Journal of Biodiversity, Bioprospecting and Development

Ling Ho and Li Heng, J Biodivers Biopros Dev
2015, 2:1
DOI: 10.4172/2376-0214.1000143

Research Article Open Access

Xylanase Production by *Bacillus subtilis* in Cost-Effective Medium Using Soybean Hull as Part of Medium Composition under Submerged Fermentation (SmF) and Solid State Fermentation (SsF)

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Received date: Dec 06, 2014, **Accepted date:** Jan 02, 2015, **Publication date:** Jan 05, 2015

Abstract

Objective: Extracellular xylanase is produced by several microorganisms mainly from bacterial species such as *Bacillus spp*. The advances knowledge in the aspect of biochemistry, physiology and genetics of *Bacillus spp* especially *B. subtilis* play an important role, giving its huge impact for developing the optimisation of xylanase production. As a result, *B. subtilis* is widely adopted as a vital model as the producer of industrial xylanase. In this context, the aim of this study is to elucidate the effect of different cost-effective undefined medium of agricultural extracts using soybean hull as part of medium composition on xylanase production by *B. subtilis* under submerged fermentation (SmF) and solid state fermentation (SsF).

Methods: In the present study, in order to elucidate the optimum carbon source, the culture of *B. subtilis* ATCC 6633 was grown under the optimised growth conditions in the medium containing soybean hull with agricultural extracts as carbon source including wheat bran, rice bran, palm kernel cake, barley husk, corn cob, sawdust and pineapple peel under SmF and SsF in shake flasks culture, respectively.

Results: Based on our results, it was found that the highest xylanase activity of 11.968 ± 1.419 U/mL was achieved in the presence of wheat bran and soybean hull after 48-hour of SmF. The production of xylanase increased to 18.875 ± 0.569 U/mL using pineapple peel and soybean hull, thus becoming the most efficient substrate for SsF.

Conclusion: We concluded that SsF was considerately a potential fermentation method for the production of xylanase. This research presents the overview study of xylanase production by *B. subtilis* using cheap alternative agricultural extracts such as wheat bran and pineapple peel with the future prospects of large scale xylanase production. Indeed, due to the economical production of xylanase, this enzyme could be used as an effective eco-friendly bio-bleaching agent in various industries especially pulp and paper.

Keywords: Submerged fermentation (SmF); Solid state fermentation (SsF); *Bacillus subtilis*; Xylanase; Undefined medium; Agricultural extracts; Soybean hull

Introduction

Hemicellulose is the second most abundant polysaccharide in plants that readily available in nature. It is a storage polymer found in seeds that is also being a structural component of cell wall in plants [1]. Hemicellulose is consisted of a combination polymer of hexoses, pentoses and sugar acids which gives great usage in the production of different antibodies, alcohols, animals feed and fuels [2]. Normally, 40% of hemicelluloses in agricultural extracts are mainly formed by pentose sugars [3]. Xylan, after cellulose is a major component of hemicelluloses of hardwood and softwood in nature. It is a heterogeneous carbohydrate, comprising of homopolymeric backbone of β-1,4 linked D-xylopyranose units and short chain branches that consisted of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues [4]. Over the years, xylanase has occupied the centre stage of all biochemical and industrial processes due to its remarkable distinctive characteristics [5]. Therefore, its biochemical reactions under ambient conditions have enable them to be eco-friendly and is often being substituted as the best alternative to replace the hazardous chemicals for better greener technology. In this context, microbial xylanases are increasing more useful as a result of its great variety of catalytic activities, high yield and productivity, ease of genetic manipulation, regular sustainability due to the absence of seasonal fluctuations as well as the rapid growth of its producers using inexpensive undefined medium [6]. Indeed, microbial xylanase production is well-known to be more convenient and safer [7].

Xylanase (endo-1,4-β-xylohydrolase) is a hydrolytic enzyme that involved in depolymerisation of xylan. Many applications in vast industrial processes such as bio-bleaching of kraft pulp, digestibility of animal feed, softening of fruits, beer and juice clarification, extraction of plant oils, bioconversion of agricultural wastes and degumming of plant fibers [5,8]. Over the last few years, industries in worldwide have gained a lot of attention and interest on the production of xylanase due to their bio-bleaching potential. According to Sanghi et al. [9], the removal of the lignin fraction occurs in two stages involving kraft process and chemical bleaching. The kraft process involves the cooking of the wood chips at high alkaline pH and temperature, some

parts of the xylan are then dissolved while the remained of other short-chain xylans precipitate in crystalline form on the surface of cellulose microfibrils. Approximately 95% of the lignin is removed during this process, but the remaining of 5% lignin imparts a brown color on the cellulose fibres [10]. After that, the subsequent chemical bleaching process is processed to produce white pulp from the residual lignin. These compounds such as chlorine, chlorine dioxide, etc that used in bleaching process are found to be toxic, mutagenic, bioaccumulation, persistent and harmful to biological systems [11]. Hence, it is crucial to replace the existing bleaching process of chlorination by an eco-friendly and cost-effective biological process using xylanase. In fact, bio-bleaching using xylanase is encouraging as it gradually reduced organic pollutants load to the environment [12,13]. Bio-bleaching of pulp with xylanase has been reported to reduce the usage of chlorine during the bleaching process, resulting in the release of lower amount of chlorinated organic compounds in the bleaching effluents, and thus minimizing the environmental pollutions [14,15]. Pre-treatment of pulp with xylanase helps in partial disruption of lignin-carbohydrate bonds, thereby enhancing the accessibility of the subsequent bleaching chemicals to the pulp [16]. Despite the fact that the use of xylanase has become well accepted in the industry, however, the large scale usage of xylanase in the paper industry worldwide is still facing some major obstacles, particularly because of the cost of the xylanase production [16].

Xylanase is produced by a variety of microorganisms including bacteria, fungi, and actinomycetes [17-19]. Bacterial strains are generally more applicable as they often offer higher xylanase activities compared to yeasts [20]. Besides that, other advantages including ease genetic and environmental manipulation that are able to increase the yield of bacteria cells [21]. Indeed, it is comparatively easier to employ using microbial cells as the host of producer because of their shorter generation time, relatively simple nutritional requirements and easier screening procedures for desired characteristics on xylanase production [6]. As a result, among the bacterial sources, *Bacillus spp* are industrially important producers of xylanase production [22,23]. According to Nagar et al. [24], as high as 695.12 U/m L of xylanase activity was generated by *B. subtilis* in SmF. Much lower production of xylanase at 2.70 U/mL and 5.74 U/mL were produced by *Thermomyces lanuginosus* and *Schizophyllum commune* in SmF according to Haltrich et al. [25] and Purkarthofer et al. [26], respectively. In SsF, according to Gessesse and Mamo [27] and Murugan et al. [28], 720 U/mL and 819 U/mL of xylanase activity were anticipated from *B. subtilis*, respectively. In contrast, only 12.65 U/mL and 23.97 U/mL were observed from *Aspergillus niger* in SsF according to Pang and Ibrahim [29] and Kavya and Padmavathi [30]. These bacteria strains are non-pathogenic and non-toxigenic bacterium where a wide range of carbon sources of agricultural extracts produces large quantities of industrially important enzymes especially xylanase. As a result, *B. subtilis* has become widely adopted as a model microorganism for the laboratory studies, which focused on the optimizing the production of xylanase using cost-effective undefined medium of agricultural extracts. Although fungi secrete high levels of extracellular xylanase, yet the presence of considerable amount of cellulase activity and their lower optimal pH have render them from pulp and paper industry [31]. Additionally, bacteria grow at a short incubation time as compared to fungi. Notably, it also produces xylanase at high pH and temperature with minimal cellulase production. Therefore, in this study, *Bacillus subtilis* ATCC 6633 was being selected to ferment different agricultural extracts of undefined medium under SmF and SsF to yield the maximum xylanase production in this study.

In the present study, the production of xylanase was conducted using SmF and SsF. SmF is currently universal in the development of industrial enzymes in most fermentation processes of industrial field. In the case of SmF, both of the microorganism and substrate are involved in submerged state in the liquid medium, where a large quantity in the form of solvent is present [32]. Since the contents are in submerged state in the liquid medium, the transfer of heat and mass are more efficient and effective [33]. Methods for the designing of fermentation equipment and for the evaluations of its performance are greatly improved by increased knowledge of factors affecting the oxygen transfer in SmF system that required some degree of aeration [34]. In fact, the production of commercially important enzymes in SmF has long been established especially xylanase. Reports have been studied on the xylanase production in SmF [9,35-37]. 80% to 90% xylanase are produced in submerged culture because of the microbial biomass and substrate that are homogeneously distributed in a liquid medium. Better understanding of scientific literature on the bacterial metabolism, characteristics and their responses have led to the raising on the development of SmF on xylanase production. Nonetheless, SsF has also been increasingly attracted many attentions in the industrial xylanase production in the recent years. In order to develop SmF and SsF, parameters including temperature, pH, substance concentrations, size of inoculum, cultivation time and aeration should be optimized and maintained. Irfan et al. [35] reported the selection of suitable medium plays a vital role in xylanase production. It is important to make the fermentation process cost-effective. Therefore, utilisation of alternative undefined medium in SmF and SsF is mandatory for the maximum xylanase production in this study. SsF is defined as the growth of microorganisms on a layer of moist solid substrate with air as the continuous phase [27]. In SsF, there is no presence of free moving water, yet it still contains sufficient moisture for the growth and metabolism of the microorganisms. On the contrary, In SmF, the microbial biomass and substrate are homogenously distributed in a liquid phase during SmF. Nevertheless, SsF is a high recovery method for the production of industrial enzymes including xylanase [25,38]. It has been reported that in many chemical reactions, the amount of products obtained in SsF are many fold higher than those obtained in SmF [39]. Therefore, in recent years, SsF has gained numerous interests from researchers worldwide and also has often been employed for the production of xylanase because of its economical and engineering advantages [38]. Therefore, in this study, our main objective was to elucidate the optimum carbon source using undefined medium containing agricultural extracts for xylanase production by *B. subtilis* ATCC 6633 through SmF and SsF in shake flasks culture, respectively.

Materials and Methods

Bacteria strain and inoculum preparation

The microorganism model that used for xylanase production in this study was *Bacillus subtilis* strain ATCC 6633. This microorganism strain is a Gram-positive and rod-shaped bacterium that was maintained on nutrient agar. Nutrient agar was prepared and autoclaved at 121°C for 15 minutes before it was poured on the sterile Petri dishes using aseptic techniques. Then, *B. subtilis* was subcultured on nutrient agar at 37°C until the formation of colonies appeared after 3-day of incubation. In this study, the first seed medium was prepared

by dissolving 4.8 g of nutrient broth into 60 mL of distilled water in a 250 mL of Erlenmeyer flask before autoclaved at 121°C for 15 minutes. After that, 1×10^6 cells/mL from a freshly grown nutrient agar were harvested and transferred into the 60 mL of nutrient broth. The culture medium was incubated in an incubator shaker at 37°C and 150 rpm for 8 to 12 hours until the log phase of cell growth was achieved. Once the log phase of cell growth was identified through the cell count using haemocytometer, the cell suspension was harvested as the first seed culture. Thereafter, a cell suspension of 1×10^8 cells/mL was required as the standard inoculum size for the growth and production of xylanase throughout the whole study of SmF and SsF. Hence, a serial dilution of cell suspension was carried out in order to obtain a final cell concentration of 1×10^8 cells/mL. The number of bacteria cells was counted by using haemocytometer under a microscope.

Optimisation of the production of xylanase by *B. subtilis* under submerged fermentation (Smf) in shake flask culture

In this study, the medium composition of the undefined medium that used in this study has been determined as the following (gram per liter): agricultural extract, 10; soybean hull, 10; peptone, 5; yeast extract, ; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.1. The culture of *B. subtilis* was grown in the medium containing various agricultural extracts as the carbon source including wheat bran, rice bran, palm kernel cake, barley husk, corn cob, sawdust, pineapple peel and soybean hull as a control. A combination of undefined medium consisted of soybean hull and one type of agricultural extract was conducted to elucidate the optimum carbon source for xylanase production by *B. subtilis*. For the first experiment, standard of 2.5 g of soybean hull combined with 2.5 g of wheat bran as the carbon sources were prepared in 250 mL volume in the 500 mL of Erlenmeyer flask for the study of SmF. The rest of the experiments were carried out as the similar fashion except for replacing the wheat bran with other agricultural extracts. A single prime carbon source of soybean hull was used as the control throughout the whole study of SmF. After the pH medium was adjusted to pH 6.5, the substrate was sent to autoclave at 121°C for 15 minutes. The carbon sources were autoclaved separately from other medium compositions. Subsequently, during the inoculation of *B. subtilis*, the culture was inoculated with 1 mL of standard inoculum size of 1×10^8 cells from the first seed culture. After that, the SmF was carried out at the optimised 37°C and 150 rpm for 96 hours in an incubator shaker. The samples were withdrawn and collected at every 12-hour interval. In the present study, sampling analysis was carried out to elucidate the maximum production of xylanase using agricultural extracts as the carbon source in SmF. All of the experiments were repeated twice in SmF and the mean value was generated from the analysis.

Sampling analysis and extraction of crude extracellular xylanase from Smf

5 mL of samples from SmF were withdrawn at every 12-hour interval for quantification of biomass concentration via optical density and measurement of pH medium. After that, the samples were centrifuged at 10,000 rpm for 15 minutes. The clarified supernatant was used as the source of extracellular xylanase for the enzymes activity and protein assays.

Optimisation of the production of xylanase by *B. subtilis* under solid state fermentation (SsF) in shake flask culture

The medium composition of the undefined medium used in this study was comprised of (gram): agricultural waste, 2.5; soybean hull, 2.5; peptone, 1.25; yeast extract, 1.25 ; KH_2PO_4, 0.25 and $MgSO_4.7H_2O$, 0.025. The medium composition was prepared in 500 mL Erlenmeyer flask with the moisture content ratio adjusted to 1:1 (substrate: water) (w/v). 2.5 g of soybean hull combined with 2.5 g of wheat bran as the carbon sources were prepared and autoclaved at 121°C for 15 minutes before combined together with other medium composition. The rest of the experiments were performed by replacing the wheat bran with other agricultural extracts. Identical agricultural extracts that used in SmF were also elucidated in SsF. A single prime carbon source of soybean hull was used as the control throughout the whole study. After cooling down from autoclaving, both of the agricultural extract and medium composition were combined and mixed well before sterile distilled water was added at the ratio of 1:1 (w/v). Thereafter, the medium was inoculated with 1 mL of standard inoculum size of 1×10^8 cells of *B. subtilis* obtained from the first seed culture. Subsequently, SsF was carried out at the optimised growth conditions of 37°C and 150 rpm for 96 hours in an incubator shaker. The extracellular xylanase was extracted using sterile distilled water under aseptic techniques at every 12-hour time intervals to elucidate the maximum production of xylanase using agricultural extracts as the carbon source in SsF. The analysis was carried out in two batches to find the average data.

Sampling analysis and extraction of crude extracellular xylanase from SsF

Sampling for SsF was conducted at the regular time interval of 12-hour. To extract the extracellular xylanase from the fermented bacteria in SsF, 5 mL of sterile distilled water was added and mixed homogenously with the substrate in the culture flask as shown in Figure 1. Thereafter, the liquid sample was withdrawn, leaving the remaining agricultural residuals in the culture flask for the rest of the fermentation. Then, the liquid sample was used for the quantification of biomass concentration and pH medium measurement. Subsequently, the remaining sample was centrifuged at 10,000 rpm for 15 minutes. The clear supernatant was subjected to xylanase activity and protein assays.

Figure 1: Culture flask containing agricultural extract as the carbon source for the production of xylanase *by B. subtilis* in SsF (left). Sterile distilled water was added into the carbon source to extract the sample from culture flask in SsF for analysis (right).

Xylanase activity assay

The xylanase activity was assayed according to the method by Bailey et al. [40] with slight modification. The detection of xylose as the reducing sugar after xylanase activity was conducted using 3,5-dinitrosalicyclic acid (DNS) method [41]. Xylan from Birchwood was used as the substrate for xylanase activity. 0.1 mL of enzyme solution was added together with 0.9 mL of 1% xylan in 0.05 M sodium phosphate buffer, pH 5.3 and incubated at 50ºC for 30 minutes. 1.5 mL of DNS reagent was added and incubated at 90ºC for 5 minutes. Thereafter, to terminate the reaction, 0.5 mL of 40% potassium sodium tartrate was added and allowed to cool down at room temperature. The amount of xylose released by the enzymatic reaction is determined by measuring its absorbance at 575 nm. To quantify the xylanase activity, one unit of xylanase activity corresponds to the amount of enzyme required to release one micromole of xylose per mL of enzyme extract per minute under the assay condition [9,40]. The activity of xylanase was measured according to the standard curve of xylose. Several concentrations of xylose from 1 to 10 mg/mL were prepared and drawn by plotting the absorbance reading against concentration.

Quantification of soluble protein concentration

During xylanase synthesis, protein that produced by *B. subtilis* was determined according to Lowry et al. [42] using Bovine Serum Albumin (BSA) as the standard curve. In this method, the absorbance for protein concentration was measured at OD 750 nm. For the preparation of BSA standard curve, several BSA concentrations from 0.01 to 0.10 mg/mL were used.

Quantification of biomass concentration: optical density at 610 nm

Waghunde et al. [43] stated that optical density (OD) is an easy and reliable method which required lesser time as compared to haemocytometer count reading. Thus, the optical density of the culture sample was measured at OD 610 nm using spectrophotometer to determine the growth of the *B. subtilis* in this study of SmF and SsF.

Measurement pH of culture medium

The pH of the culture medium was measured using pH meter with a glass electrode.

Results and Discussion

Effect of undefined medium on xylanase production by *Bacillus subtilis* in submerged fermentation (smf) under optimum growth conditions

Currently, xylanase is an essential option of environmental friendly enzyme used in numerous industrial applications, as a result, there is a rising demand for the large scale production. According to Sonia et al. [44], xylanase has a worldwide market of around USD 200 million in 2005 and the widespread use of xylanase in commercialized industrial applications requires extensive studies to optimise their production capability. Commercial mass production of xylanase can be quintessentially done by either SmF or SsF. Their effectiveness has often been consociated with process conditions and physiochemical forces of prior significance. However, the driving force has ideally produced quick and high quantity xylanase from simple and inexpensive carbon sources-containing medium [45]. Any fermentation processes including SmF and SsF proceed through the action of microorganisms which perform in the presence of medium. Therefore, the proper preparation of the medium is an essential component in the design of a fermentation process [32]. Since the medium is desired to support the functioning of microorganisms, the requirements of the medium including carbon, nitrogen, energy source, minerals, oxygen and water are decided by those of the microorganisms [32]. Defined medium is consisted of all chemical components that specifically defined and added in pure form to possess pre-defined composition and concentration. According to Rao [32], the medium used in a laboratory-scale process is reasonably composed of pure components of defined medium, but it is not affordable in the case of commercial production where the cost of production rules the economic viability. The selection of optimal medium is playing an important role on production of xylanase, making it a prerequisite to make the overall production process relatively more economical. In fact, the medium in scaling up process should possess the main characteristic of cheap and easily available at a consistent rate and quality besides being producing higher yield and productivity, maximum production of enzymes and minimum secretion of undesired products [32]. On the other hand, agricultural extracts as the undefined medium are the most abundant and renewable resources found on the earth. Accumulation of the mass of agricultural extracts in large quantities each year results not only in the deterioration of the environment, but also in the loss of potentially valuable raw material which could be processed to yield xylanase. A literature survey of Saleem et al. [46] was reported that *B. subtilis* performed well when grown on pre-treated natural substrates such as rice straw, wheat straw, sugarcane bagasse and wheat bran at 2% concentration and in which sugarcane bagasse produced 4.0 U/mL of xylanase activity after 14-hour of fermentation. On the other hand, 2.0 U/mL of xylanase activity obtained when wheat bran was used as carbon source in the growth medium. In SmF, the main focus should be on the utilization of agricultural extracts along with the development of efficient bioprocess strategies to obtain higher enzyme yield and productivity with the lowest possible cost [47]. Therefore, the replacement using agricultural extracts is mainly implied on the economical advantages for xylanase production process with regard to the high cost of commercial xylan and the low cost effortless availability of agricultural extracts such as wheat bran. As a result, according to Mullai et al. [45], many researchers have targeted on using residues from agricultural and food industry, thereby reducing the related environmental pollution. Those residues contain nearly 20% to 30% of hemicelluloses that could be efficaciously used for microbial xylanase production [48]. Therefore, in this study, experiments have been carried out to investigate and elucidate the effect of various agricultural extracts such as wheat bran, rice bran, palm kernel cake, barley husk, corn cob, sawdust, pineapple peel and soybean hull for xylanase production under optimum growth conditions of pH medium 6.5 at 37ºC and 150 rpm for 96-hour of fermentation time. These medium contained various carbon sources are employed in place with the control medium of soybean hull, since they were anticipated to produce xylanase production equal or higher than that obtained in the control medium of soybean hull. Figure 2 shows the effect of various carbon sources on xylanase production by *B. subtilis* in SmF. Our results revealed that the xylanase production reached its maximum peak at 48-hour of SmF in the presence of wheat bran as the optimum carbon source by *B. subtilis*, which possessed the maximum xylanase activity of 11.968 ± 1.419 U/mL, followed by

sawdust which produced the maximum enzyme activity equal to 10.110 ± 1.686 U/mL at 60-hour of SmF.

Worldwide consumption of wheat estimated by World Agricultural Supply and Demand Estimates (WASDE) has been reported to be 652.18 million ton in the year of 2010. Xylanase production on commercial scale could be achieved by using wheat bran as the substrate as it is an agro-economical inducer due to its 12.65% of high xylan content in dry material [49,50]. Indeed, according to Gawande and Kamat [51], 30% cellulose, 27% hemicellulose, 21% lignin and 8% ash are found in commercial wheat bran. Notably, wheat bran also contained approximately 28% protein which serves as the main source of nitrogen. Thus, no further addition of expensive nutrients are necessary, it thus, be critically important economic advantage. Hence, in this study, xylanase production in the presence of wheat bran as the carbon source increased drastically after 12-hour of fermentation before reached its peak at 48-hour of fermentation in which xylanase activity equal to 11.968 ± 1.419 U/mL was observed. Likewise, Yang et al. [52] claimed that 3% finely ground wheat bran was more economical to the optimal xylanase production at 72-hour of fermentation with enzyme activity of 22.1 U/mL. Indeed, according to Sanghi et al. [9], wheat bran produced 410 U/mL of xylanase activity by *B. subtilis* in SmF. In our study, after 84-hour of fermentation, even though, xylanase production was depreciated to 9.085 ± 3.386 U/mL, yet wheat bran was still remained as the carbon source for higher xylanase biosynthesis than other carbon sources of agricultural extracts. Thereafter, the xylanase activity continued to decline to 7.277 ± 3.736 U/mL at 96-hour of SmF. In a study by Espinar et al. [53], the lower production of xylanase during the later stages of fermentation in the medium might be due to the release of small amounts of protease from the aging cells that were gradually entering into autolysis. As a result, degradation of xylanase was anticipated in the prolonged fermentation process. In addition, it could be due to the scarcity of insoluble xylan particles in the medium [49]. Apparently, the prolonged fermentation process did not increase any xylanase enzyme yield, but further declined the enzyme activity. Similarly, Sanghi et al. [9] also reported that the reduced in xylanase yield might be due to the formation of thick cell culture suspensions and improper mixing of oxygen and carbon sources in the shake flask culture. In fact, according to Kavya and Padmavathi [30], reduced xylanase activities were observed in the non-agitated flasks, as a result of limitation in oxygen and mass transfer. On the contrary, with agitation, higher xylanase biosynthesis was observed probably due to good oxygen and nutrients supply. Notably, the volume used for fermentation has also possessed a great impact on the continuous supply of air and nutrients, growth of microorganism and production of enzyme [54,55]. Therefore, in SmF, the optimum aeration and agitation are mandatory to ensure availability of oxygen, nutrients and other essential substances to the growing cells. Kapoor et al. [56] also stated that the reduction in xylanase yield was probably due to the depletion of available nutrients to microorganisms or due to proteolysis and autolysis.

On the other hand, Buthelezi et al. [57] used sawdust and digestive bran as the cheap alternate substrates for xylanase production by Bacillus strains. According to Murugan et al. [28], 117.0 U/mL of xylanase activity by *Arthrobacter sp* was obtained using sawdust as carbon and energy sources in SmF. Sawdust is a raw material that exhibits a high polarity mainly due to the presence of different oxygen-containing functional groups such as alcohol and ester [58,59]. The cell wall of sawdust is mainly consisted of cellulose, lignin, hydroxyl groups such as tannins and active phenolic ion exchange compounds. The lignin content of hardwoods is usually in the range of 18% to 25%, whereas softwood varies between 25% and 35% [60]. Sawdust used in the present study possesses 20% of high beechwood content, therefore making it an ideal substrate for xylanase production, since beechwood has been reported as a good substrate for xylanase activity [40,61]. This could explain why high xylanase production of 10.110 ± 1.686 U/mL at 60-hour of SmF was obtained using sawdust in this study. Thereafter, the decrease in xylanase activity observed after 60-hour of fermentation could possibly due to the accumulation of metabolic wastes and protease production which might inhibit and terminate xylanase activity [27]. On the other hand, the production of xylanase using soybean hull as the only carbon source was not desirable in SmF. Notably, as proposed by He et al. [62] where they also reported that the use of soybean as the sole substrate for production of enzyme such as elastase by *Bacillus sp* was not sufficient in their study.

Other studies on the xylanase production using cheap alternative raw materials have also been reported. 52 U/mL of xylanase activity was achieved by *Bacillus sp* using oat spelt according to Anuradha et al. [1]. Moreover, according to Kapilan and Arasaratnam [63], 224.4 U/mL of xylanase activity by *Bacillus pumilus* was detected after utilising paddy husk as the recycled carbon and energy sources in their study. In fact, higher xylanase activity of 334.34 U/mL was obtained by *Streptomyces chartreusis* using corn cobs as the sole carbon source [64]. In contrast, utilisation of defined medium on xylanase production was not as preferable as undefined medium especially in the industrial production. Much lower xylanase production was anticipated from maltose as the carbon source as compared to agricultural wastes [65]. Indeed, according to Ahmad et al. [66], 60.03 U/mL of xylanase activity was produced by *Aspergillus niger* from the growth medium containing corn cob as the carbon source compared to much lower activity of 17.80 U/mL and 19.50 U/mL from maltose and lactose by *Aspergillus niger* and *Penicillium implicatum*, respectively. Thus, agricultural extracts are able to possess better uses as the carbon and energy sources in the industrial production of xylanase instead of self-decomposition that results in relatively severe water and environmental pollutions. Indeed, agricultural or crop residues are burnt openly as compared to animal wastes. In the nutshell, we concluded that wheat bran as the low cost, time-saving and highly xylan-containing hemicellulosic agro-waste is preferable to use as the optimal carbon and energy sources for the maximum production of xylanase by *Bacillus subtilis* in SmF in shake flask culture under optimised growth conditions at the pH medium 6.5 and 30ºC with the agitation speed of 150 rpm up to 48 hours of fermentation in this study. Table 1 summarises the xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SmF.

Agricultural extract	Xylanase activity assay		
	Fermentation time for maximum activity (hours)	Maximum xylanase activity (U/mL)	Optimum pH medium
Wheat bran	48	11.968 ± 1.419	6.98 ± 0.25
Rice bran	48	5.176 ± 0.597	6.76 ± 0.48
Palm kernel cake	72	5.429 ± 1.362	7.54 ± 0.19

Barley husk	48	4.947 ± 0.887	6.68 ± 0.16
Sawdust	72	10.110 ± 2.199	7.31 ± 0.12
Pineapple peel	12	6.438 ± 0.347	6.05 ± 0.63
Corn cob	48	4.789 ± 0.540	6.93 ± 0.04
Soybean hull	48	4.620 ± 0.155	7.35 ± 0.13

Table 1: Xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SmF.

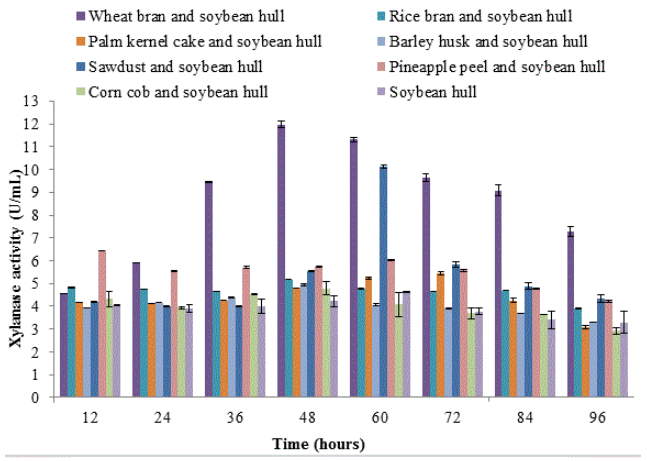

Figure 2: Production of xylanase by *B. subtilis* using various agricultural extracts as carbon source in SmF.

In the present study, the biomass concentration of *B. subtilis* was measured using optical cell density at OD 610 nm to illustrate the growth profile of *B. subtilis* during xylanase production in SmF. The growth profile of *B. subtilis* during xylanase production in SmF in this study is shown in Figure 3. Overall, the xylanase production increased after 12-hour of fermentation as the biomass concentration increased. The log phase of growth was observed before reaching the maximum activity at 48-hour of growth of the late stationary phase. After that, the production of xylanase declined parallel with the biomass concentration of *B. subtilis*. Damaso et al. [67] observed the maximum production of xylanase by *Thermomyces lanuginosus* occurred after 96-hour of fermentation. On the contrary, other studies have been reported that the maximum xylanase production by *Bacillus spp* occurred significantly at much shorter fermentation time of 24 to 48-hour compared to fungus. Shorter fermentation time offers greater and cost-effective production of xylanase which is always favorable in the industrial production. In the nutshell, xylanase production by *B. subtilis* using wheat bran to achieve its maximum at the shorter fermentation period of 48-hour in SmF was one of the most anticipated as it possessed more economical in term of capital and operational amount of cost with equal energy provided from other agro-waste sources. This was also the reason why *Bacillus spp* were more preferable than other microorganisms in xylanase production [68-70].

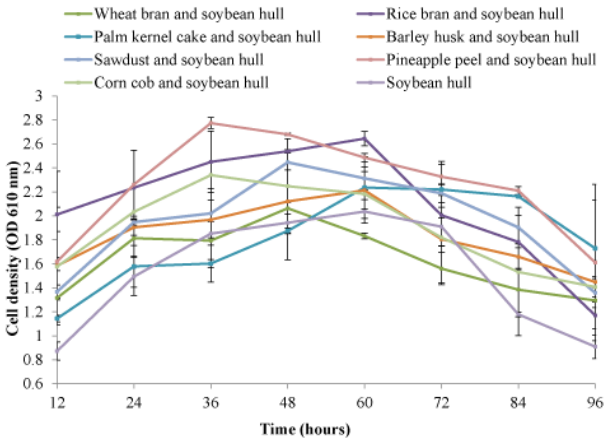

Figure 3: Growth profile of *B. subtilis* during xylanase production using various agricultural extracts as carbon source in SmF.

The pH of culture medium was also strongly affected many enzymatic processes including xylanase activity [71]. Figure 4 shows the profile of pH medium using various carbon sources in SmF by *B. subtilis*. The pH of the production medium during xylanase production was varied from pH 6 to 8.0, but appreciable xylanase activity was observed between pH medium 6.0 and 7.0, with the highest xylanase activity occurred at pH 6.9. Thereafter, higher value of pH medium was obtained at the end of fermentation due to the degradation of carbon sources, limitation of oxygen transfer in the substrate and accumulation of unwanted toxic waste products which inhibited the growth of bacteria and termination of the xylanase biosynthesis. Hence, this would probably why at the end of fermentation in the present study, higher alkaline pH was observed that caused much lower OD of *B. subtilis* expressed during its death phase of the growth profile. A study of Sanghi et al. [9] reported that the maximum xylanase activity of *B. subtilis* ASH produced 283 U/mL at pH medium 7.0 which was much higher than *B. circulans* AB-16 that produced 55 U/mL in the study by Dhillon et al. [13]. Furthermore, according to Anurdha et al. [1], mild alkaline xylanase is a good potential as bio-bleaching agent in pulp and paper industry due to the great reduction of its requirement for pH and temperature. In fact, Yang et al. [52] reported that they managed to isolate *Bacillus spp* from a hardwood kraft pulp with the optimum pH of 6.0 to 8.5. Hence, the xylanase that produced from pH 6 to 8 in the present study would be of great use in pulp and paper industry. On the other hand, soluble protein concentration also produced by *B. subtilis* during xylanase production. Figure 5 demonstrates the production of soluble protein using various agricultural extracts by *B. subtilis* during xylanase production in SmF. Sawdust was able to produce the highest maximum production of protein concentration at 16.761 ± 0.502 mg/mL whereas palm kernel cake generated the lowest maximum protein concentration production of 12.355 ± 1.077 mg/mL.

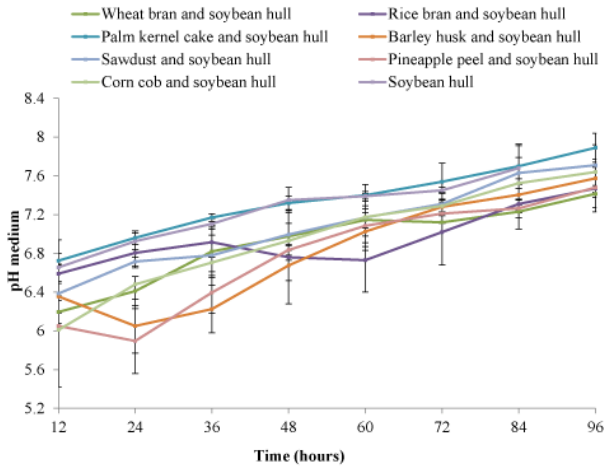

Figure 4: pH medium profile during xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SmF.

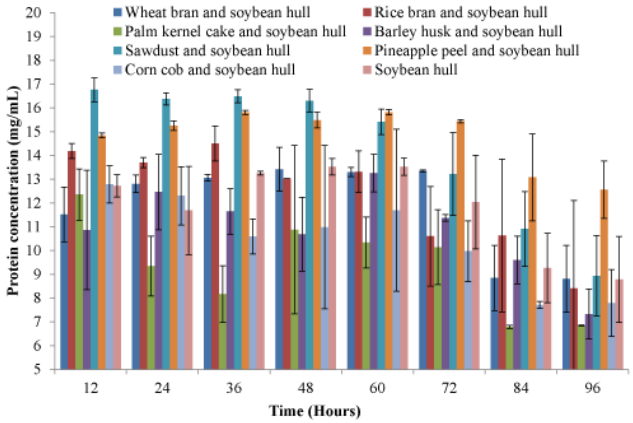

Figure 5: Protein concentration produced by *B. subtilis* during xylanase production using various agricultural extracts as carbon source in SmF.

Effect of undefined medium on xylanase activity by *Bacillus subtilis* in solid state fermentation (ssf) under optimum growth conditions

Given the potential usage of xylanase in different industrial applications, the development and optimisation of xylanase production methods with the ultimate aim of reducing the overall production cost is very crucial [27]. In this context, one alternative approach is through the application of SsF. In the present study of SsF, xylanase yield was elucidated using medium supplemented with various agricultural extracts as the carbon source. SsF is relatively more suitable and practical for the complex substrates derived from agricultural and forestry extracts. Purified xylan is an excellent substrate due to its low molecular weight that has been compared aggressively with other abundantly available and cost-effective agricultural residues. Nonetheless, utilisation of agricultural extracts allows the reduction of overall manufacturing cost of xylanase production which leads to better facilitation of this environmental friendly enzyme in pulp and paper industry, contrary to application of chemicals including chlorine for bleaching process. Figure 6 demonstrates the effect of various agricultural extracts as the carbon source on the production of xylanase by *B. subtilis* in SsF. In our results, it is possible to observe the induction effect of the hydrolysate from pineapple peel due to xylanase activity when compared to control medium with soybean hull as fermentation prolonged. From Figure 6, a sharp maximum peak of the highest xylanase activity at 48-hour of SsF equivalent to 18.875 ± 0.569 U/mL was produced in the presence of pineapple peel as the optimum carbon source compared to other agricultural extracts. Higher growth rate of the *B. subtilis* on pineapple peel was corresponded closely to the production of xylanase. The particles size of pineapple was relatively smaller than other carbon sources, thus, it possessed larger surface area to ease the diffusion of oxygen and carbon dioxide, absorption of nutrients and removal of toxic materials. Likewise, Arasaratnam et al. [72] stated smaller particle size of carbon source was satisfactory in giving bigger surface area to ease oxygen diffusion, nutrients absorption and assimilation of faster growth and better metabolic activities of the microorganisms. Apparently, smaller particle size of the carbon substrate is more preferable, providing the desirable larger surface area for adhesion and attachment of the microorganisms. As a result, higher efficiency and greater achievement in production of biomass and enzymes are anticipated. On the contrary, much poorer respiration and aeration of the microorganisms occur if the particle size is drastically micro-sized that would therefore cause retarded growth and eventually undesirable enzymes production. Nevertheless, if the particles size of carbon substrate is significantly larger, it thus would be one of the undesirable factors. Even, it possesses considerably amount of inter-particle space for respiration, it however, still provide inadequate space for microorganism attachment and adhesion. In the nutshell, particle size of pineapple peel was anticipated to be the desirable size for the xylanase production by *B. subtilis* in the present study. Furthermore, our results also indicated wheat bran was also a potential substrate for xylanase production by *B. subtilis* in SsF with the maximum xylanase production of 17.364 ± 1.973 U/mL at 60-hour of fermentation. Thereafter, the xylanase activity declined to 12.713 ± 1.882 U/mL at 72-hour and 9.618 ± 0.357 U/mL at 96-hour of fermentation might due to the formation of a thick cell suspension and improper mixing of the substrate in the culture flask. Notably, many studies used wheat bran as the carbon source for xylanase production [9,27,73,74]. In fact, Battan et al. [74] and Sanghi et al. [9] reported wheat bran was superior to other agro-wastes such as gram bran, soya bran, maize bran, wheat straw, rice husk, paddy straw, sugarcane bagasse, sawdust and groundnut shell. With the natural composition of arabinoxylan and hemicellulose found in wheat bran, suitable particle size of this substrate encourages the xylanase production by providing enough amount of void space within the substrate. Consequently, oxygen and nutrients transfer were achievable for better growth of *B. subtilis* and higher xylanase biosynthesis [75]. Notably, Ninawe and Kuhad [76] reported that wheat bran was used as an enhancer for xylanase production by *Streptomyces cyaneus* SN32. Wheat bran and wheat straw were applied as the total carbon source for xylanase production may be attributed to its hemicellulose components and favorable degradability [44]. Similarly, both *Thermoascus aurantiacus* and *Penicillium canescens* were known to use wheat straw for the production of xylanase according to Kalogeris et al. [77] and Bakri et al. [78]. Notably, *T. aurantiacus* also produced 64 U/mL of xylanase activity using wheat bran under SsF after 4 days of incubation [79]. Likewise, in the study of Gessesse and Mamo [27], *Bacillus spp*

produced up to 720 U/mL of xylanase activity when grown in SsF using wheat bran at wheat bran-to-moisture ratio (w/v) from 1:0.5 to 1:1.5 and Na_2CO_3 concentration of 10% (w/w). On the other hand, based on our result findings, they revealed lower xylanase activity was obtained from corn cob and soybean hull, producing 13.698 ± 1.591 U/mL and 11.975 ± 1.136 U/mL at 48-hour of fermentation, respectively. According to Kapilan and Arasaratnam [63], lower xylanase activity was obtained due to inadequate amount of xylan obtained from the carbon sources besides the deterioration of moisture content ratio after prolonged fermentation. These might be the reasons for the lower xylanase production by *B. subtilis* in medium containing corn cob and soybean hull as the carbon sources in this study. Table 2 demonstrates the xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SsF.

	Xylanase activity assay		Optimum pH medium
Agricultural extract	**Fermentation time for maximum activity (hours)**	**Maximum xylanase activity (U/mL)**	
Wheat bran	60	17.364 ± 1.972	6.89 ± 0.10
Rice bran	60	15.732 ± 0.658	6.57 ± 0.25
Palm kernel cake	60	11.799 ± 0.961	7.06 ± 0.08
Barley husk	60	15.071± 3.531	6.65 ± 0.02
Sawdust	48	14.376 ± 6.094	7.11± 0.21
Pineapple peel	48	18.875± 0.569	6.63 ± 0.27
Corn cob	48	13.698 ± 1.591	7.09 ± 0.05
Soybean hull	48	11.975 ± 1.136	7.19 ± 0.18

Table 2: Xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SsF.

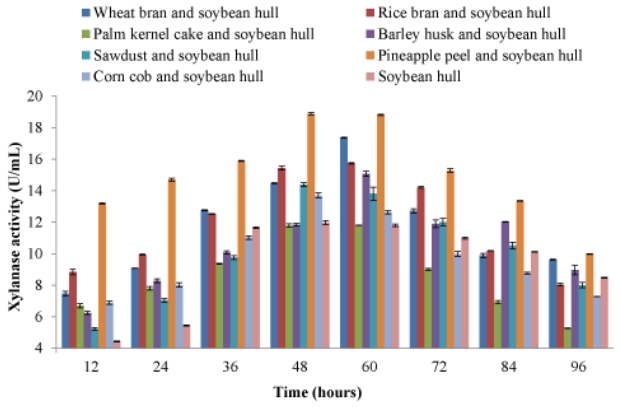

Figure 6: Production of xylanase by *B. subtilis* using various agricultural extracts as carbon source in SsF.

Ramesh and Lonsane [80] stated that the moisture of content in SsF is another crucial factor in determining the success of a fermentation process. Many studies showed a high level of xylanase production at lower solid substrate-to-moisture content ratio (w/v) by different bacterial strains under SsF [81]. Generally, solid carbon sources used in SsF are insoluble in water. Thus, water is absorbed into the substrate particles for better xylanase production. In regard to this, dissolved water causes the swelling of the substrate, and thus, facilitating the rapid absorption and good utilisation of nutrients from substrate to the microorganisms for growth, metabolic and enzymatic activity [82]. Besides that, the risk of contamination is generally reduced during SsF because it commonly operates at the water activity of below 1, as a result, the growth of undesired foreign bacteria and yeasts is minimised. The importance of moisture level of medium in SsF and its influence on the biosynthesis and secretion of enzyme are attributed to the interference of moisture in the physical properties of the solid particles [77]. Bacteria are apparently preferred the growth medium with the optimum moisture content for the maximum enzymes production [83]. If the moisture content is adjusted to above or below the optimum value, the metabolic activities of the microorganism and enzymes production are easily affected [83]. Thus, the optimal moisture content ratio was anticipated to increase the production of xylanase in SsF. In SsF, solid substrate is viewed as a solid but porous matrix which constitutes source of nutrients for the microorganisms. Solid substrate should possess the characterisation of absorbing and retaining a minimum layer of water to support a high level of water activity. The layer of water is where all of the microbes activities occur. Effective mass transfer of nutrients, oxygen, carbon dioxide and waste products occur within this thin layer of water. Due to high concentration of fermentation products, hydrolysed sugars occurred within the layer of water. This layer of water becomes viscous, thus explaining the importance of water activity rather than presence of free water in the metabolism of microorganisms [38]. Literature survey revealed that a few attempts have made on SsF processes employing inert supports. Sanghi et al. [9] stated *B. subtilis* produced a very high level of xylanase in SsF using inexpensive agro-residues of wheat bran and it was much higher than that reported by other bacterial isolated. However, the lower moisture content causes reduction in the solubility of nutrients of the substrate, low degree of swelling and high water tension which discourage the growth of microorganism and hence,

inhibiting the biosynthesis of xylanase [84]. On the contrary, increasing moisture level is believed to reduce the porosity of substrate, thus limiting the oxygen transfer and amount of void inter-particle space available in the substrate [75,85,86]. Moreover, it leads to conglomeration of the substrate or sticking of solid substrate particles to the wall of the culture flask. Consequently, the substrate becomes more vulnerable to other bacterial contamination [87]. Thus, the optimum moisture ratio of SsF in our study was set at 1:1 (w/v) of agricultural extract: water to render from under production of xylanase and contamination of undesired microorganisms.

An important aspect for industrial applications of xylanase is the need to reduce the costs of production [88]. The production cost of xylanase is always anticipated to be high and uneconomical, it thus, necessary to direct more efforts in enhancing enzyme production with in-depth emphasis of different fermentation modes that are associated with cost reduction in the industrial point of view. Hence, Gessesse and Mamo [27] postulate the great advantages of using SsF over SmF for enzymes production including lower water content of enzymes extraction, ease of control, lower probability of contamination, easier downstream processing of enzymes and among others. In fact, the selection of better solvents than the medium for enzymes extraction is another important point of research when selecting SsF as a fermentation system [89]. The production of commercially important xylanase in SmF has increased rapidly, but the popularity of this fermentation process has been represented by alternative method which known as SsF. In the recent years, research interest in batch SsF has addressed successful production of many innovative and high value products especially xylanase. SsF that carries out on the inert support raw materials allows the process of microbial growth on or in solid substrate particles. Identification and optimisation of solid substrate of raw materials have been regarded as one of the future research and development of the SsF system [82]. Generally, microorganisms are favorable to grow on a solid moistened surface which has free access to air with many void spaces within. The use of solid inert raw material support with suitable moisture content would provide optimal homogeneous aerobic conditions throughout the fermentation process and recovery of the product would also be comparatively easier [38]. In general, SsF is a simple, in term of equipment and process control, less expensive and often results in higher yields and productivity. In principle, there are no real differences between the popular SmF and SsF. Both fermentation processes are still involved the same components of the typical fermentation process including presence of fermenting microorganism, raw fermentation substrate to be acted upon by the microorganism and end products of fermentation produced [32]. Indeed, the most significant difference between SsF and SmF is the presence of the nutrients in the form of solid phase where very minimal requirement for the liquid is requested in SsF as compared to submerged culture [32]. SsF possesses many ecological, economical and environmental friendly features. Besides very restricted water consumption is required, barely minimum water effluent is also discharged during the process, perhaps, the necessity of using antifoam and application of using semi-sterile substrate in certain conditions would be made possible in the industry to enhance greater enzymes production. Subsequently, due to the low requirement of sterilisation and instrumental in SsF process, the need to process enzyme especially xylanase in the downstream processing is lessen particularly in the concentrating step. As a result, more efficient xylanase extraction method would be developed and employed in SsF. In addition, according to Holker and Hofer [90], fermentation time and enzyme degradation would possibly be diminished in SsF. However, in the absence of free aqueous phase in the SsF system, the difficulty of heat removal is anticipated whereby rapid increase of internal temperature of the microenvironment in the culture flask as well as in bioreactor would be a severe problem specifically for the production of heat-denatured enzymes like xylanase, which eventually lead to the fatal of the culture microorganism as the fermentation prolonged. In order to compensate this biological loses, the obligation of maintaining the growth temperature is always emphasized besides the cooling down of the fermentation system achieved through evaporation process especially in bioreactor. Nevertheless, higher aeration and agitation speed would sufficiently overcome the heat problem arises from the aggressive metabolic activity of the microorganism as fermentation prolonged. Despite all these problems, the development of SsF is still very encouraging for xylanase production. According to Archana and Satyanarayana [91], high titer of xylanase production was achieved using *Bacillus spp* in SsF.

Besides that, according to Sepahy et al. [71], pH of the medium is also strongly affected many enzymatic processes and influenced various transport of many components across cell membrane. Each microorganism holds its pH range for its growth and metabolic activity with the optimum value between the ranges. Figure 7 illustrates the pH medium profile of xylanase production using various agricultural extracts as the carbon source by *B. subtilis* in SsF. The pH of the production medium was varied in the range from 6.0 to 7.6, but the appreciable of xylanase activity using pineapple peel was observed at pH 6.5. Thereafter, cell lyses that caused the liberation of basic amino acids and peptides into the medium as a result of protein degradation by protease were probably responsible for a strong variation of pH culture [89].

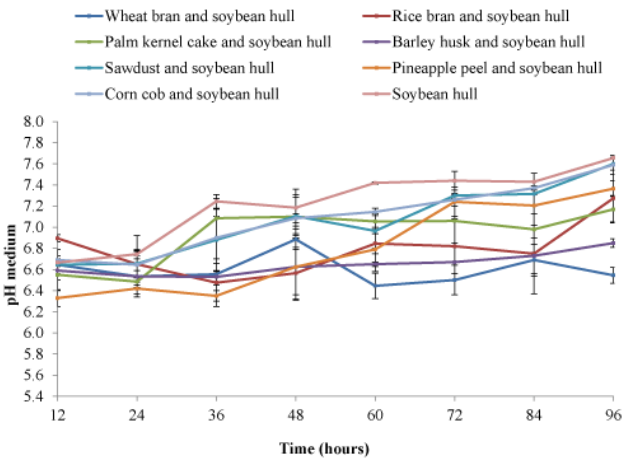

Figure 7: pH medium profile during xylanase production by *B. subtilis* using various agricultural extracts as carbon source in SsF.

The growth profile of *B. subtilis* during xylanase production in SsF is illustrated in Figure 8. Our results revealed that high growth of *B. subtilis* using agricultural extracts as the carbon source corresponded closely to the production of xylanase. As a result, higher biomass concentration was also obtained in SsF. Overall, most of the bacteria cultures in SsF adapted the growth conditions after 24-hour of fermentation. Log phase began from 24-hour reaching the maximum peak at 48-hour of the late stationary phase before declined until the

end of fermentation as demonstrated in Figure 8. The method for determining protein concentration is performed via acid hydrolysis of the protein to analyse the amino acid composition [92]. For the estimation of protein concentration, Lowry method has been used to analyse the quantitative measurement of total soluble protein in the enzyme samples. It is highly desirable and simpler, yet easily replicable methods to assess protein concentration. Figure 9 illustrates the soluble protein production by *B. subtilis* during xylanase production using various agricultural extracts as carbon source in SsF. In SsF, pineapple peel produced the highest maximum protein concentration of 29.823 ± 4.400 mg/mL whereas palm kernel cake produced the lowest maximum protein concentration of 17.180 ± 1.316 mg/mL. Table 3 illustrates the summary of xylanase production by *B. subtilis* using optimised carbon source of wheat bran and pineapple peel in SmF and SsF, respectively.

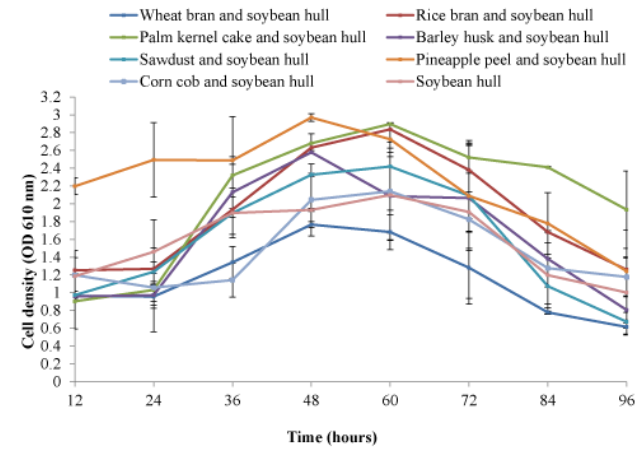

Figure 8: Growth profile of *B. subtilis* during xylanase production using various agricultural extracts as carbon source in SsF.

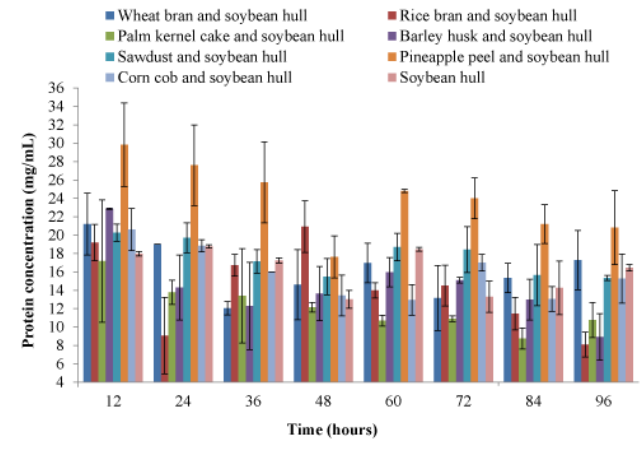

Figure 9: Protein concentration produced by *B. subtilis* during xylanase production using various agricultural extracts as carbon source in SsF.

Types of Fermentation	SmF	SsF
Optimised carbon source	Wheat bran	Pineapple peel
Growth Conditions		
Fermentation period (hours)	96	96
Incubation temperature (oC)	37	37
Standard inoculum size (total number of cells)	1×10^8	1×10^8
Initial pH medium	6.5	6.5
Agitation speed (rpm)	150	150
Analysis		
Maximum xylanase activity (U/mL)	11.968 ± 1.419	18.875 ± 0.569
Fermentation time at the maximum activity (hours)	48	48
Maximum biomass production (OD610 nm)	2.7735	2.9705
Optimum pH medium	6.98 ± 0.25	6.63 ± 0.27

Table 3: Summary of xylanase production by *B. subtilis* using optimised carbon source in SmF and SsF.

Conclusion

Xylanase has a huge usage in many industrial applications such as bio-bleaching of kraft pulp, feedstock processing, beer and juice clarification, softening of fruits, bioconversion of agricultural wastes and textile processing. Since xylanase has become a widespread in commercial production, it is necessary to develop low production cost for xylanase production besides reducing pollutions of organic agro-wastes to the environment. In terms of economical cost production, many industries in the worldwide have gained interest in finding cheap and eco-friendly alternative carbon source as the replacement. Notably, research studies have been reported on the comparison of different carbon sources and fermentation parameters in producing optimum xylanase activity by *B. subtilis* through SmF and SsF. SmF is homogenous on the distribution of nutrients and gas exchanges in addition to the efficient aeration capability in a liquid medium. However, SsF is most preferred than SmF. SsF is performed with no presence of free moving water, but contains moistened solid substrate with dissolved water as the continuous phase for the growth of the microorganisms. Therefore, SsF is a highly preferred method for the production of industrial enzymes especially xylanase. Besides that, the optimisation of xylanase production is required to develop the appropriate fermentation parameters in both fermentation processes in order to obtain the maximum xylanase activity. In this study, xylanase activity and its biomass concentration from *B. subtilis* was evaluated by DNS method and optical density via spectrophotometer, respectively. Various carbon sources from different agricultural extracts on xylanase production were also investigated. In SmF, wheat bran was found to be the optimum carbon source for xylanase activity, producing 11.968 ± 1.419 U/mL by *B. subtilis* at 48-hour of fermentation, followed by sawdust of 10.110 ± 1.686 U/mL. Meanwhile, pineapple peel was found to be the best substrate in SsF

for xylanase activity by *B. subtilis*, producing 18.875 ± 0.569 U/mL. Besides pineapple peel, wheat bran and rice bran in SsF also showed a higher xylanase activity at 17.364 ± 1.973 U/mL and 15.732 ± 0.657 U/mL, respectively. Based on the results in this study of SmF, xylanase activity was highly affected by incubation time and pH medium. In the nutshell, the optimum xylanase was observed between 48 and 72-hour. Indeed, pH of medium is strongly affected many enzymatic and metabolite processes. An increase in xylanase production was observed by initial increase of pH from 6.0 to 7.0. A further increase pH medium caused a gradual decline in xylanase yield. Nevertheless, the link between xylanase activity and agricultural extracts especially pineapple peel by *B. subtilis* is poorly understood. In order to increase xylanase activity, further work is needed. Hence, a detailed investigation is required to study the optimisation of xylanase activity by *B. subtilis* through SmF and SsF of agricultural extract as the prime carbon source.

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