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Improvement of Xylanase Production by *Bacillus subtilis* in Submerged Fermentation after UV and Chemicals Mutagenesis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: *Bacillus subtilis* is one of the most utilized industrial microorganisms for its high capability of producing large amount of extracellular enzymes including xylanase. This extracellular enzyme has been used extensively for many years in food and detergents industry. In recent years, many attempts have been made for the improvement of microbial xylanase production by inducing mutagenesis. Thus, the objectives of this study are to produce mutants of *B. subtilis* using UV and chemicals mutagenesis, subsequently to elucidate the xylanase activity and finally to identify the most effective mutagenic agent for overproduction of this enzyme.
Methodology: In this study, ultraviolet (UV) irradiation and chemical mutagens of ethyl methanesulfonate (EMS) and acridine orange (AO) have been selected because of their simplicity of handling and cost-effectiveness as compared to recombinant DNA technology. The efficiency of these mutagenesis was further assessed in submerged fermentation in shake flask culture compared with the non-treated wild type control strain.
Results: Based on our results, *B. subtilis* that had been induced with 50 µg/mL of AO for 30 min exhibited the overproduction of xylanase with 1.580±0.027 U/mL in submerged fermentation. Indeed, the highest xylanase overproduction reached the maximum peak of 1.638±0.027 U/mL after

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30 min of induction using 100 ug/mL of EMS. Apparently, when compared to the non-treated control, xylanase activity experienced the highest percentage of overproduction as much as 15.68% from EMS mutagenesis induced for 30 min in contrast to only 11.58% from *B. subtilis* that had been induced with 50 µg/mL of AO for 30 min.

Conclusion: In a nutshell, based on our results, EMS evidenced among the most promising mutagenic agent of inducing *B. subtilis* that capable of overproduction of xylanase to the greater extend as compared to UV irradiation and mutagenic agent of AO.

Keywords: Random mutagenesis; ultraviolet (UV) irradiation; ethyl methanesulfonate (EMS); acridine orange (AO); xylanase; Bacillus subtilis.

1. INTRODUCTION

Enzymes are composed of unique and complex protein based substances that found in every cell of living organisms including plant, animal and human. They are essential and function as biocatalyst in chemical reactions both inside as well as outside of living systems. The application of enzymes has been used since the ancient time for the making of cheese, yogurt, beer and vinegar [1]. In the past, the sources of enzymes were originated from fresh fruits, vegetable and grains [2]. Industrial enzymes are however, more likely to be extracted from the microorganisms as compared to animals and plants. Microbial enzymes are generally produced via fermentation in large volume of fermenters which are space and time-effective as a result of the mass production of enzymes in shorter period of time. Among all microorganisms, family of *Bacillus* is one of the main microbial enzymes producers [3]. Family of *Bacillus* has been used for decades because of its high capability in producing large amount of extracellular enzymes such as xylanase, amylase, protease, pullulanase, chitinase, lipase, among others. These enzymes are produced commercially and their production represents approximately 60% of the global market demand [4]. Among *Bacillus* spp*, B. subtilis*, an aerobic, rob-shaped gram positive bacteria is capable of producing a large amount of extracellular xylanase. Besides that, *B. subtilis* is able to grow in a wide range of growth conditions including utilising simple substrates at relatively high growth rates. *B. subtilis* is therefore, one of the most utilised industrial species for its microbial xylanase. Xylanase is a hydrolytic enzyme that catalyzes the hydrolysis of the β-1,4 backbone of xylan which is the main polysaccharide of the hemicellulose components found in plant cell walls [5]. Xylanase is an important enzyme that used in industrial applications such as pretreatment of Kraft pulp by improving bleach ability of pulp while decreasing consumption of chlorine chemical [6]. Xylanase is also used in bakery products to increase the quantity of bread by hydrolyzing arabinoxylan in dough [7]. Besides that, xylanase is widely applied in animal feedstock to improve their overall digestibility by degrading water extractable arabinoxylan in the feedstock, resulting enhanced nutrient absorptions [8]. In fact, xylanase is also utilised in juice and wine clarification by hydrolyzing pectins, starch and hemicellulosic components that cause the juice to become turbid and viscous.

B. subtilis is also one of the best understood prokaryotes in terms of molecular and cellular biology due to many of its genomes that have been sequenced [9]. Its superb genetic amenability and relatively large size genome provide powerful tool to investigate a bacterium in all possible aspects. The understanding of sequenced genomes of *Bacillus* spp has led to the development of alternative methods to greatly facilitate the new and innovative strategies to enhance the production of extracellular xylanase in large scale production [9]. In recent years, global demand for industrial xylanase was increased rapidly due to its high performance in food processing industry. Moreover, xylanase manufacturers are consistently looking for the maximum costs reduction, fastest speed processing and optimum production and yield which are always desired from a business standpoint. Therefore, many attempts have been performed for the improvement of the microbial xylanase through mutagenesis. Mutagenesis is a mutation process where the genetic information of an organism is changed in a stable manner. Mutagenesis is generally induced via ultraviolet (UV) irradiation, chemicals and other radioactive emitting substances. In the past, the construction of enzyme-producing industrial strains of *Bacillus* spp was generally based on the screening for desired properties, nevertheless, with the achievements of the last decade, basic genetic and biochemical information with the ability to

manipulate genetic materials have helped us to further improve these strains by mutagenesis for the selection of highly productive variants [10]. Most of the strains used for enzymes production have been improved through mutagenesis by UV irradiation and chemical agents. These chemicals and UV irradiation are known as mutagenic agents. Mutation occurs any time when there is a change in the nucleotide sequence of DNA. When microbial culture simultaneously exposes to mutagenic agents, mutation increases in frequency, gene duplication that occurred enables it to increase the production of the inefficient enzymes. As a result, mutations may occur in the enzymes structural gene, enabling the former inefficient enzymes to metabolize their substrates more efficiently and effectively [11]. Indeed, mutation changes the protein structure and most probably results in the deterioration of function. Therefore, changes in structural components by mutation are rarely to cause improvements unless the specific loss of function is required for the production purposes [12]. A loss of regulatory function is commonly resulted in enhanced enzymes production. Most of the bacteria wild strains which have high potential use in industrial fermentation processes are subjected to industrial strain improvement program to achieve better economical fermentation process. Hence, there are some ways to improve the strain including recombinant DNA technique, however, the advantages of random mutagenesis using UV irradiation and chemical mutagens are more overwhelming due to their simplicity and low cost procedure as compared to DNA recombinant technology [13]. Although recombinant technique made a significant contribution to the industrial enzymes productivity, random mutagenesis is still preferable, it thus, frequently selected as the favorable choice due to their reliable strain development [14]. Therefore, the objectives of this study are to produce mutants of *Bacillus subtilis* for xylanase overproduction using physical and chemical mutagenesis methods. In this study, the results of improved mutated strains are directed primarily based on their higher activity and overproduction of xylanase.

2. MATERIALS AND METHODS

2.1 Microorganism Strain and Inoculum Preparation

The microorganism model that used for xylanase overproduction in this study was *Bacillus subtilis* strain ATCC 6633. Nutrient agar was prepared and autoclaved at 121ºC for 15 minutes before it was poured on the sterile Petri dishes using aseptic techniques. Then, *B. subtilis* was subcultured on nutrient agar and incubated at 37ºC until the formation of colonies appeared after 2-day of incubation.

2.2 Random Physical Mutagenesis using Ultraviolent (UV) Irradiation: Source and Effect of UV Irradiation on the Production of Xylanase

B. subtilis on nutrient agar plates was treated with UV irradiation of the wavelength 2537 Angstroms or 254 nm in a horizontal laminar flow cabinet. In order to produce the mutants of *B. subtilis*, the nutrient agar containing *B. subtilis* were placed from the distance of 10, 20 and 30 cm from the UV light with the exposure time interval of 5 min up to 10, 20 and 30 min, respectively. The control of the experiment was conducted with non-treated *B. subtilis*. The mutants and control were then incubated overnight in an incubator at 37ºC. After incubation, bacteria cells were harvested using a spread rod. Cell count of mutants and control was then carried out using a hemacytometer. The inoculums size were standardized to 1×10^6 cells from the mutants and control before transferring into 500 mL Erlenmeyer flask containing working volume of 250 mL culture medium which was adjusted to the initial medium pH 7, respectively. The medium composition of the culture medium used was consisted of (g/L): barley husk, 20; peptone, 5; yeast extract, 5; KH_2PO_4, 1 and $MgSO_47H_2O$, 0.1. The inoculums were inoculated and incubated in an orbital rotary shaker of 37ºC agitated at 150 rpm for 72 h of submerged fermentation in shake flask culture. Xylanase activity assay was performed every 12 h intervals. All the analysis was also conducted on the non-treated control of UV mutagenesis.

2.3 Random Chemical Mutagenesis using Ethyl Methane Sulfonate (EMS): Effect on the Production of Xylanase

B. subtilis grown on nutrient agar was harvested and suspended into sterile distilled water. 9 mL of cell suspension treated with 1 mL of 100 µg/mL EMS in sodium phosphate buffer (pH 7) was used as the sample for EMS mutagenesis. Subsequently, the mutagenesis sample was incubated at 37ºC at 150 rpm for 15, 30 and 45 min, respectively. On the other hand, another 9

mL of cell suspension mixed with 1 mL sodium phosphate buffer (pH 7) was used as the non-treated control for EMS mutagenesis. 2 mL of mutagenesis samples and non-treated control were withdrawn at various time intervals of 15, 30 and 45 min, respectively. The samples were then centrifuged at 4500 rpm for 30 min at 4ºC. The supernatant were discarded and the cell pellet was washed three times with sterile distilled water. Then, the samples were centrifuged at 4500 rpm for 2 min at 4ºC followed by suspended in 3 mL of sodium phosphate buffer (pH 7). Thereafter, serial dilution was performed and cell count was conducted using haemocytometer. 1×10^6 cells were standardized for the inoculation onto a new nutrient agar. The inoculation was carried out using spread plate technique. The nutrient agar with non-treated control and mutagenesis samples of *B. subtilis* were incubated at 37ºC overnight. After incubation, 1×10^6 cells from both mutagenesis samples and non-treated control were inoculated into the working volume of 250 mL culture medium which was adjusted to the initial medium pH 7, respectively. The medium composition of the culture medium used was identical with the culture medium used in UV irradiation. The culture medium was then incubated in the orbital rotary shaker at 37ºC agitated at 150 rpm for 84 h of submerged fermentation in shake flask culture. Xylanase activity assay was carried out every 12 h. All the analysis was also performed on the non-treated control. The EMS mutagenesis was also repeated using 150 µg/mL of EMS.

2.4 Random Chemical Mutagenesis using Acridine Orange (AO): Effect on the Production of Xylanase

B. subtilis grown on nutrient agar was harvested and suspended into sterile distilled water. 9 mL of cell suspension mixed with 1 mL of sodium phosphate buffer (pH 7) was used as the non-treated control of mutagenesis. On the other hand, another 9 mL of cell suspension added into 1 mL of 10 µg/mL AO in sodium phosphate buffer (pH 7) was used as the sample for AO mutagenesis. Subsequently, the mutagenesis sample was incubated at 37ºC for 15, 30 and 45 min, respectively. 2 mL of samples were withdrawn at various time intervals from control and mutagenesis samples. Subsequently, the samples were centrifuged at 4500 rpm for 30 min at 4ºC. The supernatant were discarded. The cell pellet was washed three times with sterile distilled water. The samples were subsequently centrifuged at 4500 rpm for 2 min at 4ºC followed by suspended in 3 mL of sodium phosphate buffer (pH 7). Serial dilution was carried out and cells were counted using haemocytometer. 1×10^6 cells were standardized for the inoculation onto a new nutrient agar. Then, the nutrient agar plates with non-treated control and mutagenesis samples of *B. subtilis* were incubated at 37ºC overnight, respectively. After incubation, 1×10^6 cells from non-treated control and mutagenesis samples were subcultured in 250 mL culture medium with the same medium composition as in the method mentioned in UV irradiation and EMS mutagenesis before incubated at 37ºC agitated at 150 rpm for 84 h of submerged fermentation in shake flask culture. Sampling analysis including xylanase activity assay was carried out at every 12 h interval. All the analysis was also conducted on the non-treated control. The AO mutagenesis was repeated again using 50 µg/mL of AO.

2.5 Sampling and Analysis

All non-treated control and mutagenesis samples were withdrawn and collected every 12 h. The samples were subsequently centrifuged at 4500 rpm for 30 min at 4ºC. The supernatant containing xylanase was transferred and activity assay was then carried out and quantified.

2.6 Xylanase Activity Assay

Xylanase activity was measured according to Bailey et al. [15] using 3,5-dinitrosalicyclic (DNS) to detect the concentration of xylose. 1% of beechwood xylan was dissolved into 0.05 M of sodium phosphate buffer (pH 5.3) at 50ºC. 0.1 mL of the supernatant was added into 0.9 mL of xylan substrate. It was subsequently incubated at 50ºC for 30 min. 1.5 mL of DNS was added into the mixture and incubated at 90ºC for 5 min. Then, 0.5 mL of 40% Rochelle salt was added and cooled before the absorbance at 575 nm was measured using a spectrophotometer. The activity of xylanase was measured according to the xylose standard curve. The xylose standard curve of the absorbance reading at 575 nm against concentration was plotted. To quantify the activity of xylanase, one unit of xylanase activity is defined as the amount of enzyme required to release one µmole of xylose as the reducing sugar from 1 mL of enzyme extract per min under assay conditions.

2.7 Protein Assay

The total amount of soluble protein in the supernatant was measured according to Lowry method [16]. The absorbance reading of the protein samples was measured at 750 nm using spectrophotometer before quantified using the BSA standard curve.

2.8 Quantification of Biomass Concentration and Medium pH

The cell count of *B. subtilis* was performed using haemocytometer. The pH of the culture medium was measured using pH meter to study the profile of pH during the growth and overproduction of xylanase by *B. subtilis* after UV irradiation and chemicals mutagenesis in submerged fermentation.

2.9 Data Analysis

In this study, all of the random mutagenesis experiments involving UV irradiation, mutagenesis of EMS and AO were conducted in triplicate. The mean value of the sampling analysis including xylanase activity, protein concentration, biomass concentration and medium pH that were generated from the experiments was used to elucidate the overproduction of xylanase by *B. subtilis* after UV irradiation, mutagenesis of EMS and AO, respectively. The standard error was also calculated and represented on all the Figures in this study.

3. RESULTS AND DISCUSSION

3.1 Effect of UV Irradiation on the Production of Xylanase by *Bacillus subtilis*

In the past, although genetic engineering has made a significant contribution to bacteria strain improvement, but random mutagenesis is still a cost-effective procedure for reliable short-term strain development and is frequently preferred as the method of choice [17]. In the present study, both UV irradiation and chemicals induced random mutagenesis are elucidated to investigate the effectiveness in improving xylanase overproduction by *B. subtilis. Bacillus* spp are one of the best characterised members of the gram positive bacteria, also being one of the main microbial enzymes producers, have a wide range of industrial applications making them the more preferable microorganisms to produce extracellular enzymes [3]. Few attempts are thus, made for the improvement of enzymes production via mutagenesis [11]. Production of mutants by UV irradiation and chemicals mutagenesis is relatively inexpensive. It is thus, an attractive genetic strategy for microbes, since genotype can be mutagenised and the distribution of mutations is random in the genome [18]. Unlike recombinant DNA technology which requires highly efficient microbes transformation systems, chemicals and UV irradiation forms of mutagenesis do not rely on any transformations that are very beneficial in term of time and cost savings. In this experiment, wild type *B. subtilis* was subcultured on nutrient agar under the optimum growth conditions as the non-treated control. On the other hand, mutants of *B. subtilis* were obtained after exposed to UV irradiation with a wavelength of 254 nm. Both the non-treated control and mutants of bacteria were then subcultured and incubated overnight at 37ºC after it were inoculated into 250 mL of culture medium in 500 mL Erlenmeyer flask via submerged fermentation using barley husk as carbon source. Notably, the amount of xylanase production by microbes is influenced by carbon as substrate source. The role of the carbon as the inducer or repressor such as glucose or xylose is important in xylanase production [19]. Besides xylanase is an enzyme liable to induction, it is preferably synthesized by microbes in culture medium containing xylan or hemicellulose residues [20]. It is therefore, barley husk from agricultural residue was utilised as the xylanase inducer for its overproduction after random mutagenesis in this study. This study was carried out for 72 h of submerged fermentation while sampling was performed every 12 h interval. The sampling at every 12 h interval for *B. subtilis* was chosen due to its shorter reproduction period as compared to other microorganisms. Based on our results in Fig. 1, the maximum xylanase production of 1.366±0.025 U/mL was obtained by non-treated control of *B. subtilis*, which unfortunately, was the highest compared to other mutants. There was relatively 28.33% lesser of xylanase activity obtained from the mutant *B. subtilis* of 2.7±0.09 × 10^8 cells/mL that had been exposed to UV from a distance of 20 cm for 20 min as compared to its non-treated control. This mutant produced its highest xylanase activity of only 0.979±0.036 U/mL at medium pH 6.64±0.115 with the total protein production of 1.21±0.033 g/mL at 36 h of fermentation. In contrast, the non-treated control

of *B. subtilis* which showed the highest xylanase activity of 1.366±0.025 U/mL, reached the maximum peak at 36 h of fermentation, producing 3.5±0.11 × 10^8 cells/mL with the total soluble protein concentration of 1.567±0.013 g/mL at medium pH 6.81±0.028. Santiago et al. [21] mentioned in their studies that the increase in xylanase activity of mutant strain was attributed to possible changes in the gene promoter encoding for xylanase after UV exposure. UV induced mutations are closely related to DNA damage where the UV light has strong genotonic effects to damage DNA, resulting the induction of mutations or termination of enzymes activity. UV induces specific types of mutation including base substitutions of cytosine (C) to thymine (T) at di-pyrimidine sites and CC to TT tandem base substitutions. Solaiman et al. [22], Surendra et al. [23], Venkata [24], Lipika et al. [17] and Ashajyothi et al. [25] exposed *Bacillus* species strains to UV irradiation to induce random mutagenesis in order to study the effect of UV mutagenesis on xylanase, amylase, protease and lipase overproduction. According to Surendra et al. [23], the exposure of the wild type *B. subtilis* to UV irradiation conducted for a time interval of 5 to 60 min for amylase overproduction where the distance between the UV and sample adjusted to a distance ranging from 5 to 55 cm produced satisfactory results of mutagenesis. Thus, the optimum range of time including 10, 20 and 30 min from the distance of 10, 15 and 20 cm were selected and elucidated in this study. According to Lipika et al. [17], they showed that UV induced random mutagenesis yield mutants with enhanced xylanase activity. Based on their results, the UV treated mutant showed the maximum of apparently, 230% increment in xylanase activity as compared to non-treated control of *B. subtilis.* Unfortunately, in this study, the UV irradiation might deregulate the transcription of the mRNA corresponding to xylanase, resulting the reduction in its activity and productivity.

The protein concentration of *B. subtilis* was detected using Lowry method as shown in Fig. 2. Fig. 2 illustrates the correlation of the maximum amount of cell count and protein concentration from non-treated control and mutagenesis samples occurred at 36 h of fermentation. Indeed, we anticipated that protein concentration increased with the increase of cell number from the samples in shake flask culture. The highest protein concentration was found to be at 36 h with its concentration of 1.841±0.0127 g/mL at medium pH 6.38±0.115, producing 2.9±0.88 cells/mL of mutants that were exposed to UV for 20 min from a distance of 15 cm. Notably, protein overproduction increased by relatively 18.01% as compared to 1.56±0.013 g/mL at the medium pH 6.81±0.028 by 3.5±0.115 cells/mL of non-treated control. This result is supported by the study of Surendra et al. [23], where they investigated the overproduction of the extracellular enzymes including amylase released by *B. subtilis* after UV induced mutagenesis. Based on their result findings, the mutants possessed higher protein production of 618 mg/L as compared to parent strain that produced only 292 mg/L, thus, indicating the enzyme activity was affected and improved by UV mutagenesis. According to Surendra et al. [23], mutants showed the presence of addition proteins with higher molecular weight, indicating the increment of protein expression and thus, results in overproduction after UV exposure. On the other hand, Fig. 3 shows that the profile of medium pH of non-treated control and UV mutagenesis samples increased towards low alkaline condition as the fermentation prolonged to 72 h of submerged fermentation. This is due to the nature of *B. subtilis* that grow optimal in neutral culture medium as shown at 36 h of fermentation. Indeed, based on our results, the medium pH of all UV mutated samples were found in the range of 6.3±0.115 to 6.81±0.028 at 36 h of fermentation in which the xylanase activity was the highest from all samples at this time. Xylanase activity possessed the highest at 36 h and decreased thereafter due to the diminishing of nutrients and substrates available for its activity. Likewise, Bisaria and Ghose [26] stated that neutral medium pH favored bacterial enzymes production by *B. subtilis.*

3.2 Effect of Ethyl Methane Sulfonate (EMS) on the Production of Xylanase by *Bacillus subtilis*

EMS is a well-known mutagenic agent, whose mode of action is attributed to alkylation to nitrogen position 7 of guanosine of the DNA molecule, leading to transition of mutation [27]. In this study, the non-treated control of *B. subtilis* was grown on medium culture in submerged fermentation under optimum growth conditions whereas the mutants of *B. subtilis* were attained after being exposed to two different concentrations of EMS at 100 and 150 µg/mL, respectively. This study was carried out for 72 h of fermentation with sampling every 12 h interval.

Based on our results, out of all EMS induced mutants of *B. subtilis,* 100 µg/mL of EMS produced the highest xylanase activity of 1.638±0.027 U/mL as compared to 150 µg/mL EMS induced mutants and non-treated control that produced 1.344±0.0929 and 1.416±0.0248 U/mL, respectively. The gene responsible for the production of xylanase was overexpressed due to mutation, as a result, it increased its activity. Fig. 4 shows the highest xylanase overproduction reached 1.638±0.027 U/mL, producing 3.6±0.173 × 10^8 cells/mL with total protein of 1.66±0.004 g/mL at medium pH 6.77±0.069 was attained after induction of 100 µg/mL EMS for 30 min. Apparently, there was 15.68% higher of xylanase overproduction as compared to only 1.416±0.0248 U/mL by 4.6±0.15 × 10^8 cells/mL of non-treated control with total protein of 1.53±0.006 g/mL at medium pH 6.85±0.099. In contrast, 150 µg/mL EMS induced mutants produced xylanase activity of 1.344±0.0929 U/mL, which was relatively 5.08% lesser than non-treated control that produced its activity of 1.416±0.024 U/mL. This results is supported by Chusnul et al. [28], they conducted similar experiments to investigate the effectiveness of EMS by inducing *Bacillus* sp to 50, 100 and 150 µg/mL of EMS for 30 and 60 min to produce improved strain for the enhancement of xylanase yield. According to Chusnul et al. [28], the xylanolytic mutants possessed the highest xylanase activity of 15.057 U/mL by 50 µg/mL EMS induced mutants, which was literally 61.61% higher as compared to their non-treated counterparts of 9.317 U/mL. Based on our observation, EMS was a strong mutagenic agent that induced permanent changes in DNA structure, involving frame shift mutation. We also anticipated that the concentration of EMS increases, xylanase production decreases, and hence, 150 µg/mL EMS exhibited lesser xylanase activity production of 1.344±0.0929 U/mL in mutants.

Chemical mutagenic agents that possess the ability to induce mutation have been a major driving force in genetics study field for the past 75 years [29]. Among all various mutagens that have been used to induce mutations, such as EMS, are particular effective. In fact, EMS mutagenesis causes mispairing in their complementary bases, primarily in G/C to A/T transition, thereby, introducing base changes after replication [30,31]. This base mispairing would alternately lead to permanent DNA changes. Indeed, EMS mutagenesis results in high point mutational density with only low levels of chromosome break that would cause aneuploidy and dominant lethality. Therefore, EMS has been employed as one of the two chemical mutagenic agents used in this study. EMS is an alkylating agent which carries alkyl groups in reactive form. The transfer of methyl or ethyl groups to the bases could lead to altered base-paring and changes in transitions, as a result, it induces all kinds of mutations including transition, transversion and frame shift [32]. EMS was successfully demonstrated improvement of xylanase production in the study by Sangkharak et al. [33]. Likewise, according to Haq et al. [34], they treated *Bacillus* sp with EMS to induce random mutagenesis in order to study its effects on xylanase overproduction. In their study, they exposed wild type bacteria of *Bacillus* sp to EMS for time interval of 10 to 60 min with its concentration prepared in the range of 50 to 300 ug/mL. Similarly, according to Chusnul et al. [28], EMS treated mutants produced xylanase activity of 15.057 U/mL which was literally 61.61% higher than xylanase activity of 9.317 U/mL produced by non-treated control. In fact, EMS is also capable of inducing overproduction of other enzymes including amylase, protease and lipase by *Bacillus* spp. In the study by Haq et al. [35], the highest amylase production of 102.78 U/mL was attained from EMS treated mutants of *Bacillus sp* as compared with non-treated *Bacillus* that produced only 73.98 U/mL which was 38.9% lesser than the mutants. Nadeem et al. [36] stated that EMS treated mutants of *Bacillus* sp produced the highest protease activity of 88 U/mL, which was apparently 29.72% higher as compared to non-treated control that produced only 67.84 U/mL. Additionally, Ravindranath et al. [37] employed EMS to enhance extracellular lipase production from 6.0 to 20.4 U/mL with the tremendous increment of 340% as compared to the non-treated control. On the other hand, Fig. 5 displays the maximum overproduction of protein of 1.664±0.004 g/mL at medium pH 6.77±0.069, producing 3.6±0.17 × 10^8 cells/mL of mutants that had been induced with 100 µg/mL of EMS for 30 min. In this regard, the protein overproduction increased about 8.55% as compared to 4.6±0.15 × 10^8 cells/mL of non-treated control that produced 1.533±0.006 g/mL of protein at medium pH of 6.85±0.099. Fig. 6 displays the increase of medium pH of non-treated control and mutagenesis samples, as a result of natural growing characteristic of *B. subtilis*. Consequently, based on our results, the maximum xylanase overproduction of 1.638 ± 0.027 U/mL by 100 µg/mL EMS induced mutants

was anticipated to produce during neutral medium pH condition at 36 h of submerged fermentation. Similarly, Horikishi [38] suggested that the optimal medium pH of commercial xylanase from *B. subtilis* was found within the neutral pH range. In fact, the pH of all EMS mutated samples at 36 h was attained in the range of 6.77±0.069 to 6.85±0.099 in which the xylanase activity was produced at the highest peak at 36 h.

3.3 Effect of Acridine Orange (AO) on the Production of Xylanase by *Bacillus subtilis*

In the present study, AO has been reported as a simple approach for inducing genetic alterations by introducing mutation in bacterial strains [39]. AO is an aromatic compound that intercalates within the pairs of bases in DNA favoring insertions and deletions of nucleotide bases upon replication. The resulting mutation causes a shift in the translated reading frame of the coded information in the mRNA transcript, thus leading to an altered sequence of amino acids at the point of the insertion and deletion [40]. Some studies reported that antibiotic activity was enhanced in *B. subtilis* using mutagenesis induced with AO [41]. Nevertheless, there are very scarce studies reported the enhanced enzymes activity by mutated *B. subtilis* that induced by AO. In addition, nearly no report is available on the enhanced production of xylanase in *B. subtilis*, even though, AO mediated mutation is relatively simple approach for inducing genetic alterations in bacterial strains [39]. In this study, we therefore, used AO for the first time to enhance the production of xylanase activity through random mutagenesis. In other words, we used AO to induce mutation to detect if the random mutation could enhance the production of microbial xylanase. In this study, the non-treated *B. subtilis* was grown on culture medium in submerged fermentation under optimum growth conditions whereas the mutants of *B. subtilis* were obtained after being exposed to two different concentrations of AO at 10 and 50 µg/mL with different exposures time of 15, 30 and 45 min, respectively. According to Arshad et al. [39], AO caused growth inhibition effect where the minimum inhibitory concentration of AO was 10 µg/mL. Apparently, the bacterial growth was gradually suppressed by increasing concentration of AO from 10 to 100 µg/mL. Based on the results, the highest concentration that possessed the permissible growth rate of AO induced mutants was 50 µg/mL [39]. Therefore, an optimum range of time from 15, 30 and 45 min with the concentrations of AO at 10 and 50 µg/mL were selected and tested in this study. This study was thus, carried out for 72 h of fermentation required sampling every 12 h interval.

Based on Fig. 7, the highest xylanase production of 1.580±0.027 U/mL was attained at medium pH of 6.59±0.078 with the total protein of 1.698±0.018 g/mL at 36 h by 3.8±0.29 $\times 10^8$ cells/mL of mutants *B. subtilis* that had been induced with 50 µg/mL of AO for 30 min. Xylanase activity was anticipated to be higher with approximately 11.58% than non-treated control that produced 1.416±0.024 U/mL by 4.6±0.15 $\times$ 10^8 cells/mL with the total protein of 1.533±0.006 g/mL occurred at the medium pH of 6.85±0.099. Based on our results, the decrease of xylanase activity after 36 h of submerged fermentation was due to the correlation between the two factors in which the number of bacteria cell present in fermentation was directly proportional to the xylanase activity produced by *B. subtilis*. There was a study conducted by Arshad et al. [39] where they proved the similar effectiveness of 50 µg/mL AO as the optimal concentration in the overproduction of penicillin G acylase (PGA) by *E. coli*. Apparently, their study indicated that the bacteria growth was gradually suppressed as the concentration of AO increased from 10 to 70 µg/mL. In fact, 80 to 100 µg/mL of AO was proven lethal to the growing bacteria, thereby, a dilution of 10 to 50 µg/mL of AO was being selected in this study. On top of that, the study also indicated that 50 µg/mL of AO induced mutants *E. coli* has two-fold increment in PGA production of 12.7 mg in contrast to non-treated control strain that produced only 6.3 mg. In our results, mutants of *B. subtilis* that induced with 10 µg/mL of AO also showed increment in xylanase activity of 1.466±0.0495 U/mL as compared to 1.416±0.024 U/mL from non-treated control, it was however, not as high as mutants induced with 50 µg/mL AO. Therefore, 10 µg/mL of AO is not efficient and effective enough in giving rise to high frequency of mutants that able to enhance higher xylanase activity. In contrast, 50 µg/mL of AO was the optimal concentration used to overproduce xylanase by *B. subtilis* in this study. Notably, AO is capable of intercalating the pairs of bases in DNA, thus resulting mutation in translated reading frame of the coded information in the mRNA transcript, affecting the enzyme production gene [40]. Nonetheless, the

enhancement in enzyme production obtained by AO induced mutagenesis of *Bacillus* spp has seldom been reported in the literature [41]. As a result, the exact mechanism responsible for the enhancement of enzyme activity is still remained not clear.

Fig. 8 displays the maximum protein concentration of 1.698±0.018 g/mL at pH 6.59±0.078, producing 3.8±0.29 × 10^8 cells/mL of *B. subtilis* mutants that had been induced with 50 µg/mL of AO for 30 min. Apparently, protein overproduction increased about 10.76% as compared to 1.533±0.006 g/mL of protein from 4.6±0.15 × 10^8 cells/mL of non-treated control at medium pH of 6.85±0.099. According to Yousry et al. [42], when *Streptomyces nasri* was induced with 50 µg/mL of AO, they showed relatively higher protein production of almost two-fold as compared to control parent strain. On the other hand, Fig. 9 illustrates the medium pH of non-treated control and mutagenesis samples were slightly increased to the low alkaline growing condition of *B. subtilis* as submerged fermentation proceeded. Based on our result findings, the maximum xylanase activity and cell count occurred at 36 h of fermentation where the medium pH of AO mutated samples were found in the range of 6.59±0.078 to 6.85±0.099 in which they matched the optimum medium pH of xylanase according to Horikishi [38]. Apparently, according to Horikishi [38], the neutral pH range was proven to be the optimum medium pH for xylanase production by *B. subtilis* in his study.

In a nutshell, *B. subtilis* induced by 100 µg/mL of EMS and 50 µg/mL of AO for 30 min produced the overexpression of xylanase activity with 1.638±0.027 U/mL by 3.6±0.17 × 10^8 cells/mL and 1.580±0.027 U/mL by 3.8±0.29 × 10^8 cells/mL, respectively. Indeed, EMS induced *B. subtilis* mutants experienced the highest percentage of increment for the overproduction of xylanase activity with 15.68% as compared to AO treated mutants with only 11.58% increment. In contrast, UV exposed mutants exhibited decrease of approximately 28.33% of xylanase activity. Literally, the UV irradiation might down regulate the transcription of the mRNA corresponding to xylanase, resulting decline in xylanase expression [21].

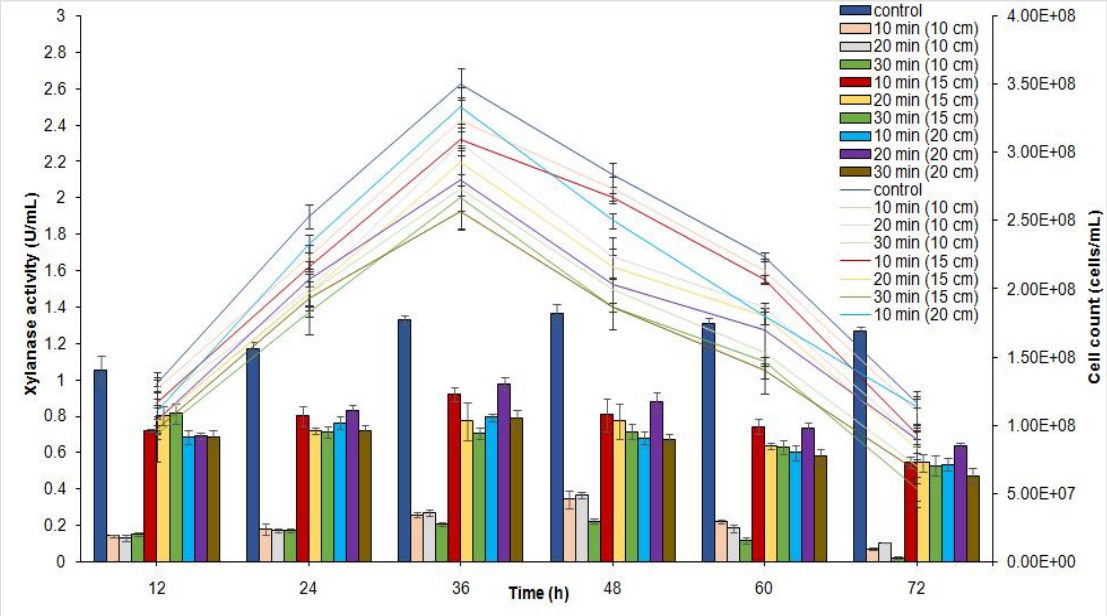

Fig. 1. Xylanase production from non-treated control and mutants of *B. subtilis* after UV exposure from a distance of 10, 15 and 20 cm at various time intervals. Xylanase activity is presented as column chart while cell count is presented as line graph

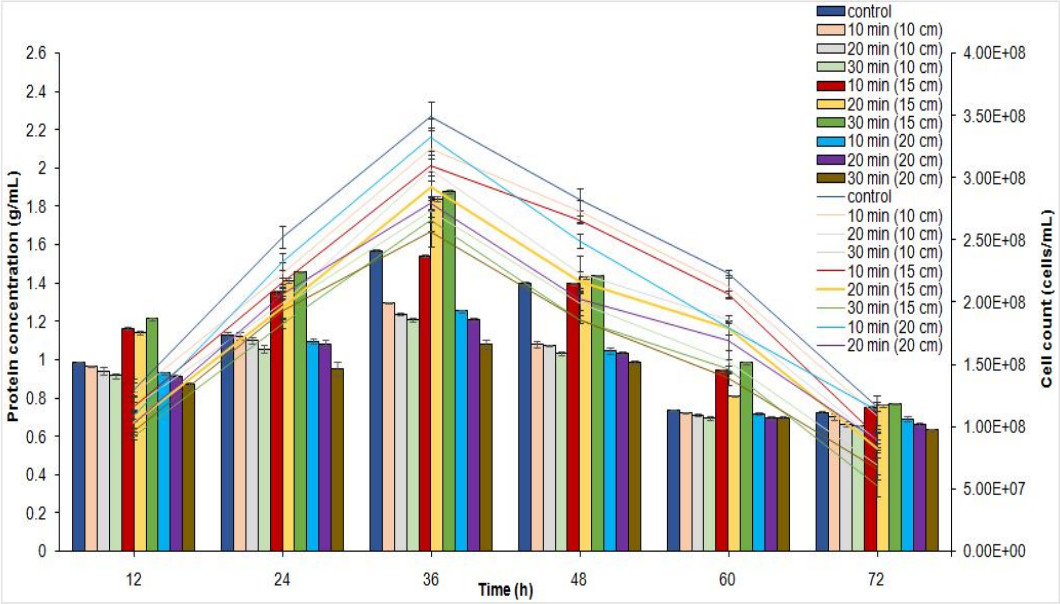

Fig. 2. Total protein production from non-treated control and mutants of *B. subtilis* after UV exposure from a distance of 10, 15 and 20 cm at various time intervals. Protein concentration is presented as column chart while cell count is presented as line graph

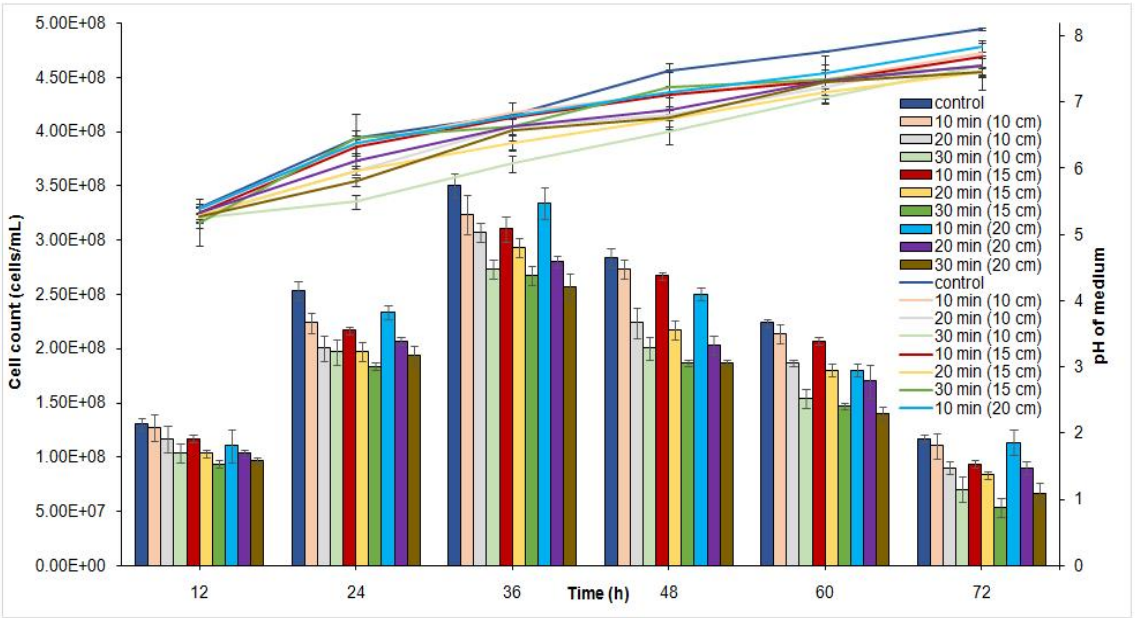

Fig. 3. Medium pH profile of non-treated control and mutants of *B. subtilis* after UV exposure from a distance of 10, 15 and 20 cm at various time intervals. Cell count is presented as column chart while pH medium is presented as line graph

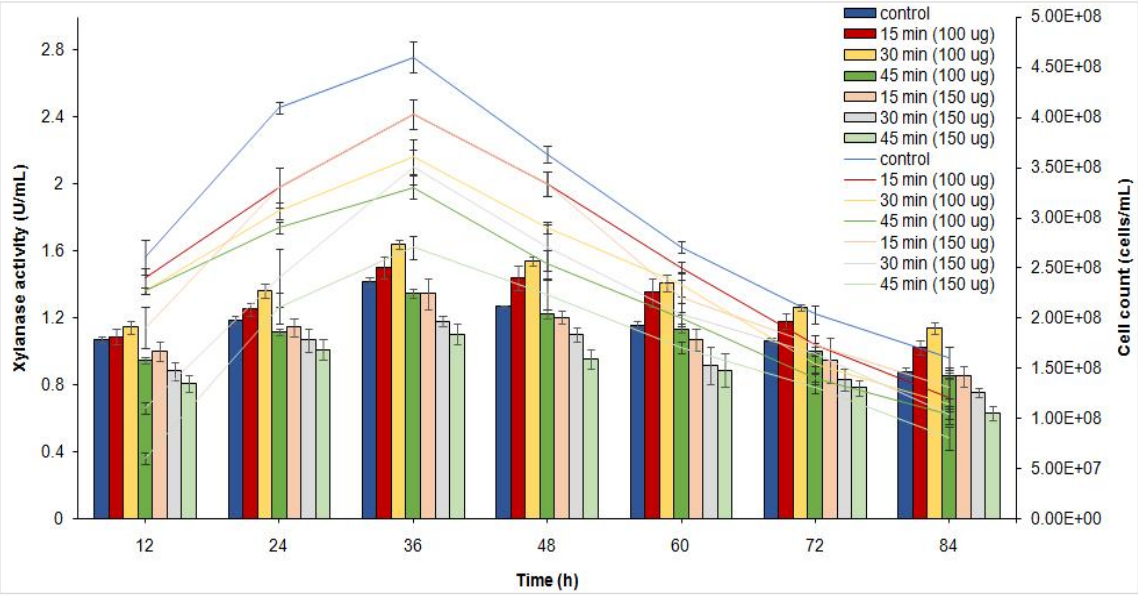

Fig. 4. Xylanase overproduction from non-treated control and mutants of *B. subtilis* after exposure of 100 and 150 µg/mL EMS at various time intervals. Xylanase activity is presented as column chart while cell count is presented as line graph

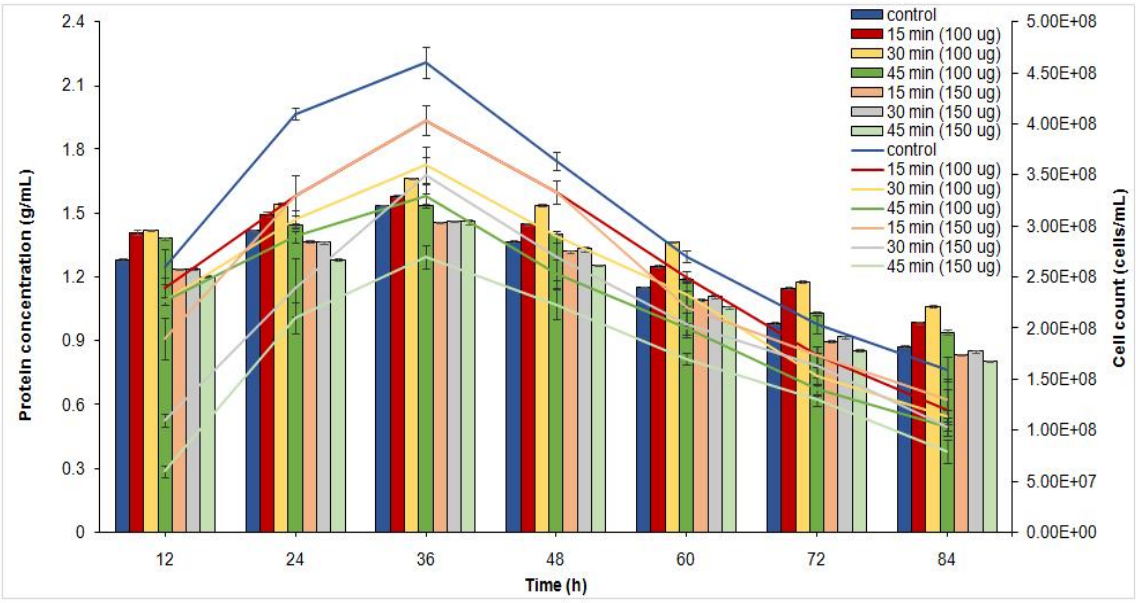

Fig. 5. Total protein production from non-treated control and mutants of *B. subtilis* after exposure of 100 and 150 µg/mL EMS at various time intervals. Protein concentration is presented as column chart while cell count is presented as line graph

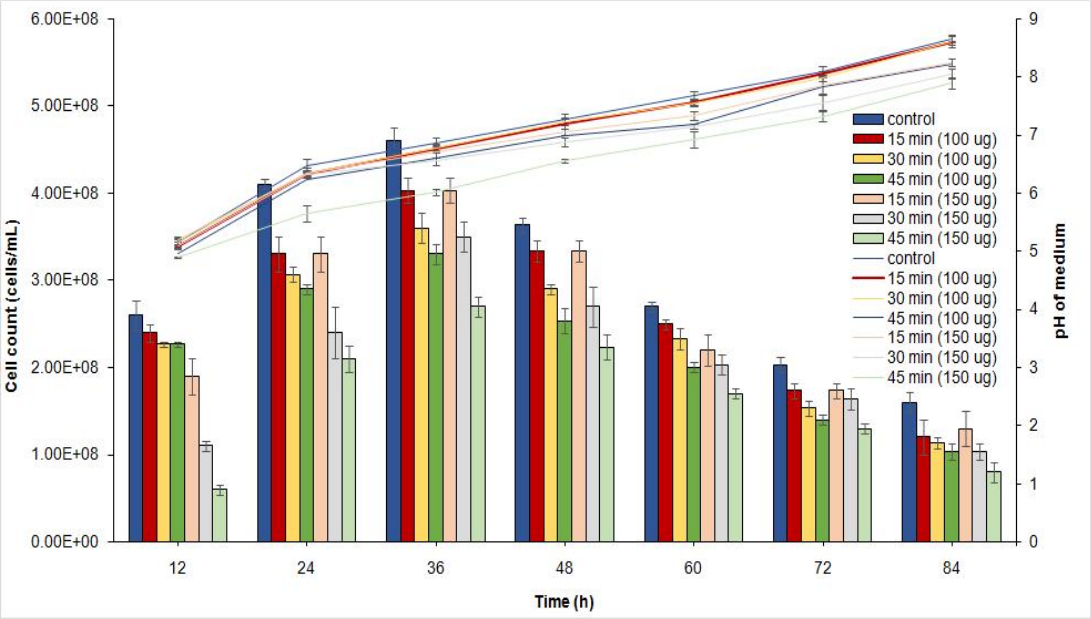

Fig. 6. Medium pH profile from non-treated control and mutants of *B. subtilis* after exposure of 100 and 150 µg/mL EMS at various time intervals. Cell count is presented as column chart while pH medium is presented as line graph

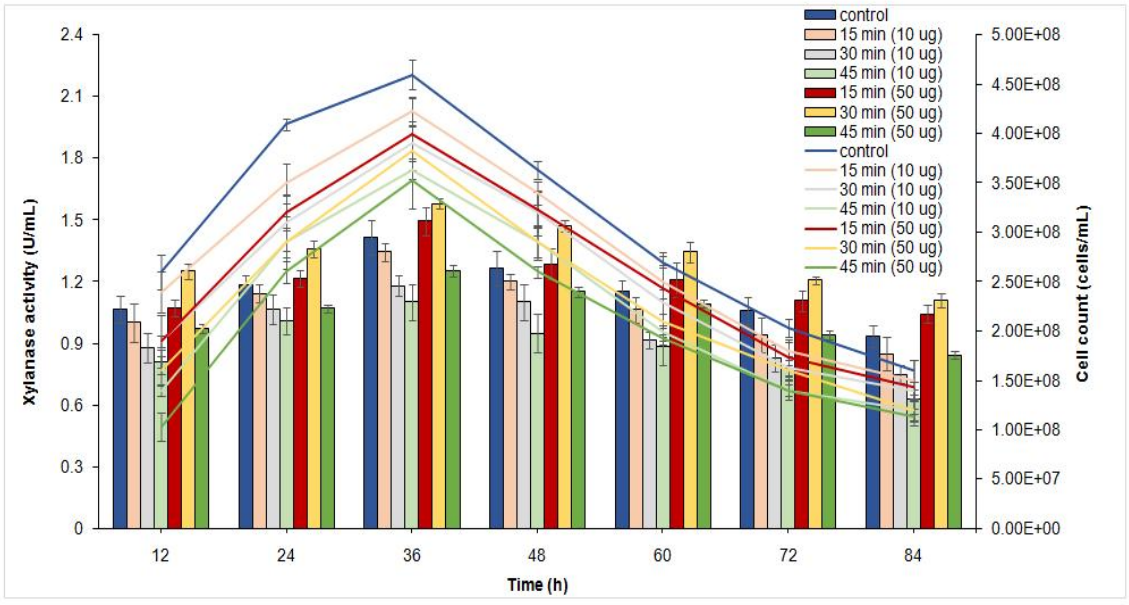

Fig. 7. Xylanase overproduction from non-treated control and mutants of *B. subtilis* after exposure of 10 and 50 µg/mL AO at various time intervals. Xylanase activity is presented as column chart while cell count is presented as line graph

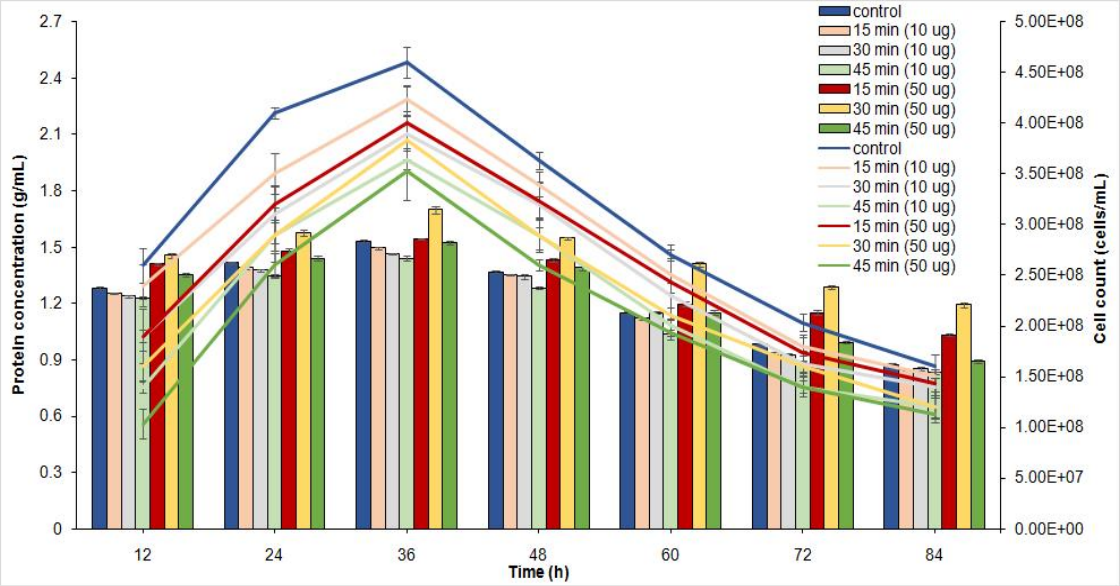

Fig. 8. Total protein production from non-treated control and mutants of *B. subtilis* after exposure of 10 and 50 µg/mL AO at various time intervals. Protein concentration is presented as column chart while cell count is presented as line graph

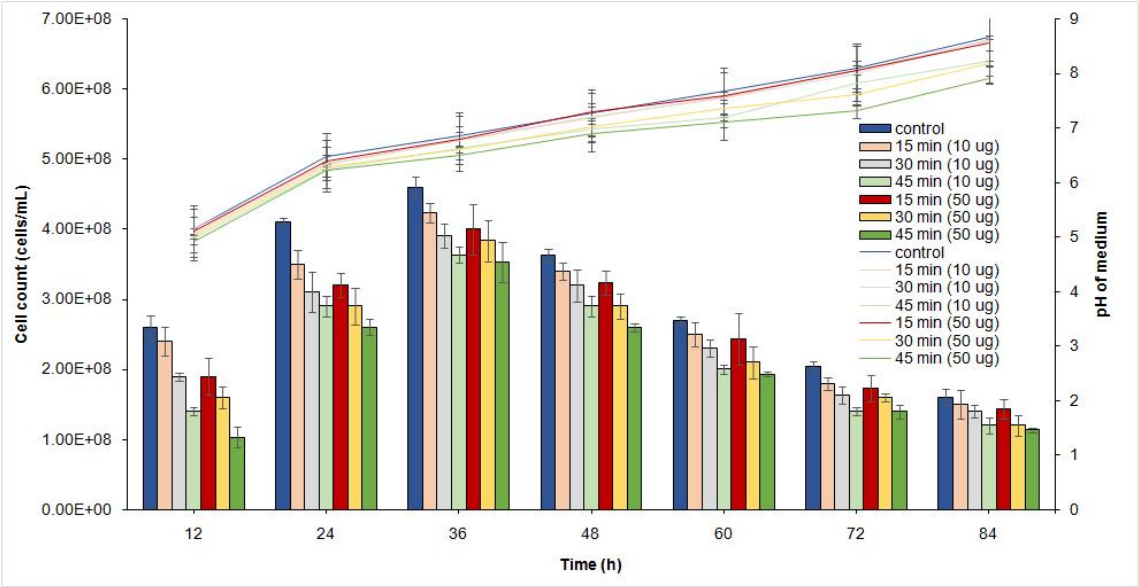

Fig. 9. Medium pH profile from non-treated control and mutants of *B. subtilis* after exposure of 10 and 50 µg/mL AO at various time intervals. Cell count is presented as column chart while pH medium is presented as line graph

4. CONCLUSION

In recent years, the global demand for industrial enzymes increases rapidly due to their high commercial performance in food industry. In addition, many enzyme manufacturers are looking for an alternative to reduce the costs of the production with increase yield and productivity. Such attributes are thus, tremendously beneficial to the industrial applications. Consequently, several attempts have been made for the improvement of the

microbial enzymes through mutagenesis. Random mutagenesis has been suggested to be one of the most successful methods, where in this study, it proved chemicals mutagenesis of EMS and AO was capable of enhancing xylanase activity. The effectivenes of EMS and AO induction for the strain improvement of *B. subtilis* for the overproduction of xylanase has been demonstrated well in this study under optimum growth conditions of 37ºC at 150 rpm for 84 h of submerged fermentation in shake falsk culture. According to our results, the optimum time of xylanase production from *B. subtilis* mutants occurred at 36 h of fermentation. Various mutants showed improved xylanase activity regardless of the types of mutagenic agents of EMS and AO. We suggested that the increase of xylanase activity was probably due to the increase of gene copy number, as a result of rapid DNA amplification in the transcription of mRNA corresponding to xylanase, leading to an increment in over expression and hyper production of xylanase. Notably, *B. subtilis* that had been induced with 100 µg/mL of EMS for 30 min and 50 µg/mL of AO for 30 min exhibited the overproduction of xylanase with 1.638±0.027 and 1.580±0.027 U/mL, respectively. We suggested that these results indicate that chemicals mutagenesis was able to mutate *B. subtilis* to produce greater expression of xylanase in contrast to UV mutagenesis that was not effectively generate the positive mutants by destroying the DNA of xylanase that might cause the down regulation or termination of xylanase activity. In fact, EMS induction considered as the most favorable approach of mutagenesis in terms of enhancing xylanase production in this study. Notably, the outcome of this result findings provide an in depth information relating to the application of mutagenesis methods with different parameters to generate mutants of *B. subtilis* that are competent of producing high amount of xylanase. In conclusion, different types of chemicals mutagenesis are able to improve the targeted enzyme production compared to UV mutagenesis. In order to meet the increasing demands of xylanase in global market, further study on mutagenesis and research on rapid amplification of DNA in mRNA transcripts that results in the high xylanase of gene copy number should be continued from this study. The understanding of the mutated gene sequence that causes the xylanase overproduction combines with recombinant DNA technology is anticipated to increase its specific enzyme activity. Moreover, researches on other types of mutagenesis such as site-directed mutagenesis and optimisation of carbon source in medium formulation for xylanase production would absolutely advantageous as they could increase the enzyme activity besides reducing the production costs which is essential in term of industrial point of view.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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