British Microbiology Research Journal
14(1): 1-17, 2016, Article no.BMRJ.22959
ISSN: 2231-0886, NLM ID: 101608140

SCIENCEDOMAIN *international*
www.sciencedomain.org

Overproduction of Xylanase from Mutants of *Bacillus subtilis* with Barley Husk as the Prime Carbon Source under Submerged Fermentation after Random Mutagenesis Using Ethyl Methane Sulfonate (EMS) and Acridine Orange (AO)

Hooi Ling Ho[1*] and Ajounmah Maryann Chinonso[1]

[1]Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, Cheras 56000, Kuala Lumpur, Malaysia.

Authors' contributions

This work was carried out in collaboration between both authors. Author HLH designed and supervised the study, edited and approved the manuscript. Author AMC performed the experiments and analysis. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/22959
Editor(s):
(1) Giuseppe Blaiotta, Department of Food Science, Via Universita, Italy.
(2) Joao Lucio Azevedo, University of Sao Paulo, Department of Genetics, Brazil.
(3) Grzegorz Cieslar, Department and Clinic of Internal Diseases, Angiology and Physical Medicine, Medical University of Silesia, Poland.
(4) Alok K. Upadhyay, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA.
Reviewers:
(1) Anonymous, Central University of Gujarat, Gandhinagar, India.
(2) Pedro Fernandes, Universidade de Lisboa, Portugal.
(3) Anonymous, National University of Lesotho, Lesotho.
(4) Armando Zepeda Bastida, Universidad Autonoma del Estado de Hidalgo, Mexico.
(5) Yordanka Tsankova Tasheva, University "Prof. Dr A.Zlatarov"- Burgas, Bulgaria.
(6) Maulin P. Shah, Industrial Waste Water Research Lab, India.
Complete Peer review History: http://sciencedomain.org/review-history/13964

Original Research Article

Received 6th November 2015
Accepted 18th March 2016
Published 31st March 2016

ABSTRACT

Aims: Xylanase (EC 3.2.1.8) 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

**Corresponding author: E-mail: hohooiling@gmail.com;*

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 using 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, mutant strains of *B. subtilis* ATCC 6633 were identified as the potent 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 initiated 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±0.02 U/mL was attained by the mutant MXB 4 of *B. subtilis* which was approximately 29.4% increase in xylanase activity than the wild type with 1.36±0.003 U/mL. Furthermore, MXB 1, MXB 2, MXB 3 and MXB 5 also exhibited comparatively higher maximum xylanase activity of 1.64±0.009 U/mL, 1.73±0.05 U/mL, 1.74±0.02 U/mL and 1.66±0.02 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 0.82±0.02 mg/mL and 0.82±0.03 mg/mL, respectively. Both demonstrated increment of 49.1% in protein production than the wild type which possessed relatively lower concentration of 0.55±0.01 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±0.2 as shown by the mutant MXB 4 in comparison with wild type at pH 6.47±0.3.

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 under submerged fermentation using barley husk as the prime carbon source. 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 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 generally exhibit high catalytic activity. Furthermore, they are highly biodegradable and economically feasible biocatalysts 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 (EC 3.2.1.8) due to its pivotal roles in vast biotechnological applications. The biodegradation ability of xylanase on plant cell wall proves this enzyme

possesses essential function in many significant applications [2]. Xylanase has also been used in 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 such as 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 enzyme is one of the 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 successful production of large amount of xylanases. Many lignocellulolytic substrates such as barley husks, corncobs, sugarcane bagasse and wheat bran have been studied. A good number of them have performed significantly better than purified xylan with respect to the yields of xylanase in large-scale production. Maximum xylanase production using cassava bagasse as the carbon source was reported by Sugumaran et al. [4]. Indeed, Ecaterina et al. [5] also 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. Notably, many studies have also been carried out using barley husk for production of ethanol [7]. Nonetheless, only a few studies have been conducted using *B. subtilis* subsp. spizizenii ATCC 6633 for the xylanases production. Therefore, in the present study, we embarked on the following objectives to qualitatively confirm the activity of xylanase from the mutants of *B. subtilis* 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 as the wild type in this study was obtained from American Type Culture Collection (ATCC), Manassas, USA. *B. subtilis* ATCC 6633 was subcultured on nutrient agar and incubated at 37℃ until bacterial colonies developed. Subsequently, the bacteria was maintained and stored at 4℃. On the other hand, a 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 in submerged fermentation 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 for xylanase production under submerged fermentation compared to their parental wild type as shown in Table 1, respectively.

Table 1. Mutagens concentrations used for generation of mutants

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

[a]*Ethyl methane sulphonate (EMS)*
[b]*Acridine orange (AO)*

2.2 Preparation of Standard Inoculums

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 at 37℃ at 150 rpm for 24 h, respectively. The test tubes were then vortex 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 inoculums of 2.0×10^6 cells. On the other hand, the same standard inoculum of 2.0×10^6 cells of the wild type of *B. subtilis* was also used as the control in this study. The standard inoculum used in the submerged fermentation for xylanase production was counted using Haemocytometer in this study.

2.3 Qualitative Confirmation of Xylanase Activity from Mutants of *B. subtilis* Using Plate Hydrolysis Assay

The qualitative 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; $MgSO_4 \cdot 7H_2O$, 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℃ until the mutant cultures developed and covered the thin layer on the surface of the agar after 2 to 3 days of incubation. 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 distaining 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. Thereafter, the mutant strains and wild type were subjected to quantitative confirmation using xylanase activity assay on the samples obtained from submerged fermentation in this study.

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): yeast extract, 5; peptone, 5; $MgSO_4 \cdot 7H_2O$, 0.1; K_2HPO_4, 1 and pre-treated barley husk, 20 employed as the prime cheap alternative carbon source for the production of xylanase. In order to treat the barley husk, it was first being homogenized using a food grinder to obtain smaller and smoother structure before dried in an oven at 65℃ until the constant weight achieved. Then, the pre-treated barley husk was mixed with other medium components except for yeast extract and peptone in 150 mL distilled water before adjusted its initial pH to 6.5. On the other hand, yeast extract and peptone as the nitrogen sources were dissolved in 100 mL distilled water and likewise adjusted its initial pH to 6.5. Thereafter, both the carbon and nitrogen sources were autoclaved at 121℃ for 15 minutes separately to prevent Maillard reaction. Then, both the medium were mixed well after cooling down at room temperature to obtain the standard working volume of 250 mL in a 500 mL Erlenmeyer flask. Subsequently, 2.0×10^6 cells of mutants used as the standard inoculum were inoculated into 250 mL medium and incubated in the rotary incubation shaker at 150 rpm at 37℃ 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 barley husk as the sole carbon source under the 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℃. 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 to liberate xylose

as the reducing sugar. The detection of xylose after xylanase activity was conducted using 3,5-dinitrosalicyclic acid (DNS). 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℃ for 30 min. 1.5 mL of DNA was added to the reaction mixture and incubated at 90℃ 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 count the number of cells of wild type and mutants of *B. subtilis* found in the fermentation samples.

2.9 Measurement of Medium pH

The medium pH used for the submerged fermentation was adjusted to 6.5 by using 1 M NaOH and 1 M HCl. The pH of medium was measured using a pH meter.

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 Qualitative Confirmation of Xylanase Activity from Mutants of *B. subtilis* Using Plate Hydrolysis Assay

Based on our results, all mutants of *B. subtilis* ATCC 6633 were identified with their 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 Fig. 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 ring zone. The discoloration of the clear ring 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 2 to 3 days of incubation at 37℃. The presence of discoloration around the mutants colonies on the xylan agar occurred after incubation proved the secretion of xyl anase 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 important for industrial applications. When *Bacillus* spp 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 ring 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 ring zone formation on xylan agar after incubated with *B. cereus* [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 and confirmation 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]. Notably, higher xylo-oligosaccharides and xylotetraose were also identified after hydrolysis using birchwood and oat spelt xylan [15]. 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 [16]. 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 [17]. Based on the results in the present study, we 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. On the other hand, according to Meddeb-Mouelhi et al. [18], they suggested that methods that relied on the interactions between a dye of Congo red and a polymeric substrate such as birchwood xylan for indirect detection of enzyme hydrolysis might require the use of relevant controls and independent confirmation of enzymatic activities assay. Hence, the mutants used in this study were thereafter scaled up in the submerged fermentation to quantitatively confirm the xylanase activity in the fermentation samples using the enzyme activity assay according to Bailey et al. [9].

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 known for fermentation of aerobic microorganisms especially in the production of xylanase [21].

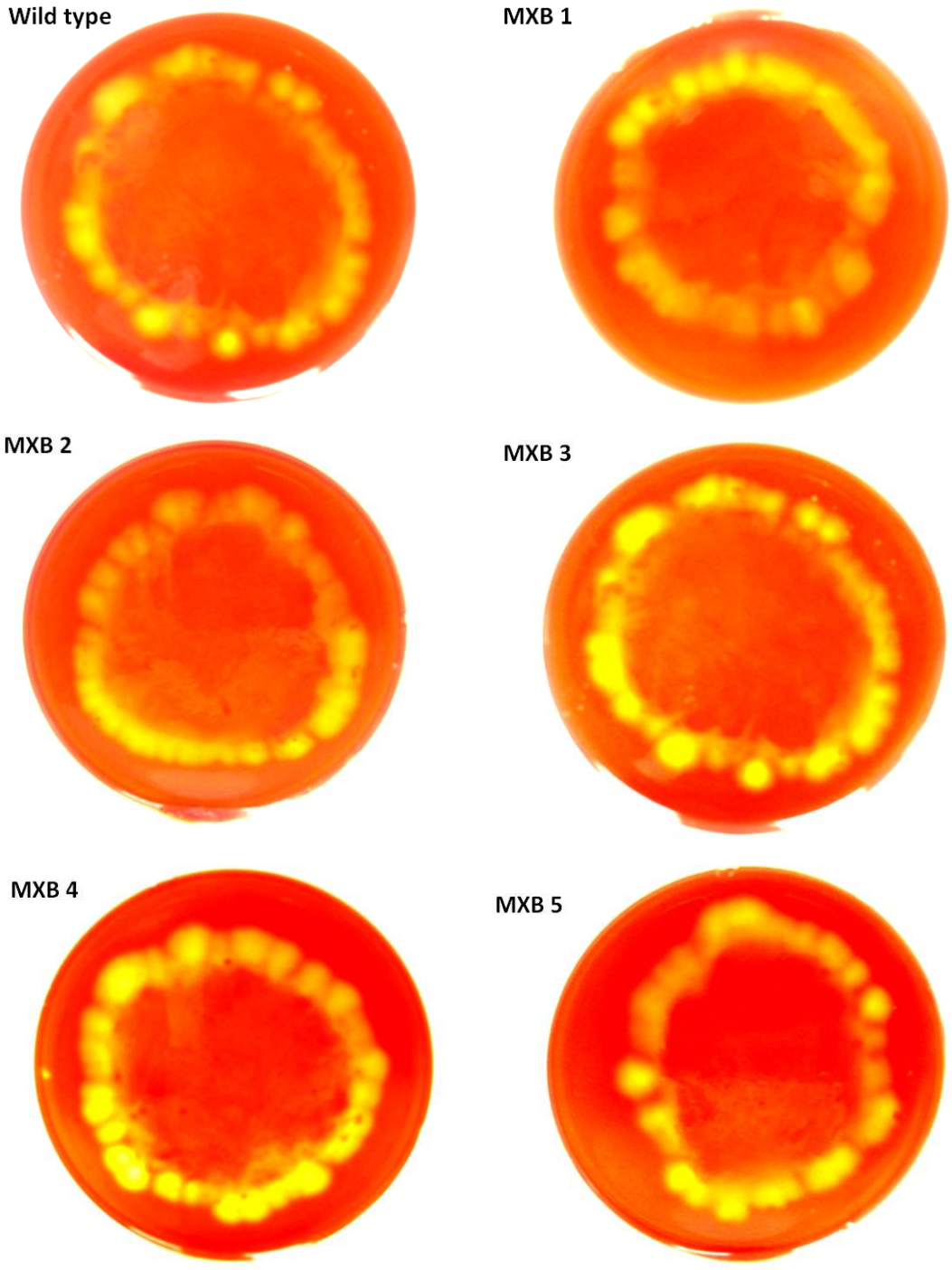

Fig. 1. Xylan plate hydrolysis assay of wild type and mutants of *B. subtilis* ATCC 6633

The discoloration of Congo red dye occurred after 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. 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.

Based on the results, 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±1.53 × 10^6 cells/mL for wild type, 1.16 × 10^8 ±5.8 × 10^6 cells/mL for MXB 1, 1.32 × 10^8±2.3 × 10^7 cells/mL for MXB 2, 1.13 × 10^8±1.47 × 10^7 cells/mL for MXB 3, 1.27 × 10^8±2.14 × 10^7 cells/mL for MXB 4 and 1.21 × 10^8±1.41 × 10^7 cells/mL for MXB 5 were attained at 24 h of fermentation, respectively. Apparently, the cells of wild type and mutants of *B. subtilis* grown at the fastest rate at 24 h of fermentation resulting in the most rapid xylan hydrolysis of barley husk. Subsequently, xylanase activity was decreased 1.29±0.002 U/mL to 1.05±0.01 U/mL for wild type, 1.49±0.04 U/mL to 1.17±0.01 U/mL for MXB 1, 1.51±0.02 U/mL to 1.23±0.005 U/mL for MXB 2, 1.60±0.07 U/mL to 1.25±0.02 U/mL for MXB 3, 1.61±0.09 U/mL to 1.30±0.03 U/mL for MXB 4 and 1.49±0.002 U/mL to 1.22±0.03 U/mL for MXB 5 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 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.

Due to the uneconomical use of pure xylan for industrial xylanase production, this study is also investigated the employment of xylan-rich low-cost agricultural residue such as barley husk. The finding obtained from this study is similar 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] detected 7.5 U/mL of xylanase activity from

B. subtilis using cassava bagasse as carbon substrate 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 Fig. 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.

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 that possess significant effects on yield of xylanases are optimised 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. Several 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 Fig. 3. Based on the results, all of the mutants were observed to

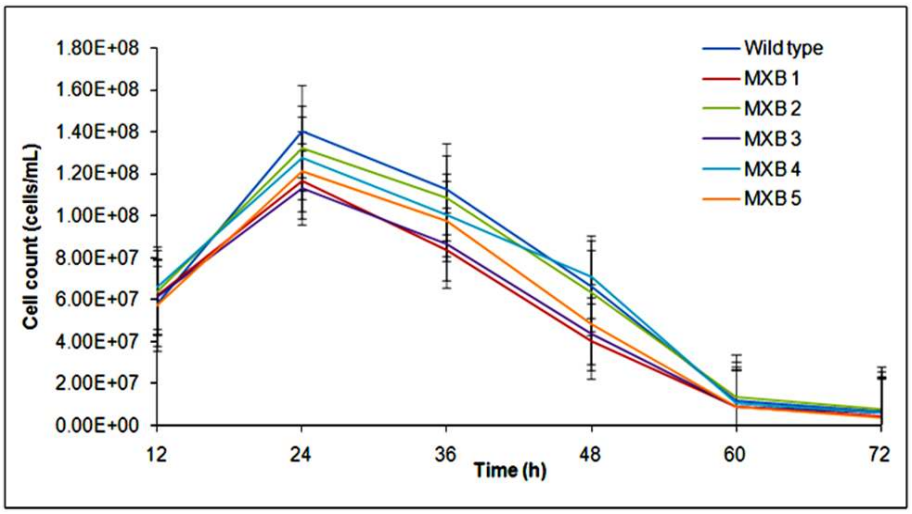

Fig. 2. Growth profile of wild type and mutants of *B. subtilis* ATCC 6633 during the production of xylanase using barley husk under submerged fermentation

All experiments were conducted in triplicate while the error bars indicate the standard deviation.

possess their own maximum xylanase activity under the same growth conditions at 24 h of fermentation. Notably, the maximum xylanase activity of 1.76 ± 0.02 U/mL by the mutant MXB 4 of *B. subtilis* was attained compared to the xylanase production of the wild type with 1.36±0.003 U/mL. Indeed, this maximum xylanase activity from the MXB 4 was increased by approximately 29.4% 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±0.09 U/mL, 1.73±0.05 U/mL, 1.74±0.02 U/mL and 1.66±0.02 U/mL in comparison with their parental wild type of 1.36±0.003 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 prime 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, Desphpande et al. [49] studied a mixed culture of *Aspergillus niger* and *Trichodermareesei* QM, whereby the mutant number 414 stimulated 2-fold increase in xylanase production in the fermentation medium. 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 be ascribed 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.

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 Fig. 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±0.02 mg/mL with the cell count of $1.32 \times 10^8 \pm 2.3 \times 10^7$ cells/mL and 0.82±0.03 mg/mL with $1.27 \times 10^8 \pm 2.14 \times 10^7$ cells/mL, respectively. This was followed by the mutant of MXB 3 with its protein production of 0.81±0.02 mg/mL from cell count of $1.13 \times 10^8 \pm 1.47 \times 10^7$ cells/mL. On the contrary, wild type of *B. subtilis* showed an extracellular protein concentration of 0.55±0.01 mg/mL. Indeed, mutants of MXB 2 and MXB 4 exhibited 49.1% 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].

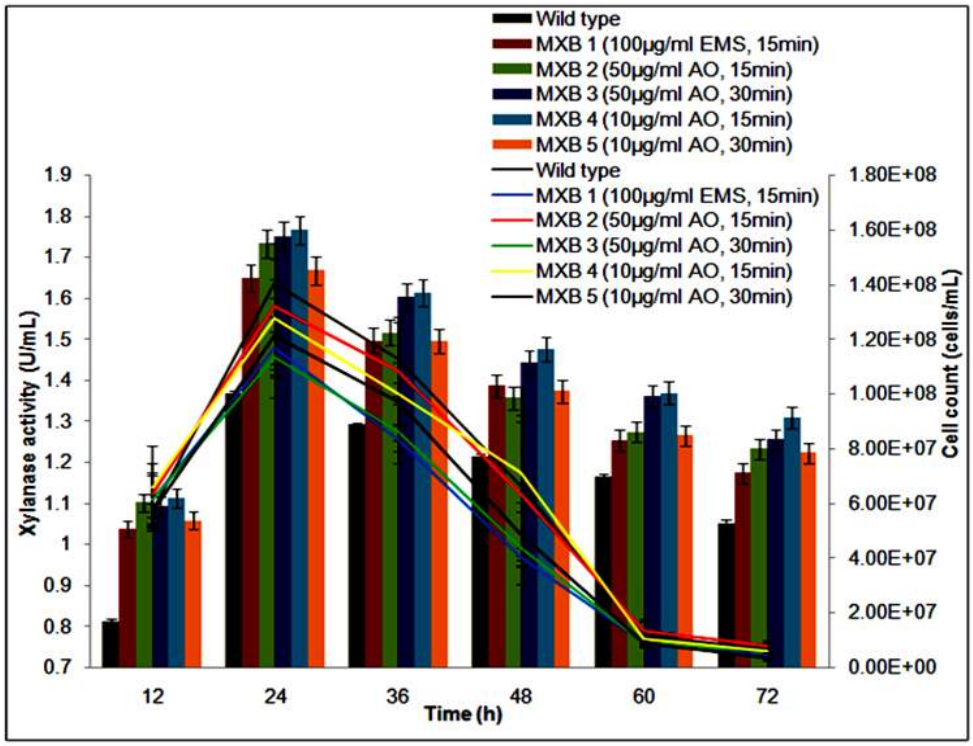

Fig. 3. Production of xylanase by wild type and mutants of *B. subtilis* ATCC 6633 using barley husk under submerged fermentation

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

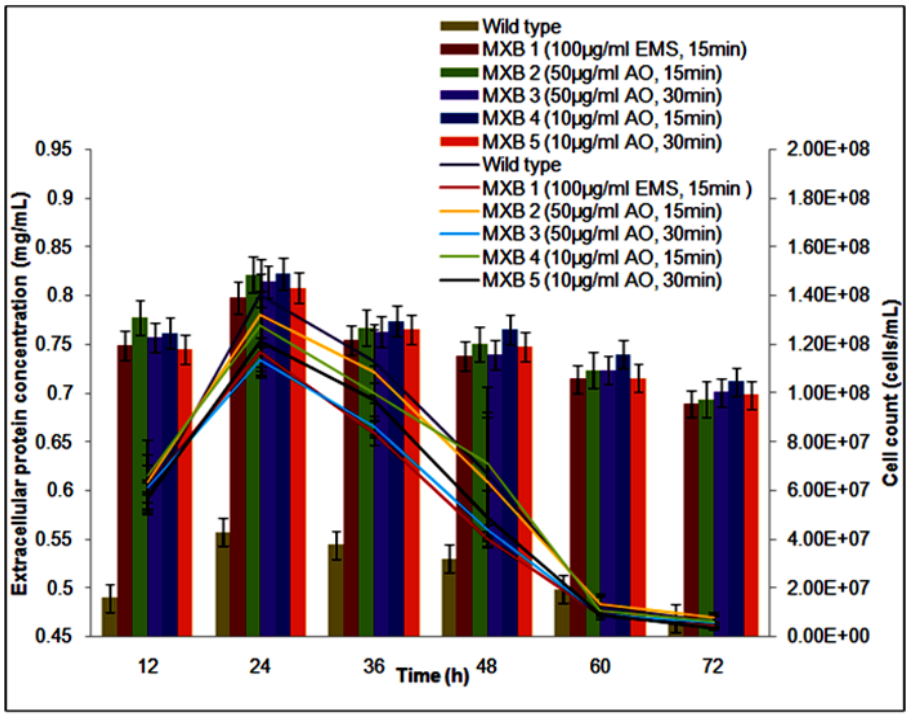

Fig. 4. Total extracellular protein production by wild type and mutants of *B. subtilis* ATCC 6633 using barley husk under submerged fermentation

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 Fig. 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 6.09±0.2 to 6.5±0.3 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±0.003 U/mL by the wild type of *B. subtilis* ATCC 6633 was attained at the near neutral medium pH of 6.47±0.3 compared to the mutant of MXB 4 with the xylanase activity of 1.76±0.02 U/mL at slight acidic pH of 6.1±0.2. In fact, the maximum xylanase activity exhibited at slight acidic medium pH of 6.2±0.1, 6.09±0.2, 6.1±0.2, and 6.2±0.1 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±0.2 to 7.9±0.3. 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

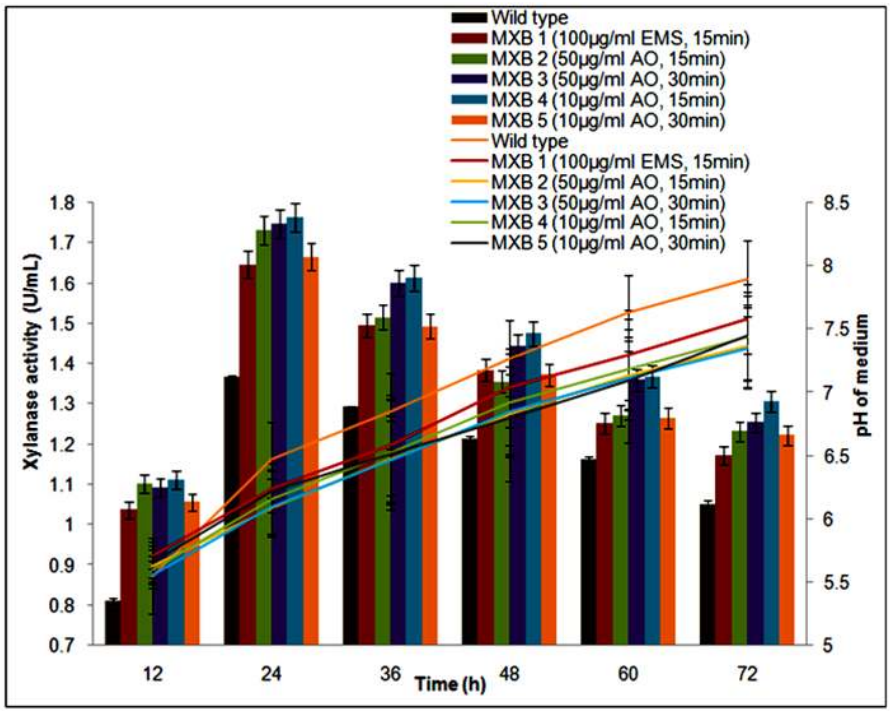

Fig. 5. Profile of medium pH during growth of wild type and mutants of *B. subtilis* ATCC 6633 using barley husk under submerged fermentation

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

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.

4. CONCLUSION

In this study, all mutants of *B. subtilis* ATCC 6633 were verified to produce xylanase after xylan hydrolysis as detected by Congo red dye staining on xylan agar. The extracellular protein concentration and xylanase activity of all mutants particularly MXB 4 increased after 24 h of fermentation compared to their parental strain. In fact, MXB 4 attained as the highest xylanase mutant producer after subjected the wild type to 10 µg/mL of AO for 15 min. MXB 4 possessed higher maximum xylanase activity of 1.76 U/mL compared to the xylanase production by the wild type with 1.36 U/mL at medium pH 6.47. AO induced visible and lethal mutations in the xylanase genes, causing alkylation of guanine residues which resulted in permanent lesions within the DNA. Hence, AO could be considered

as one of the effective mutagens to enhance overexpression and overproduction of xylanase enzyme in *B. subtilis*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Motta F, Andrade C, Santana M. 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. 2013;251-275.
2. Ghazi S, Sepahy AA, Azin M, Khaje K, Khavarinejad R. UV mutagenesis for the overproduction of xylanase from *Bacillus mojavensis* PTCC 1723 and optimization of the production condition. Iranian Journal of Basic Medical Sciences. 2014;17(11): 844-853.
3. Kuhad RC, Singh A. Lignocellulose bio-technology: Current and future prospects. Critical Reviews in Biotechnology. 1993; 13:151-172.
4. Sugumaran KR, Kumar BK, Mahalakshmi M, Ponnusami V. Cassava bagasse-low cost substrate for thermo-tolerant xylanase production using *Bacillus subtilis*. International Journal of ChemTech Research. 2013;5(1):394-400.
5. Ecaterina DP, Lonut SA, Gabriela GC, Domnica C. Fodder yeast development optimisation using as main carbon source barley husks hydrolysed. Romanian Biotechnological Letters. 2014;19(1):9085-9095.
6. Soliman HM, Sherief AA, Tanash BEL. Production of xylanase by *Aspergillus niger* and *Thrichoderman viride* using some agriculture residues. International Journal of Agricultural Research. 2012;7:46-57.
7. Palmarola-Adrados B, Galbe M, Zacchi G. Pretreatment of barley husk for bioethanol production. Journal of Chemical Technology and Biotechnology. 2005; 80(1):85-91.
8. Ho HL, Chor XK. Improvement of xylanase production by *Bacillus subtilis* in submerged fermentation after UV and chemicals mutagenesis. Journal of Advances in Biology and Biotechnology. 2015;3(2):42-57.
9. Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology. 1992;23(3):257-270.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951;193:265-275.
11. Annamalai N, Thavasi R, Jayalakshmi S, Balasubramanian T. Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated form marine environment. Indian Journal of Biotechnology. 2009;8(3):291-297.
12. Nagar S, Mittal A, Gupta VK. A cost effective method for screening and isolation of xylan degrading bacteria using agro waste material. Asian Journal of Biological Sciences. 2012;5(8):384-394.
13. Roy N, Habib MR. Isolation and characterization of xylanase producing strain of *Bacillus cereus* from soil. Iranian Journal of Microbiology. 2009;1(2):49-53.
14. Kamble RD, Jadhav AR. Isolation, purification and characterization of xylanase produced by a new species of *Bacillus* in solid state fermentation. International Journal of Microbiology. 2012; 1-8.
15. Yoon KH. Cloning of the *Bacillus subtilis* AMX-4 xylanase gene and characterization of the gene product. Journal of Microbiology and Biotechnology. 2009; 19(12):1514-1519.
16. Cho NC, Bai S. Purification and characterization of xylanase from *Bacillus* sp. strain DSNC 101. Journal of Microbiology and Biotechnology. 1997; 7(6):386-390.
17. Bernier R, Desrochers M, Jurasek L, Paice MG. Isolation and characterization of a xylanase from *Bacillus subtilis*. Applied Environmental Microbiology. 1983;46(2): 511-514.
18. Meddeb-Mouelhi F, Moisan JK, Beauregard M. A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. Enzyme and Microbial Technology. 2014;66:16-9.
19. Damiano VB, Bocchini DA, Gomes E, Da Silva R. Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus kraft pulp. World Journal of Microbiological Biotechnology. 2003;19(2): 139-144.
20. Nakamura L, Roberts MS, Cohan FM. Relationship between the *Bacillus subtilis*

clades associated with strains 168 and W23: a proposal for *B. subtilis* subsp. subtilis and *B. subtili*s subsp. spizizenii. International Journal of Systematic Bacteriology. 1999;49:1211-1215.
21. Garcia-Kirchner O, Munoz-Aguilar M, Perez-Villalva R, Huitron-Vargas C. Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. Applied Biochemistry and Biotechnology. 2002; 1105-1114.
22. Corral OL, Villasenor-Ortega F. Xylanases. Advances in Agricultural and Food Biotechnology. 2006;305-322.
23. Sinha S. Studies on the production of acid protease by submerged fermentation. International Journal of Food Engineering. 2009;5(1).
24. Subramaniyan S, Prema P. Cellulase-free xylanases from *Bacillus* and other microorganisms. FEMS Microbiology Letters. 2000;183(1):1-7.
25. Bansal N, Soni R, Janveja C, Soni SK. Production of xylanase-cellulase complex by *Bacillus subtilis* NS-7 for the biodegradation of agro waste residues. Lignocellulose Journal. 2012;1(3):196-209.
26. Buthelezi SP, Olaniran AO, Pillay B. Sawdust and digestive bran as cheap alternate substrates for xylanase production. African Journal of Microbiology Research. 2011;5(7):742-752.
27. Atta S, Ali S, Akhtar M, Haq I. Determination of some significant batch culture conditions affecting acetyl-xylan esterase production by *Penicillium notatum* NRRL-1249. Biomedcentral Biotechnology. 2011;11(1):52-58.
28. Haq I, Tasneem M, Raana K, Khan A, Mukhtar H, Javed M. Optimization of cultural conditions for the production of xylanase by chemically mutated strain of *Aspergillus niger* GCBCX-20. International Journal of Agriculture and Biology. 2004;6(6):1115-1118.
29. Kulkarni N, Shendye A, Rao M. Molecular and biotechnological aspects of xylanases. FEMS Microbiology Reviews. 1999; 23:411-456.
30. Porsuk I, Özakin S, Bali B, Ince Yilmaz E. A cellulase-free, thermoactive, and alkali xylanase production by terrestrial *Streptomyces* sp. CA24. Turkish Journal of Biology. 2013;37(3):370-375.
31. Thomas L, Joseph A, Gottumukkala LD. Xylanase and cellulase systems of *Clostridium* sp.: An insight on molecular approaches for strain improvement. Bioresource Technology. 2014;158:343-350.
32. Dhiman SS, Dayanand K, Sadashiv SJ, Haw JR, Chan KY, Lee JK. Characterization of a novel xylanase from *Armillaria gemina* and its immobilization onto SiO_2 nanoparticles. Applied Microbiology and Biotechnology. 2013;97: 1081-1091.
33. Chapla D, Dholakiya S, Madamwar D, Amita S. 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. 2013;91(4):682-692.
34. Sharma N, Ranchan SH, Pathania SH. 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. 2013;19(1):28-36.
35. Su X, Han Y, Dodd D, Moon YH, Yoshida S, Mackie RI, Cann IK. Reconstitution of a thermostable xylan-degrading enzyme mixture from the bacterium *Caldicellulosiruptor bescii*. Applied and Environmental Microbiology. 2013;79(5): 1481-1490.
36. Bhasin A, Razdan K, Gupta N, Sethi N. Production and purification of alkali stable xylanase from *Bacillus* sp. International Journal of Current Microbiology and Applied Sciences. 2014;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. Xylanase production by *Aspergillus niger* LPB 326 in solid-state fermentation using statistical experimental designs. Food Technology and Biotechnology. 2008;46(2):183-189.
38. Bhosale HJ, Sukalkar SR, Zaker S, Kadam TA. Production of xylanase by *Streptomyces rameus* grown on agricultural wastes. Journal of Biotechnology, Bioinformatics and Bioengineering. 2011;1(4):505-512.
39. Jänis J, Rouvinen J, Vainiotalo P, Turunen O, Shnyrov VL. 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. 2008;42(1): 75-80.

40. Kosugi A, Murashima K, Doi RH. Characterization of xylanolytic enzymes in *Clostridium cellulovorans*: Expression of xylanase activity dependent on growth substrates. Journal of Bacteriology. 2001;183(24):7037-7043.
41. Roy S, Dutta T, Sarkar TS, Ghosh S. Novel xylanases from *Simplicillium obclavatum* MTCC 9604: Comparative analysis of production, purification and characterization of enzyme from submerged and solid state fermentation. Springer Plus. 2013;2(1):1-10.
42. Hiremath KS, Patil CS. Isolation, production and characterization of alkalo thermostable xylanase from newly isolated *Bacillus* sp. International Journal of Biotechnology Applications. 2011;3:48-51.
43. Tork S, Aly MM, Alakilli SY, Al-Seeni MN. Production and characterization of thermostable xylanase from *Bacillus subtilis* XP10 isolated from marine water. African Journal of Biotechnology. 2013; 12(8): 780-790.
44. Amore A, Parameswaran B, Kumar R, Birolo L, Vinciguerra R, Marcolongo L, Ionata E, Cara FL, Pandey A, Faraco V. Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in brewer's spent grain saccharification. Journal of Chemical Technology and Biotechnology. 2014;90:573-581.
45. Irfan M, Nadeem M, Syed Q, Baig S. Effect of medium composition on xylanase production by *Bacillus subtilis* using various agricultural wastes. American-Eurasian Journal of Agricultural and Environmental Sciences. 2012;12(5):561-565.
46. Heck JX, Hertz PF, Ayub MA. Cellulase and xylanase productions by isolated Amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. Brazilian Journal of Microbiology. 2002;33(3):213-218.
47. Khodayari F, Cebeci Z, Ozcan BD. Optimization of xylanase and alpha-amylase production by alkaline and thermophilic *Bacillus* Isolate KH-13. Journal of Entomology and Zoology Studies. 2014;2(5):295-303.
48. Kumar KS, Manimaran A, Permaul K, Singh S. Production of beta-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. Journal of Bioscience and Bioengineering. 2009; 107(5):494-498.
49. Desphpande SK, Dhotmange MG, Chakrabarti T, Shastri PN. Production of cellulose and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). Indian Journal of Chemical Technology. 2008;15:449-456.
50. Tasneem M, Khan A, Ashraf H, Haq I. Xylanase biosynthesis by chemically mutated strain of *Aspergillus niger*. Journal of Food Technology. 2003;1(4):178-181.
51. Azeri C, Tamer U, Oskay M. 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. 2010;9(1):63-72.
52. Kanauchi M, Chijimi A, Ohnishi-Kameyama M, Bamforth CW. An investigation of two xylan-degrading enzymes and a novel xylanase inhibitor in malted barley. Journal of the Institute of Brewing. 2013; 119(1-2):32-40.
53. Jacob J, Pescatore A. Using barley in poultry diets: A review. The Journal of Applied Poultry Research. 2012;21(4):915-940.
54. Ferre H, Broberg A, Duus JO, Thomsen KK. A novel type of arabinoxylan arabinofuranohydrolase isolated from germinated barley. European Journal of Biochemistry. 2000;267(22):6633-6641.
55. Lazaridou A, Chornick T, Biliaderis CG, Izydorczyk MS. 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. 2008;48(2):304-318.
56. Westers L, Westers H, Quax WJ. *Bacillus subtilis* as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. Biochimica et Biophysica Acta (BBA): Molecular Cell Research. 2004;1694(1):299-310.
57. Ammoneh H, Harba M, Akeed Y, Al-Halabi M, Bakri Y. Isolation and identification of local *Bacillus* isolates for xylanase biosynthesis. Iranian Journal of Microbiology. 2014;6(2):127-132.
58. Sá-Pereira P, Mesquita A, Duarte JC, Barros MRA, Costa-Ferreira M. Rapid

production of thermostable cellulase-free xylanase by a strain of *Bacillus subtilis* and its properties. Enzyme and Microbial Technology. 2002;30(7):924-933.
59. Chaturvedi S, Singh R, Khurana SP. 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. 2013;4(4):92-100.
60. Özcan BD, Coşkun A, Özcan N, Baylan M. Some properties of a new thermostable xylanase from alkaphilic and thermophilic *Bacillus* sp. Isolate DM-15. Journal of Animal and Veterinary Advances. 2011; 10(2):138-143.
61. Panbangred W, Shinmyo A, Kinoshita S, Okada H. Purification and properties of endoxylanase produced by *Bacillus pumilus*. Agricultural and Biological Chemistry. 1983;47(5):957-963.
62. Kapilan R, Arasaratnam V. A novel *Bacillus pumilus* strain producing thermostable alkaline xylanase at and above 40℃. Malaysian Journal of Biochemistry and Molecular Biology. 2010;18:7-15.
63. Kaur H, Dutt D, Tyagi C. Production of novel alkali-thermo-tolerant cellulase-poor xylanases from *Coprinopsis cinerea* HK-1 NFCCI-2032. BioResources. 2011;6(2): 1376-1391.
64. Sooch BS, Kauldhar BS. Influence of multiple bioprocess parameters on production of lipase from *Pseudomonas* sp. BWS-5. Brazilian Archives of Biology and Technology. 2013;56(5):711-721.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13964