultural residuals to elucidate the optimum carbon source on xylanase production by *A. brasiliensis* in SsF.

Optimisation of the initial moisture content ratio of the optimum carbon source on xylanase production by *Aspergillus brasiliensis* in solid state fermentation (SsF)

The moisture content ratio of the substrate is one of the critical factors influencing the outcome of SsF. Thus, it is necessary to elucidate the maximum production of xylanase in this study. The optimisation of medium formulation on xylanase production by *A. brasiliensis* in SsF was conducted in the stepwise manner where one parameter was investigated at a time. Using the optimum carbon source of agricultural residuals that investigated from the earlier experiments, the initial substrate to moisture ratio of its optimised carbon source was examined using different ratio ranging from 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 to 1:4 in SsF, respectively.

Optimisation of nitrogen source and its concentration on xylanase production by *Aspergillus brasiliensis* in solid state fermentation (SsF)

Using the optimum substrate to moisture content ratio of the optimised carbon source that was obtained from the earlier experiments, the elucidation of optimum nitrogen source was carried out with the addition of 2% of various nitrogen sources, respectively. In this respect, organic and inorganic nitrogen sources were used to further enhance the growth of *A. brasiliensis* and thus, synthesis of xylanase in SsF. Hence, in order to determine the optimum nitrogen source, peptone, malt extract, urea, yeast extract, ammonium chloride, ammonium nitrate, ammonium sulfate and sodium nitrate were elucidated, respectively. Thereafter, using the optimised nitrogen source obtained earlier in SsF, the optimisation of nitrogen concentration was examined ranging from 2, 4, 6, 8 to 10% for the maximum production of xylanase by *A. brasiliensis*, respectively.

Sampling and enzyme extraction

Sampling was conducted at the regular time interval of 24 h from the culture flasks for analysis up to 120 h of SsF. To extract the extracellular xylanase from the fermented mycelial substrate in SsF, small volume of sterile distilled water was added and mixed homogenously with the entire substrate in the culture flask. Thereafter, the liquid sample was withdrawn, leaving the agricultural residuals aside in the culture flask for the rest of the fermentation. Then, the liquid sample was used for spore count. Subsequently, the remaining sample was centrifuged at 10,000 rpm for 15 minutes. The clear supernatant was subjected to xylanase activity and protein assays.

Xylanase activity assay

Xylanase activity was assayed according to Bailey et al. [10] with slight modification. 1% xylan in 0.05 M sodium phosphate buffer, pH 5.3 was used as the substrate for the xylanase activity assay. 0.9 mL substrate solution was added into 0.1 mL supernatant. The mixture was vortexed and incubated at 50°C for 30 minutes. After incubation, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added into the mixture. The mixture was then vortexed and incubated at 90°C for 5 minutes. Colour changes were observed when the xylose that liberated from the enzymatic reaction occurred between xylan and xylanase reacted with DNS. 0.5 mL of 40% Rochelle salt was added immediately to the mixture to terminate the enzymatic reaction. The absorbance reading was measured spectrophotometrically at 575 nm. Xylanase activity was measured according to the xylose standard curve. One unit of xylanase activity is defined as one micromole of xylose released per minute per mL of enzyme solution under the assay condition.

Quantitative of biomass concentration and measurement of medium pH

Spores of *A. brasiliensis* were estimated using a haemocytometer. The spore suspension from the sample was withdrawn and serially diluted before transferred to the haemocytometer for spore count. The pH of the samples was measured using a pH meter.

Quantitative of soluble protein concentration

The concentration of total soluble protein produced by *A. brasiliensis* during xylanase synthesis in SsF was determined at the absorbance reading of 750 nm according to Lowry et al. [11] using BSA as the standard curve.

Results and Discussion

Optimisation of carbon source using agricultural residuals on xylanase production by Aspergillus brasiliensis in solid state fermentation (SsF):

Wheat bran as the optimised carbon source

Various agricultural residuals such as wheat bran, rice bran, barley, soybean hulls, sugarcane bagasse, PKC and sawdust were investigated for the optimisation of carbon source by *A. brasiliensis* in SsF. Based on our results, wheat bran exhibited the optimum xylanase activity of 6.7091 U/mL at 48 h compared to the other agricultural residuals as shown in Figure 1. Based on our results, the production of xylanase by *A. brasiliensis* increased rapidly towards 48 h using wheat bran as the prime carbon source. The fungi hydrolysed arabinoxylan and hemicelluloses from wheat bran enormously when the xylanase activity was at its peak at 48 h. As a result, higher degradation of the substrate produced maximum concentration of xylose, which in turn, increased the availability of nutrients and thus, drastically stimulated the growth of *A. brasiliensis* from 48 h to reach its maximum peak at 72 h as shown in Figure 2. It was likely that under SsF, which was basically a static-state fermentation where TCA cycle were less dominant, thus, the xylan fermentation leading to xylose and subsequently hexose mono-phosphate pathway might be operated enormously by xylanase activity at 48 h of SsF. As a result, there was a steep increment in biomass production of *A. brasiliensis* from 48 to 72 h as reflected by the maximum spore production at 72 h. However, due to the condition of SsF that was lacking of free moving water, xylose might not be able to homogenously distribute and use instantly during the early stage of SsF. Consequently, a short span of time had been acquired by the fungi to consume xylose before it achieved its maximum spore production at 72 h when wheat bran was used as the carbon source in the present study. Notable differences in xylanase production from each of the agricultural residual were believed to be influenced by the quantity of the available arabinoxylan and hemicelluloses in the medium. The maximum activity of xylanase by *A. brasiliensis* was found to be in wheat bran in this study. The significant production of xylanase when wheat bran was used as the prime carbon source, besides due to its arabinoxylan and hemicelluloses nature, it might also attribute to the favourable degradability and particles size of the wheat bran.

Particles size of substrate in SsF exerts critical influential on xylanase and energy production in the microorganisms. Since it affects the surface area to volume ratio of the particles, indeed, it evaluates how accessible of the mass transfers to the microorganisms. As a result, the particles size of the substrate regulates the quantity of void space within the substrate. It ultimately governs the rate of oxygen transfer that affects the growth of microorganisms. In fact, the substrate in SsF should also possess an optimum particles size to enhance the heat and carbon dioxide removal from the microorganisms. It was suggested that the promising optimum growth could be achievable with smaller particles size of the substrate in SsF. The highest xylanase activity of 9868 U/g from *Thermoascus aurantiacus* was obtained using particles size of 0.3 to 0.45 mm of wheat straw compared to lower activities using other sizes in SsF [12]. Our results coincides with those of Okafor et al. [13], whereby they produced the highest xylanase activity of 6.47 U/mL using wheat bran

at 96 h by *A. niger* ANL 301. Again, Singh et al. [14] also observed the highest production of xylanase utilising wheat bran, producing 727.78 and 227.99 U/mL at 144 h by *Coprinellus disseminates* strain SH-1 and SH-2, respectively. Apparently, the highest xylanase activity was harvested from wheat bran compared to other carbon sources due to its significantly high nutrients composition that was comprised of 40% arabinoxylan and 28% protein. It was suggested that minerals and amino acids in the wheat bran elevated the synthesis of xylanase by *A. brasiliensis*. According to Pal and Khanum [15], wheat bran emerged as the most ideal agricultural residual for enzymes production. The porosity in wheat bran which consists of small pores size allows larger surface area for oxygen transfer, nutrients intake and heat dispersion. Additionally, they also found that wheat bran supplemented medium yielded the highest xylanase activity of 9.5 U/mL compared to 9.2 and 8.9 U/mL by *Aspergillus flavus* DFR-6 using oat bran and pineapple peel, respectively. Similar results were also observed by Alam et al. [16]. They investigated xylanase production by *Thermomyces lanuginosus* and *Thermoascus aurantiacus* using various agricultural extracts such as wheat bran, rice bran and sugarcane bagasse, respectively. Based on their results, wheat bran emerged as the most favourable substrate for the optimum production of xylanase on the 7 day with the increment of 3 fold, producing 1787.7 U/g compared to 424.7 U/g from rice bran by *Thermomyces lanuginosus*. Surprisingly, around 326.83% increment or 542.5 U/g of xylanase production by *Thermoascus aurantiacus* was detected using wheat bran compared to relatively low activity of 127.1 U/g obtained using rice bran. In the same study, wheat bran produced again, the maximum xylanase activity with the increment of 116.82% by *T. lanuginosus* when compared to 824.5 U/g from sugarcane bagasse. Interestingly, 85.40% increment of xylanase activity was greatly obtained from wheat bran by *T. aurantiacus* compared to only 292.6 U/g from sugarcane bagasse. In a nutshell, wheat bran of agricultural residuals was found to be the most optimum carbon source on the production of extracellular xylanase by different types of fungi in SsF.

On the other hand, in this study, the maximum xylanase activity obtained from soybean hulls, sugarcane bagasse, rice bran, barley, sawdust and PKC were 6.5682, 5.0223, 4.8612, 3.9534, 3.9130 and 3.6633 U/mL, respectively. Relatively lesser xylanase activity that obtained from other agricultural residuals compared to wheat bran by *A. brasiliensis* was probably attributed to the presence of lesser amount of hemicellulose in other agricultural residuals such as only 17.21% of hemicellulose was found in soybean hulls when compared to 40% arabinoxylan from wheat bran [17]. Arabinoxylan of wheat bran has been proven to induce the maximum xylanase production by *Bacillus spp.* SPS-O [18]. Other possibility of lesser xylanase activity was, however, due to the synthesis of cellular materials using the agricultural residuals rather for xylanase synthesis. Agricultural residuals such as sawdust possessed relatively lesser xylanase activity by *A. brasiliensis* because of its composition that is majorly comprised of complex polymers of lignin which is highly unhydrolysed by xylanolytic enzymes. In this study, PKC as the least preferable carbon source was found to produce the lowest xylanase activity by *A. brasiliensis*. Ramanchandran et al. [19] stated that PKC possessed poor amino acid profile, lacking of important amino acids such as lysine, methionine and tryptophan that were critically used as the building blocks for enzymes synthesis. The low production of xylanase might also attributed to the fact that PKC contained mainly 78% polysaccharides mannas which were hard and highly crystalline, thus, it required alkaline or sodium chloride pre-treatment before it used as the solid substrate [20]. According to their results, PKC is composed of only 6% xylan and 18% crude fibre which are highly unhydrolysed by xylanolytic enzymes. Therefore, in the present study, wheat bran as the carbon source exerted tremendous positive influential impact on the xylanase production by *A. brasiliensis* under SsF. Our results suggested that xylan degradation in SsF stimulated the xylose production and thus, initiated the biomass production of *A. brasiliensis* when wheat bran as the agricultural residual was used as the main carbon source. Agricultural residuals have been reported to induce xylanase synthesis significantly [21]. Our findings again, suggested that the nature of the agricultural residuals play an important role in the regulation of enzymes especially xylanase synthesis, due to the differences in their molecular structures and chemicals composition. Thus, based on our results, wheat bran was determined to be the most optimum and preferable agricultural residuals for xylanase production by *A. brasiliensis* in SsF.

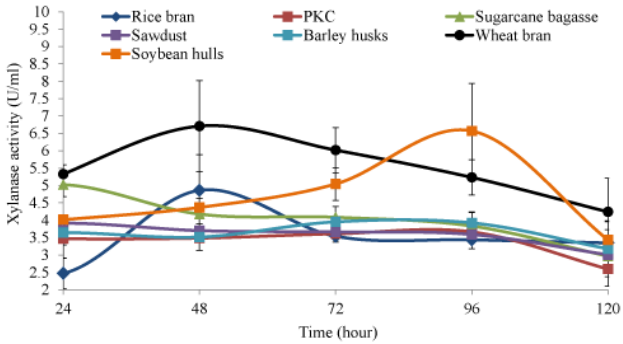

Figure 1: Time course of xylanase production by *Aspergillus brasiliensis* using different agricultural residuals as the carbon source in SsF.

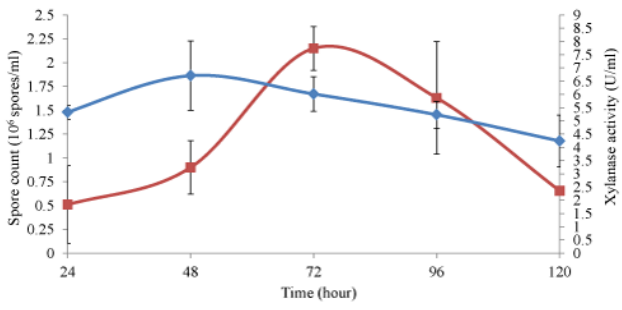

Figure 2: Correlation between xylanase activity and spore count of *Aspergillus brasiliensis* using wheat bran as the optimised carbon source in SsF. Symbols: spore count of *A. brasiliensis* (■) and xylanase activity (♦).

Soluble protein production from various agricultural residuals

Lowry method was used to determine the concentration of soluble protein excreted by *A. brasiliensis* during xylanase production in the present study. Based on our results in Figure 3, wheat bran exhibited the optimum soluble protein concentration of 18.3299 mg/mL at 48 h compared to the other agricultural residuals. Likewise, Okafor et al. [13] reported that wheat bran produced the optimum soluble protein concentration of 1.14 mg/mL by *A. niger* ANL 301 at 96 h. Practically, wheat bran was known to have vast nutritive values that aided in relatively high level of the soluble protein production by *Aspergillus spp*. It was suggested that soluble protein was utilised and breakdown by *Aspergillus spp* to form amino acids which undergo deamination process to produce ammonium ions that eventually became a source of nitrogen for the fungi to thrive and secret proteins [22]. In this study, the maximum soluble protein concentration produced from rice bran, PKC, soybean hulls, barley, sugarcane bagasse and sawdust showed comparatively lower than wheat bran, producing 15.6272, 12.1656, 13.8462, 10.5509, 8.8358 and 6.9404 mg/mL, respectively. In this respect, each of the agricultural residual in this study exerts the potential in producing relative amount of soluble protein. According to Hardini [22], the soluble protein concentration produced using rice bran as the carbon source was relatively high, producing 75.20% at 72 h by *A. niger* that was isolated from Food Research and Development Institute, Agricultural Department, Bogor. In the present study, soluble protein produced by *A. brasiliensis* using rice bran as the substrate was the second optimum after wheat bran. When rice bran was used as the carbon source, some hydrolytic enzymes were produced and secreted to breakdown rice bran into soluble protein for the growth of *A. brasiliensis*. Nonetheless, the soluble protein concentration from rice bran was seen to drop after 48 h of the SsF. It was probably because of the fungi utilised the soluble protein for others cellular activity.

On the other hand, in this study, sugarcane bagasse exhibited relatively lower soluble protein concentration than wheat bran. Likewise, Sasi et al. [23] reported that sugarcane bagasse was found to produce the lowest soluble protein concentration of 0.013 g/mL compared to rice bran and wheat bran by *A. flavus* that was isolated from Muthupettai mangrove, India. Wheat bran contained high quantity of fibre and sparsely concentration of nutrients whereas the complex lignin structure of sawdust restricted the utilisation of carbohydrates by *A. brasiliensis*, making it unattainable. Hence, it led to the nutrition exhaustion and ultimately lesser production of xylanase when compared to wheat bran in SsF. As a result, in this study, *A. brasiliensis* produced the lowest amount of soluble protein using sawdust in the medium of SsF. Sawdust that predominantly composed of lignin was surrounded by complex polymers of cellulose and therefore, making it unavailable to enzymatic degradation, hence, low xylanase activity was obtained [24].

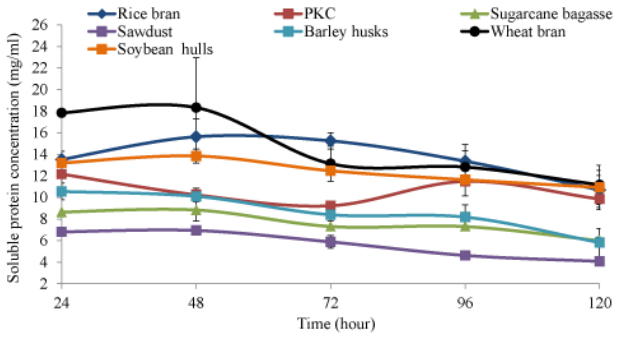

Figure 3: Time course of soluble protein production by *Aspergillus brasiliensis* using different agricultural residuals as the carbon source in SsF.

Medium pH profile from various agricultural residuals

The initial substrate medium pH has a great influence on the enzymatic reactions due to the metabolic activities of microorganisms. The initial medium pH was adjusted to 6.5 in this study. Based on our results, wheat bran showed the decrement of medium pH to 5.05 at 48 h as shown in Figure 4. On the other hand, the medium pH of sawdust, soybean hulls and barley decreased to pH 5.62, 5.07, 4.1 and 4.36 at 96 h, respectively. The medium pH of PKC, rice bran and sugarcane bagasse decreased to pH 5.62, 5.95 and 4.17 at 96 h. According to Highina et al. [25], enzymatic reactions are correlated with the medium pH. Enzymes are comprised of amino acid residues of basic, neutral or acid side groups that possess a particular charge either positive or negative depending on the medium pH.

Based on our results, low medium pH occurred during SsF was probably because of the secretion of xylanase into the medium in addition to the production of acidic metabolites such as citric acids by *A. brasiliensis* [26]. As a result, the medium pH of 5.05 was observed when the synthesis of xylanase exhibited its maximum peak at 48 h using wheat bran as the prime carbon source. In other words, xylanase was liberated out into the fermentation medium when the low medium pH occurred. Our results suggested that slightly acidic nature of xylanase was produced by *A. brasiliensis*. Indeed, this result also indicated that the maximum xylanase production was obtained at the imperceptible acidic medium pH. On the contrary, at high alkaline condition, xylanase synthesis was inactivated. According to Yang et al. [27], high level of xylanase production with more than 10,000 U/g was obtained by *Paecilomyces thermophila* J18 at the medium pH 5.0. On the contrary, the rise in medium pH after prolonged SsF suggested that the metabolic activities of fungi are halted due to the diminishing of nutrients in the medium resulted from the lower activity of xylanase. Moreover, the production of alkaline compounds by the microorganisms was also attributed to the alkaline condition in the medium pH. As the SsF prolonged, the medium pH was suggested to be continuously raised, the activity of xylanase was inhibited probably due to the denaturation and configuration changes of xylanase.

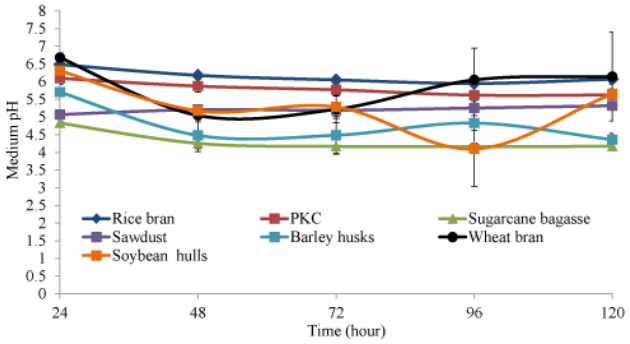

Figure 4: Medium pH profile of using different agricultural residuals as the carbon source for the xylanase activity and growth of *Aspergillus brasiliensis* in SsF.

Spore production from various agricultural residuals

Compared to the other agricultural residuals, the maximum spore production of 5.51×10^6 spores/mL was found to be the optimum at 96 h using soybean hulls in SsF as shown in Figure 5. Soybean hulls contain essential nutrients that lead to a surge in the biomass production [28]. According to Corredor et al. [29], soybean hulls liberated up to 72% of hexose sugars after pre-treatment using diluted acid and modified steam explosion system. Thus, in this study, hexose sugar of soybean hulls might assist in the growth of *A. brasiliensis*. Additionally, 70% of the protein content in soybean hull could also be attributed to the growth of fungi. In the present study, fairly high maximum spore production obtained from rice bran, wheat bran, barley, PKC, sawdust and sugarcane bagasse, producing 4.67×10^6 spores/mL at 72 h, 2.15×10^6 spores/mL at 72 h, 1.59×10^6 spores/mL at 72 h, 1.59×10^6 spores/mL at 72 h, 7.0×10^5 spores/mL at 72 h and 5.9×10^5 spores/mL at 72 h, respectively. According to Ravinder et al. [30] and Hoebler et al. [31], relatively high spore production by *A. niger* using rice bran and barley were detected as a result of high protein concentration in the medium. Likewise, Cavalcante et al. [32] reported that wheat bran was one of the preferable substrate for high spore production because it possessed better water retention, greater porosity and higher nutrients content. Wheat bran is abundant with nutrients that able to be absorbed in the fungi and also capable of retaining sufficient water for the fungi to flourish even in the SsF. On the other hand, Ramanchandran et al. [19] detected the lowest protein concentration using PKC due to the poor amount of essential amino acids including lysine, methionine and tryptophan. Consequently, this may be the reason why in this study, PKC was found to yield relatively lesser protein concentration and thus, lower spore count of *A. brasiliensis* when compared to the optimised soybean hulls. When the growth of *A. brasiliensis* was diminished, as a result, the xylanase activity was at the minimum, yielding only 3.6633 U/mL at 96 h of SsF.

In the present study, sawdust and sugarcane bagasse were the least preferable carbon sources for the growth of *A. brasiliensis*. Sawdust is comprised mostly of complex polymers of lignin whereas sugarcane bagasse majority are consisted of extremely inhomogeneous materials with 30 to 40% of unhydrolysed fibre which are derived from the core of plants. However, with the optimum pre-treatment using acid hydrolysis process, sawdust and sugarcane bagasse would be able to liberate sufficient nutrients for spore production [33]. Nonetheless, sawdust and sugarcane bagasse were not introduced to pre-treatment using acid, therefore, it may be the reason of low spore count in this study.

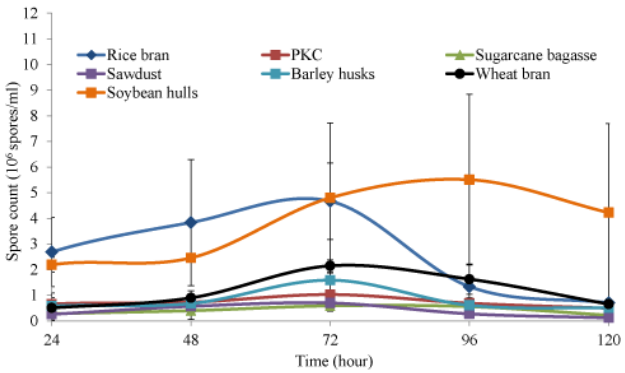

Figure 5: Growth profile of *Aspergillus brasiliensis* using different agricultural residuals as the carbon source in SsF.

Optimisation of the initial moisture content ratio of the optimum carbon source on xylanase production by *Aspergillus brasiliensis* in solid state fermentation (SsF)

The optimum initial substrate to moisture content ratio of 1:1 for the maximum xylanase production

Various initial substrate to moisture content ratio of 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 and 1:4 were investigated using wheat bran as the optimised carbon source for the maximum xylanase production by *A. brasiliensis* in SsF. The optimisation of initial moisture ratio of substrate in SsF is elucidated because it is one of the crucial parameters that commonly influenced the growth and xylanase production by *A. brasiliensis*. Since SsF is involved in the biological processes in which microorganisms grow on solid materials with limited moisture level, thus, the moisture content ratio of the substrate is a crucial factor which drastically influences the fermentation process of xylanase. Based on our results, the initial substrate to moisture content ratio of 1:1 using wheat bran as the optimised carbon source was the most suitable for the maximum xylanase production by *A. brasiliensis*, producing 6.7115 U/mL at 48 h of SsF as shown in Figure 6. The degradation of wheat bran using xylanase from *A. brasiliensis* to produce xylose as one of the important nutrients occurred gradually until it reached its maximum peak at 48 h to generate the maximum amount of spore at 72 h. Indeed, low initial moisture ratio of 1:1 using optimised carbon source was the most preferable for the significant activity of xylanase by *A. brasiliensis* under SsF in the present study. In this respect, the moisture content of the substrate is regulated by the capability of substrate to hold water content, the types of end-product and the characteristics of the microorganisms. The inter-particles nutrients transfer from the solid substrate to the growing microorganisms depend on the characteristic and moisture content of the substrate. It was suggested that when the moisture content is at the optimum level, it causes the swelling of the substrate, thereby, facilitating rapid absorption of nutrients from the substrate to the microorganisms. Some studies even showed the maximum production of xylanase by using lower moisture content ratio of substrate. According to Pal and Khanum [15], the optimum xylanase production was obtained from wheat bran using relatively low initial substrate to

moisture content ratio of 1:0.7, producing 2596 U/gds by *A. niger* DFR-5. In addition, Gessesse and Mamo [34] reported that the low initial moisture ratio of 1:1 was found to yield the optimum xylanase activity of 720 U/gds by *Bacillus spp.* AR-009. Likewise, Kavya and Padmavathi [35] also reported another low initial moisture ratio of 1:0.75 yielded the optimum xylanase activity of 9.38 U/mL by *A. niger* that was isolated from garden soil samples in Banglore, India. Therefore, we concluded that the best initial moisture content ratio for xylanase production in SsF was ranging from 1:0.7 to 1:1. Nevertheless, the reduction in enzymes synthesis occurred with higher and lower moisture contents than the optimal in the solid substrate are anticipated as the oxygen and mass transfers are not accessible to the microorganisms.

Relationship between the initial substrate to moisture content ratio with xylanase and spore production

Nonetheless, in the present study, using the initial moisture content ratio of 1:1 of wheat bran, *A. brasiliensis* was able to thrive exponentially, producing 2.15×10^6 spores/mL at 72 h of SsF. With the present of enriched oxygen concentration in the substrate, the spore production was observed to increase three folds [36]. There was a close linear relationship between the moisture content ratio of substrate and increase of spore production of *A. brasiliensis*. Low moisture ratio of substrate encourages the spore germination of fungi and thus stimulates the xylanase synthesis. This moisture ratio ensures the sufficient porosity of the substrate, and thus, creates the significant oxygen transfer to the fungi and encourages the removal of carbon dioxide, heat, water vapour and volatile components during the metabolic activity of fungi, which eventually leads to the increase in growth and xylanase production under SsF. Therefore, the increasing xylanase activity was more pronounced when low substrate to moisture ratio of 1:1 was used in the study. According to Hassouni et al. [37], the optimum spore germination rate was observed in all species of *Aspergillus* when 85% of initial moisture content ratio of substrate in SsF occurred. In addition, 65% of initial moisture content ratio was adequate enough to acquire full spore germination rate of *A. niger*. In fact, the optimum moisture content ratio for the growth of the microorganisms is governed by the characteristics of the microorganisms and types of substrate used for the growth. The moisture content ratio of solid substrate plays an important role in SsF because it influences the biosynthesis of xylanase, secretion of different metabolites including citric acid and growth of microorganisms. Nonetheless, the reduction in xylanase production at higher and lower moisture content ratio than the optimum occur as the result of substrate porosity involving the volume of pores space in substrate, changes in the structure of substrate particles size including the thickness of substrate layer and volume of the gas within the solid substrate. As a result, the decline in xylanase production may cause the retarded growth of the microorganisms, however, it is very much subjected to the selection of microorganisms, aeration rate of fermentation, quantity of carbon dioxide as well as the particular amount of oxygen needed for product synthesis. Aeration rate of fermentation is in fact, another important parameter that greatly affects the spore germination, besides the humidity of the fermentation medium. Indeed, Hassouni et al. [37] reported that with the optimum aeration rate and 98% of relative humidity, the spore germination was able to exhibit its optimum and also capable to minimise the incubation period for the maximum spore germination.

In this study, the second maximum xylanase activity of 6.4652 U/mL was obtained with the initial substrate to moisture content ratio of 1:1.5 followed by the ratio of 1:3.5, producing 6.3808 U/mL, 1:2, producing 6.2324 U/mL, 1:4, producing 6.1514 U/mL, 1:2.5, producing 5.4394 U/mL and 1:3, producing 5.0783 U/mL, respectively. Different moisture content ratio than the optimum could be unfavourable for the biomass production and xylanase activity by *Aspergillus spp.* When the much lower initial moisture content ratio of substrate than the optimum is used, the reduction in the solubility of nutrients from wheat bran occurs as a result of lesser swelling of the solid substrate, consequently, reducing the utilisation of nutrients by the fungi. On the contrary, the further increased of the initial moisture content ratio of carbon source than the optimum, it alters the morphology and texture of wheat bran by decreasing its porosity, hence, causing the interruption of the oxygen transfers to the fungi, forming the disturbance of heat and mass transmission through the culture and resulting the decrease of air exchange, which in turn, reduced the activity of xylanase as well as the microbial growth of fungi in SsF [38]. In the present study, using the optimum initial moisture content ratio of substrate in SsF, wheat bran is tended to swell incomparably and thus creates a larger surface for the hyphae of fungi to survive for the better absorption of nutrients from wheat bran and removal of heat from the fungi [39]. In addition, the effect of oxygen saturation at the optimised initial moisture ratio of substrate has the optimum impact on the xylanase as well as biomass production by *A. brasiliensis*. Oxygen possesses the essential functions in SsF; to supply for aerobic metabolism of fungi and to provide good ventilation in culture flask. Sufficient oxygen supply is one of the critical culture conditions for the maximum xylanase production in SsF.

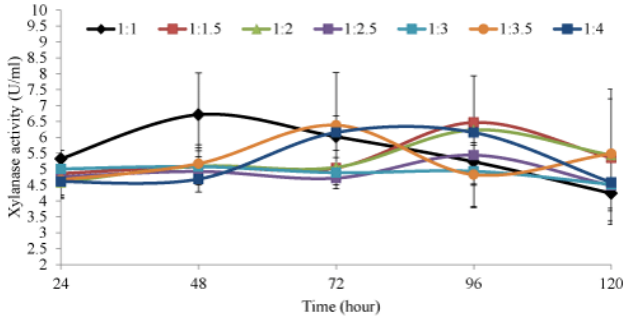

Figure 6: Time course of xylanase production by *Aspergillus brasiliensis* using different initial moisture content ratios of wheat bran as the optimised carbon source in SsF.

Soluble protein production from various initial substrate to moisture content ratios

On the other hand, when the initial substrate to moisture content ratio of wheat bran was set to 1:3, it was the most suitable for the optimum production of soluble protein by *A. brasiliensis*, producing 18.7110 mg/mL at 48 h as shown in Figure 7. Suitable initial moisture content level of substrate has been known to enhance the protein production. In this study, the maximum soluble protein concentration using the initial moisture content ratios of 1:1, 1:2.5, 1:2, 1:3.5, 1:1.5 and 1:4 were 18.3299, 18.122, 17.4636, 16.043, 15.4193 and 15.0693 mg/mL, respectively. On the contrary, the initial moisture ratio of 1:4

was observed to produce the lowest soluble protein concentrations. When the initial moisture ratio of substrate increased, it altered the morphology of wheat bran by reducing its porosity. The decrease of porosity in wheat bran which in turn, led to the weaker oxygen diffusion, thus poorer heat dissipation occurred and caused the denaturation of soluble protein in SsF [40].

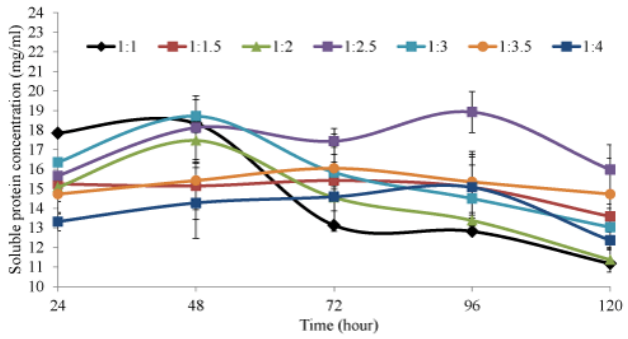

Figure 7: Time course of soluble protein production by *Aspergillus brasiliensis* using different initial moisture content ratios of wheat bran as the optimised carbon source in SsF.

Spore production from various initial substrate to moisture content ratios

The correlation between the xylanase activity and spore count using wheat bran to moisture content ratio of 1:1 is shown in Figure 8. Again as the xylanase activity was at its optimum at 48 h, more arabinoxylan and hemicelluloses from wheat bran were hydrolysed to produce vast amount of xylose to activate the growth of the fungi. Hence, the steep rise in spore production of *A. brasiliensis* was observed from 48 to 72 h. Nonetheless, due to the nature culture condition of SsF that was limited with free-moving water, therefore, xylose was not able to disperse homogenously in the culture flask. As a result, the fungi required longer time to consume xylose to achieve its maximum spore count. On the other hand, the initial moisture content ratio of wheat bran using 1:2, 1:2.5, 1:3, 1:4, 1:1.5 and 1:3.5 were observed to yield the maximum spore count of 9.3×10^5, 6.8×10^5, 6.4×10^5, 3.7×10^5, 3.2×10^5 and 3.1×10^5 spores/mL, respectively as shown in Figure 9. The initial substrate to moisture ratio of 1:3.5 was observed to produce the lowest spore count. In this respect, it is important to provide satisfactory moisture ratio in SsF because the over-supplied of moisture may lead to poor microbial growth and ultimately affect the biological and physiological activities including xylanase synthesis of *Aspergillus spp* [40]. Higher initial substrate to moisture ratio may alter the morphology of wheat bran by reducing its porosity which made it unfavourable to supply the essential nutrients for the growth of *A. brasiliensis*. In contrast, the lower initial moisture content ratio of the substrate causes the insufficient nutrients absorption by the fungi and therefore, creates the undesirable environment for the growth and xylanase synthesis in SsF.

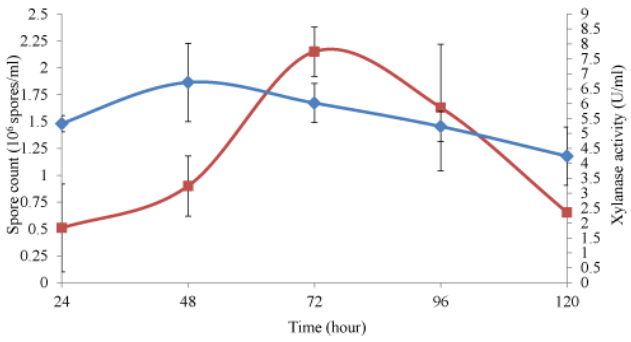

Figure 8: Correlation between xylanase activity and spore count of *Aspergillus brasiliensis* using the optimised substrate to moisture content ratio of wheat bran at 1:1 in SsF. Symbols: spore count of *A. brasiliensis* (▪) and xylanase activity (♦).

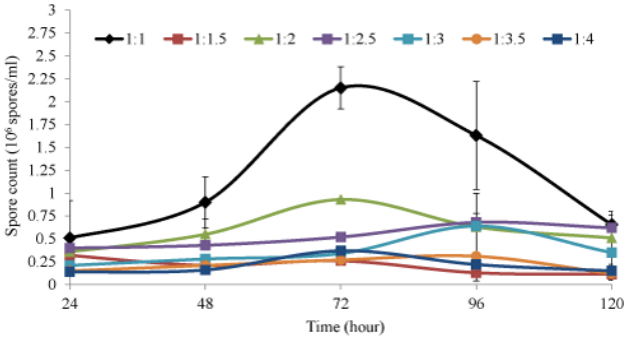

Figure 9: Growth profile of *Aspergillus brasiliensis* using different initial moisture content ratios of wheat bran as the optimised carbon source in SsF.

Medium pH profile from various initial substrate to moisture content ratios

There is a strong influence of initial medium pH on xylanase production. Based on our results, the initial medium pH 6.5 was decreased to 5.05 when the maximum xylanase activity of 6.7115 U/mL was achieved at 48 h using wheat bran with the initial substrate to moisture ratio of 1:1 as shown in Figure 10. When the initial moisture content of 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 and 1:4 of wheat bran were used, the medium pH were decreased to pH 5.69 at 120 h, 5.92, 6.28 and 5.63 at 48 h, 6.0 at 72 h and 5.74 at 96 h, respectively. *A. brasiliensis* utilised the nutrients of wheat bran for its optimum cellular growth, maximum enzymes and acids production when the optimum oxygen concentration and moisture content were achieved in SsF. The liberation of organic acids to the fermentation caused the formation of acidic condition [38]. In general, the optimal xylanase production was attained at the slight acidic medium pH. However, as the fermentation prolonged, the transition from acidic to alkaline condition might occur due to the gradual depletion of nutrients, besides the accumulation of unwanted toxic wastes, which made it unfavourable for *A. brasiliensis* to synthesize enzymes [41].

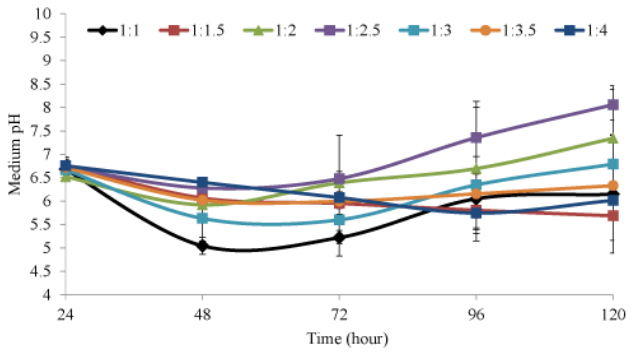

Figure 10: Medium pH profile of using different initial moisture content ratios of wheat bran as the optimised carbon source for the xylanase activity and growth of *Aspergillus brasiliensis* in SsF.

Optimisation of nitrogen source on xylanase production by *Aspergillus brasiliensis* in solid state fermentation (SsF)

Yeast extract as the optimised nitrogen source

To further optimise the xylanase production by *A. brasiliensis* in SsF, various organic nitrogen sources of yeast extract, peptone, urea and malt extract and inorganic nitrogen sources of $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and NH_4Cl were investigated to establish the optimisation of nitrogen source in enzyme production. Using the optimum initial moisture ratio of 1:1 of wheat bran as the optimised carbon source, yeast extract exhibited the optimum xylanase activity of 8.6382 U/mL at 48 h as shown in Figure 11. Nitrogen sources are generally supplied as the additional nutrients on the xylanase production. According to Pal and Khanum [42], yeast extract was notably improved xylanase activity by *A. flavus* DFR-6, producing 13.8 U/mL compared to the other nitrogen sources such as peptone, tryptone, skim milk, casein peptone, soy peptone, urea and beef extract. Based on our results, yeast extract contained most of the amino acids that were required for the growth of *A. brasiliensis*, followed by the optimum xylanase production. Likewise, Bajaj et al. [43] also revealed that yeast extract was the optimum nitrogen source for xylanase synthesis of 6500 U/mL by *Streptomyces spp.* SU9. Similarly, Muthezhilan et al. [44] reported that yeast extract produced the highest xylanase activity of 3.5 U/mL by *Penicillium oxalium* that was isolated from Pitcha-varam mangrove forest when compared to peptone, urea, sodium nitrate, ammonium sulphate, ammonium nitrate, meat extract and beef extract, respectively. In addition, Abbas et al. [45] observed by using yeast extract, the highest xylanase production occurred, producing 90 U/mL by *A. niger* that was isolated from soil. Likewise, another study by Nikhil et al. [46], they found out that when the millet bran was used as the carbon source, the optimum xylanase production of 1417.6 U/gds/min was obtained using yeast extract compared to 1395.1 U/gds/min using $(NH_4)_2SO_4$ as nitrogen source by *A. niger flavus* FPDN1. Surprisingly, in the present study, the production of xylanase increased with the addition of yeast extract in the medium up to 28.75%. The correlation between the xylanase activity by *A. brasiliensis* and spore count using yeast extract as the optimum nitrogen source is shown in Figure 12. Again, with the xylanase production attained at its maximum peak at 48 h, it resulted in the maximum production of spore by *A. brasiliensis* at 96 h. Yeast extract was detected as the optimum nitrogen source supplemented with wheat bran at the moisture ratio of 1:1 for the maximum production of xylanase by *A. brasiliensis* in SsF in this study. Based on our results, the maximum xylanase production using other nitrogen sources of urea, peptone, malt extract, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl and $NaNO_3$ were 8.4864, 8.0916, 6.4415, 6.2661, 6.1143, 5.3685 and 4.9602 U/mL, respectively. Apparently, organic nitrogen sources were found to accelerate the growth of *A. brasiliensis*, thus enhanced the xylanase activity compared to the inorganic nitrogen sources. Butt et al. [47] confirmed the organic nitrogen source of urea produced the optimum xylanase activity of 1790 U/g by *A. niger* GCB-15 compared to $(NH_4)_2SO_4$ and $NaNO_3$, respectively. Some studies even showed a very low activity of xylanase produced using inorganic nitrogen sources. In fact, Soliman et al. [48] reported that the inorganic nitrogen source of NH_4Cl and $(NH_4)_2SO_4$ yielded xylanase activity of only 20.2 and 29.7 U/g by *A. niger* and *Trichoderma viride* that were isolated from fermented rice straw, respectively. Notably, organic nitrogen sources possesses higher amount of available nitrogen components such as amino acids, peptides, vitamins and other nutrients in comparison with the inorganic nitrogen sources. Slaughter [49] described the significant lower production of xylanase in ammonium salts due to the ammonium ions that were not easily assimilated compared to the other amino acids. Therefore, low production of xylanase activity using inorganic nitrogen sources occurred as a result of the limited assimilation rate of ammonium ions by *A. brasiliensis* in this study. These findings revealed that the addition of organic nitrogen source is more effective in promoting the microbial xylanase synthesis, indicating the source of nitrogen should be organic for better results. On the contrary, the hydrolysed arabinoxylan and hemicelluloses from wheat bran complemented with the nutrients from yeast extract was the most optimum substrate for the xylanase production in SsF. Since xylan is unable to penetrate into the fungi, it is suggested that the low molecular weight degradation products of wheat bran such as xylose are absorbed into the cells to induce the production of xylanase and growth of the fungi.

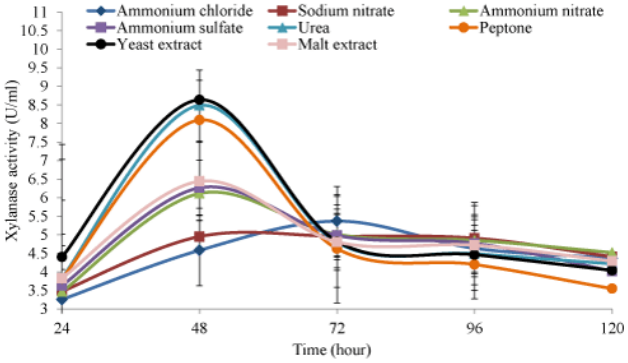

Figure 11: Time course of xylanase production by *Aspergillus brasiliensis* using different nitrogen sources combined with the optimised carbon source of wheat bran in SsF.

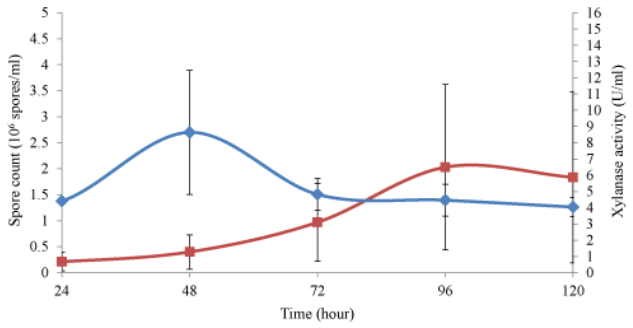

Figure 12: Correlation between xylanase activity and spore count of *Aspergillus brasiliensis* using yeast extract as the optimised nitrogen source in SsF. Symbols: spore count of *A. brasiliensis* (▪) and xylanase activity (♦).

Soluble protein production from various nitrogen sources

Using the optimum initial moisture ratio of 1:1, yeast extract exhibited the maximum soluble protein concentration of 23.0007 mg/mL at 48 h as shown in Figure 13. In this study, the maximum soluble protein production using malt extract, urea, peptone, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$ were 22.0617, 21.8607, 21.4241, 18.3229, 17.6369, 16.6424 and 15.9113 mg/mL, respectively. Yeast extract comprises of specific co-factors and amino acids that are essential for *A. brasiliensis* to excrete soluble protein. Absorption of organic nitrogen molecules are more energetically efficient than inorganic nitrogen, in fact, making it easier for the organic nitrogen sources to consume by microorganisms [50]. Likewise, Gundampati and Debnath [51] agreed that organic nitrogen sources have vast amino acids, vitamins, minerals and inconclusive growth factors to support the growth and enzymes production by *Aspergillus spp*. Relatively higher soluble protein production of 0.036 g/mL by *A. flavus* that isolated from Muthupettai mangrove, India obtained when the medium supplemented with yeast extract in rice bran compared to inorganic nitrogen source of $(NH_4)_2SO_4$ [23].

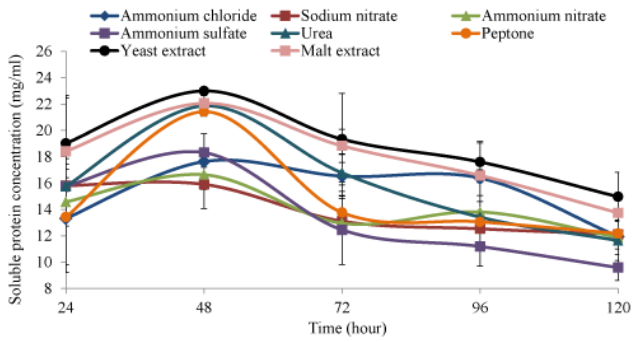

Figure 13: Time course of soluble protein production by *Aspergillus brasiliensis* using different nitrogen sources combined with the optimised carbon source of wheat bran in SsF.

Spore production from various nitrogen sources

Using the optimum initial moisture ratio of 1:1 of optimised wheat bran, yeast extract exhibited the optimum spore count of 2.03×106 spores/mL at 96 h as shown in Figure 14. In this study, the maximum spore count for $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, $NH4NO_3$, malt extract, urea and peptone were reported to be 7.1×10^5, 6.8×10^5, 6.6×10^5, 6.45×10^5, 4.35×10^5, 4.3×10^5 and 3.25×10^5 spores/mL, respectively. According to Oshoma et al. [52], yeast extract increased the spore count to 2.75 g/L by *A. niger* that was obtained from the culture collection of the Microbiology Laboratory of the University of Benin, Nigeria. Based on their findings, yeast extract contained some essential nutrients required for the optimum growth of *A. niger*.

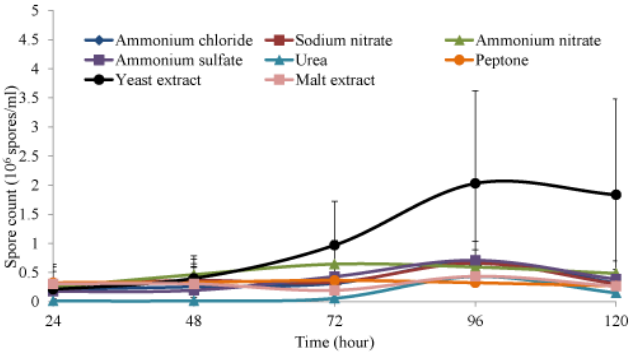

Figure 14: Growth profile of *Aspergillus brasiliensis* using different nitrogen sources combined with the optimised carbon source of wheat bran in SsF.

Medium pH profile from various nitrogen sources

In this study, the initial medium substrate pH was adjusted to pH 6.5. In Figure 15, the medium pH containing $NaNO_3$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and malt extract declined to pH 5.15, 4.85, 5.15, 5.09 and 5.12 at 72 h, respectively. The medium pH containing peptone was, however, decreased to 5.25 at 48 h whereas the medium pH of urea and yeast extract were dropped to pH 5.53 and 5.12, respectively. The nitrogenous salts decreased the pH of the culture medium by the formation of enzymes and acids that allowed *Aspergillus spp* to proliferate to an extent [53]. Thereafter, the pH of the medium was increased especially at 120 h, suggesting that the nitrogen sources in the culture medium were diminished after being utilised by *A. brasiliensis*. The diminishing of carbon and nitrogen sources in SsF was eventually led to the depletion of the biomass of *A. brasiliensis*. As a result, the production of enzymes and organic acids especially citric acid were reduced which in turn, resulted the increment in medium pH as the SsF prolonged [41]. Due to the limitation in oxygen and nutrients transfer as the SsF continued, it gave a negative impact on the proliferation of fungi and thus, the reduction of xylanase synthesis.

In a nutshell, *A. brasiliensis* exerts preference to grow and produce xylanase in slight acidic condition. The fungi possesses the growth capability, even it displays its limitation in the extreme acidic and alkaline conditions. This characteristic is advantageous to the fermentation process, whereby most of the microorganisms that responsible for the fermentation contamination are halted, thus, no

others foreign microorganisms were able to contaminate the culture at the acidic condition throughout the SsF.

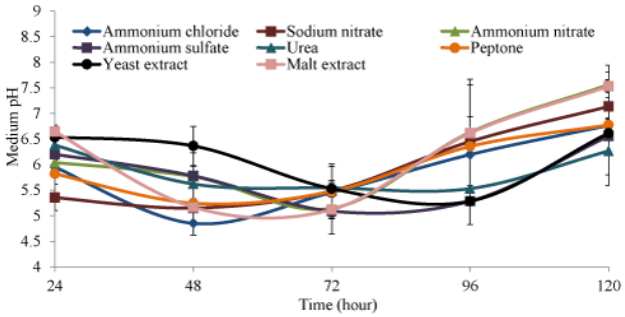

Figure 15: Medium pH profile of using different nitrogen sources combined with the optimised carbon source of wheat bran for the xylanase activity and growth of *Aspergillus brasiliensis* in SsF.

Optimisation of nitrogen concentration on xylanase production by *Aspergillus brasiliensis* in solid state fermentation (SsF)

2% yeast extract as the optimised nitrogen concentration

2, 4, 6, 8 and 10% of the yeast extract as the optimised nitrogen source were investigated for the optimisation of nitrogen concentration for the xylanase production by *A. brasiliensis* in SsF. Using the optimised wheat bran at the moisture ratio of 1:1, 2% yeast extract was observed to yield the highest xylanase activity, producing 8.6382 U/mL at 48 h as shown in Figure 16. In this study, the maximum xylanase activity obtained from 4, 6, 8 and 10% yeast extract were 2.0718, 1.4403, 1.6332 and 1.9773 U/mL, respectively. 2% yeast extract used in SsF excreted sufficient amino acids and proteins for *A. brasiliensis* to proliferate, hence higher xylanase activity was anticipated. Additionally, nitrogen source is crucial as the building blocks for the cellular proteins, besides utilised for the metabolic activity [54]. The correlation between the xylanase activity and spore count using 2% yeast extract is shown in Figure 12. The xylanase activity using 2% yeast extract at its highest peak was observed at 48 h. High xylanase activity resulted in large production of xylose which stimulated the growth of the fungi to achieve its maximum spore production at 96 h. In our study, lower and higher amount of yeast extract than the optimum decreased the production of xylanase. According to Ul-Haq et al. [55] and Xu et al. [56], the further increased of nitrogen concentration exhibited toxic effects on the growth of fungi. High amount of nitrogen supplemented medium promoted a rise in temperature and eventually lead to the denaturation of enzymes. Thus, in this study, higher than 2% yeast extract were observed to produce lower xylanase activity. The potential of overheating had been generated, as a result of excess concentration of nitrogen source used. Indeed, it initiated the amassing of wheat bran to form larger clumps in the culture flask during prolonged SsF. From this study, we concluded that nitrogen concentration especially 2% yeast extract has significant positive effect on the growth and xylanase production by *A. brasiliensis* in SsF.

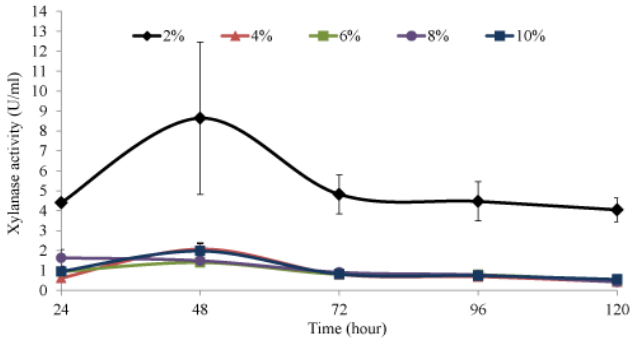

Figure 16: Time course of xylanase production by *Aspergillus brasiliensis* using different concentrations of yeast extract combined with the optimised carbon source of wheat bran in SsF.

Soluble protein production from various nitrogen concentrations

Using the optimised wheat bran at the moisture ratio of 1:1, 2% yeast extract was observed to yield the highest soluble protein concentration of 23.007 mg/mL at 48 h as shown in Figure 17. In this study, the maximum soluble protein concentration obtained from 4, 6, 8, and 10% yeast extract were 16.3548, 16.0603, 16.4484 and 17.9279 mg/mL, respectively. The soluble protein secretion occurs predominantly at the hyphal tips of the fungi [57]. High soluble protein concentration obtained in culture medium containing 2% yeast extract was probably due to the presence of other soluble proteins produced during xylanase synthesis by *A. brasiliensis* in SsF. Furthermore, *Aspergillus spp.* is generally known to produce some extracellular proteins such as cell-wall hydrolysing enzymes that degrade polymers of substrate for easy absorption of nutrients by the fungi [33].

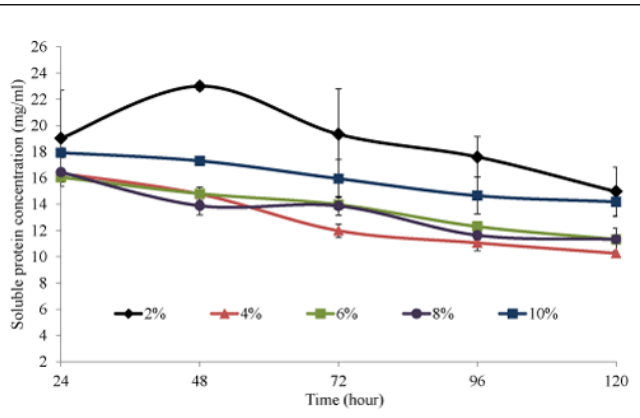

Figure 17: Time course of soluble protein production by *Aspergillus brasiliensis* using different concentrations of yeast extract combined with the optimised carbon source of wheat bran in SsF.

Spore production from various nitrogen concentrations

In this study, the maximum spore count of 2, 6, 8 and 10% yeast extract were 2.03×10^6, 8.20×10^5, 1.10×10^6, and 8.70×10^5 spores/mL,

respectively. Using the optimised carbon source, 4% yeast extract was observed to yield the highest spore count of 3.13×10^6 spores/mL at 72 h, however, it declined as SsF prolonged as shown in Figure 18. Supplementation of high concentration of nitrogen source in general, was used as a stimulus for the growth of *A. brasiliensis* at the early stage of fermentation. The nitrogen source was immediately utilised for the germination of spores, where high concentration of yeast extract was known to accumulate big amount of spores [57,58]. Nonetheless, as the SsF prolonged, the biomass production of *A. brasiliensis* reduced due to the insufficient mixing and aeration in the culture flasks that gradually led to the disturbance in the transfers and removals of heat, oxygen, carbon dioxide and nutrients in wheat bran even operated with agitation speed of 150 rpm. Oxygen deprivation was probably anticipated when the spores of *A. brasiliensis* and yeast extract were aggregated to form large sticky pellets at one particular site in the culture flask. Subsequently, it induced cell autolysis and eventually terminated the synthesis of enzymes.

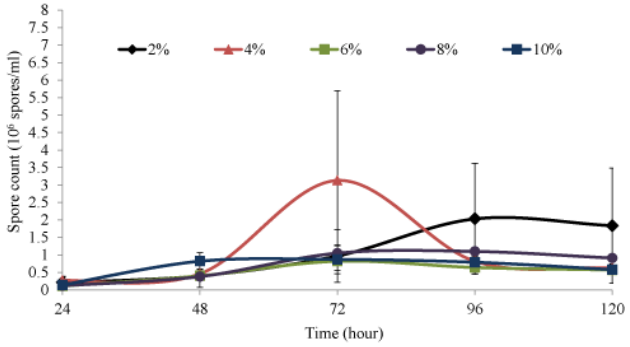

Figure 18: Growth profile of *Aspergillus brasiliensis* using different concentrations of yeast extract combined with the optimised carbon source of wheat bran in SsF.

Medium pH profile from various nitrogen concentrations

The initial medium pH 6.5 decreased depending on the concentrations of yeast extract in the medium as shown in Figure 19. Medium supplemented with 10% yeast extract was decreased to pH 5.87 at 48 h. Whereas, medium supplemented with 4, 6 and 8% yeast extract were decreased to pH 5.27, 5.45 and 5.48, respectively at 72 h. In general, all the medium pH was decreased to acidic condition with lower biomass production as SsF prolonged. On the other hand, medium supplemented with 2% yeast extract was decreased to pH 5.29 at 96 h from pH 5.87 at 48 h. Notably, the initial medium pH has a major influence on the production of enzymes. The rapid decrease of medium pH was possibly due to the utilisation of the sufficient amount of nitrogen source that led to the production of the extracellular metabolites such as enzymes and organic acids into the culture medium, forming the acidic condition in the culture flask [59]. On the contrary, the increase in medium pH at 96 h was the result of poor oxygen and heat transfers as SsF continued in batch fermentation in the culture flask, which in turn, causing a decline in production of biomass. Additionally, according to Upadhyay et al. [60], nitrogen supplementation was observed to increase the temperature of the substrate that eventually destroyed the mycelium and hence, reduced the spore production of fungi.

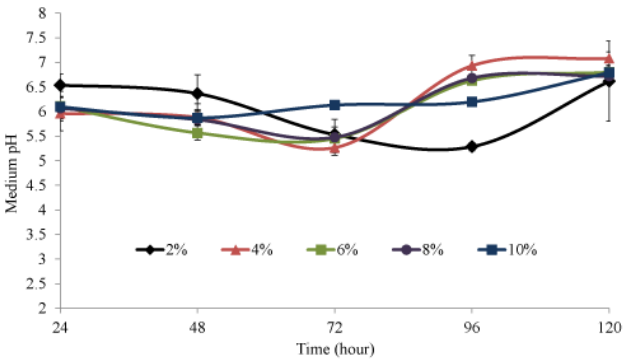

Figure 19: Medium pH profile of using different concentrations of yeast extract combined with the optimised carbon source of wheat bran for the xylanase activity and growth of *Aspergillus brasiliensis* in SsF.

Optimum production of fungal xylanase by solid state bioprocessing of wheat bran as the potential industrial substrate in the shorter production time of SsF

Xylanase is among the most important enzymes in the present day technology. Xylanase was commercialised worldwide, generating USD $200 million per annual [61]. Many methods have been established to overcome the overuse of chlorine as a bleaching agent in pulp and paper industry due to its toxicity to the environment. By replacing the chlorine with the enzymatic reaction using xylanase, it has been proven that xylanase was managed to reduce the bleaching cost with little to no capital investment [62]. As a result, it reduces the production cost of paper making. Additionally, the commercial xylanase used in pulp and paper industry was increased sharply in 3 years span. Thus, the production of xylanase is considerably as one of the important blooming enzyme manufacturing industries in the recent years. Therefore, the economic aspect of the fermentation process focusing on the substrate should be seriously taken into account for the xylanase production to be commercially applicable. Apparently, in the industrial point of view, it is very crucial to produce the maximum xylanase production using the lowest possible costs of production especially in batch SsF system. Our results indicated the suitability of using cheap and abundantly available agricultural residuals such as wheat bran as solid substrate in SsF. Agricultural residuals appear to be accumulated in the agro-industrial yards, leaving very less to zero significant uses in industry and commercial sectors, yet attributes to serious environmental disaster. Indeed, by utilising wheat bran which composed of carbohydrates, inorganic and organic nutrients, vitamins and minerals from by-product of agricultural industry, it is a reasonable alternative substrate for the maximum production of xylanase. This approach is notably economical and environmentally sound because it reduces the amount of agricultural wastes production. Furthermore, the maximum utilisation of this agricultural waste in SsF would also contribute more efficiency in solid waste management and recovery in long run. Thus, it would be very beneficial to maximise the usage of agricultural residuals in xylanase production. Therefore, we suggested that wheat bran to be used for large-scale production of xylanase in SsF system. Besides minimising the expensive costs of pure xylan and reducing the costs of dewatering system, SsF with agricultural residuals as the

carbon source would remarkably result in the shortest production time possible and thus, lessen the risk of contamination, making SsF, a promising technology for xylanase production in the future. Interestingly, based on our findings, the maximum xylanase synthesis of 8.6382 U/ml was attained in the shorter fermentation time of 48 h under SsF compared to other studies which required 96 h of SsF for the maximum production of xylanase by *Arthrobacter spp* [63]. According to Seyis and Aksoz [64], the costs of production have a linear relationship with the production time. Therefore, to minimize the production costs, xylanase should be produced in the shortest possible time. As a result, it is exciting to report that *A. brasiliensis* in our study was anticipated to produce the maximum production of xylanase at much shorter fermentation time if the scaling-up of industrial SsF is performed.

Conclusion

This study was focused on the elucidation of different medium formulation for the optimisation of xylanase activity by *A. brasiliensis* in SsF under the optimised growth conditions of initial medium pH 6.5 at the incubation temperature of 30°C with the agitation speed of 150 rpm. The outcomes of this study revealed that wheat bran as one of the agricultural residuals was the optimum carbon source for the maximum xylanase activity by *A. brasiliensis*, producing 6.7091 U/mL as a result of rapid utilisation and absorption of hydrolysed arabinoxylan and hemicelluloses in SsF. On the contrary, PKC was likely to be the least preferable solid substrate because of their poor xylanase synthesis. On the other hand, different initial substrate to moisture content ratios were evaluated and our results indicated that 1:1 was the optimised moisture content ratio in wheat bran for the xylanase activity by *A. brasiliensis*, producing 6.7115 U/mL. In order to enhance higher xylanase synthesis by *A. brasiliensis*, the supplementation of nitrogen source in the medium was necessary during SsF. Interestingly, when yeast extract was used, relatively higher xylanase activity was attained, producing 8.6382 U/mL. In fact, the concentration of yeast extract also influenced the activity of xylanase in this study. When 2% yeast extract was used, it attributed to much higher activity, producing 28.75% more compared to the medium without yeast extract. In conclusion, the optimum medium formulation and culture conditions for xylanase activity by *A. brasiliensis* under SsF was achieved using 10 g of wheat bran as the optimum carbon source with the moisture ratio of 1:1, supplemented with 2% yeast extract as the optimised nitrogen source grown at the optimum culture conditions adjusted with the initial medium pH 6.5 at 30°C at 150 rpm up to 48 h.

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