

Original research Articles

OVERPRODUCTION OF XYLANASE FROM MUTANTS OF *Bacillus subtilis* USING BARLEY HUSK AS THE PRIME CARBON SOURCE IN SUBMERGED FERMENTATION AFTER RANDOM MUTAGENESIS OF ETHYL METHANE SULFONATE (EMS) AND ACRIDINE ORANGE (AO)

ABSTRACT

Aims: Xylanase also known as endo-1,4- β -xylanohydrolase is a type of hydrolytic enzyme participated in the hydrolysis of hemicelluloses particularly in xylan to generate xylose and xylo-oligosaccharides. Due to its enormous potentials, xylanase is frequently used in biobleaching of kraft pulp, clarification of fruit juice, extraction of plant oils, processing of animal feeds, softening of fruits, degradation of agricultural wastes and plant fibers and manufacturing of chemicals including biofuel, ethanol and xylitol. These applications of xylanase avoid the use of chemicals that are expensive, mutagenic and highly non-biodegradable. Interestingly, in recent years, the applications of xylanase in biobleaching and bioprocessing of paper pulp have gained numerous attentions and interests in the industry over the world. Therefore, couple of lignocellulolytic substrate as the alternative cheap carbon source and strain improvement for overproduction of microbial xylanase is implemented as a more potent approach in improving its yield and productivity in submerged fermentation. As a result, the main aim of the present study was primarily involved in the overproduction of xylanase by five mutant strains of *Bacillus subtilis* subsp. spizizenii ATCC 6633 designated as the MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 in submerged fermentation using barley husk as the prime carbon source.

Methodology: In order to attain the mutants, *B. subtilis* was subjected to random mutagenesis of ethyl methane sulfonate (EMS) and acridine orange (AO) in the earlier study before screened for the overproduction of xylanase in the present investigation.

Results: Based on the present investigation, we targeted to verify and produce xylanase from mutant strains of *B. subtilis* ATCC 6633 as the potent bacterial xylanase producers using cheap agro-industrial residue of barley husk as the sole carbon source under submerged fermentation. Furthermore, extracellular protein production and profile of medium pH during growth of wild type and mutants of *B. subtilis* under submerged fermentation were also elucidated. Based on the result findings, the time course of xylanase biosynthesis by the mutants of *B. subtilis* revealed that the enzyme production was materialized starting from the logarithmic to stationary growth phase whereby the maximum xylanase activity was achieved after 24 h of fermentation. In fact, all mutant strains of *B. subtilis* were successfully synthesized relatively higher production of xylanase than their parental wild type in submerged fermentation using barley husk as the prime carbon source. Notably, the maximum xylanase activity of 1.76 U/mL was attained by the mutant MXB 4 of *B. subtilis* which was approximately 1.3-fold increase in xylanase activity than the wild type with 1.36 U/mL. Furthermore, MXB 1, MXB 2, MXB 3 and MXB 5 also

exhibited comparatively higher maximum xylanase activity of 1.64 U/mL, 1.73 U/mL, 1.74 U/mL and 1.66 U/mL compared to their parental wild type. Indeed, the statistical single factor analysis of variance (ANOVA) on xylanase production revealed there was significant difference in the mean of the xylanase production by the wild type and mutant strains of *B. subtilis* ($p < 0.05$). On the other hand, the total maximum extracellular protein production was achieved by mutant strains of MXB 2 and MXB 4 with protein concentration of 0.82 mg/mL. Both demonstrated increment of 1.49-fold in protein production than the wild type which possessed relatively lower concentration of 0.55 mg/mL. Besides that, the profile of medium pH on xylanase activity by mutants and wild type of *B. subtilis* was also elucidated in the present study. The highest xylanase activity was attained at slight acidic pH of 6.1 as shown by the mutant MXB 4 in comparison with wild type at pH 6.47.

Conclusion: In a nutshell, the result findings suggested the mutant strains of *B. subtilis* ATCC 6633 particularly MXB 4 as the most potent xylanase producer in submerged fermentation using carbon source from agricultural extract of barley husk. Mutant MXB 4 of *B. subtilis* is anticipated to be beneficial in various xylanase applications especially in the processing of animal feeds and food industry.

Keywords: Overproduction, xylanase, random mutagenesis, *Bacillus subtilis*, submerged fermentation

1. INTRODUCTION

Enzymes are the biocatalysts function with crucial roles in all stages of metabolism and biochemical reactions. In recent years, a tremendous upsurge in the utilization of enzymes as industrial catalysts has been observed. These enzymes often offer immense benefits in comparison to the use of conventional chemical catalysts for numerous reasons. Enzymes are generally exhibit high catalytic activity. Furthermore, they are highly biodegradable and economically feasible that commonly produced in large amounts without any danger threats to the environment [1]. Amongst these enzymes, there has been drastic increase in the production of enzymes such as xylanase due to its pivotal roles in vast biotechnological applications. The biodegradation characteristic of xylanase on plant cell wall described this enzyme possesses one essential character along with many significant applications [2]. Xylanase has also proven plausible in the clarification of fruit juices and wines, production of modified xylan as bulking agent for food processing, conversion of xylan into monomeric products such as sweetener liked xylitol as well as modification of cereal flour to enhance volume, texture and staling properties of bread [1]. In fact, biobleaching using xylanase is crucial especially in the manufacturing of pulp paper by reducing and releasing lignin component. As a result, it increases the brightness of pulp which involves the hydrolytic breakdown of xylan. Notably, xylan is a heterogeneous polysaccharide that consists of a linear backbone of β -1,4-D-xylopyranoside residues with short side-chain branches [3]. Xylan is the most abundant, natural non-cellulosic polysaccharide present mainly in the secondary cell wall in plants constituting about 20% to 35% of the total dry weight in tropical plant biomass. Xylan hydrolyzing enzymes are crucial industrial enzymes. Consequently, intense researches in modern years have focused on xylanases

production by many types of microorganisms. On the other hand, the choice of the substrate is of great importance for the selection and successful production of large amount of xylanases. Many lignocellulolytic substrates such as barley husks, corncobs, sugarcane bagasse and wheat bran have been studied and a good number of them have performed significantly better than isolated purified xylans with respect to the yields of xylanase in large-scale production processes. Maximum xylanase production and activity using cassava bagasse as the carbon source was reported by Sugumaran et al. [4]. Indeed, Ecaterina et al. [5] mentioned that barley husk comprised of various polysaccharides with hemicelluloses as the most important component suitable for xylanase hydrolysis. In fact, a study was conducted by Soliman et al. [6] where they reported that the maximum xylanase activity of 8.66 g/L was produced by *Bacillus mojavensis* A21 using barley bran as the carbon source. In accordance, many studies have also been evaluated using barley husk for production of ethanol [7]. Nonetheless, only a few studies have been conducted regarding *B. subtilis* subsp. *spizizenii* ATCC 6633 in respect to xylanase production. Therefore, in the present study, we embarked on the following objectives to verify the production of xylanase from the mutants of *B. subtilis* after random mutagenesis of ethyl methane sulfonate (EMS) and acridine orange (AO) using plate hydrolysis assay and to elucidate overproduction of xylanase from the mutants of *B. subtilis* under submerged fermentation using the prime carbon source of barley husk compared with its parental wild type of *B. subtilis*.

2. MATERIALS AND METHODS

2.1 Mutants and wild type of *Bacillus subtilis* subsp. *spizizenii* ATCC 6633

Bacillus subtilis subsp. *spizizenii* ATCC 6633 was obtained from American Type Culture Collection (ATCC), Manassas, USA. *B. subtilis* ATCC 6633 was subcultured on nutrient agar and incubated at 30°C until bacterial colonies developed. Subsequently, the bacteria was maintained and stored at 4°C. On the other hand, total of five mutants of *B. subtilis* were produced after random mutagenesis using EMS and AO as the chemical mutagens to induce the random mutation in wild type of *B. subtilis* subsp. *spizizenii* ATCC 6633 for xylanase production according to the method mentioned by Ho and Chor [8]. Notably, in this study, the mutant strains of *B. subtilis* were designated as MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 as shown in Table 1, respectively.

Table 1. Mutagens used for random mutagenesis of *B. subtilis* ATCC 6633

Mutant	Mutagen concentration	Exposure time (min)
MXB 1	100 µg/mL EMS ^a	15
MXB 2	50 µg/mL AO ^b	15
MXB 3	50 µg/mL AO ^b	30
MXB 4	10 µg/mL AO ^b	15
MXB 5	10 µg/mL AO ^b	30

^aEthyl methane sulphonate (EMS)

^bAcridine orange (AO)

2.2 Preparation of standard inoculum

In this study, mutant cells were harvested from overnight nutrient agar grown with the mutant cultures of *B. subtilis*. A loopful of MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 mutant cultures was aseptically transferred into five different test tubes containing nutrient broth and incubated overnight at 37°C at 150 rpm for 24 h, respectively. The overnight test tubes were then vortexed to produce a homogeneous cell suspension. Serial dilution of the cell suspension of the mutant cultures was conducted using sterile distilled water to prepare the standard inoculum. On the other hand, the standard inoculum of the wild type of *B. subtilis* used as the control was also performed using the similar steps. 2.0×10^6 cells were used as the standard inoculum in the submerged fermentation for xylanase production by wild type and mutants of *B. subtilis* in this study.

2.3 Confirmation of xylanase activity from mutants of *B. subtilis* using plate hydrolysis assay

The confirmation of xylanase activity from five mutants of *B. subtilis* ATCC 6633 was conducted using plate hydrolysis assay. To detect the activity of xylanase, beechwood xylan agar adjusted at medium pH 6.5 with the following composition (g/L): beechwood xylan, 10; peptone, 5; yeast extract, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; K_2HPO_4 , 1 and agar, 15 was used in this confirmation test. To begin, 20 μL of the five mutant cultures were aseptically pipetted on the xylan agar, respectively. Using sterile hockey stick, the mutant cultures were spread uniformly on the agar. The agar plates were then incubated at 37°C until the mutant cultures developed and covered the surface of the agar. The plates were then subjected to staining with 3 mL of 1% w/v Congo red dye before allowed to stand for 15 min at room temperature. Subsequently, 1 M NaCl was used as the destaining reagent to drain off the excess dye from the agar. Meanwhile, wild type of *B. subtilis* was used as the control of this experiment to detect the production of xylanase. All experiments were performed in triplicate. At the end of the experiments, the appearance of clear ring zone of discoloration on the xylan agar occurred due to the presence of xylanase activity where xylan substrate was hydrolyzed by xylanase secreted by the mutants of *B. subtilis* ATCC 6633.

2.4 Xylanase production by wild type and mutants of *B. subtilis* under submerged fermentation using barley husk as the prime carbon source

The medium composition used for the xylanase production by mutants and wild type of *B. subtilis* ATCC 6633 in 250 mL working volume comprised of (g/L): peptone, 5; yeast extract, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; K_2HPO_4 , 1 and barley husk, 20 employed as the prime cheap alternative carbon source for the production of xylanase. Then, the pH of medium was adjusted to 6.5 before autoclaved at 121°C for 15 min. Subsequently, 2.0×10^6 cells of mutants used as the standard inoculum were inoculated into 250 mL medium in 500 mL Erlenmeyer flasks and incubated in the rotary incubation shaker at 150 rpm at 37°C for 72 h of submerged fermentation. On the other hand, wild type of *B. subtilis* was also inoculated and incubated for the production of xylanase as comparison. Samples were withdrawn and collected at every 12 h interval. In the present study, sampling analysis was carried out to elucidate the maximum

production of xylanase by mutants and wild type of *B. subtilis* using agricultural extract of barley husk as the sole carbon source in submerged fermentation. All the experiments were performed in triplicate and the mean value was generated from the analysis.

2.5 Sampling and analysis

10 mL samples of submerged fermentation were collected aseptically at regular interval of 12 h. The cell count and pH of the medium were measured from the collected samples. Then, the samples were centrifuged at 9000 rpm for 20 min at 4°C. Subsequently, the supernatant was collected and used for the quantification of extracellular protein concentration and xylanase activity based upon its specific substrate of beechwood xylan.

2.6 Xylanase activity assay

The xylanase activity was estimated according to Bailey et al. [9] with slight modification using 1% beechwood xylan in 0.05 M sodium phosphate buffer, pH 5.3 as the substrate. The detection of xylose as the reducing sugar after xylanase activity was conducted using 3, 5-dinitrosalicylic acid. To determine the xylanase activity, 0.1 mL of supernatant was added into 0.9 mL of 1% beechwood xylan substrate. The reaction was then incubated at 50°C for 30 min. 1.5 mL of 3, 5-dinitrosalicylic acid was added to the reaction mixture and incubated at 90°C for 5 min followed by the addition of 0.5 mL of 40% potassium sodium tartrate and subsequently allowed to cool at room temperature. The amount of xylose was determined by measuring the absorbance of the mixture at 575 nm via spectrophotometer. To quantify the xylanase activity, one unit of xylanase enzyme activity is defined as the amount of xylanase that catalyses the release of one micromole xylose per mL of enzyme extract per min under the standard assay conditions. 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, measured and plotted into a graph of the absorbance reading versus against concentrations. The activity of xylanase was quantified according to the standard curve of xylose. The results represent the mean value of xylanase activity from three independent experiments obtained from the fermentation samples of the wild type and mutants of *B. subtilis*. Standard deviation was calculated for the xylanase activity obtained at 12 h interval from the wild type and mutants of *B. subtilis*.

2.7 Quantification of total extracellular protein concentration

Total extracellular protein concentration was determined by Lowry method using Bovine Serum Albumin (BSA) as the standard [10]. 0.2 mL of supernatant was subjected to the quantification of total protein concentration by measuring the optical density at 750 nm.

2.8 Quantification of cell number of wild type and mutants of *B. subtilis*

Haemocytometer was used to estimate the number of cells of wild type and mutants of *B. subtilis* found in the fermentation samples.

2.9 Measurement of medium pH

The pH of culture medium was measured using a pH meter. The pH meter was calibrated with special calibration solution. The medium pH was adjusted by using 1 M NaOH and 1 M HCl.

2.10 Statistical analysis

In the present study, the data of the xylanase production by wild type and mutants of *B. subtilis* in the submerged fermentation in this study was statistically evaluated using single factor analysis of variance (ANOVA) at a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Confirmation of xylanase activity from mutants of *B. subtilis* using plate hydrolysis assay

Based on the results, all mutants of *B. subtilis* ATCC 6633 were detected with the ability to produce xylanase. The xylan plate hydrolysis assay provided unequivocal evidence that all mutants of *B. subtilis* ATCC 6633 produced extracellular xylanase activity as shown in Figure 1. The clear ring zone of discoloration was evidently formed surrounding the cultures and Congo red dye was in fact, added on the xylan agar for better observation of the clear zone. The discoloration of the clear zone was large enough where they were easily observed and detected with the naked eyes. Indeed, the ring zones of discoloration on the xylan agar incubated with mutants appeared after overnight incubation at 30°C. The presence of discoloration around the mutants colonies on the xylan agar occurred after overnight incubation proved the secretion of xylanase which particularly hydrolyzed its beechwood xylan substrate as a result of xylan hydrolysis. The xylan hydrolysis might be varied depends on their different tones of discoloration. Some of bacteria species might produce darker zone color compared to those from *Bacillus* spp. Members of the genus *Bacillus* have proven to possess the ability to produce a wide variety of extracellular enzymes in which xylanases are notably peculiar significance and industrial importance. When *Bacillus* spp that isolated by Annamalai et al. [11] were subjected to screening, they detected that the xylan binding xylanases could eliminate side chains from the xylan backbones. As a result, halo clear zones with the sizes of 18 mm to 24 mm in diameter around the bacteria colonies were observed on the xylan agar, thus, proving their xylanolytic abilities to hydrolyse xylan backbone which led to the formation of complex xylo-oligosaccharides with different degrees of composition and polymerization [12]. Xylo-oligosaccharides are composed of a mixture of oligosaccharides obtained from the breakdown of β -1,4-linkages of xylan-containing substrates by xylanase enzyme. Furthermore, an approximate study was also carried out amongst twelve strains of *Bacillus* spp in Rajshahi University, Bangladesh. One of which was characterized and found to be *B. cereus* isolated from soil. Their findings revealed the presence of

transparent-clear zone formation on xylan agar after incubated with *B. cereus* overnight [13]. Additionally, a 33 mm zone of clearance occurred around a bacteria colony on xylan agar after staining with Congo red dye, hence, proving the presence of xylanolytic ability that was belonged to *Bacillus arseniciselenatis* strain DSM-15340 [14].

Congo red dye was noted to interact only with intact 1,3- and 1,4- β -D-glucans. Pure beechwood xylan substrate used in this study was employed in the agar for the qualitative identification of xylan degrading microorganisms. Thus, the molecular mass of the substrate decreases simultaneously as the enzymatic reaction occurs [12]. Xylanase activity confirmation could be prepared with xylan extracted from different sources such as beechwood, oat spelt and birchwood. All demonstrate xylan hydrolysis process by producing xylose and xylo-oligosaccharides regardless of whether xylose is produced by xylan hydrolysis is based upon the origin of the xylanase [15, 16]. Notably, higher xylo-oligosaccharides and xylotetraose were also identified after hydrolysis using birchwood and oat spelt xylan [16]. Indeed, *Bacillus* sp. strain DSNC 101 released higher oligosaccharides, xylotriose and xylobiose, but a minor quantity of xylose was also observed, thus suggesting that the enzyme was an endoxylanase [17]. Furthermore, *B. subtilis* generated traces of xylose, xylobiose and xylotriose following hydrolysis of xylan but elicited no reducing sugar from cellulose and it thus described as true xylanase [18]. Based on the results in the present study, we could suggested that all mutant strains of *B. subtilis* ATCC 6633 released some xylo-oligosaccharides and xylose after xylanase hydrolysis of xylan as detected from the plate hydrolysis assay.

Wild type

MXB 1



MXB 2

MXB 3



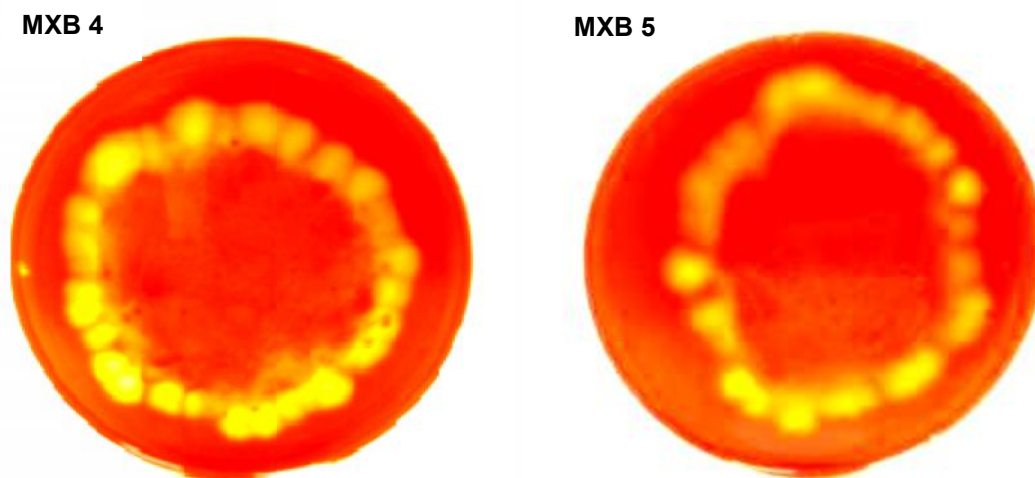


Figure 1. Xylan plate hydrolysis assay of wild type and mutants of *B. subtilis* ATCC 6633. The discoloration of Congo red dye occurred after overnight incubation of wild type and mutants of *B. subtilis* on xylan agar. The appearance of the clear ring zone as a result of discoloration of Congo red dye was observed on the xylan agar after the incubation of mutants of MXB 1, MXB 2, MXB 3, MXB 4 and MXB 5 of *B. subtilis* overnight. Wild type of *B. subtilis* ATCC 6633 as the control of the experiments also shows the presence of clear ring zone of discoloration. The clear ring zone formation occurred after the xylanase hydrolysis of xylan proving the secretion of xylanase from the mutant cultures.

3.2 Growth of wild type and mutants of *B. subtilis* under submerged fermentation using barley husk as the prime carbon source

Microbial enzymes production is usually growth-associated. The highest enzyme production from different microorganisms has been found to accelerate during stationary growth phase but deteriorate thereafter during the death phase [19]. In the present study, we disclosed the xylanase overproduction by mutant strains of *B. subtilis* subsp. *spizizenii* ATCC 6633 as the potent bacterial xylanase producer using cost effective sustainable agro-industrial residues of barley husk as the prime carbon source under submerged fermentation. *B. subtilis* ATCC 6633 is a subspecies of *B. subtilis*. ATCC nucleic acid data sheet describes it as *Bacillus subtilis* subsp. *spizizenii* strain NRS 231 created by Nakamura et al. [20] Based on the results, wild type and mutants of *B. subtilis* ATCC 6633 grown exponentially from 12 h to 24 h before crossed the threshold of the stationary growth phase. When *B. subtilis* grown in the batch submerged fermentation, the xylanase production and cell count typically changed constantly as a result of the metabolism of the bacterial cells. Data showed that the maximum xylanase activity from the wild type and mutants of *B. subtilis* was detected at 24 h of fermentation. Notably, the extracellular xylanase production was commenced after 12 h of inoculation of *B. subtilis* in submerged fermentation. Submerged fermentation has been exploited to play pivotal roles in attaining improved xylanase yields. Submerged fermentation allows the increased regulation of fermentation conditions including adequate and effective transfer of heat and mass. In fact, it is the preferred production method to obtain more purified enzymes [21, 22, 23]. As such, approximately all xylanases manufactured worldwide are produced under submerged fermentation [1]. Submerged fermentation also allows higher enzyme titres besides being

able to efficiently control the fermentation conditions. Apparently, it is diversely known for fermentation of aerobic microorganisms especially in the production of xylanase [21].

Based on the results observation, a rapid simultaneous increase of xylanase production was detected with a continuous increase of the log growth phase. The maximum cell count of 1.40×10^8 cells/mL for wild type, 1.16×10^8 cells/mL for MXB 1, 1.32×10^8 cells/mL for MXB 2, 1.13×10^8 cells/mL for MXB 3, 1.27×10^8 cells/mL for MXB 4 and 1.21×10^8 cells/mL for MXB 5 were attained at 24 h of fermentation, respectively. Apparently, the cells of wild type and mutants of *B. subtilis* grew at the fastest rate at 24 h of fermentation resulting in the most rapid xylan hydrolysis of barley husk. Subsequently, xylanase activity was decreased with increase in fermentation time from 36 h to 72 h. The xylanase production observed during the later stage of growth could be attributed to the reflection of minute amounts of xylanase liberated from the bacterial cells which were undergoing autolysis [24]. Nonetheless, the gradual decline of xylanase yield was associated with the bacterial cells which entered the death phase. Diminishing amount of nutrients, presence of growth inhibitors, cellular fragmentation and liberation of intracellular residues such as protease into the fermentation medium could be the reasons to inhibit and terminate xylanase activity [25, 26, 27]. In addition, according to Haq et al. [28], it might also due to the presence of crystalline residuals left in the medium that could not be hydrolysed by the bacteria for further xylanase production after the digestion of susceptible portion of xylan molecules was carried out. Indeed, the result findings in the present study revealed that there was a direct relationship between secretion of extracellular xylanase and cell count of *B. subtilis*. An increase in cell count resulted in a simultaneous increase in xylanase secretion into the culture medium which suggested that xylose was actively utilized by the wild type and mutants of *B. subtilis* during the growth phase. Several studies of xylanase production with similar patterns have been reported. Indeed, the maximum xylanase production from *B. subtilis* was detected during the stationary growth phase according to Bansal et al. [25].

Microbial xylanases from bacteria such as *B. subtilis* are the preferred catalysts for xylan breakdown due to their mild reaction conditions, high substrate specificity, minimal loss of substrate and nominal side product generation and it therefore has been considered worthy in diverse applications in the processing of food, animal feeds and paper pulp [1, 29]. Many microorganisms including fungi and bacteria have demonstrated their readiness to hydrolyse xylans by synthesising 1,4- β -D endo-xylanases (enzyme of this study) which cause the partial degradation of plant cell wall as a result of the softening occurred at the penetration region of cell wall. Indeed, microbial xylanolytic enzyme systems which hydrolyse xylan by synthesizing 1,4- β -D endo-xylanases and sometimes with β -xylosidases were also being reported in actinomycetes [30, 31], fungi [32, 33] and bacteria [34, 35]. There are several important xylanolytic enzyme producers include *Bacillus* [36], *Aspergillus* [37], *Streptomyces* [38], *Trichoderma* [39], *Clostridia* [40] together with a few of novel xylanases from *Simplicillia* [41] and *Armillaria* [32]. Among all the microorganisms, *B. subtilis* has been evaluated as a dominant xylanase producer [42]. Bacillaceae as the

interesting industrial microorganisms have been widely used than other bacteria. *B. subtilis* is described as a rod-shaped catalase-positive bacterium. In biotechnological applications for xylanase production, this microorganism is regarded as one of the most popular microorganisms exhibiting excellent fermentation properties. Therefore, *B. subtilis* is currently being utilised as the best characterized Gram-positive bacterium for xylanase production. *B. subtilis* has shown its viability to use various agricultural residues as sources of carbon in diverse medium compositions for biomass and xylanase production. Various agricultural wastes such as wheat straw, sugarcane bagasse, corn cobs, rice husk, soybean meal and wheat bran in varying medium components were evaluated to enhance growth and to produce high titres of xylanase.

Based on the uneconomical use of pure xylan for industrial xylanase production, the current inducement of this study is also investigated the deployment of xylan-rich low-cost agricultural residue such as barley husk. The finding obtained from this study is homogenous to the published reports from *Bacillus* species in attempt to employ agricultural residues as sources of carbon to boost xylanase production. In a study by Tork et al. [43], the highest maximum xylanase activity of 1.06 U/mL produced by *B. subtilis* XP10 was attained using barley bran as an inducer. Sugumaran et al. [4] exhibited 7.5 U/mL of xylanase activity from *B. subtilis* using cassava bagasse as carbon substrate achieved after optimisation of culture medium. Similar result was described by Sharma et al. [34]. They reduced the enzyme production cost of *B. coagulans* B30 by utilizing microwave treated forest agro-residuals as carbon sources for xylanase production. The biosynthesis of xylanase on xylan or xylan containing agro-residues suggested that xylan plays a pivotal role in effective induction of xylanase by *Bacillus* strains. Furthermore, Amore et al. [44] evaluated an appreciable xylanase activity produced by *Bacillus amyloliquefaciens* XR44A for its ability to hydrolyse brewer's spent grain which yielded the maximum xylanase activity of 10.5 U/mL following pre-treatment of grain with aqueous ammonia soaking under optimised conditions. Likewise, Irfan et al. [45] also investigated vast agricultural-based substrates for xylanase production from bacterial strain of *B. subtilis* BS05. As a result, the maximum xylanolytic activity of 439.5 ± 2.84 U/mL was attained using sugarcane bagasse in the production medium constituents containing sucrose and yeast extracts. On the other hand, *Bacillus subtilis* BL53 showed the highest specific activity of xylanase within 72 h of cultivation using industrial soybean residue as the carbon source [46]. Furthermore, the use of barley husk as the optimum alternative cheap substrate for xylanase production by *B. subtilis* was detected in a study conducted by Soliman et al [6]. Therefore, carbon source of barley husk was used in the present study to elucidate the production of xylanase by wild type and mutant strains of *B. subtilis* ATCC 6633. Below here in Figure 2 shows the growth profile of wild type and mutants of *B. subtilis* ATCC 6633 during the production of xylanase using barley husk as the prime substrate in the present study.

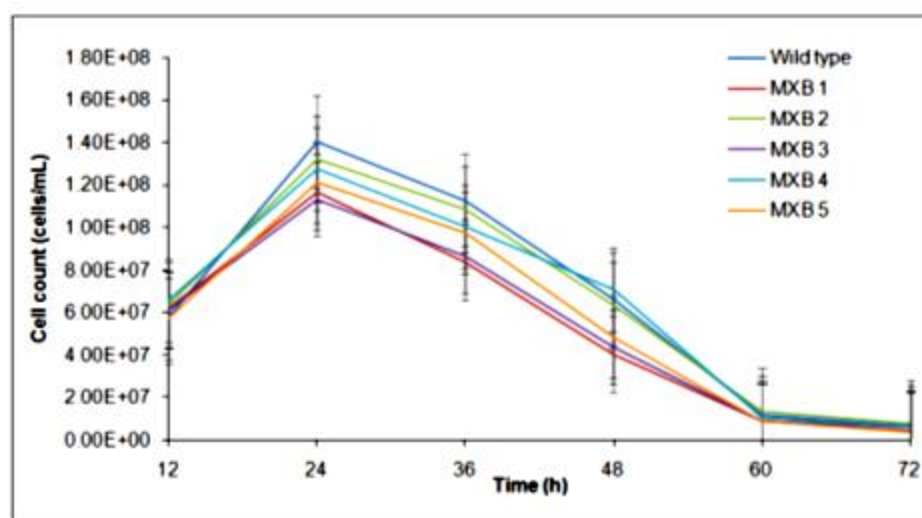


Figure 2. Growth profile of wild type and mutants of *B. subtilis* ATCC 6633 during the production of xylanase in submerged fermentation. All experiments were conducted in triplicate while the error bars indicate the standard deviation.

3.3 Xylanase production by wild type and mutants of *B. subtilis* under submerged fermentation using barley husk as the prime carbon source

Submerged fermentation has brought out industrial-level production of xylanase. According to Corral and Villasenor-Ortega [22], significant fermentation conditions such as temperature, medium pH, oxygen saturation and agitation of the culture medium which possess significant effects on yield of xylanases are optimized in submerged fermentation. Almost all industrial xylanases production worldwide is conducted under submerged fermentation [1]. Study on the time course is a crucial determining factor which exhibits the efficacy of the fermentation process in association with enzymes formation. Copious studies divulged the maximum xylanase production occurred at 36 h to 48 h after inoculation [2, 11]. In this study, submerged fermentation supported the growth of the wild type and mutants of *B. subtilis* utilizing free flowing liquid substrate of barley husk to produce xylanase is shown in Figure 3. Based on the results, all of the mutants were observed to possess their own maximum xylanase activity under the same growth conditions at 24 h of fermentation. Notably, the maximum xylanase activity of 1.76 U/mL by the mutant MXB 4 of *B. subtilis* was attained compared to the xylanase production of the wild type with 1.36 U/mL. Indeed, this maximum xylanase activity from the MXB 4 was increased by approximately 1.3-fold compared to the wild type. Furthermore, MXB 1, MXB 2, MXB 3 and MXB 5 also exhibited relatively higher maximum xylanase activity of 1.64 U/mL, 1.73 U/mL, 1.74 U/mL and 1.66 U/mL in comparison with their parental wild type of 1.36 U/mL.

In the present study, the data of the xylanase production by wild type and mutants of *B. subtilis* ATCC 6633 in the submerged fermentation in this study was statistically evaluated using single factor analysis of variance (ANOVA) at a significance level of $p < 0.05$. Based on the results, we observed that there were

variations in the sum of square (SS) in xylanase production, producing values of 0.098316 and 0.010815 between and within the groups of wild type and mutants of *B. subtilis* in the present study. Furthermore, there were also differences in the mean square (MS) of xylanase production between and within the groups of wild type and mutants with the values of 0.098315 and 0.002704, respectively. MS was quantified by dividing the SS with the degree of freedom (df). In addition, since the F-statistics value of 36.363187 is larger than F-critical value of 7.708647 and p value of 0.003812 is less than the alpha value of 0.05, we rejected the null hypothesis. As such, we concluded that there was significant difference in the mean values for the production of xylanase between the mutants and wild type of *Bacillus subtilis* ATCC 6633 using barley husk as the carbon source under submerged fermentation.

Other related approaches in attempt to increase xylanase production using mutant strains have also been conducted and elucidated. Khodayari et al. [47] performed related study by using *Bacillus* sp. KH-13 to improve xylanase production. In their experiments, the chemical mutagen of ethidium bromide was used to induce mutation of the bacterial strain. As a result, the mutant known as *Bacillus* sp. KH13-M4 produced xylanase activity of 2.58 U/mL which was around 1.36-fold higher than its parental strain. Additionally, UV mutagenesis was also subjected to *Bacillus mojavensis* PTCC 1723 to overproduce xylanase under the optimised growth conditions [2]. In their study, the selected mutants were successfully produced 3.45-fold more xylanase enzyme than the wild strain. Likewise, Kumar et al. [48] also performed the overproduction of extracellular xylanase by *Thermomyces lanuginosus* SK after subjected to N-methyl-N-nitro-N-nitrosoguanidine and UV irradiation. As a result, the mutant *T. lanuginosus* MC 134 possessed 1.5-fold increase in xylanase yield using oat spelt xylan as the substrate when compared to the wild type strain. On the other hand, Deshpande et al. [49] studied a mixed culture of *Aspergillus niger* and *Trichoderma reesei* QM, whereby the mutant number 414 stimulated 2-fold increase in xylanase production in the fermentation medium. Random mutagenesis in earlier study also showed improved efficacy for strain improvement of *B. subtilis* ATCC 6633 by enhancing its xylanase productivity several fold in comparison to the wild type [8]. Chemical mutagens of EMS and AO were probably caused by alkylation of guanine residues in the mutants of *B. subtilis*. As a result, the permanent lesions within the gene of xylanase were anticipated in the mutants that eventually formed clusters of closely linked mutations. It would therefore potentially increase and improve xylanase production [50]. Furthermore, the increased production of xylanase in mutants of *B. subtilis* particularly MXB 4 with the highest maximum xylanase activity could also owing to the ability of the mutant strain in gaining easier access of the hemicelluloses of xylan in barley husk [51]. In essence, the results from the present study indicated the use of mutant strains was a constructive method for significant xylanase overproduction utilising lignocellulosic material of barley husk as the substrate. Barley husk is a by-product of barley germination. The cell wall of barley tissue is primarily consisted of arabinoxylan found in the husk [52]. The husk which also known as the hull represents 10% to 30% of the barley kernel is composed of hemicellulose, cellulose and lignin with a minimal quantity of barley [53]. Indeed, it is predicted that the complete xylan

degradation of barley requires endoxylanases and β -xylosidases in association with arabinofuranosidases in order to liberate arabinose and xylose [54]. Furthermore, huge differences in the digestibility and solubility of barley cell wall polysaccharides are apparently depended on the composition of the growing barley grain [55]. In essence, one of the advantages of using barley husk as a substrate includes the production of high xylanase titres. Barley grain possesses a characteristic of softwood xylan that is known for a high content of β -glucans ranging from 2.5% to 11.3%. In fact, arabinoxylans and β -glucans are the principal structural residues of the cell wall in the different tissues that make up the barley grain which is suitable for enhanced xylanase production.

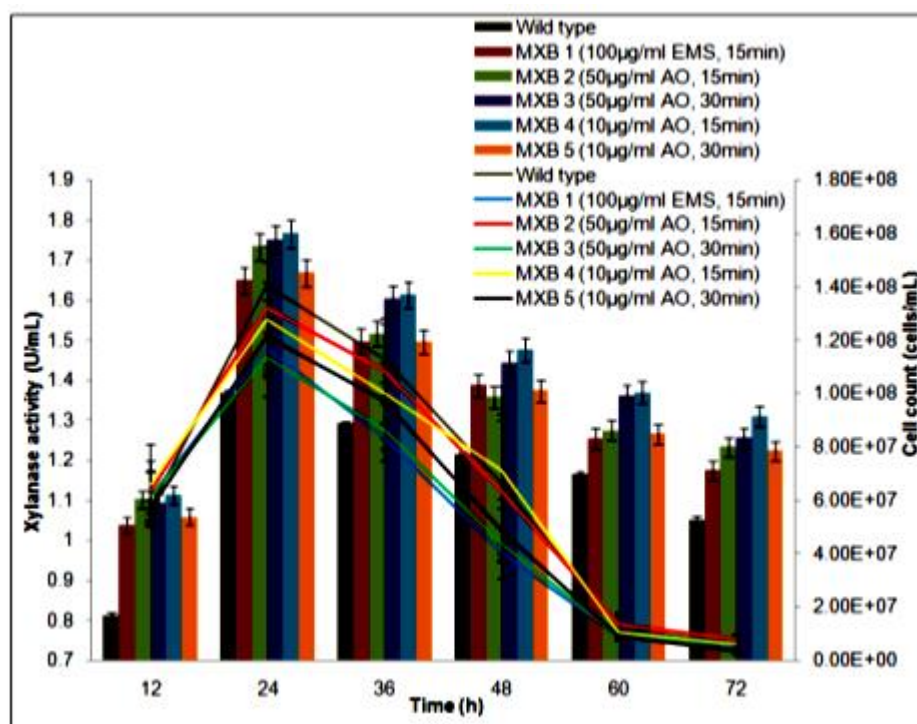


Figure 3. Production of xylanase by wild type and mutants of *B. subtilis* ATCC 6633 using barley husk in submerged fermentation. All experiments were conducted in triplicate and all assays were carried out at 12 hour interval. The error bars indicate the standard deviation. Xylanase activity is represented as column charts while cell count is represented as line graphs.

3.4 Extracellular protein production by wild type and mutants of *B. subtilis* under submerged fermentation using barley husk as the prime carbon source

Protein is one of the biological metabolites produced by *Bacillus* spp during the fermentation using agricultural residues as the carbon sources. Based on Figure 4, the maximum level of extracellular protein secretion from the wild type and mutants of *B. subtilis* was attained after 24 h of fermentation. In the present study, the mutant strains of MXB 2 and MXB 4 possessed the highest maximum extracellular protein concentration of 0.82 mg/mL with the cell count of 3.36×10^8 cells/mL and 3.13×10^8 cells/mL, respectively. This was followed by the mutant of MXB 3 with its protein production of 0.81 mg/mL from cell

count of 3.32×10^8 cells/mL. On the contrary, wild type of *B. subtilis* showed an extracellular protein concentration of 0.55 mg/mL. Indeed, mutants of MXB 2 and MXB 4 exhibited 1.49-fold increment in protein secretion than the wild type under the same conditions. Thereafter, there was gradual decline in protein production in the medium along with reduction in the bacteria growth rate. In the course of this study, low yields of extracellular protein concentration produced by wild type and mutants of *B. subtilis* ATCC 6633 were probably due to the protease secretion [41]. Notably, *Bacillus* spp secrete high levels of extracellular protein into the medium. Nonetheless, they also secreted high amounts of proteases which hydrolyse the peptide bonds of proteins. *Bacillus* spp have also been found to be the principal producer of protease which results in shortcoming of protein production [56].

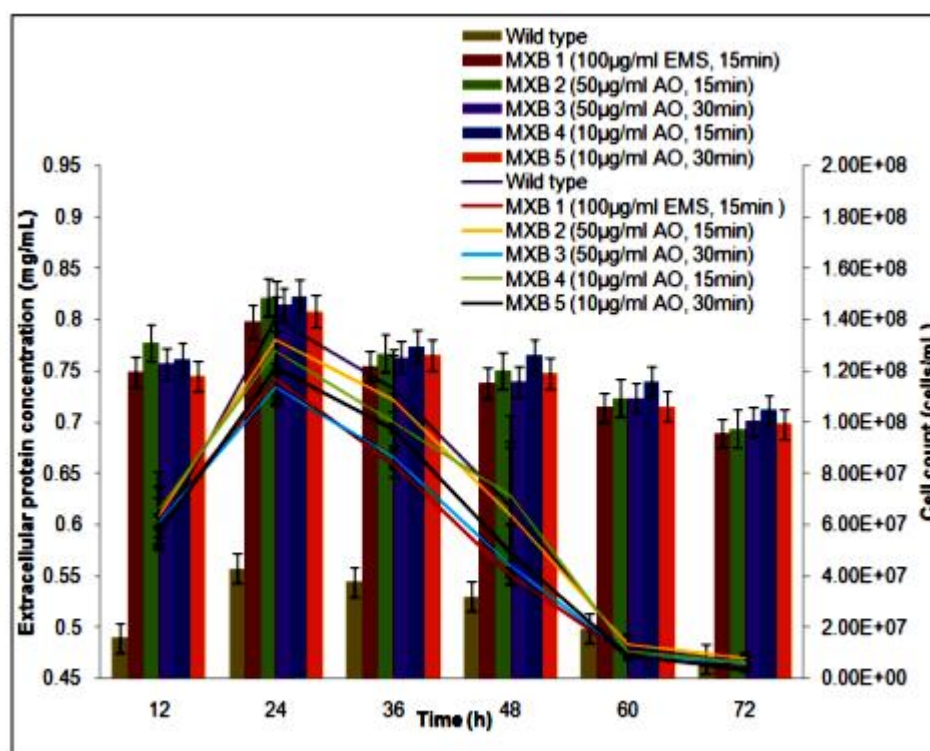


Figure 4. Total extracellular protein production by wild type and mutants of *B. subtilis* ATCC 6633 using barley husk in submerged fermentation. All assays were done at 12 hour interval and conducted in triplicate. The error bars indicate the standard deviation. The total extracellular protein concentration is represented as column charts while cell count is represented as line graphs.

3.5 Profile of medium pH during growth of wild type and mutants of *B. subtilis* under submerged fermentation

The influence of medium pH on xylanase production is shown in Figure. 5. Medium pH is among the most important parameters in fermentation process. The changes of medium pH are depended on the medium composition. The mutants in this study achieved the maximum cell count at medium pH range of 6.1 to

6.5 after 24 h of inoculation. In fact, simultaneous increase in xylanase activity with its increase in medium pH occurred at 24 h of fermentation when barley husk as the source of carbon was used for the xylanase production by wild type and mutants of *B. subtilis* ATCC 6633. Apparently, the maximum xylanase production of 1.36 U/mL by the wild type of *B. subtilis* ATCC 6633 was attained at the near neutral medium pH of 6.47 compared to the mutant of MXB 4 with the xylanase activity of 1.76 U/mL at slight acidic pH of 6.1. In fact, the maximum xylanase activity exhibited at slight acidic medium pH of 6.2, 6.0, 6.1, and 6.2 for MXB 1, MXB 2, MXB 3 and MXB 5, respectively. On the contrary, when the fermentation was prolonged after 72 h, relatively lower xylanase production from the wild type and mutants of *B. subtilis* ATCC 6633 was observed at the medium pH from 7.5 to 7.9. Based on the result findings, we suggested that mutants of *B. subtilis* achieved their maximum xylanase production at the slight acidic medium pH while wild type produced xylanase at the near neutral pH under submerged fermentation. Similar optimum pH for *Bacillus* spp has been observed in many studies for xylanase production. Ammoneh et al. [57] reported the optimum medium pH of 7 was needed for the maximum production of xylanase by *B. pumilus* SY30A and medium pH 6 for *B. subtilis* SY185C and SY190E, respectively. In another study, *B. subtilis* was reported to produce the optimum xylanolytic enzymes of 12.0 U/mL at medium pH 6.0 within 18 h of fermentation [58]. Furthermore, the maximum xylanases production by *B. licheniformis* [59], *Bacillus* sp DM-15 [60] and *B. pumilus* [61] exhibited at the optimum medium pH of 6.5. Nonetheless, the optimum medium pH of a few bacterial xylanases has also been detected at the alkaline medium pH. The optimum pH for xylanase production by *Bacillus* sp KH-13 with its ethidium bromide mutant *Bacillus* sp KH13-M4 occurred at medium pH 9.5 besides *B. pumilus* showed its optimum activity at pH 9.0 [47, 62]. In this study, the medium pH might probably influence the transport of xylanase across the cell membrane. Knowing that enzymes are globular proteins, the ionic characteristic of the carboxylic and amino acid groups on the xylanase surface are predisposed to be influenced by changes in pH, as such, the catalytic attribute of the xylanase was strikingly influenced [63]. In the present study, fermentation that occurred at relatively higher and lower medium pH affect the xylanase production by wild type and mutants of *B. subtilis* ATCC 6633. Variations in the external pH of the medium possess the ability to alter the ionization of nutrient molecules by reducing the availability of nutrients to the microorganisms. As a result, the drastic changes in pH impair microbial cells by disrupting their plasma membranes, limiting their accessibility of substrate and interfering their metabolisms for growth and xylanase secretion [64]. Therefore, different mutants of *B. subtilis* ATCC 6633 displayed their distinctive pH profile for its optimal growth and xylanase activity using barley husk as the source of carbon in the submerged fermentation.

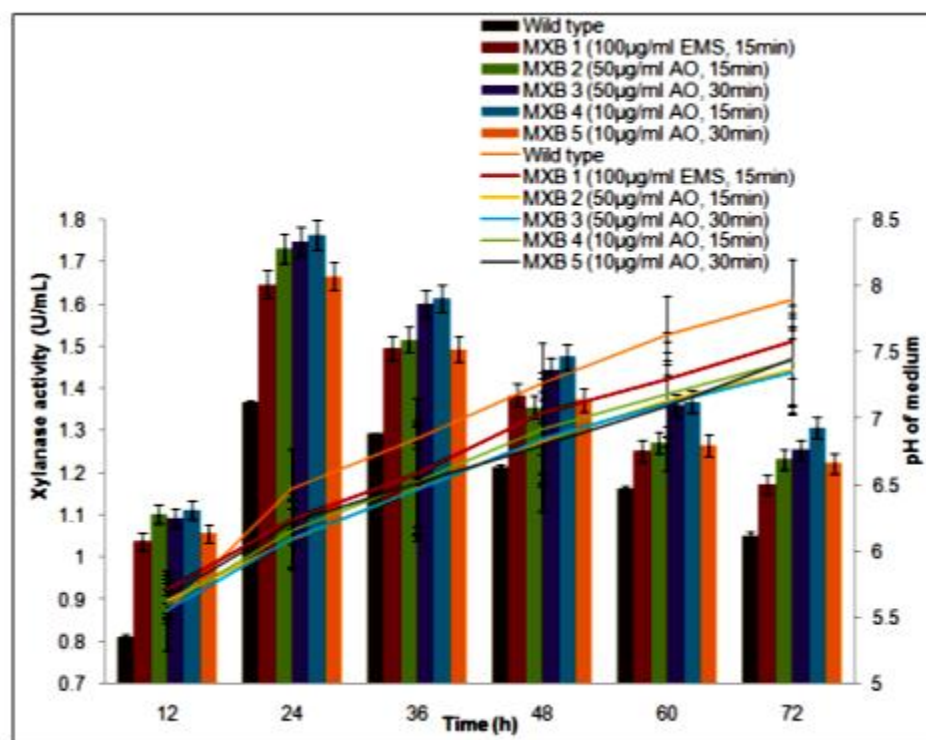


Figure 5. Profile of medium pH during growth of wild type and mutants of *B. subtilis* ATCC 6633 under submerged fermentation. pH of the medium was quantified at 12 hour interval. All assays were conducted in triplicate. The error bars indicate the standard deviation. The xylanase activity is represented as column charts while medium pH is represented as line graphs.

4. CONCLUSION

This work was investigated to verify, determine and analyse xylanase production by wild type and mutants of *B. subtilis*. It presents an attempt made to improve xylanase secretion of mutants of *B. subtilis* after random chemicals mutagenesis of EMS and AO using barley husk as the primary cheap xylan-rich source of carbon in submerged fermentation. The outcome obtained in the present study demonstrated that all mutants of *B. subtilis* ATCC 6633 were verified to produce xylanase after xylan hydrolysis as detected by Congo red dye staining on the xylan agar. The extracellular protein concentration and xylanase activity of all the mutants particularly MXB 4 increased after 24 h of fermentation compared to their parental strain. The result findings might attribute to the fact that EMS and AO induced visible and lethal mutations in the xylanase genes, causing alkylation of guanine residues which resulted the permanent lesions within the DNA and thereafter brought about clusters of closely linked mutations and thus improved the expression of the genes coding for xylanase production. Hence, EMS and AO could be considered as effective mutagens causing the overexpression and overproduction of xylanase enzyme. In fact, in comparison to the wild type, these mutants established relatively higher xylanase activity in submerged fermentation. Notably, five mutants of *B. subtilis* ATCC 6633 were successfully showed increase of the maximum xylanase activity after 24 h of inoculation in comparison to the wild type parental

strain. The highest xylanase producing mutant of MXB 4 attained after subjected the wild type to 10 µg/mL of AO for 15 min. MXB 4 possessed the maximum xylanase activity of 1.76 U/mL in the medium compared to the xylanase production of the wild type with 1.36 U/mL at medium pH 6.47.

Therefore, by employing combined approaches of strain improvement using random mutagenesis and economical source of substrate are potentially be cost effective in overproduction of xylanase for industrial perspective. Cheaper and efficient secretion and production of xylanases are in fact crucial to increase industrial production level. Thus far, many approaches on the enhancement of xylanase activity have been limited to the improvement of its alkaliphilicity and thermostability. Therefore, improved techniques are implemented to find more potent fungal or bacterial strains for xylanase production. Notably, the cost of production medium in the industry also affects the economics of the fermentation process. Decrease in production cost could be achieved by using lower cost and easily available agricultural residual materials. Barley husk is produced as a by-product of barley. It is also known to be rich in carbohydrates serving as a carrier in submerged fermentation, supporting the growth of microorganisms by providing nutrients in enhancing the production of enzymes. Furthermore, submerged fermentation is a preferred fermentation approach since it allows for increase regulation of fermentation conditions, conversion of batch to continuous fermentation, adequate and effective release of heat that are essential for the growth of microorganisms and production of enzymes. Divergence microorganisms with varying potentials with the capability of synthesizing xylanase enzyme have been reported recently. One of which would be belonged to *Bacillus* spp. *B. firmus* and *B. subtilis* have been observed to produce high xylanolytic activity. Nevertheless, the production of xylanase by wild types *Bacillus* spp possesses certain limitations in submerged fermentation even after the optimization of medium formulation and growth conditions are established. Hence, the strain improvement approaches have been employed to improve xylanase production. Quantitative enhancement which involves strain improvement using random mutagenesis has been regarded as the initial approach due to its relatively lower cost of process. In many studies, chemical mutagens including N-methyl-N-nitro-N-nitroso guanidine (MNNG) promoted the increase of xylanase productivity by several folds when compared to the parental strain. Besides that, physical mutagenic agents involving UV irradiation has been routinely implemented to produce overproducing mutants. Additionally, protein engineering, recombinant DNA technology and site directed mutagenesis which aid in improving the desirable traits of xylanase have also been explored and conducted to generate mutants with the positive motivating characteristics. Therefore, in the previous study, several attempts of mutagenesis have been made to produce overproducing xylanase mutants of *B. subtilis* in order to enhance the production of the enzyme and to reduce the cost of production as far as the industrial production is concerned. In a nutshell, the result findings suggested that the mutant strains of *B. subtilis* ATCC 6633 particularly MXB 4 as the most potent xylanase producer in submerged fermentation using carbon source from barley husk is applicable in various industrial applications especially in animal feeds and food processing industry.

5. REFERENCES

1. Motta F, Andrade C, Santana M (2013) A review of xylanase production by the fermentation of xylan: Classification, characterization and applications. Sustainable degradation of lignocellulosic biomass techniques, application and commercialization. Open Intech 251-275
2. Ghazi S, Sepahy AA, Azin M, Khaje K, Khavarinejad R (2014) UV mutagenesis for the overproduction of xylanase from *Bacillus mojavensis* PTCC 1723 and optimization of the production condition. Iranian Journal of Basic Medical Sciences 17(11): 844-853
3. Kuhad RC, Singh A (1993) Lignocellulose bio-technology: current and future prospects. Critical Reviews in Biotechnology 13:151-172
4. Sugumaran KR, Kumar BK, Mahalakshmi M, Ponnusami V (2013) Cassava bagasse-low cost substrate for thermo-tolerant xylanase production using *Bacillus subtilis*. International Journal of ChemTech Research 5(1): 394-400
5. Ecaterina DP, Lonut SA, Gabriela GC, Domnica C (2014) Fodder yeast development optimisation using as main carbon source barley husks hydrolysed. Romanian Biotechnological Letters 19(1): 9085-9095
6. Soliman HM, Sherief AA, Tanash BEL (2012) Production of xylanase by *Aspergillus niger* and *Trichoderma viride* using some agriculture residues. International Journal of Agricultural Research 7: 46-57
7. Palmarola-Adrados B, Galbe M, Zacchi G (2005) Pretreatment of barley husk for bioethanol production. Journal of Chemical Technology and Biotechnology 80(1): 85-91
8. Ho HL, Chor XK (2015) Improvement of xylanase production by *Bacillus subtilis* in submerged fermentation after UV and chemicals mutagenesis. Journal of Advances in Biology and Biotechnology 3(2): 42-57
9. Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23(3): 257-270
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275

11. Annamalai N, Thavasi R, Jayalakshmi S, Balasubramanian T (2009) Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated form marine environment. Indian Journal of Biotechnology 8(3): 291-297
12. Nagar S, Mittal A, Gupta VK (2012) A cost effective method for screening and isolation of xylan degrading bacteria using agro waste material. Asian Journal of Biological Sciences 5(8): 384-394
13. Roy N, Habib MR (2009) Isolation and characterization of xylanase producing strain of *Bacillus cereus* from soil. Iranian Journal of Microbiology 1(2): 49-53
14. Kamble RD, Jadhav AR (2012) Isolation, purification and characterization of xylanase produced by a new species of *Bacillus* in solid state fermentation. International Journal of Microbiology 1-8
15. Beric T, Urdaci MC, Stankovic S, Knevzevic-Vukvcevic J (2009) RAPD analysis of genetic diversity and qualitative assessment of hydrolytic activities in a collection of *Bacillus* sp. isolate. Archives of Biological Sciences 61(4): 645-652
16. Yoon KH (2009) Cloning of the *Bacillus subtilis* AMX-4 xylanase gene and characterization of the gene product. Journal of Microbiology and Biotechnology 19(12): 1514-1519
17. Cho NC, Bai S (1997) Purification and characterization of xylanase from *Bacillus* sp. strain DSN 101. Journal of Microbiology and Biotechnology 7(6): 386-390
18. Bernier R, Desrochers M, Jurasek L, Paice MG (1983) Isolation and characterization of a xylanase from *Bacillus subtilis*. Applied Environmental Microbiology 46(2): 511-514
19. Damiano VB, Bocchini DA, Gomes E, Da Silva R (2003) Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus kraft pulp. World Journal of Microbiological Biotechnology 19(2): 139-144
20. Nakamura L, Roberts MS, Cohan FM (1999) Relationship between the *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*. International Journal of Systematic Bacteriology 49: 1211-1215
21. Garcia-Kirchner O, Muñoz-Aguilar M, Pérez-Villalva R, Huitrón-Vargas C (2002) Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. Applied Biochemistry and Biotechnology 1105-1114

- 715 22. Corral OL, Villaseñor-Ortega F (2006) Xylanases. Advances in Agricultural and Food Biotechnology
716 305-322
717
- 718 23. Sinha S (2009) Studies on the production of acid protease by submerged fermentation. International
719 Journal of Food Engineering 5(1)
720
- 721 24. Subramaniyan S, Prema P (2000) Cellulase-free xylanases from *Bacillus* and other microorganisms.
722 FEMS Microbiology Letters 183(1): 1-7
723
- 724 25. Bansal N, Soni R, Janveja C, Soni SK (2012) Production of xylanase-cellulase complex by *Bacillus*
725 *subtilis* NS-7 for the biodegradation of agro waste residues. Lignocellulose Journal 1(3): 196-209
726
- 727 26. Buthelezi SP, Olaniran AO, Pillay B (2011) Sawdust and digestive bran as cheap alternate substrates
728 for xylanase production. African Journal of Microbiology Research 5(7): 742-752
729
- 730 27. Atta S, Ali S, Akhtar M, Haq I (2011) Determination of some significant batch culture conditions
731 affecting acetyl-xylan esterase production by *Penicillium notatum* NRRL-1249. Biomedcentral
732 Biotechnology 11(1): 52-58
733
- 734 28. Haq I, Tasneem M, Raana K, Khan A, Mukhtar H, Javed M (2004) Optimization of cultural conditions
735 for the production of xylanase by chemically mutated strain of *Aspergillus niger* GCBCX-20.
736 International Journal of Agriculture and Biology 6(6): 1115-1118
737
- 738 29. Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS
739 Microbiology Reviews 23: 411-456
740
- 741 30. Porsuk I, Özakin S, Bali B, Ince Yilmaz E (2013) A cellulase-free, thermoactive, and alkali xylanase
742 production by terrestrial *Streptomyces* sp. CA24. Turkish Journal of Biology 37(3): 370-375
743
- 744 31. Thomas L, Joseph A, Gottumukkala LD (2014) Xylanase and cellulase systems of *Clostridium* sp.: An
745 insight on molecular approaches for strain improvement. Bioresource Technology 158: 343-350
746
- 747 32. Dhiman SS, Dayanand K, Sadashiv SJ, Haw JR, Chan KY, Lee JK (2013) Characterization of a novel
748 xylanase from *Armillaria gemina* and its immobilization onto SiO₂ nanoparticles. Applied Microbiology
749 and Biotechnology 97: 1081-1091
750

33. Chapla D, Dholakiya S, Madamwar D, Amita S (2013) Food and bioproducts processing characterization of purified fungal endoxylanase and its application for production of value added food ingredient from agroresidues. *Food and Bioproducts Processing* 91(4): 682-692
34. Sharma N, Ranchan SH, Pathania SH (2013) Production, purification and characterization of cellulose free-xylanase by *Bacillus coagulans* B30 using lignocellulosic forest wastes with different pretreatment methods. *Journal of Agroalimentary Processes Technologies* 19(1): 28-36
35. Su X, Han Y, Dodd D, Moon YH, Yoshida S, Mackie RI, Cann IK (2013) Reconstitution of a thermostable xylan-degrading enzyme mixture from the bacterium *Caldicellulosiruptor bescii*. *Applied and Environmental Microbiology* 79(5): 1481-1490
36. Bhasin A, Razdan K, Gupta N, Sethi N (2014) Production and purification of alkali stable xylanase from *Bacillus* sp. *International Journal of Current Microbiology and Applied Sciences* 3(3): 365-377
37. Maciel GM, de Souza Vandenberghe LP, Haminiuk CWI, Fendrich RC, Della Bianca BE, da Silva Brandalize TQ, Pandey A, Soccol CR (2008) Xylanase production by *Aspergillus niger* LPB 326 in solid-state fermentation using statistical experimental designs. *Food Technology and Biotechnology* 46(2): 183-189
38. Bhosale HJ, Sukalkar SR, Zaker S, Kadam TA (2011) Production of xylanase by *Streptomyces rameus* grown on agricultural wastes. *Journal of Biotechnology, Bioinformatics and Bioengineering* 1(4): 505-512
39. Jänis J, Rouvinen J, Vainiotalo P, Turunen O, Shnyrov VL (2008) Irreversible thermal denaturation of *Trichoderma reesei* endo-1,4-beta-xylanase II and its three disulfide mutants characterized by differential scanning calorimetry. *International Journal of Biological Macromolecules* 42(1): 75-80
40. Kosugi A, Murashima K, Doi RH (2001) Characterization of xylanolytic enzymes in *Clostridium cellulovorans*: expression of xylanase activity dependent on growth substrates. *Journal of Bacteriology* 183(24): 7037-7043
41. Roy S, Dutta T, Sarkar TS, Ghosh S (2013) Novel xylanases from *Simplicillium obclavatum* MTCC 9604: comparative analysis of production, purification and characterization of enzyme from submerged and solid state fermentation. *Springer Plus* 2(1): 1-10

- 787 42. Hiremath KS, Patil CS (2011) Isolation, production and characterization of alkalo thermostable
788 xylanase from newly isolated *Bacillus* sp. International Journal of Biotechnology Applications 3: 48-51
789
- 790 43. Tork S, Aly MM, Alakilli SY, Al-Seeni MN (2013) Production and characterization of thermostable
791 xylanase from *Bacillus subtilis* XP10 isolated from marine water. African Journal of Biotechnology
792 12(8): 780-790
793
- 794 44. Amore A, Parameswaran B, Kumar R, Birolo L, Vinciguerra R, Marcolongo L, Ionata E, Cara FL,
795 Pandey A, Faraco V (2014) Application of a new xylanase activity from *Bacillus amyloliquefaciens*
796 XR44A in brewer's spent grain saccharification. Journal of Chemical Technology and Biotechnology
797 90: 573-581
798
- 799 45. Irfan M, Nadeem M, Syed Q, Baig S (2012) Effect of medium composition on xylanase production by
800 *Bacillus subtilis* using various agricultural wastes. American-Eurasian Journal of Agricultural and
801 Environmental Sciences 12(5): 561-565
802
- 803 46. Heck JX, Hertz PF, Ayub MA (2002) Cellulase and xylanase productions by isolated Amazon *Bacillus*
804 strains using soybean industrial residue based solid-state cultivation. Brazilian Journal of
805 Microbiology 33(3): 213-218
806
- 807 47. Khodayari F, Cebeci Z, Ozcan BD (2014) Optimization of Xylanase and alpha-amylase production by
808 alkaline and thermophilic *Bacillus* Isolate KH-13. Journal of Entomology and Zoology Studies 2(5):
809 295-303
810
- 811 48. Kumar KS, Manimaran A, Permaul K, Singh S (2009) Production of beta-xylanase by a *Thermomyces*
812 *lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. Journal
813 of Bioscience and Bioengineering 107(5): 494-498
814
- 815 49. Deshpande SK, Dhotmange MG, Chakrabarti T, Shastri PN (2008) Production of cellulose and
816 xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state
817 fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). Indian Journal of Chemical Technology
818 15: 449-456
819
- 820 50. Tasneem M, Khan A, Ashraf H, Haq I (2003) Xylanase biosynthesis by chemically mutated strain of
821 *Aspergillus niger*. Journal of Food Technology 1(4): 178-181
822

51. Azeri C, Tamer U, Oskay M (2010) Thermoactive cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in biobleaching of kraft pulp. African Journal of Biotechnology 9(1): 63-72
52. Kanauchi M, Chijimi A, Ohnishi-Kameyama M, Bamforth CW (2013) An investigation of two xylan-degrading enzymes and a novel xylanase inhibitor in malted barley. Journal of the Institute of Brewing 119(1-2): 32-40
53. Jacob J, Pescatore A (2012) Using barley in poultry diets: A review. The Journal of Applied Poultry Research 21(4): 915-940
54. Ferre H, Broberg A, Duus JO, Thomsen KK (2000) A novel type of arabinoxylan arabinofuranohydrolase isolated from germinated barley. European Journal of Biochemistry 267(22): 6633-6641
55. Lazaridou A, Chornick T, Biliaderis CG, Izydorczyk MS (2008) Composition and molecular structure of polysaccharides released from barley endosperm cell walls by sequential extraction with water, malt enzymes and alkali. Journal of Cereal Science 48(2): 304-318
56. Westers L, Westers H, Quax WJ (2004) *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1694 (1): 299-310
57. Ammoneh H, Harba M, Akeed Y, Al-Halabi M, Bakri Y (2014) Isolation and identification of local *Bacillus* isolates for xylanase biosynthesis. Iranian Journal of Microbiology 6(2): 127-132
58. Sá-Pereira P, Mesquita A, Duarte JC, Barros MRA, Costa-Ferreira M (2002) Rapid production of thermostable cellulase-free xylanase by a strain of *Bacillus subtilis* and its properties. Enzyme and Microbial Technology 30(7): 924-933
59. Chaturvedi S, Singh R, Khurana SP (2013) Isolation and identification of xylanolytic enzyme from an effective strain *Bacillus licheniformis* isolated from the decaying wood. International Journal of Applied Biology and Pharmaceutical Technology 4(4): 92-100
60. Özcan BD, Coşkun A, Özcan N, Baylan M (2011) Some properties of a new thermostable xylanase from alkaphilic and thermophilic *Bacillus* sp. Isolate DM-15. Journal of Animal and Veterinary Advances 10(2):138-143

- 860 61. Panbangred W, Shinmyo A, Kinoshita S, Okada H (1983) Purification and properties of endoxylanase
861 produced by *Bacillus pumilus*. Agricultural and Biological Chemistry 47(5): 957-963
862
- 863 62. Kapilan R, Arasaratnam V (2010) A novel *Bacillus pumilus* strain producing thermostable alkaline
864 xylanase at and above 40°C. Malaysian Journal of Biochemistry and Molecular Biology 18: 7-15
865
- 866 63. Kaur H, Dutt D, Tyagi C (2011) Production of novel alkali-thermo-tolerant cellulase-poor xylanases
867 from *Coprinopsis cinerea* HK-1 NFCCI-2032. BioResources 6(2): 1376-1391
868
- 869 64. Sook BS, Kauldhar BS (2013) Influence of multiple bioprocess parameters on production of lipase
870 from *Pseudomonas* sp. BWS-5. Brazilian Archives of Biology and Technology 56(5): 711-721
871
872