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Xylanase Production by *Bacillus subtilis* Using Carbon Source of Inexpensive Agricultural Wastes in Two Different Approaches of Submerged Fermentation (SmF) and Solid State Fermentation (SsF)

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Abstract

Xylanase has become an attractive enzyme due to its enormous economical roles especially as bio-bleaching agent in pulp and paper industry. Thus, in order to reduce the cost of production, the cheapest alternative carbon source is desirable under submerged (SmF) and solid state fermentation (SsF). Therefore, the objective of the study is to involve the use of two approaches of SmF and SsF to identify their potential ability on the production of xylanase by *Bacillus subtilis* ATCC 6633 using sustainable cost effective agricultural wastes to replace the expensive xylan as the prime carbon source. Seven defined, undefined and minimal Medium A to G in SmF were investigated to determine the optimum medium formulation. Thereafter, the carbon source was replaced with various agricultural wastes in SmF and SsF, respectively. On the other hand, the replacement of xylan with agricultural wastes as the alternative carbon source in SmF and SsF is particularly essential in industrial production. Based on our results, xylanase activity of 11.099 ± 1.127 U/mL was detected from the undefined Medium F. Nonetheless, higher xylanase activity of 11.646 ± 4.163 U/mL was obtained after the carbon source was replaced with barley husk in SmF. Notably, when wheat bran was substituted in SsF, 2.50×10^9 cells/mL of biomass concentration and 22.071 ± 0.186 U/mL of xylanase activity were obtained at 48 h of fermentation. These findings successfully displayed significant potential of scaling up for industrial xylanase production using inexpensive agricultural wastes in both approaches of SmF and SsF. In the nutshell, the production of xylanase using agricultural wastes of barley husks and wheat bran as the alternative carbon and energy source in SmF and SsF was more economically advantages and environmentally conscious than the use of expensive xylan substrate in term of lowering the costs of capital and operation in the industrial point of view.

Keywords: *Bacillus subtilis*; Xylanase activity; Xylan; Agricultural wastes; Submerged fermentation (SmF); Solid state fermentation (SsF)

Introduction

Nowadays, numerous types of commercial enzymes have been introduced to the market. Xylanase is one of the existing enzymes that have been focused on the research and development due to its potential values in various industries especially pulp and paper, baking, food and beverages, textile and animal feeds production [1]. Bacterial species such as *Bacillus subtilis* is one of the preferable microorganisms in the production of xylanase according to Annamalai et al., [2], Harbak and Thygesen [3] and Sa et al., [4]. In fact, xylanase produced from bacteria with genus *Bacillus* reported by Archana and Satyanarayana [5] exerts higher thermostability which is beneficial as bio-bleaching agent in the pulp and paper industry.

Xylanase production by *B. subtilis* is enhanced by replacing carbon source with sustainable and cost-effective lignocelluloses or agricultural wastes. The main components of agricultural wastes are consisted of 30-40% cellulose, 20-40% hemicellulose and 20-30% lignin that are able to contribute to massive production of xylanase. Indeed, lignocellulose wastes are widespread and found abundantly in nature, thus, they are the largest renewable and fermentable carbohydrates; therefore, making use of them in various industries is of great interest [6]. Due to their similar structure polymers as xylan, these lignocellulose wastes are suitable to use as the prime carbon source for xylanase production. Xylanase is important in the bioconversion of hemicelluloses into their constituent sugars such as xylose. Xylan is the major structural polysaccharides in plants which belonged to the group of hemicelluloses. Nonetheless, the use of pure xylan is expensive as carbon source for the industrial production of xylanase. Therefore, the replacement of pure xylan substrate with inexpensive lignocellulose residuals is greatly favorable to produce xylanase especially in large scale production. For commercial applications, xylanase should be able to produce rapidly in large quantities. Natural xylan sources such as sugarcane bagasse, wheat bran, rice bran and corn cob that are abundantly available in many countries are the potential raw materials used as the carbon source for xylanase production [7]. Only a few studies on the optimisation of carbon sources for the maximum xylanase production by bacteria have been conducted. The application of using agricultural wastes as the carbon source for the industrial xylanase production by *B. subtilis* was scarce and not comprehensively studied, compared and reported in submerged fermentation (SmF) and solid state fermentation (SsF), although these agricultural residuals are simple and cost-effective to yield xylanase. By reusing and recycling these organic agricultural wastes, converting these wastes into resources, it become better alternative replacement as the carbon source for industrial xylanase production. In fact, the application of agricultural wastes aids in reducing the cost of production besides providing enormous amounts of nutrients for the growth besides acting as the inducer for xylanase production. Therefore, the objective of this study is to compare and determine the optimum medium formulation using different agricultural wastes as the carbon source for the production of

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xylanase by *B. subtilis* in SmF and SsF. The cost-effective production of xylanase using agricultural wastes is greatly importance in this study.

Materials and Methods

Bacteria strain and inoculum preparation

Bacillus subtilis ATCC 6633 was subcultured on nutrient agar and incubated at 37°C. Cell suspension was prepared and cell count was determined using Haemocytometer. Serial dilutions were conducted to obtain cell suspension of 1×10^8 to use as the standard inoculum size for the growth of *B. subtilis* in 250 mL culture medium.

Optimisation of medium formulation on xylanase production by *B. subtilis* in SmF

To elucidate the potential use of agricultural wastes as the optimised carbon source, the optimisation study of medium formulation on xylanase production was conducted in stepwise manner. This study was started with the determination of medium formulation followed by optimisation of carbon source using different agricultural wastes in SmF and SsF, respectively. To begin, there are three different types of medium including defined, undefined and minimal Medium A to G were examined in 250 mL working volume in 500 mL culture flask to determine the optimum medium formulation in SmF. The composition of defined Medium A was consisted of (g/L): glucose, 2.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2; Na_2CO_3, 5; $(NH_4)_2SO_4$, 0.5; NH_4NO_3, 0.5; $Na_2HPO_4.12H_2O$, 0.5 and KCl, 0.5. On the other hand, defined Medium B from Sa et al., [8] was used and consisted of (g/L): glucose, 2; yeast extract, 2; $Na_2HPO_4.12H_2O$, 1; $MgSO_4.7H_2O$, 0.5; Na_2CO_3, 1; $CaCO_3$, 2 and NaCl, 1. Undefined Medium C used in this study was consisted of (g/L): rice flour, 5; peptone, 10; yeast extract, 5; NaCl, 3; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.2 and Na_2CO_3, 10. Undefined Medium D was consisted of wheat flour, 20; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.1. The minimal Medium E from Rusli et al., [9] was used and comprised of (g/L): glucose, 1; NH_4Cl, 1; KH_2PO_4, 3; $NaHPO_4.2H_2O$, 6 and $MgSO_4.7H_2O$, 1. On the other hand, Medium F was comprised of (g/L): wheat bran, 20; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.1. Likewise, Medium G was consisted of (g/L): rice bran, 5; peptone, 10; yeast extract, 5; NaCl, 3; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.2 and Na_2CO_3, 10. The entire medium was adjusted to pH 6.5 before autoclaved at 121°C for 15 min. Thereafter, 1×10^8 cells of standard inoculum size was inoculated into culture medium and incubated at 37°C at 150 rpm for 96 h. Once the optimised medium formulation was determined, then, the carbon source from the optimised medium formulation was replaced with different agricultural wastes in the subsequent experiments to elucidate the maximum xylanase production in SmF and SsF, respectively.

Optimisation of carbon source using agricultural wastes on xylanase production by *B. subtilis* in SmF

The carbon source of the optimised medium formulation determined from Medium A to G obtained from the earlier experiments was replaced with different agricultural wastes including wheat bran, rice bran, soybean hull, barley husk, palm kernel cake, pineapple skin, corn cob and combined wheat bran with soybean hull at ratio 1:1 (w/w), respectively. Pure xylan from beechwood was also elucidated as the carbon substrate for the xylanase production as the control of this study in SmF. For the pre-treatment of agricultural wastes, these substrates were dried at 80°C in oven until their constant weights were achieved. Thereafter, these agricultural wastes were crushed, sieved and autoclaved separately at 121°C for 15 min in 250 mL working volume after their pH of the medium was adjusted to 6.5. The standard inoculum size of 1×10^8 cells was used to inoculate to the medium before incubated at the optimum growth conditions of 37°C at 150 rpm for 96 h.

Sampling analysis and extraction of crude extracellular xylanase from SmF

5 mL of samples of SmF were withdrawn from the culture flasks at 12 h interval throughout 96 h of fermentation. Then, they were used for cell count and medium pH measurement. On the other hand, the clarified supernatant obtained after centrifugation at 10,000 rpm for 15 min was used for extracellular xylanase activity and protein assays.

Optimisation of carbon source using agricultural wastes on xylanase production by *B. subtilis* in SsF

To elucidate the optimised carbon source for the maximum production of xylanase by *B. subtilis* in SsF, the carbon source of the optimised medium formulation obtained from the earlier experiments was replaced with different agricultural wastes of wheat bran, rice bran, soybean hull, barley husk, palm kernel cake, pineapple skin, corn cob and combined wheat bran with soybean hull at ratio 1:1 (w/w). These substrates were then dried at 80°C until their constant weights were achieved. Subsequently, they were crushed using an electric blender to homogenise into smaller residual particles before sieved to obtain the substrates with consistency in size and moisture content. Agricultural wastes were autoclaved at 121°C for 15 min separately. Then, sterile water with the optimum pH 6.5 was added in the culture flask to achieve the substrate to moisture content ratio of 1:1 (substrate: water) (w/v) in 500 mL culture flask under SsF. The culture flask was then covered with a non-absorbent cotton wool to prevent evaporation and contamination. After inoculated with 1×10^8 cells, the culture flask of SsF was carried out at 37°C at 150 rpm for 96 h. All of the experiments in SsF were carried out in the similar growth conditions as SmF except for the moisture content ratio which was set to 1:1 (substrate: water) (w/v) in the study of SsF.

Sampling analysis and extraction of crude extracellular xylanase from SsF

In order to extract the extracellular xylanase in SsF, 5 mL of sterile distilled water was added and mixed homogenously with the substrate in the culture flask. Thereafter, the liquid sample was withdrawn, leaving the agricultural wastes in the culture flask for the rest of the fermentation. Then, the liquid sample was used for cell count and measurement of medium pH. Subsequently, the remaining sample was centrifuged and the clear supernatant was subjected to xylanase activity and protein assays.

Xylanase activity and protein assays

Xylanase activity was measured according to Bailey et al., [10] using 3, 5-dinitrosalicyclic (DNS) assay. 1% beechwood xylan was dissolved into 0.05 M sodium phosphate buffer (pH 5.3) at 50°C. 0.1 mL of the supernatant was added into 0.9 mL of 1% xylan substrate in 0.05 M sodium phosphate buffer (pH 5.3) and incubated at 50°C for 30 min. 1.5 mL DNS was added into the mixture and incubated at 90°C for 5 min. Thereafter, to terminate the reaction, 0.5 mL of 40% Rochelle salt was added and allowed to cool down at room temperature. The amount of xylose released by the reaction is determined by measuring its absorbance at 575 nm. The activity of xylanase was measured according to the xylose standard curve. In this study, to quantify the xylanase activity, one unit (U) of xylanase activity is defined as the

amount of enzyme required to release one µmole of xylose per mL of enzyme extract in SmF and SsF per min under assay condition. On the other hand, the soluble protein produced by *B. subtilis* during xylanase activity was determined according to Lowry et al., [11] using Bovine Serum Albumin (BSA) as the protein standard. The absorbance reading of protein assay was measured using a spectrophotometer at 750 nm.

Quantification of biomass concentration and medium pH

The cell concentration of *B. subtilis* was calculated using Haemocytometer. The pH of the culture medium was measured using pH meter to study the profile of medium pH during the growth and production of xylanase by *B. subtilis* in SmF and SsF, respectively.

Data analysis

In this study, all of the experiments on the xylanase production under SmF and SsF were conducted in duplicate. The mean values of the sampling analysis including xylanase activity, protein assay, biomass concentration and medium pH that generated from the experiments were used to elucidate the production of xylanase by *B. subtilis* under SmF and SsF, respectively. The means were represented on all the Figures in this study.

Results and Discussion

Optimisation of medium formulation on xylanase production by *B. subtilis* in SmF

Every microorganism requires suitable and sufficient nutrient for growth and other cellular processes. Total of seven types of defined, undefined and minimal medium in SmF were used in the present study to determine the optimum medium formulation for the maximum xylanase production by *B. subtilis* prior to the replacement of carbon source with various agricultural wastes. Significant difference in term of the xylanase activity was observed from the different types of medium in the study. Based on the trend of xylanase activity obtained from Medium A to G, the maximum xylanase activity was detected between 48 to 72 h as shown in Figure 1. Based on our results, Medium F was identified as the optimum medium formulation for *B. subtilis* to produce the maximum xylanase activity of 11.099 ± 1.127 U/mL at 96 h in SmF even though relatively high xylanase activity of 10.985 ± 0.560 U/mL was already detected at 72 h of SmF. A tremendous rising trend was observed starting from 12 to 96 h using wheat bran in Medium

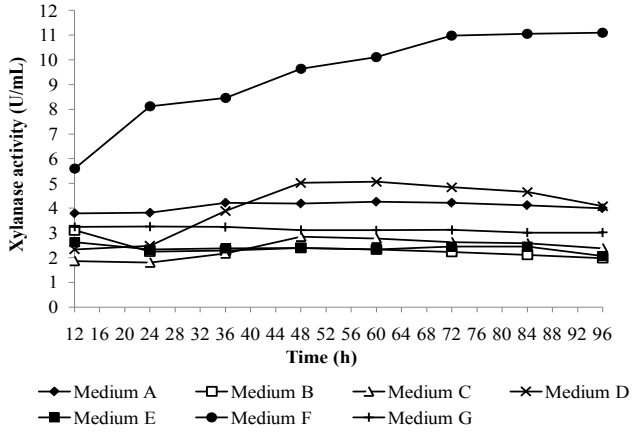

Figure 1: Time course of xylanase production by *B. subtilis* using different medium formulation in SmF.

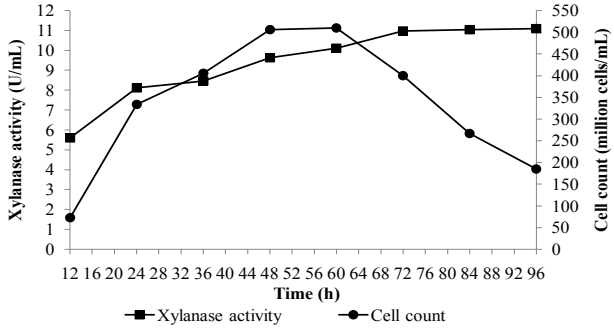

Figure 2: Correlation of xylanase activity and cell count of *B. subtilis* using the optimised medium formulation of Medium F in SmF.

F where about 20 to 40% of hemicelluloses in wheat bran enhanced the production of xylanase. On the contrary, insignificant increase of xylanase production was observed in the other medium as compared to Medium F. The highest xylanase yield by Medium F was most probably due to the affinity of *B. subtilis* to this medium as compared to the other medium. Medium F is the medium categorised as the undefined medium which consists of complex nutrients. The maximum yield of xylanase production in Medium F was due to the presence of wheat bran as the carbon source which mostly consisted of hemicellulose and cellulose components.

The correlation of xylanase activity using Medium F with its biomass production of *B. subtilis* is shown in Figure 2. Medium F exhibited the highest cell count of 5.10×10^8 cells/mL at 60 h, while the highest xylanase activity of 11.099 ± 1.127 U/mL was also detected at 96 h. The production of xylanase was excreted abundantly at the end of stationary phase proving that xylanase was not needed during the initial stage of cell growth in Medium F in SmF. In this respect, the interaction between growth metabolism and enzyme secretion is often critically influenced by growth limiting nutrient concentrations. As a result, medium formulation with the critical components should be optimised to produce the maximum yield of enzymes and minimal yield of undesired products. In these regards, our data showed a range of positive main effect values, indicating that the presence of yeast extract and peptone in the growth medium positively affects the production of xylanase. Thereby, we suggested the combination of yeast extract and peptone as the nitrogen source was anticipated to be the xylanase inducer for *B. subtilis* to produce more activity. This was supported by Nagar et al., [12] stated that organic nitrogen including yeast extract and peptone produced higher xylanase activity of 448.99 and 348.47 U/mL compared with inorganic compounds such as KNO_3, $(NH_4)_2SO_4$ and $NaNO_3$. Additionally, Battan et al., [13] also reported that combination of yeast extract and peptone resulted in higher xylanase production. Moreover, Sanghi et al., [14] reported the release of ammonium ion from peptone stimulated growth of *B. subtilis* ASH, thus producing higher xylanase activity in SmF. According to Sanghi et al., [14], the combination of yeast extract and peptone as nitrogen source stimulated *B. subtilis* ASH to produce higher xylanase activity of 413 U/mL compared to single nitrogen source of yeast extract and peptone which produced 380 and 362 U/mL, respectively. Likewise, Subramaniyan et al., [15] also reported the highest xylanase production was obtained by *Bacillus sp* in a medium consisting of yeast extract and peptone at 0.25%. In these regards, the optimum medium formulation with essential growth limiting nutrients is an important operation mode to optimise and increase the productivity especially xylanase.

On the other hand, Medium D, A, G and C possessed xylanase activity of 5.068 ± 2.065, 4.255 ± 0.118, 3.256 ± 0.003 and 2.848 ± 0.918 U/mL, respectively. Lower xylanase activity observed from the rest of the medium was probably due to the different composition of the medium that were less favorable by *B. subtilis*. 5.068 ± 2.065 U/mL of xylanase activity at 60 h was observed from Medium D, which possessed the second highest after wheat bran in Medium F. Medium A exerted the closest maximum xylanase as Medium D, which was 4.255 ± 0.118 U/mL at 60 h. In contrast, Medium E possessed relatively lower xylanase activity of 2.396 ± 0.101 U/mL at 48 h. Medium B which categorised as the defined medium possessed the lowest xylanase activity of 2.389 ± 0.013 U/L. Glucose as the carbon source in Medium A has negative effect on the xylanase production. Due to the absence of hemicelluloses from plant materials as carbon source, *B. subtilis* in Medium A was not facilitated to produce sufficient xylanase activity. We recommended that when glucose was used as the sole carbon source, it was preferentially consumed to support the initial microbial growth. Repression by glucose is common for catabolite extracellular enzymes by some microorganisms. Thus, glucose was not suitable to use as the carbon source for xylanase production. Most journals have been cited of using wheat bran as the optimum carbon source for xylanase production. However, these authors did not provide details of the process. From our study, we suggested two independent pathways that might involve in the growth and xylanase production. Extracellular xylanase is controlled by catabolite repression. Catabolite repression caused by glucose is more dominant in glucose-containing medium compared to medium with wheat bran in our study. Glucose exerts a repression effect on xylanase expression where glucose is preferentially used for growth of *B. subtilis*, while wheat bran is acting as the xylanase inducer. When the catabolite repression overrides the induction of xylanase in *B. subtilis*, as a result, lower xylanase production is observed in medium containing glucose. Nonetheless, when the absence of glucose occurs, it terminates catabolite repression, thus promoting better xylanase activity especially in undefined medium containing wheat bran. Repression by glucose for catabolite extracellular enzymes including xylanase by some microorganisms is rather common. Hence, we infer that growth and xylanase synthesis by *B. subtilis* are possibly regulated independently by different pathways as proven in this study when the microorganism was cultured using glucose and wheat bran. Moreover, the chemical compositions in Medium A have added up the costs of operation and production which would be expensive in the industrial scale of xylanase production.

On the other hand, Medium E which was categorised as the minimal medium provided minimal nutrient for the growth of *B. subtilis* without the presence of amino acid. Medium E which consisted of glucose and some inorganic compounds were more favorable in culturing specific lines of auxotrophic recombinants instead of enzymes production. Similarly, Medium B that displayed the lowest activity was probably due to the presence of glucose as the simple carbon source ready for growth and reproduction of bacteria without facilitating secretion of xylanase from *B. subtilis*. Based on our results, the optimised medium formulation by *B. subtilis* that produced the highest xylanase activity was detected to be the undefined Medium F. With nearly half of wheat bran in Medium F comprised of hemicellulose materials supplemented with the presence of amino acids as the organic nitrogen source under optimal growth conditions are suitable to promote more xylanase activity in SmF. Medium A to F exhibited certain effect on the biomass production. Maximum cell count of all seven medium was observed between 48 to 72 h, respectively. The maximum cell count of 7.73×10^8 cells/mL was determined from Medium D at 48 h, followed by 5.53×10^8, 5.10×10^8, 4.53×10^8, 3.69×10^8 and 3.45×10^8 cells/mL obtained from Medium B, F, C, A and G between 48 to 72 h. However, the lowest cell count of 5.8×10^7 cells/mL at 36 h was observed from Medium E. In order to determine the soluble protein production by *B. subtilis* in the samples during xylanase production, Lowry method was used. Indeed, the maximum protein concentration of 0.013±0.0007 g/mL was observed at 24 h from Medium G. Medium D and F demonstrated similar protein concentration of 0.011 ± 0.003 and 0.010 ± 0.003 g/mL at 36 h. Additionally, Medium B exerted 0.0024 ± 0.00001 g/mL at 24 h. The lowest protein concentration was observed from Medium A.

On the other hand, the highest pH of 8.73 ± 0.015 at 96 h was achieved in Medium C followed by pH 7.75 ± 0.072 at 96 h in Medium F during the maximum production of xylanase. Medium G, B, F, A and D exhibited high medium pH of 8.69 ± 0.12, 8.49 ± 0.17, 7.75 ± 0.072, 7.52 ± 0 and 7.20 ± 0.09 at 96 h, respectively. However, the lowest pH of 6.39 ± 0.035 was exhibited from Medium E at 36 h. Bisaria and Ghose [16] stated higher medium pH was favored for bacterial xylanase production. Medium pH at the beginning was gradually shifted to higher pH to become slightly alkaline. Similar pH trend shifted towards slight alkaline condition was also observed by Kapoor et al., [17]. In fact, the maximum xylanase activity was exhibited at pH from 6 to 8 in this study. Similarly, Battan et al., [13] reported that there was no bacterial xylanase production at pH 4, however, xylanase activity started only when pH changed to 5 and achieved its maximum activity at alkaline condition at pH 8. In fact, our result indicated similarity where the maximum xylanase activity of 11.099 ± 1.127 U/mL from Medium F occurred at pH 7.75 ± 0.072 at 96 h. Similar result was also reported by Battan et al., [13]. Even though, Medium B possessed high medium pH of 8.49 ± 0.17, but the maximum xylanase activity was considerably the lowest among all because when glucose was used as the carbon source, it did not facilitate the synthesis of xylanase in the medium because there was no hemicellulose and cellulose components found in the medium. Instead, it promoted the growth of *B. subtilis*.

Optimisation of carbon source using agricultural wastes on xylanase production by *B. subtilis* in SmF

Majority of the previous studies on xylanase production were concentrated mainly on the enzyme activity and characterization rather than economical aspect of the fermentation process. Nonetheless, the economics of the production should also be taken into account for commercial applications in various industries especially in pulp and paper. Indeed, the use of xylan would be expensive to produce this xylanolytic enzyme. Therefore, more researches should be carried out, focus on the economical production of xylanase. There is limited number of studies on this subject found in the literature, as a result, in this study, medium formulation of Medium F which showed the highest xylanase activity from the earlier experiment was selected by replacing its carbon source with low-cost agricultural wastes. High xylanase production using inexpensive and abundantly available agricultural wastes as carbon source are important to various industries. Agricultural wastes as an economical carbon source for the fermentation process are composed of organic and inorganic nutrients and vitamins as well as carbohydrates. For that reason, xylanase production by *B. subtilis* using different types of agricultural wastes including wheat bran, rice bran, soybean hull, barley husk, palm kernel cake, pineapple skin, corn cob and combined wheat bran with soybean hull at ratio 1:1 (w/w) were compared with xylan in this study. Carbon source and concentration in the medium formulation appear to exert a profound effect on the xylanase production behavior of bacteria as it is one of the crucial elements of the microbial fermentation medium

with the major role on the overall growth and metabolism. Thereby, optimal carbon source and concentration are of great importance to achieve the optimum quantity of xylanase in fermentation process. In this respect, each agricultural waste was added into the medium at a concentration of 20 g/L according to the medium formulation of the optimised Medium F. Xylan extracted from beech wood was used as the control of the study in SmF.

Different types of xylan as sole carbon source have been investigated in previous studies as the substrate to induce xylanase production. Beechwood xylan was used as the substrate in xylanase production by *Bacillus thermoleovorans* K-3d and *Bacillus flavothermus*, respectively [18]. Likewise, birchwood, larchwood and oatspelt xylan were used in the study of xylanase production by *Clostridium absonum* CFR-702 [19]. In fact, Battan et al., [13] reported that 1% w/v of purified birchwood xylan as carbon source produced higher xylanase activity than 1% w/v wheat bran. Based on our results, the maximum xylanase activity of 17.4250 ± 3.930 U/mL at 48 h was observed from xylan as the carbon source as shown in Figure 3. Beechword xylan demonstrated a remarkable increase of xylanase production of 17.4250 ± 3.930 U/mL at 48 h. Thereafter, a decreasing trend was observed which reached to 8.8272 ± 1.007 U/mL at 96 h. Even though 96 h was the end of the fermentation period, xylanase production from beechwood xylan by *B. subtilis* was still considered high compared to some of the agricultural wastes. Nonetheless, xylan as the substrate is not economical for industrial xylanase production, thus an alternative carbon source for xylanase production was elucidated using agricultural wastes. In order to perform scaling up of xylanase production, reusing and recycling of agricultural wastes are more preferable carbon source to promote xylanase activity. Generally, substrates have contributed approximately 30 to 40% of the overall production costs in microbial enzymes production [20]. In this respect, the substitution of xylan with low cost agricultural wastes was studied and compared in order to reduce the costs of industrial xylanase production. As a result, it was our major objective to produce the highest xylanase production using the optimised medium formulation with alternative agricultural wastes as the prime carbon source. When using agricultural wastes as the carbon source in SmF, barley husk produced the maximum xylanase activity of 11.646 ± 4.163 U/mL at 84 h. Then, the activity decreased to 10.325 ± 3.488 U/mL at 96 h. A similar result was obtained from Soliman et al., [21] where they reported that the maximum xylanase activity of

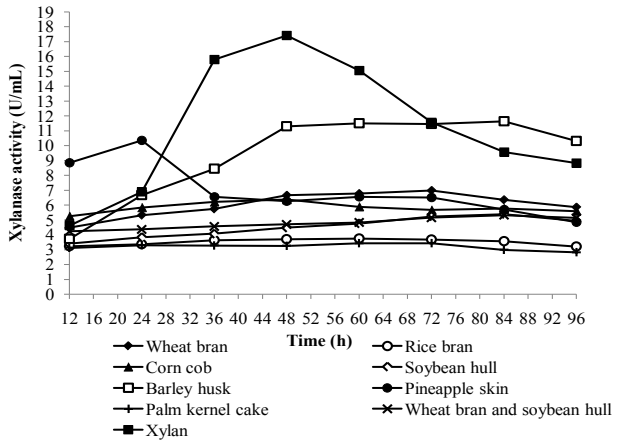

Figure 3: Time course of xylanase activity by *B. subtilis* in SmF using different agricultural wastes of wheat bran, rice bran, corn cob, soybean hull, barley husk, pineapple skin, palm kernel cake and combined wheat bran with soybean hull (1:1). Xylan was used as carbon source as comparison.

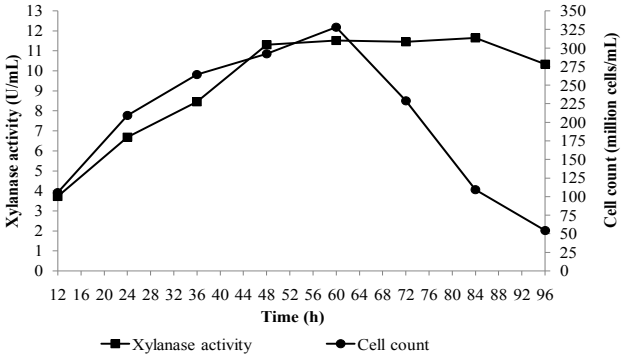

Figure 4: Correlation of xylanase activity and cell count of *B. subtilis* using the optimised barley husk in SmF.

12.5 ± 0.13 and 11.0 ± 0.13 U/g were produced from barley bran by *Aspergillus niger* and *Trichoderma viride*, respectively. Haddar et al. [22] also reported that the maximum xylanase activity of 18.66 g/L was produced by *Bacillus mojavensis* A21 using barley bran. In addition, the highest xylanase activity of 10 U/mL produced by *Bacillus sp* LB-4 using barley straw was also reported by Gandarillas et al., [23].

Figure 4 illustrates the correlation of the xylanase activity using barley husk with its biomass production of *B. subtilis*. The highest cell count from barley husk of 3.28×10^8 cells/mL was detected at 60 h. Even though the maximum cell count was observed at 60 h, increase of xylanase production was detected from 48 h to 84 h, then decreased from 84 to 96 h indicated no xylanase production towards the end of fermentation period. From our study, barley husk produced the maximum xylanase activity in SmF. Even though the concentration of biomass was depleted, nonetheless, the production of xylanase was still continued to sustain at the maximum level indicating that xylanase was produced at the end of tropophase when barley husk was used. Besides that, utilising the barley husk for the production of xylanase is more economical and environmental friendly because it reduced the decomposition of agricultural wastes to the surrounding. In other words, the use of barley husk in the production of xylanase would decrease the costs of production in an environmentally sound manner. On the contrary, pineapple skin produced high xylanase activity of 10.359 ± 5.029 U/mL at 24 h of SmF. Although barley husk and pineapple skin exerted similar high xylanase activity, a noticeable difference could be observed from the xylanase production trend from 24 to 96 h. Barley husk showed an increasing trend of xylanase activity from 24 h onwards, reaching its maximum at 84 h. Unlike barley husk, xylanase production by pineapple skin dropped rapidly to 4.850 ± 1.514 U/mL at 96 h after achieving the maximum xylanase activity at 24 h. On the other hand, the moderate xylanase activity was observed from wheat bran, corn cob, soybean hull and combined wheat bran and soybean hull (1:1) with the production of 6.976 ± 3.129 U/mL at 72 h, 6.359 ± 0.156 U/mL at 48 h, 5.385 ± 0.727 U/mL at 84 h and 5.322 ± 2.360 U/mL at 84 h, respectively. A few researchers reported wheat bran was one of the best agricultural wastes as the carbon source on xylanase production in SmF. Indeed, Poorna and Prema [24] reported 430 U/g of xylanase activity was observed in SmF by *B. pumilus* using wheat bran. Azin et al., [25] also reported combination of wheat bran and wheat straw at the ratio of 7:3 produced the maximum xylanase activity of 479.7 U/g by *Trichoderma longibrachiatum*.

Rice bran and palm kernel cake possessed lower xylanase activity compared to other agricultural wastes. The maximum xylanase activity

obtained from rice bran was 3.741 ± 0.573 U/mL at 60 h, whereas palm kernel cake displayed the lowest xylanase activity of 3.428 ± 0.287 U/mL at 72 h. Even though, palm kernel cake is abundantly available in Malaysia, however, it did not possess sufficient amount of xylanase activity. Researches on palm kernel cake as carbon source was relatively less due to its low sustainability in other countries. The lowest xylanase activity from palm kernel cake was most probably due to its inaccessible hemicellulose components as compared to the other agricultural wastes. Our result was similar to a study by Lee [26] who proved that the relatively low xylanase activity of 3.2 U/g by *Aspergillus niger* was found using palm cake meal. The maximum soluble protein concentration of 0.0174 ± 0.00156 g/mL by *B. subtilis* at 60 h in SmF was obtained from pineapple skin. The combined wheat bran and soybean hull (1:1), palm kernel cake, barley husk, beechwood xylan, soybean hull and corn cob produced the protein concentration of 0.0144 ± 0.00059 g/mL at 12 h, 0.0135 ± 0.00059 g/mL at 12 h, 0.0131 ± 0.00343 g/mL at 24 h, 0.0129 ± 0.00134 g/mL at 60 h, 0.0127 ± 0.00021 g/mL at 24 h and 0.0114 ± 0.00076 g/mL at 36 h, respectively. The lowest protein concentration was demonstrated by wheat bran with 0.0107 ± 0.00305 g/mL at 48 h. The maximum cell count of 7.65×10^8 cells/mL was observed from purified beechwood xylan at 60 h. Cell count of 3.58×10^8, 3.55×10^8, 3.30×10^8, 3.28×10^8 and 3.08×10^8 cells/mL were yielded from rice bran, palm kernel cake, pineapple skin, barley husk and soybean hull. On the other hand, the cell count of corn cob was found to be the lowest, which was 2.28×10^8 cells/mL. pH of the medium affects the cell growth significantly besides influencing the enzymes production. According to Bajpai [27], subculture of microorganism at unfavorable medium pH limits the cell growth, substrate accessibility and eventually xylanase production. Optimum pH of 6 to 8 has been reported for promoting better growth of *B. subtilis*. Towards the end of fermentation, medium pH increased to 6.62 ± 0.69 at 84 h in barley husk where the maximum xylanase activity was detected. An increased in pH trend was also observed from medium containing wheat bran, rice bran, corn cob, soybean hull, palm kernel cake, xylan and medium with combined wheat bran and soybean hull (1:1) which possessed pH of 7.48 ± 0.225, 6.94 ± 0415, 7.48 ± 0.025, 7.85 ± 0.045, 7.58 ± 0.12, 7.37 ± 0.005 and 7.75 ± 0.02 during their optimum xylanase activity, respectively. Hashemi et al., [28] reported the increase of medium pH in SmF was due to the deposit of by-products of ammonium and urea from nitrogen source after the exhaustion of carbon sources occurred in the medium.

In conclusion, based on our results, barley husk was selected as the optimised agricultural waste as the reasonable alternative carbon source in SmF suitable for industrial xylanase production by *B. subtilis*. Therefore, the formulation of the optimum medium for the production of xylanase by *B. subtilis* in SmF in this study was consisted of (g/L): barley husk, 20; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.1.

Optimisation of carbon source using agricultural wastes on xylanase production by *B. subtilis* in SsF

Sugarcane bagasse, wheat bran, rice bran and corn cob are abundantly available in many countries and thus, they are reused and recycled as the carbon source for enzymes production. Pandey [29] stated that agricultural wastes are the best substrate in SsF for microbial xylanase production. The main components of agricultural wastes are consisted of cellulose, hemicellulose and lignin that able to contribute to massive enzymes production. Similar lignocellulose wastes are therefore used as carbon source for xylanase production. However, due to the scarce information on xylanase production by *Bacillus spp* using agricultural wastes in SsF is available, thus we focused on the reuse of cheap and sustainable lignocellulose materials from agricultural wastes to elucidate production of xylanase in SsF. Production of xylanase by *B. subtilis* from different agricultural wastes as the prime carbon source in SsF was conducted using the optimised medium formulation of Medium F as determined from the earlier experiment. 5 g of agricultural wastes as the carbon substrate with peptone, 1.25 g; yeast extract, 1.25 g; KH_2PO_4, 0.25 g and $MgSO_4.7H_2O$, 0.025 g were prepared with the moisture content ratio adjusted to 1:1 (substrate: water) (w/v) in this study of SsF.

Based on our results, wheat bran was found to be the optimum carbon source with the maximum xylanase production of 22.071 ± 0.186 U/mL at 48 h as shown in Figure 5. A remarkable increase of the xylanase production was observed from 24 to 48 h before it dropped to 5.524 ± 0.179 U/mL at the end of fermentation. Figure 6 illustrates the correlation of xylanase activity and biomass production using wheat bran in SsF. The optimum carbon source of wheat bran produced xylanase activity of 22.071 ± 0.186 U/mL at 48 h with the highest cell count of 2.50×10^9 cells/mL. Decreased trend of both the xylanase production and cell count was observed thereafter signifying xylanase production was diminishing due to the poor growth of *B. subtilis* towards the end of SsF. Hyper bacterial xylanase production

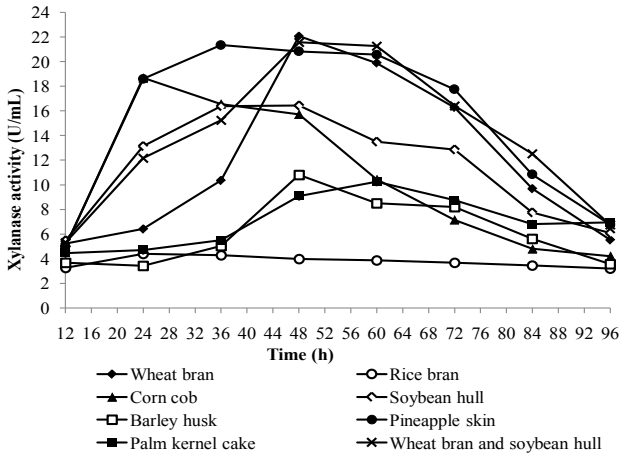

Figure 5: Time course of xylanase activity by *B. subtilis* in SsF using different agricultural wastes of wheat bran, rice bran, corn cob, soybean hull, barley husk, pineapple skin, palm kernel cake and combined wheat bran with soybean hull (1:1).

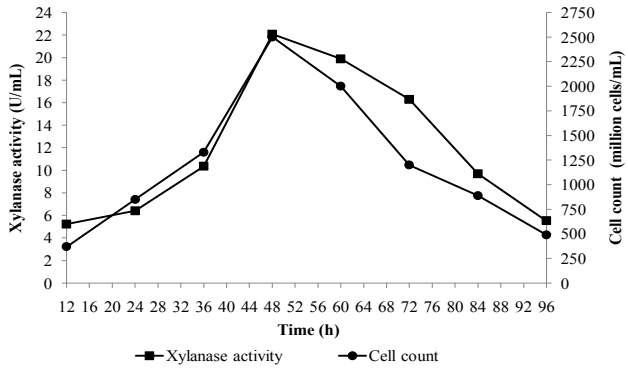

Figure 6: Correlation of xylanase activity and cell count of *B. subtilis* using the optimised wheat bran in SsF.

using wheat bran in SsF is very rare. Only a handful of researches were investigated on xylanase activity by *Bacillus spp* using wheat bran in SsF in recent years. Gessesse and Mamo [30] reported the maximum xylanase production of 720 U/g by *Bacillus sp* AR-009 using wheat bran as the carbon source. Likewise, wheat bran was also reported to produce the maximum xylanase activity of 26.8 U/g by *Bacillus licheniformis A99* in SsF. In addition, according to Poorna and Prema, 35156 U/g of the maximum production of xylanase by *Bacillus pumilus* was reported in SsF using wheat bran. Therefore, our results indicated the significant of wheat bran to replace xylan as the industrial substrate for bulk xylanase production.

On top of that, the use of pure substrate such as xylan is relatively costly as carbon source for the industrial production of xylanase, hence lower cost of agricultural residuals was used for xylanase production by *B. subtilis*. As a result, the replacement of xylan with inexpensive lignocelluloses residuals as the carbon source is greatly preferable to produce xylanase in large scale especially in SsF. Indeed, the preference of wheat bran as the carbon source in the present study was due to its ability to retain moist condition as a result of the large particle surface areas of wheat bran [31]. The moisture content of substrate in SsF is one of the critical factors affecting the results of SsF. Several factors such as water-retention capability of substrate, types of end-products and requirement of microorganisms are among the factors influencing the moisture content of substrate in SsF. The inter-particles mass transfer with the solid substrate to the growing microorganisms depends on the substrate characteristics and its moisture content. Increase of the moisture content of substrate causes the swelling of the substrate, reduces the concentration gradient of carbon within the substrate and thereby, facilitating better absorption of nutrients by microorganisms. As a result, the ability to retain the optimum moist condition of substrate enhances the growth of microorganisms and thus facilitating the xylanase activity in SsF.

Particles size of substrate is also influencing the surface area to volume ratio of the substrate which determines the size of fraction of the substrate that accessible to microorganisms. According to Mitchell et al., [32], a particle size decreases as its constant geometry of surface area to volume ratio increases. Therefore, it is critical to expose the surface area of substrate to xylanase rather than the actual amount of xylanase present in SsF. In addition, the size of the substrate also determines the amount of air occupied in the void spaces between particles of substrate. Thereby, the substrate should contain optimal size of substrate particles to enhance the optimum mass transfer of nutrients and oxygen. The rate of nutrients and oxygen transfers has profound effect on the growth and xylanase production in SsF. In this regards, smaller particles of substrate facilitate greater growth and xylanase production. As a result, even other hemicelluloses have been found to support xylanase production, but the highest xylanase production was obtained from wheat bran. Sa et al., [4] reported wheat bran has a stimulating effect on xylanase activity. In fact, the presence of lignin and protein composition in wheat bran enables it to serve as both carbon and nitrogen sources [33].

Xylanase activity from combined wheat bran and soybean hull (1:1) possessed the second highest of 21.582 ± 0.047 U/mL compared closely to 22.071 ± 0.186 U/mL from wheat bran at 48 h. Interestingly, the xylanase production profile of wheat bran throughout the fermentation in this study was very similar to the combined wheat bran and soybean hull (1:1). High xylanase activity of 16.446 ± 4.434 U/mL was obtained in SsF when using soybean hull as the sole carbon source in our study. Assamoi et al., [34] reported high xylanase production of 12096 U/g by *Penicillium canescens* 10-10c was produced from 100% soya oil cake. According to Poorna and Prema [24], 19551 U/g of xylanase activity by *Bacillus pumilus* using soybean as agricultural waste was obtained in SsF. Additionally, pineapple skin also demonstrated relatively high xylanase activity of 21.353 ± 0.054 U/mL at 36 h. Nevertheless, pineapple skin showed high xylanase activity at 12 h, then dropped to 18.603 ± 0.860 U/mL at 36 h. The decreasing profile of pineapple skin was observed starting from 60 h before dropped tremendously to the lowest at 96 h with its xylanase activity of 6.782 ± 0.297 U/mL. On the other hand, palm kernel cake and rice bran displayed very low xylanase activity. Maximum activity of only 10.241 ± 6.010 U/mL at 60 h was possessed from palm kernel cake by *B. subtilis* in our study. Similarly, *Trichoderma sp* was reported producing the basal level of xylanase activity at 8.61 U/g using palm kernel cake in SsF [35]. Interestingly, by combining palm kernel cake with other agricultural residuals, the xylanase activity was found greatly enhanced. The combination of rice husk and palm kernel cake at ratio 1:1 (w/w) by Pang et al. [35] demonstrated higher xylanase production of 68.17 U/g was obtained than using palm kernel cake alone.

Moderate xylanase production from corn cob, soybean hull and barley husk were observed with the xylanase activity of 18.690 ± 1.340 U/mL at 24 h, 16.446 ± 4.434 U/mL at 48 h and 10.801 ± 0.591 U/mL at 48 h, respectively. Gupta and Kar [7] reported moderate production of xylanase at 9.88 U/mL was obtained by *Bacillus sp* using corn cob in SsF. In our research findings, rice bran demonstrated the lowest xylanase activity of 4.400 ± 0.067 U/mL at 24 h of SsF. Rice bran showed a critical situation where the decreasing trend of activity occurred at 36 h and thereafter no significant xylanase production was observed. This was similar to the study from Poorna and Prema [24] indicated that rice bran possesed the lowest xylanase activity of 170 U/g compared to maximum activity of 19551 U/g from soybean flasks and 35156 U/g from wheat bran by *Bacillus pumilus* in SsF, respectively. Archana and Satyanarayana [5] also reported the least xylanase activity by *Bacillus licheniformis* A99, producing 10.2 U/g from rice husk. In addition, according to Ko et al., [36], the maximum xylanase activity of 13.72 U/mL by *Paenibacillus campinasensis BL11* from rice straw in SsF was detected.

The maximum xylanase activity was produced from wheat bran by *B. subtilis* in SsF, as a result of having the maximum cell count of 2.50×10^9 cells/mL at 48 h, followed by the combined wheat bran and soybean hull (1:1) that produced 2.00×10^9 cells/mL at 48 h. In contrast, rice bran that produced the lowest cell count of 7.0×10^7 cells/mL at 24 h exhibited the lowest xylanase activity. Pineapple skin, corn cob, barley husk, soybean hull and palm kernel cake exhibited the average cell count of 1.34×10^9, 1.27×10^9, 1.04×10^9, 1.01×10^9 and 7.40×10^8 cells/mL, respectively. Soluble protein concentrations secreted by *B. subtilis* in SsF using different agricultural wastes as carbon source were very similar to each other. The highest protein concentration of 0.0206 ± 0.00002 g/mL at 24 h was secreted by rice bran. Pineapple skin, wheat bran, soybean hull, combination wheat bran and soybean hull (1:1), corn cob, barley husk and palm kernel cake exerted 0.0205 ± 0.00097 g/mL at 48 h, 0.0192 ± 0.00011 g/mL at 48 h, 0.0201 ± 0.00085 g/mL at 36 h, 0.0200 ± 0.0008 g/mL at 48 h, 0.0199 ± 0.00107 g/mL at 24 h, 0.0189 ± 0.00139 g/mL at 48 h and 0.0188 ± 0.00225 g/mL at 36 h, respectively. The maximum protein concentration of these carbon sources was detected between 24 to 48 h.

On the other hand, medium pH reached 6.52 ± 0.13 when the maximum xylanase activity was produced from wheat bran at 48 h. Decresing pH trend can be observed after 48 h and continued to

decrease to pH 5.12 ± 0.11 at 96 h. A similar trend was observed from rice bran, corn cob, soybean hull, barkey husk, pineapple skin, palm kernel cake and combined wheat bran and soybean hull (1:1) where pH dropped to 6.28 ± 0.02, 6.75 ± 0.16, 7.11 ± 0.11, 6.33 ± 0.14, 6.41 ± 0.39, 6.51 ± 0.06 and 7.02 ± 0.06 at 96 h after reaching a peak pH value. pH of the medium is highly affected the xylanase production because many enzymatic processes and components transportation across the cell membrane are depended on pH [37]. Kapoor et al., [17] also reported xylanase production by *Bacillus pumilus* MK001 was found highly depended on the medium pH.

In conclusion, wheat bran was the optimised agricultural waste that could possibly be upscale for industrial xylanase production by *B. subtilis* in SsF. Therefore, the formulation of the optimum medium obtained from this study for the production of xylanase by *B. subtilis* in SsF was consisted of (g/L): wheat bran, 20; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.1.

Production of xylanase using barley husk and wheat bran as the optimised carbon source in SmF and SsF

The optimum carbon source of barley husk and wheat bran in SmF and SsF was identified to exhibit the maximum xylanase activity of 11.646 ± 4.163 and 22.071 ± 0.186 U/mL at 84 h and 48 h, respectively. Lignocellulose residuals including wood, grasses, waste paper, food industry residues, municipal solid wastes and agricultural wastes such as straw, peelings, corn cobs, stalks, nutshells, seeds, sugar bagasse, wheat bran, barley husk and sewage are commonly substituted as the carbon and energy source for enzymes production. Due to its ease accessibility and cost-effective advantages, sugars from lignocellulose residuals are frequently utilised compared to expensive limited resources such as xylan substrate in industry to manufacture enzymes including xylanase. Although the xylanase production from beechwood xylan exerted high enzymatic activity in this study, nonetheless, the costs of production were tremendously overwhelming and unbearable. Agricultural wastes used as the prime carbon source for xylanase production by *B. subtilis* in SmF and SsF were proven to be possible with better prospect of scaling up.

SmF and SsF are the most common techniques used to produce xylanase. Each of these fermentation approaches possesses its own advantages and disadvantages. SmF is the method of culturing the microorganisms in fermentation broth with air supply under optimised cultural conditions. On the other hand, SsF is the fermentation method of culturing the microorganisms on the humid solid substrates without emerging in the culture broth. SsF has always been an attractive substitute for xylanase production due to the lower capital investment, energy demand and operation cost [38]. In fact, SsF provides a closeness of the substrate similar to the natural habitat of most of the microorganisms, thus inducing them to grow and produce enzymes and metabolites such as flavor and organic acids [39]. Likewise, Dominguez et al., [40] also stated that microorganisms could penetrate deep into the intracellular void spaces of the moistened carbon source to closely associate and absorb nutrients and energy to enhance enzymes production. As a result, significant product yield and productivity in SsF are anticipated. On top of that, the time of fermentation to achieve its maximum production of xylanase has a profound effect on the cost of production. The earlier the maximum production of xylanase is achieved, the lower the costs of operation and production of xylanase is needed. The decrease of xylanase activity after its maximum peak might due to the denaturation of the enzyme and deterioration of biomass concentration after prolonged fermentation. It is therefore, necessary to terminate the production of xylanase after its maximum activity is achieved for direct recovery of the enzyme thereafter. In other words, it also fastens the downstream processing of xylanase purification.

Conclusion

Different types of medium were investigated on the production of xylanase in this study. Medium formulation of Medium F was found to be the optimum for *B. subtilis*, producing the maximum xylanase activity of 11.099 ± 1.127 U/mL. After the medium formulation of the Medium F was selected as the optimum, its carbon source composition was resubstituted with various agricultural wastes. In order to identify the capability of SmF and SsF on xylanase production by *B. subtilis,* the effects of these agricultural wastes used as the carbon source were elucidated compared to the expensive beechwood xylan as the control. From our result findings, the highest xylanase activity of 11.646 ± 4.163 U/mL was detected from barley husk at 84 h in SmF. On the other hand, when wheat bran was used in SsF, 2.50×10^9 cells/mL of biomass concentration and 22.071 ± 0.186 U/mL of xylanase activity were obtained at 48 h of fermentation. In the nutshell, *B. subtilis* grown in the SmF and SsF were managed to produce xylanase using the agricultural extracts of barley husk and wheat bran as the alternative cost-effective carbon source. We could anticipate that both SmF and SsF were suitable for scaling up in the industrial production. Besides that, the xylanase produced by *B. subtilis* in this study was found to be temperature-stable where it would possess great potential in food industry for bread making and juice clarification and also as the bio-bleaching agent in the pulp and paper industry.

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