*Journal of Advances in Biology & Biotechnology*
*3(3): 117-131, 2015; Article no.JABB.2015.039*
*ISSN: 2394-1081*

**SCIENCEDOMAIN** *international*
www.sciencedomain.org

# Fungal Strain Improvement of *Aspergillus brasiliensis* for Overproduction of Xylanase in Submerged Fermentation through UV Irradiation and Chemicals Mutagenesis

**Hooi Ling Ho[1*] and Kuan Fai Ho[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. Both authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/JABB/2015/17274
*Editor(s):*
(1) James W. Lee, Department of Chemistry and Biochemistry, Old Dominion University, USA.
*Reviewers:*
(1) Refaz Ahmad Dar, Biotechnology Division, Indian Insitute of Integrative Medicine, India.
(2) Ali Mohamed Elshafei, Department of Microbial Chemistry, Division of Genetic Engineering and Biotechnology, National Research Centre, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=930&id=39&aid=9030

***Original Research Article***

***Received 6th March 2015***
***Accepted 31st March 2015***
***Published 28th April 2015***

## ABSTRACT

**Aims:** One of the major outlooks in biotechnology is to enhance enzymes production using various strains of microorganisms. Filamentous fungi such as *Aspergillus brasiliensis* has been found to be the most promising strain as it produces extracellular enzymes such as xylanase which is easily extracted as compared to other intracellular enzymes. Xylanase has been involved in many industrial applications such as pulp and paper, baking, detergent, food and beverage. Hence, the main objectives of this study are to improve the production of xylanase by *A. brasiliensis* using physical and chemicals mutagenesis and thus to determine the most effective mutagenesis approach for the overproduction of xylanase.
**Methodology:** In this study, ultraviolet (UV) irradiation and chemical mutagens including ethyl methane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were selected as the random mutagenesis methods due to their cost-effectiveness as compared to recombinant DNA technology.
**Results:** The wild type strain was able to produce 3.097±0.089 U/mL of xylanase at 48 h of

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

submerged fermentation. In contrast, the highest xylanase overproduction of 4.86±0.095 U/mL was achieved from mutant of *A. brasiliensis* after being exposed to UV for 20 min from a distance of 10 cm. In fact, this UV exposed *A. brasiliensis* mutant experienced the highest percentage of increment with 56.93% on the overproduction of xylanase as compared to the mutants with the exposure of 150 µg/mL of EMS for 90 min and 150 µg/mL of MNNG for 30 min with the increment of only 1.34% and 17.14%, respectively.

**Conclusion:** In conclusion, UV mutagenesis was among the most effective mutagenic approach in inducing the overproduction of xylanase by *A. brasiliensis* compared to EMS and MNNG in this study, respectively.

*Keywords: Random mutagenesis; ultraviolet (UV) irradiation; Ethyl Methane Sulfonate (EMS); N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); xylanase; Aspergillus brasiliensis; submerged fermentation.*

## 1. INTRODUCTION

Xylanase is a type of glucosidases which is involved in catalyzing endohydrolysis of 1, 4-ß-D-xylosidic linkages found in xylan substrate. It is belonged to an extensive group of enzyme which is implicated in the synthesis of xylose. Xylose is a primary carbon source involved in cell metabolism as well as cell infection. Xylanase is produced by various types of organisms such as bacteria, protozoa, algae, fungi, *anthropods* and *gasthropods*. Nonetheless, one of the major outlooks of biotechnology is to improve xylanase production using different strains of microorganisms including bacteria, yeast and fungi. *Aspergillus brasiliensis* has therefore become one of the most promising strains for xylanase production as compared to bacteria and yeast. Interestingly, *A. brasiliensis* is able to produce 15 types of extracellular xylanases [1]. Indeed, *A. brasiliensis* is one of the most common fungal species among the genus of *Aspergillus. A. brasiliensis* produces white and yellowish colonies that are covered by its dark spores are produced asexually. Xylanases that produced by *A. brasiliensis* are mostly belonged to extracellular enzymes. Extracellular enzymes are usually present outside of the microbial cells after they are secreted. Extracellular enzymes are usually soluble in water and readily to be extracted and purified from the culture medium. The immense majority of xylanases that are excreted into the extracellular environment occurred as a result of impenetrability of large amount of substrates into the cells. Thus, current belief indicates that xylanase production is urged by the means of the product obtained from their mechanisms [1]. Hence, xylanase purification could become more stable and cheaper in the production costs. According to Khan et al. [2] and Dai et al. [3], they suggested that *A. brasiliensis* was able to produce 225 and 218.5 U/mL of xylanase under their basic growth conditions, respectively. Both researches have also shown that xylanase produced by fungi is more efficient than other microorganisms because this fungal enzyme is mainly constituted of extracellular enzyme which is easier to extract and recover as compared to the intracellular enzymes [4]. The reasons on why numerous researches have been focused and conducted on increasing enzymes yield because these enzymes are biological proteins which are essential in sustaining the life of various microorganisms. They are highly specified catalysts which governs billions of biological pathways of a microorganism. Apart from that, enzymes have also been widely developed and used in the industrial field as different enzymes are used to catalyze different reactions. Moreover, enzymes such as xylanase are widely used in food processing, animal feed production, laundry and automated dish washing detergents, textile, pulp and paper. Hence, many articles have been published regarding the industrial applications of xylanase. Subramaniyan and Prema [5] and Dhiman et al. [6] are a few of the many authors that published articles on the enzymatic applications of microbial xylanases. Study of synergistic action on multiple forms and mechanism of xylanase make it possible to be utilised for bio-bleaching of kraft pulp and bio-scouring of fabrics [6]. In the food processing industry, xylanase is a fibre degrading enzyme that possesses dramatically effect on dough by improving its elasticity. Additionally, xylanase also plays a significant role as filtration improvers. Slow filtration of the mash or final beer often causes the generation of viscous polysaccharides such as xylans and glucans. Pre-treatment with xylanase would degrade these viscous polysaccharides and thus, increases the filtration rates. As a result, it prevents the fouling of filtration membranes.

Besides that, xylanase is also applied in removing of arabinoxylan and starch hazes from fruit juices. In addition, xylanase is commonly added into animal feeds to reduce the content of non-starch polysaccharides, thereby decreasing the intestinal viscosity of the animals and hence, enhancing the utilisation of proteins and starch within the animals. Xylanase has also been readily utilised in the paper-making industry. Nonetheless, xylanase is unfortunately limited by its characteristic and thus, it would easily denature by some harsh external conditions, such as high temperature, extreme pH and presence of some specific organic solvents. As a result, efficient xylanase production is mandatory and it could also achieve by strain improvement through random mutagenesis. Mutagenesis is defined as the changes of genetic information of an organism in a stable and heritable manner, which could either occur naturally or perform experimentally using chemicals or ultraviolet (UV) irradiation. There are two types of mutagenesis involved in this study, UV irradiation and chemicals mutation using two types of chemicals, ethyl methyl sulfonate (EMS) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Different concentrations of chemical mutagens exposure to fungi increase enzymes production rate. In fact, by exposing fungi to UV at different exposure time, some mutants are able to yield higher enzymes production [4]. The main influence of UV irradiation is to change the structure of pyrimidine (cytosine and thymine) bases of DNA, which eventually leads to the formation of pyrimidine dimers. Such dimers influence the pattern of the DNA double helix strands and hence, distort the replication process [4]. Therefore, in common cases of UV mutation, the effects are usually harmful to the microorganisms but in some special cases, it may cause better adaptation of the microorganisms to the extreme environment with enhanced biocatalytic performance [4]. Occasionally, mutations improve enzymes yield by several folds than their parental strains. It has been proven that with mutation, it may urge certain genes to be more efficient. Butt et al. [7] had exposed *A. brasiliensis* to UV irradiation at different time intervals from 5 to 60 min for better xylanase production. In fact, by comparing various carbon sources and substrates in their study, sucrose and wheat bran were so far able to attain the maximum production of xylanase. Wheat bran is consisted of 8.04% cellulose with the rest of xylan and lignin. The large amount of xylan increases the surface area of wheat bran as the optimum substrate for xylanase synthesis [8]. In fact, strains treated with UV irradiation for 45 min possessed the maximum yield of xylanase up to 1800 U/g under solid substrate fermentation with wheat bran as the sole substrate. Hence, the results showed that mutants were able to hyperproduce xylanase in addition to the presence of wheat bran. Likewise, chemical mutagens may also enhance the efficiency of certain genes through deletion and duplication mechanisms. Chemical mutagens such as EMS and MNNG lead to the alkylation of guanidine residues that produce resident lesions within the DNA molecules and hence, result in the occurrence of clusters of closely linked mutations. Apart from that, such effects may also increase the expression of genes that are responsible for encoding enzymes production in fungi [8]. Notably, Tasneem et al. [9] conducted a research to increase the synthesis of xylanase by chemically mutated *A. brasiliensis*. MNNG was the chemical mutagenic agent involved in the experiment. The results showed that one of the strains, *A. brasiliensis* GCBCX-20 which exposed to 20 min of MNNG mutagenesis had improved the xylanase production. This xylanase production from the mutants was found to be 1.7-fold higher than its parental strain. Subsequently, Tasneem et al. [9] concluded that mutation could increase the efficiency of xylanase producing genes through duplication and deletion mechanism. The main reason of this study was conducted because most of the industrial processes that utilised xylanase are produced by particular strain of microorganisms where they are commercially competent in xylanase production especially in the industrial point of view. Therefore, strain improvement through mutagenesis would provide an easier way out to produce higher amounts of efficient xylanase to meet the huge increasing demands of its industrial applications. Moreover, *A. brasiliensis* is also an ideal fungal strain to work on as it is able to produce up to 19 types of enzymes including xylanase. In fact, the general hydrolytic enzymes such as xylanase which is commonly used in the industry is naturally produced by *A. brasiliensis*. Therefore, the objectives of the study are to improve the production of xylanase by *A. brasiliensis* using UV irradiation and chemicals mutagenesis of EMS and MNNG and subsequently, to determine the most effective mutagenesis method in the strain improvement of *A. brasiliensis* for the overproduction of xylanase.

## 2. MATERIALS AND METHODS

### 2.1 Microorganism Strain and Inoculum Preparation

*A. brasiliensis* ATCC 16404 was used in this study for the overproduction of xylanase via UV irradiation and chemicals mutagenesis. *A. brasiliensis* was then subcultured on the potato dextrose agar (PDA) before incubated in an incubator at 30ºC for 72 h. Subsequently, the brown spongy coating of *A. brasiliensis* was observed on the PDA.

### 2.2 Ultraviolet (UV) Irradiation on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

The 72 h growth of *A. brasiliensis* on PDA was treated with the UV irradiation of the wavelength 2537 Angstroms or 254 nm in a horizontal laminar flow cabinet. In order to perform the UV mutagenesis, the agar plates of *A. brasiliensis* were placed from a distance of 10 cm away from the UV at the time intervals of 10, 20 and 30 min, respectively. The control experiment was conducted with non-treated *A. brasiliensis* without the exposure of UV. The mutant and control strains were then incubated for 48 h in an incubator at 30ºC. After incubation, spores were harvested using a spread rod. Spore count was then carried out using a hemacytometer. The inoculums size was standardized to $1\times10^6$ spores before transferring to a 250 mL Erlenmeyer flask containing 150 mL of potato dextrose broth (PDB) which was adjusted to its initial pH 6.5. Subsequently, the culture medium was incubated in the orbital shaker at 30ºC agitated at 150 rpm for 144 h of submerged fermentation in shake flask culture. Sampling analysis including xylanase activity assay was carried out every 24 h interval. All the analysis was also conducted on the non-treated control used as comparison. The UV mutagenesis was performed in triplicate and the mean value of the analysis was quantified to investigate the overproduction of xylanase after mutagenesis.

### 2.3 Chemical Mutagenesis of Ethyl Methane Sulfonate (EMS) on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

The 72 h growth of *A. brasiliensis* on PDA was harvested and resuspended into 20 mL of sterile distilled water. 1 mL of sodium phosphate buffer (pH 7) was then mixed with 9 mL of spore suspension to use as the non-treated control of EMS mutagenesis. On the other hand, 1 mL of 150 µg/mL of EMS in sodium phosphate buffer (pH 7) was added into another 9 mL of spore suspension to use as the sample for EMS mutagenesis. Subsequently, both the non-treated control and mutagenesis samples were incubated at 30ºC for various time intervals of EMS treatment. 2 mL of samples were withdrawn from both control and mutagenesis samples at 30, 60 and 90 min intervals, respectively. After incubation, 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. Then, the samples were centrifuged again at 4500 rpm for 2 min at 4ºC followed by resuspension of cell pellet in 10 mL of sodium phosphate buffer (pH 7). Thereafter, serial dilution was carried out and spores were counted using hemacytometer to obtain $1\times10^6$ spores to use as the standard inoculum size for the subculture on a new PDA. The subculturing was carried out by using spread plate technique. The PDA with mutagenesis samples of *A. brasiliensis* were incubated at 30ºC for 48 h. Likewise, the control of the experiment that was conducted without the EMS mutagenesis was also incubated at 30ºC for 48 h. After incubation, inoculation of $1\times10^6$ spores from both mutagenesis and non-treated control samples were transferred into the working volume of 150 mL of PDB which its initial medium pH was adjusted to 6.5. Subsequently, the inoculums were incubated in an orbital rotary shaker at 30ºC agitated at 150 rpm for 144 h of submerged fermentation in shake flask culture. Xylanase activity assay was carried out every 24 h interval. All the analysis was also conducted on the non-treated control as the comparison. The EMS mutagenesis was repeated again using the concentration of 200 µg/mL. The EMS mutagenesis was performed in triplicate to obtain the mean value of the analysis that was used to investigate the xylanase overproduction by *A. brasiliensis* after the chemical mutagenesis of EMS.

### 2.4 Chemical Mutagenesis of N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG) on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

The 72 h growth of *A. brasiliensis* on PDA was harvested and resuspended in 20 mL of sterile

distilled water. 1 mL of sodium phosphate buffer (pH 7) was then mixed with 9 mL of spore suspension to use as the non-treated control. On the other hand, 1 mL of 150 µg/mL of MNNG in sodium phosphate buffer (pH 7) was added into another 9 mL of spore suspension to use as the sample for MNNG mutagenesis. Then, both the non-treated control and mutagenesis samples were incubated at 30ºC for various time intervals of MNNG treatment. 2 mL of samples were withdrawn from both control and mutagenesis samples at 30, 60 and 90 min intervals, respectively. After incubation, 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. Subsequently, the samples were centrifuged again at 4500 rpm for 2 min at 4ºC followed by resuspension of cell pellet in 10 mL of sodium phosphate buffer (pH 7.0). Thereafter, serial dilution was performed and spores were counted using hemacytometer to acquire the standard inoculums size of $1×10^6$ of spores before subcultured on a new PDA. The subculturing was attained by using spread plate technique. The PDA with mutagenesis samples of *A. brasiliensis* were incubated at 30ºC for 48 h with the non-treated control of the experiment conducted without the MNNG treatment. After incubation, inoculation of $1×10^6$ spores from both mutagenesis samples and non-treated control into PDB which was adjusted to pH 6.5 were carried out before incubated in an orbital rotary shaker at 30ºC agitated at 150 rpm for 144 h of submerged fermentation in shake flask culture, respectively. Xylanase activity analysis of *A. brasiliensis* was carried out every 24 h interval. All the analysis was also conducted on the non-treated control as the comparison. The MNNG mutagenesis was repeated again using the concentration of 200 µg/mL. The MNNG mutagenesis was conducted in triplicate to obtain the mean of the analysis to investigate the xylanase overproduction by *A. brasiliensis* after the mutagenesis of MNNG.

## 2.5 Sampling and Analysis

Both the mutagenesis and non-treated control samples were withdrawn and collected every 24 h. The samples were then centrifuged at 4500 rpm for 30 min at 4ºC. The supernatants were analyzed for the xylanase activity assay.

## 2.6 Xylanase Activity Assay

Xylanase activity assay was measured according to Bailey et al. [10] using 3,5-dinitrosalicyclic (DNS) to detect the concentration of xylose. 1% of beechwood xylan that was used as the sole substrate was added into 0.05 M of phosphate buffer saline (pH 5.3) at 50ºC until it was completely dissolved. 0.1 mL of the supernatant was added into 0.9 mL of xylan substrate. It was then incubated at 50ºC for 30 min. 1.5 mL of DNS was added into the mixture and incubated at 90ºC for 5 min. Subsequently, 0.5 mL of 40% Rochelle salt was added and cooled down before the absorbance at 575 nm was measured using a spectrophotometer. The activity of xylanase was measured according to the xylose standard curve. One unit of xylanase activity is defined as the amount of enzyme required to release one µmole of xylose as the reducing sugar per min under assay condition.

## 2.7 Protein Assay

The quantification of the total amount of soluble protein in the supernatant was measured according to Lowry method using bovine serum albumin (BSA) as the standard [11]. The BSA standard curve was plotted according to the absorbance reading at 750 nm.

## 2.8 Quantification of Biomass Concentration

Spores count of *A. brasiliensis* was carried out using hemacytometer.

## 2.9 Measurement of Medium pH

The pH of samples was measured using a pH meter to study the changes of medium pH profile during the growth of *A. brasiliensis* and its production of xylanase. pH meter was calibrated using buffer pH 4, 7 and 10.

## 2.10 Data Analysis

In this study, all of the random mutagenesis experiments involving UV irradiation, mutagenesis of EMS and MNNG 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 *A. brasiliensis* after UV irradiation, mutagenesis of EMS and MNNG, respectively. Each value in the Figures represents the mean for independent mutagenesis experiments performed in triplicate. The standard error was also calculated and represented on all the Figures in this study.

## 3. RESULTS AND DISCUSSION

### 3.1 Effect of Ultraviolet (UV) Irradiation on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

Filamentous fungi are the most preferred source of commercial enzymes production because the activity of enzymes produced is more efficient as compared to those extracted from yeast and bacteria [4]. In this study, wild type *A. brasiliensis* was maintained on PDA under optimum growth condition of 30ºC in an incubator as the non-treated control while the mutants of fungi generated after being exposed under UV irradiation with a wavelength of 2537 Angstroms or 254 nm from a distance of 10 cm for 10, 20 and 30 min of exposure time, respectively. Both the wild type and the mutants of fungi were then subcultured and incubated for 48 h before they were inoculated into 150 mL of PDB for various analysis including xylanase activity, soluble protein concentration, medium pH and spore count. Based on our result findings, *A. brasiliensis* mutant which exposed to UV for 10 min possessed relatively higher xylanase activity of 3.49±0.034 U/mL with the total protein concentration of 0.175±0.0034 g/mL by total number of $138.67 \pm 1.53 \times 10^6$ spores/mL at medium pH 4.63±0.03 at 48 h of submerged fermentation. There was about 12.69% increment of xylanase production as compared to $144.67 \pm 0.58 \times 10^6$ spores/mL of wild type that produced only 3.097±0.089 U/mL of xylanase activity with total protein production of 0.205±0.0037 g/mL at pH 4.75±0.05 at 48 h of fermentation in shake flask culture. Surprisingly, when *A. brasiliensis* exposed to UV for 20 min, it produced the maximum xylanase activity of 4.86±0.095 U/mL with the total protein concentration of 0.214±0.007 g/mL by $177.67 \pm 2.08 \times 10^6$ spores/mL at pH 4.47±0.02 at 48 h of fermentation with the drastical increment of 56.93% on xylanase production as compared to its wild type parent strain as shown in Fig. 1. Fig. 1 illustrates the highest xylanase overproduction by the mutants of *A. brasiliensis* that were obtained after being exposed to UV for 20 min. Rana et al. [12] also conducted a similar experiment for the production of amyloglucosidase (AMG) by exposing *A. brasiliensis* to UV irradiation for 5 to 30 min. The experiment aims to determine the enhancement on the production of AMG by observing the hydrolysis of starch into glucose, releasing on the petri dish. Based on their results, the mutant strain of *A. brasiliensis* GCBU-25 isolated after 25 min of UV exposure possessed the maximum synthesis of AMG up to 136.1 U/mL/min, which was around two-fold increment in AMG production as compared to its parental strains. Nevertheless, the mutant of the fungal strains experienced complete death after UV exposure of 40 min. The enhancement of AMG production might due to the increase of gene copy number and improvement in gene expression. On the other hand, Irfan et al. [4] treated *A. brasiliensis* under UV mutagenesis for carboxymethylcellulase (CMCase) production in submerged fermentation. The main effect of UV irradiation is to alter the structure of pyrimidine which results in the formation of thymine dimer whereby these dimmers would affect the structure of the DNA double helix chain. Eventually, it stops any further replication process. As a result, in most cases of UV mutation, it may lead to improve adaptation of a microorganism to its environment with enhanced biocatalytic performance. Thus, the results of the experiment indicated that UV mutagenesis increased CMCase activity up to two-fold as compared to its parental strains. Therefore, UV irradiation was found to be the best method for improvement of fungal strains like *A. brasiliensis* for higher enzymes yield. According to Lipika et al. [13], they showed that UV induced random mutagenesis yielded 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 the non-treated control of *B. subtilis.* Notably, xylanase activity of both mutants and wild type in this study were at its maximum peak at 48 h of submerged fermentation which was the same as reported in the study conducted by Tasneem et al. [9]. The xylanase activity increased with the time course of study due to the rapid hydrolysis of xylan in the medium. However, the further increase of the fermentation time after 48 h had resulted in the decrease in xylanase activity. Based on our result findings, we suggested that long fermentation time decreases xylanase production. This is due to the susceptible portion of xylan molecules that was rapidly degraded, as a result, only the crystalline portion which was not utilised by the microorganism was left to produce xylanase. The rapid degradation of susceptible xylan molecules gradually led to the minimal substrate amount available for the activity of xylanase as suggested by Tasneem et al. [9]. Moreover, by referring to Fig. 1, the

maximum spore count of UV induced mutants occurred at 48 h of fermentation. Therefore, we also suggested that the xylanase activity increased with the number of spores present in the mutagenesis samples.

On the other hand in Fig. 2, the results showed that the medium pH of mutated and non-treated wild type control samples decreased as the fermentation time increased. This result is similar to the study of O'Donelle et al. [14] as *A. brasiliensis* was known for its acidification of the culture medium because the degradation of xylan would lead to the production of various acids including citric acid which resulted in the decrease of pH. Moreover, by comparing to Fig. 1, the xylanase activity was at the highest at 48 h of fermentation in which the pH of samples fell between the range of 4.47±0.023 to 4.75±0.047. Likewise, this result is also similar to the study of Uhlig, [15] whereby the optimal medium pH of commercial xylanase from *A. brasiliensis* strain was found occurred at the range of 4.0 to 5.0. On the other hand, the protein production of *A. brasiliensis* was detected using Lowry method as shown in Fig. 3. The highest protein production was found to occur at 48 h of fermentation with its concentration of 0.243±0.0029 g/mL at medium pH 4.51±0.02 by 143.33±5.51×$10^6$ spores/mL of *Aspergillus* mutant that had been exposed to UV for 30 min. There was about 18.54% increment of protein overproduction as compared to 144.67±0.58×$10^6$ spores/mL of non-treated wild type that produced 0.205±0.0022 g/mL of soluble protein at medium pH 4.75±0.05 at 48 h of fermentation. Likewise, our result is similar to the study of Brakhage and Brulle [16], in which UV exposed *Aspergillus* was capable of producing greater amounts of protein as maximum as 20% when compared to wild type strain. In addition, Fig. 3 also showed that both the maximum protein production and spore count of mutants and non-treated wild type control were attained at 48 h of submerged ferrmentation. Therefore, we concluded that protein concentration increased with the number of spores present in the samples.

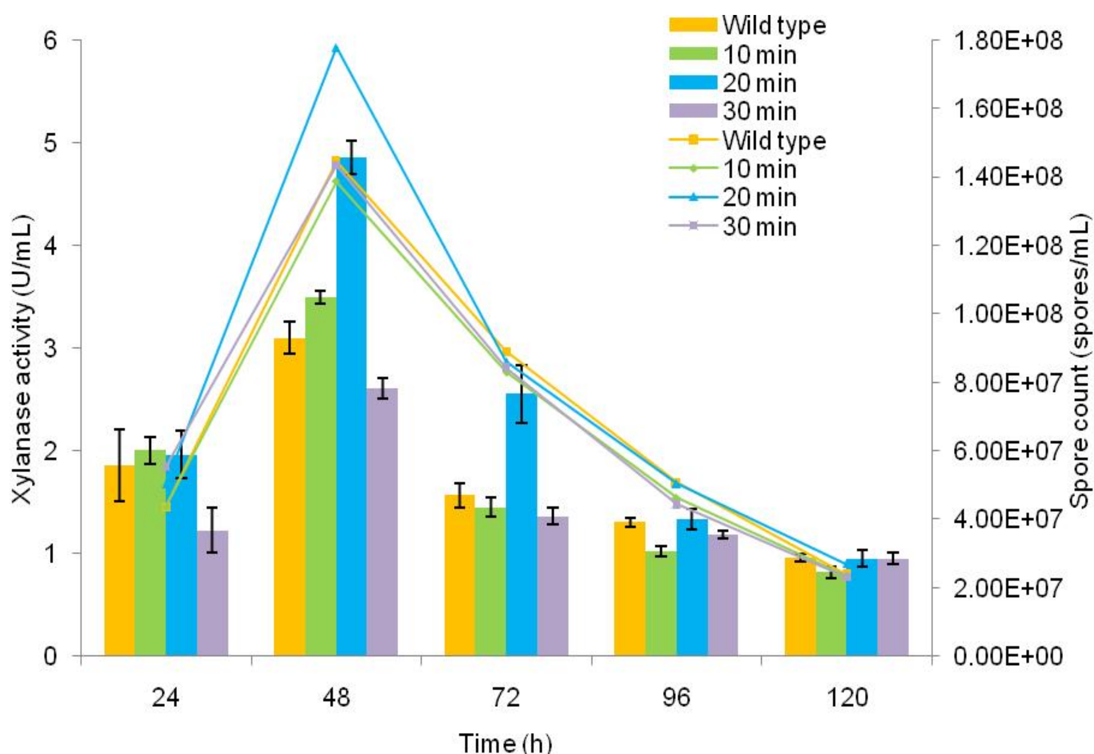


**Fig. 1. Xylanase overproduction from wild type and mutants of *A. brasiliensis* after being exposed to UV from a distance of 10 cm at time intervals of 10, 20 and 30 min, respectively. xylanase activity is presented as column chart while spore count is presented as line graph**

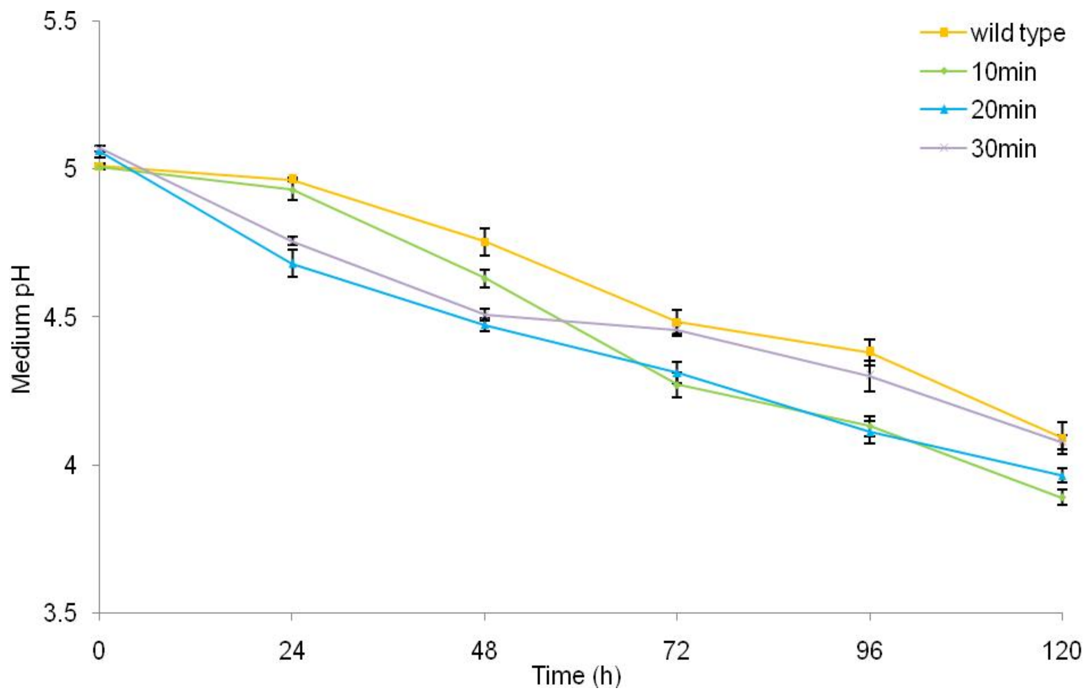


**Fig. 2. Medium pH profile of wild type and mutants of *A. brasiliensis* after being exposed to UV from a distance of 10 cm at time intervals of 10, 20 and 30 min, respectively**

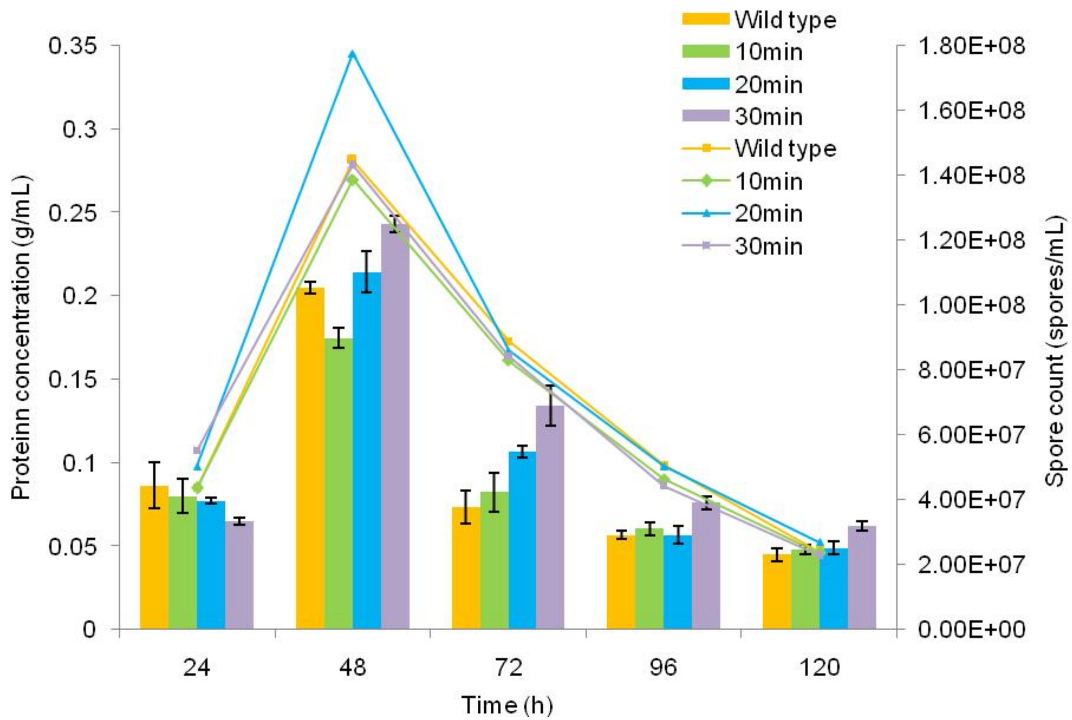


**Fig. 3. Protein production from wild type and mutants of *A. brasiliensis* after being exposed to UV from a distance of 10 cm at time intervals of 10, 20 and 30 min, respectively. protein concentration is presented as column chart while spore count is presented as line graph**

### 3.2 Effect of Ethyl Methane Sulfonate (EMS) Mutagenesis on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

Fig. 4 demonstrated the greatest xylanase overproduction by mutants of *A. brasiliensis* which was induced with 150 µg/mL of EMS for 90 min. Mutants of *A. brasiliensis* which induced with 150 µg/mL EMS for 90 min produced the maximum xylanase activity of 4.55±0.085 U/mL with the total soluble protein of 0.175±0.0045 g/mL by $180 \pm 5.29 \times 10^6$ spores/mL at pH 4.33±0.06 at 48 h of fermentation. There was about 1.34% increment of xylanase production as compared to the $149.33 \pm 2.08 \times 10^6$ spores/mL of wild type that produced 4.49±0.118 U/mL of xylanase activity at medium pH 4.44±0.04 with the total soluble protein of 0.158±0.0043 g/mL at 48 h of fermentation. It has been reported that EMS is capable of improving xylanase activity in the study of Hanim, et al. [17] to generate its maximum production of 15.057 U/g. Likewise, study of Jiang et al. [18] stated that EMS treatment together with UV iradiation was capable of enhancing xylanase activity of *Trichorderma viride* up to 42.37 U/mL. According to Jiang et al. [18], the gene responsible for the production of xylanase was overexpressed due to mutation, as a result, it increased its expression and activity. In addition, Chusnul et al. [19] also reported that the xylanolytic mutants possessed the highest xylanase activity of 15.057 U/mL after being induced with 50 µg/mL EMS, which was literally 61.61% higher as compared to the activity from their non-treated counterparts of 9.317 U/mL. 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 [20]. Among all various mutagens that have been used to induce mutations, EMS is particularly 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 [21,22]. 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.

In addition, since 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-pairing and changes in transitions, as a result, it induces all kinds of mutations including transition, transversion and frame shift [23]. EMS was successfully demonstrated improvement of xylanase production in the study by Chusnul et al. [19]. 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. [24], 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 its mutants. On the other hand, Nadeem et al. [25] 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. Likewise, Pradeep and Narasimha [26] performed an experiment with the treatment of EMS on the strain of *A. brasiliensis* for overproduction of cellulase. In their study, cellulase produced by the mutants was compared with the parental strain. Indeed, a lignocellulosic agricultural waste of pea seed husk was used as the substrate for the overproduction of cellulase. Based on their results, they illustrated an increase in cellulase overproduction of 18.03 U by EMS treated *A. brasiliensis* GNEM7.

On the other hand, Fig. 5 shows the medium pH of mutants and non-treated control samples decreased as the fermentation proceeded. This was due to the nature of *A. brasiliensis* in producing the citirc acid that resullted the acidifying culture condition after prolonged fermentation [14]. The study of Uhlig, [15] also suggested that the optimal medium pH of commercial xylanase by an *A. brasiliensis* strain was found within the range of 4.0 to 5.0. Interestingly, this study is relevant to our results, whereby the pH of EMS mutated samples at 48 h of submerged fermentation that occurred in the range of 4.0 to 5.0 possessed the highest xylanase activity obtained from the mutant samples. Fig. 6 shows the maximum amount of spore count and protein production from the mutants and non-treated control samples occurred at 48 h of fermentation. Such outcomes are relevant to the suggestion that the protein concentration increased with the number of spore present in the samples. The highest protein overproduction was found to be 0.189±0.0051 g/mL at medium pH 4.23±0.07 by

144.33±3.79×$10^6$ spores/mL of *A. brasiliensis* mutant that had been induced with 200 µg/mL of EMS for 90 min. Protein overproduction increased about 19.62% as compared to 149.33±2.08×$10^6$ spores/mL of wild type that produced 0.158±0.0043 g/mL of protein concentration at the medium pH 4.44±0.04. According to the study of Shivanna et al. [27], where EMS mutated *A. niger* was able to produce higher amounts of protein with improved specific activity of 1.26 U/mg as compared to its parental strain due to the alteration of gene, resulting in improving its ability of protein yield in mutant strain.

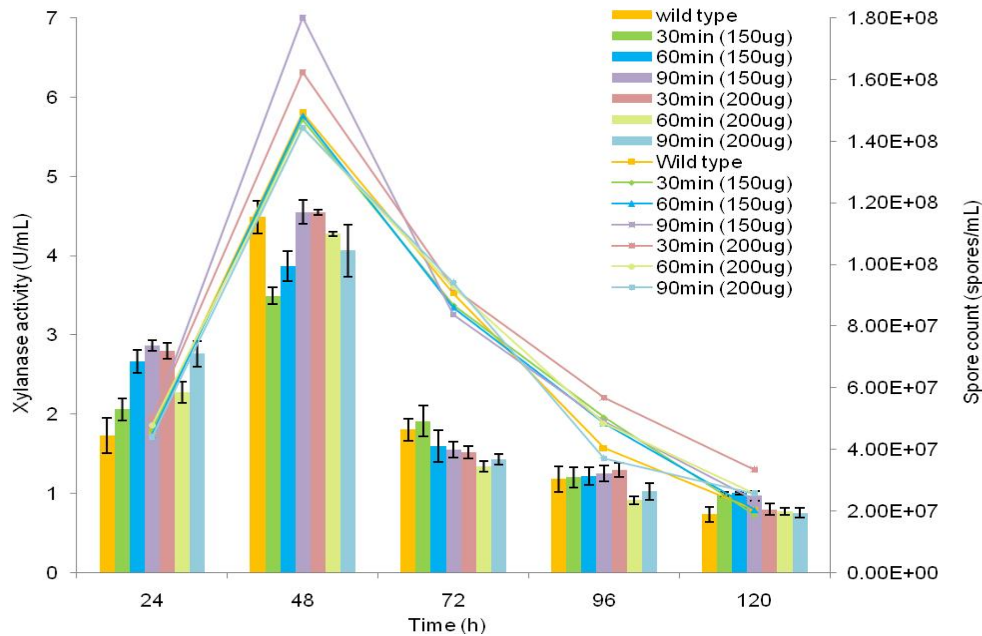


**Fig. 4. Xylanase overproduction from wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of EMS at time intervals of 30, 60 and 90 min, respectively. xylanase activity is presented as column chart while spore count is presented as line graph**

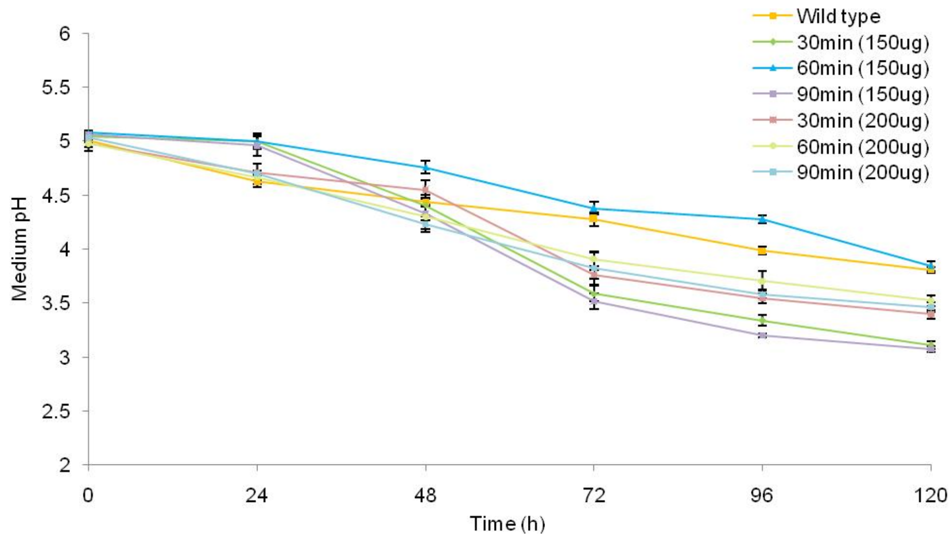


**Fig. 5. Medium pH profile of wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of EMS at time intervals of 30, 60 and 90 min, respectively**

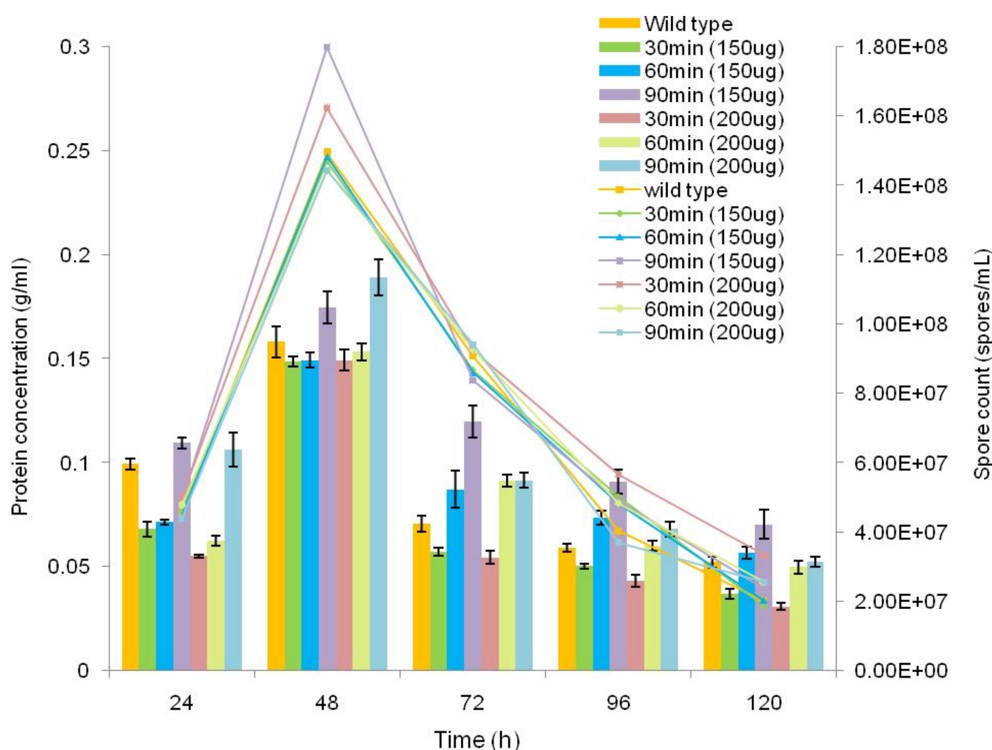


**Fig. 6. Protein production from wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of EMS at time intervals of 30, 60 and 90 min, respectively. protein concentration is presented as column chart while spore count is presented as line graph**

### 3.3 Effect of N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG) Mutagenesis on Overproduction of Xylanase by *A. brasiliensis* under Submerged Fermentation in Shake Flask Culture

It has been reported that MNNG causes the alkylation of guanine residues that produced permanent lesions within the DNA molecule which lead to cluster of closely linked mutations. Such variety may be the results of genetic redundancy or differential post translational processing. As a result, it improves the expression of genes coding for xylanase synthesis [9]. Therefore, this research outcome explained the improvement of xylanase activity in our study, as shown in Fig. 7, it demonstrates the maximum xylanase overproduction by *A. brasiliensis* which was induced with 150 µg/mL of MNNG for 30 min in this study. *A. brasiliensis* mutant which induced with 150 µg/mL of MNNG for 30 min possessed the maximum xylanase activity of 3.28±0.048 U/mL with the total soluble protein of 0.119±0.0026 g/mL by 161.67±2.89×$10^6$ spores/mL at pH 4.77±0.04 at 48 h of submerged fermentation in shake flask culture. It was approximately 17.14% improvement of xylanase production as compared to the 150±3.61×$10^6$ spores/mL of wild type that produced only 2.8±0.04 U/mL of xylanase activity at medium pH 4.75±0.04 with the total protein of 0.114±0.0007 g/mL at 48 h of submerged fermentation in shake flask culture. Similarly, in the study of Haq et al. [8], they stated that the MNNG treatment on *A. brasiliensis* was able to enhance xylanase activity to about 2.7-fold higher than its wild type parent strain. In addition, Vu et al. [28] performed a combined mutation technique on overproduction of cellulase by *Aspergillus* sp. The strain was treated with two repeated rounds of γ-irradiation of $Co^{60}$ and UV irradiation with four repeated treatment of MNNG. The cellulase production of the fungal strain was subsequently determined. It was found that *Aspergillus* sp XTG-4 possessed as the best mutant strain with improved stable cellulase production of 37.84 U/mL up to 3.20-fold of increment as compared to the wild type strain. The stability of *Aspergillus* sp XTG-4 for cellulase synthesis and production was successfully maintained by successive

subculturing on PDA for seven months. After each session of subculturing, the mutant was tested for its ability to produce cellulase through solid state fermentation. In fact, their results proved that the mutant managed to retain the similar yield of cellulase in a stable manner with approximately 37.84 U/mL after being subcultured for 19 times. Hence, this result findings prove that MNNG induced mutation is capable of maintaining stable inheritability of xylanase production to the next generations over time. Therefore, we suggested that the MNNG induced mutants in our study might able to overexpress and reproduce xylanase continuously over generations that were relatively essential in industrial applications. On the other hand, by referring to Fig. 7, it also demonstrates the positive correlation between the maximum xylanase activity and spore count occurred at 48 h of fermentation where the number of spores present in the fermentation was directly proportional to the xylanase activity produced by mutants and non-treated control of *A. brasiliensis.*

Fig. 8 displays the decreasing of medium pH of all mutants and non-treated control samples due to the acidifying nature of *A. brasiliensis*. Hence, we anticipated that MNNG induced mutants were able to overproduce xylanase during acidic medium pH condition at 48 h of fermentation. On the other hand, the protein production of MNNG mutated *A. brasiliensis* was tested using Lowry method as shown in Fig. 9. The greatest protein concentration was detected at 48 h of fermentation in shake flask culture. Such observation was significant to the suggestion that the protein production increased with the number of spores found in the samples. The highest protein overproduction was found to be 0.119±0.0026 g/mL by 161.67±2.89×$10^6$ spores/mL of *A. brasiliensis* mutant at pH 4.77±0.04 that had been induced with 150 µg/mL of MNNG for 30 min. It was about 4.39% increment of protein overproduction as compared to 150±3.61×$10^6$ spores/mL of wild type that produced 0.114±0.0007 g/mL of protein concentration at medium pH 4.75±0.04. Notably, this results is similar to the study of Gromada and Fiedurek [29], in which their study shows that the MNNG induced *A. brasiliensis* mutants produced greater amounts of protein overproduction as compared to wild type parent strain whereby the maximum protein production of 3.78 mg/mL was observed. This study proved that MNNG was capable of increasing protein production by *A. brasiliensis* to greater extend depending on the parameters involved in the mutation.

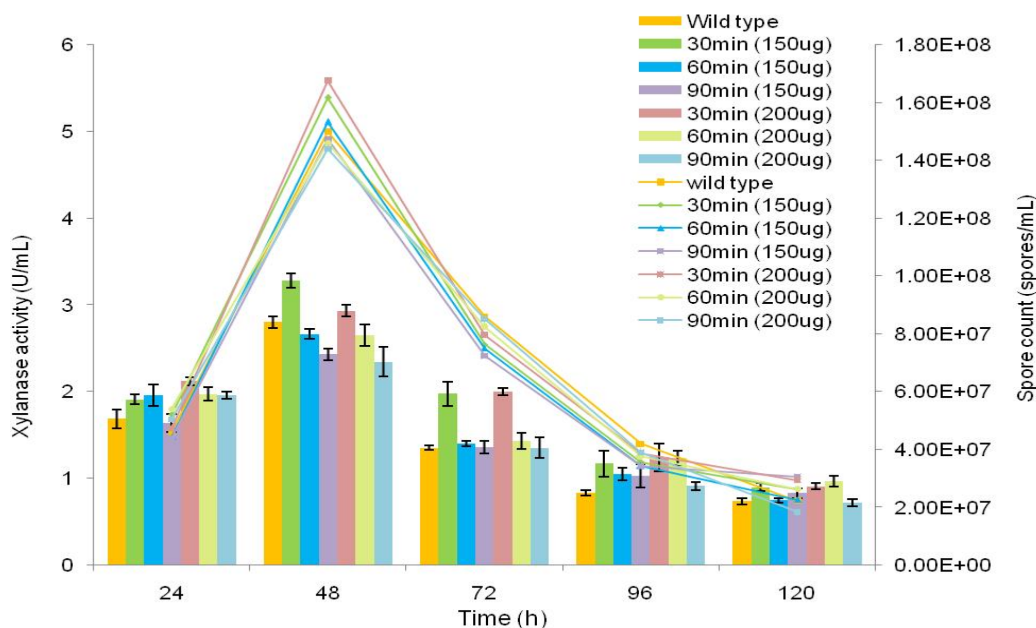


**Fig. 7. Xylanase overproduction from wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of MNNG at time intervals of 30, 60 and 90 min, respectively. xylanase activity is presented as column chart while spore count is presented as line graph**

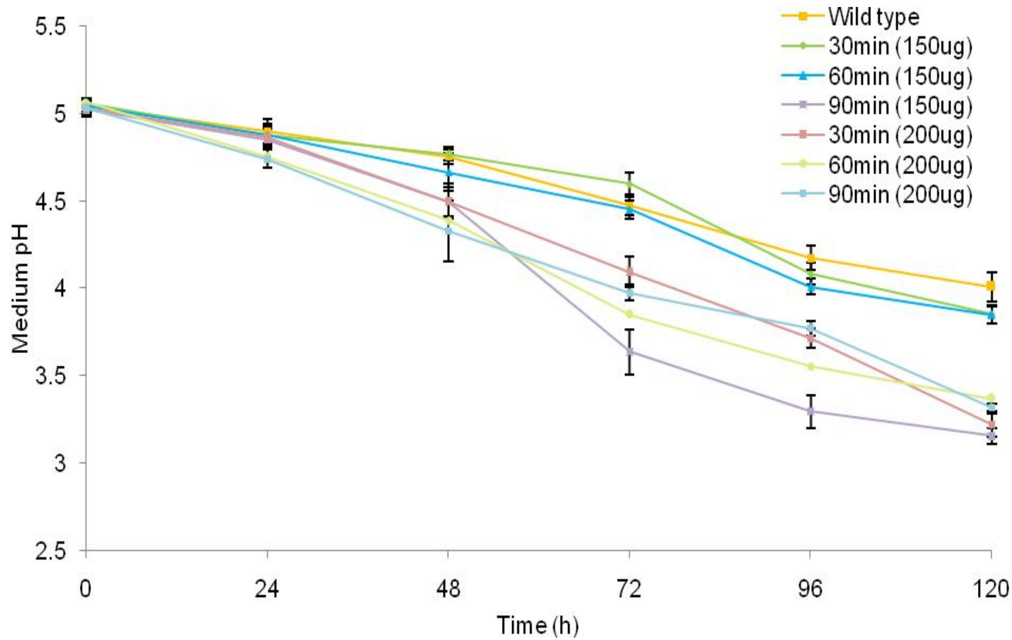


**Fig. 8. Medium pH profile of wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of MNNG at time intervals of 30, 60 and 90 min, respectively**

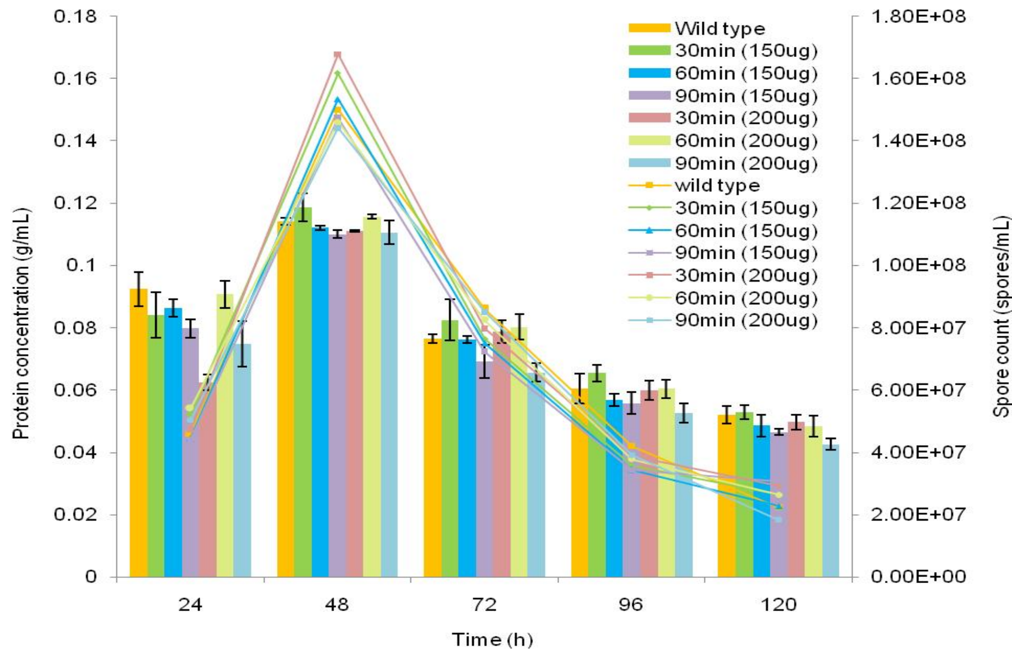


**Fig. 9. Protein production from wild type and mutants of *A. brasiliensis* after being induced with 150 and 200 µg/mL of MNNG at time intervals of 30, 60 and 90 min, respectively. protein concentration is presented as column chart while spore count is presented as line graph**

## 4. CONCLUSION

The use of xylanase and its applications are important in biotechnology industry as various researches have been conducted to improve xylanase production using different strains of microorganisms in submerged fermentation. Mutagenesis has been suggested to be one of

the most successful method and this study proved that both UV irradiation and chemicals mutagenesis of EMS and MNNG were capable of enhancing xylanase activity. Such improved strains could reduce the costs of the production and such attributes are thus, tremendously beneficial to the industrial applications. The effectivenes of UV, EMS and MNNG treatment for the strain improvement of xylanase by *A. brasiliensis* were demonstrated in this study under the optimum growth conditions of 30ºC at 150 rpm in 250 mL of Erlenmeyer flask containing 150 mL of PDB with its adjusted initial medium at pH 6.5. Most various mutants showed improved xylanase activity regardless of the types of mutation where different types of mutagenesis influence the xylanase production differently. It might due to the increase in gene number of the DNA of *A. brasiliensis* which eventually led to the overexpression and hyperproduction of xylanase. Based on our result findings, *A. brasiliensis* that had been exposed for 20 min under UV irradiation, induced with 150 µg/mL of EMS for 90 min and 150 µg/mL of MNNG for 30 min possessed different status of overexpression of this enzyme, producing 4.86±0.095, 4.55±0.085 and 3.28±0.048 U/mL, respectively. Even though both UV and chemicals mutagenesis were very effective in improving activity of xylanase but the UV irradiation provided better xylanase overproduction of 56.93% while MNNG-induced mutants possessed only 17.14% increment while EMS mutagenesis attained only 1.34%, respectively. Notable, these results indicate that UV mutagenesis was able to improve *A. brasiliensis* to produce greater amount of xylanase as compared to chemicals mutagenesis of EMS and MNNG, respectively. UV irradiation is capable of modifying the structure of pyrimidine, lead to the formation of thymine dimer which is able to distort the structure of DNA helix. In common cases, UV mutation is not beneficial but sometimes, it may lead to improved adaptation of the fungi to its environment with better biocatalytic performance. Surprisingly, the optimum time for the maximum xylanase production in this study occurred at 48 h of submerged fermentation. The outcome of this study provides in-depth information relating to the applications of different mutagenesis methods to generate mutants of *A. brasiliensis* that were competent of producing high amounts of xylanase. More mutagenesis researches should therefore, be conducted in order to meet the increasing demands of xylanase in the global market. Nonetheless, comprehensive investigation on the mutagenesis for overproduction of xylanase using site-directed mutagenesis, screening of potential xylanase producing strain and optimisation of substrate composition would absolutely advantageous as these researches could reduce the costs of production as well as maximizing the yield of xylanase which are crucial in term of industrial production.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Collins T, Gerday C, Feller G. Xylanases, Xylanase families and extremophilic xylanases. Fems Microbiology Reviews. 2005;3-23.
2. Khan A, Haq IU, Butt WA, Ali S. Isolation and screening of *Aspergillus niger* isolates for xylanase biosynthesis. Biotechnology. 2003;2:185-190.
3. Dai XJ, Liu MQ, Jin HX, Jing MY. Optimization of solid state fermentation *Aspergillus niger* JL-15 for xylanase production and xylooligosaccharides preparation. Czech J. Food Science. 2011; 557-567.
4. Irfan M, Javed J, Syed Q. UV mutagenesis of *Aspergillus niger* for enzyme production in submerged fermentation. Journal of Pak. J. Biochem. Mol. Biol. 2011;44:137-140.
5. Subramaniyan S, Prema P. Biotechnology of microbial xylanases: Enzymology, molecular biology and application. Critical Reviews in Biotechnology. 2002;22(1):33-46.
6. Dhiman SS, Sharma J, Battan B. Industrial applications and future prospects of microbial xylanases: A review. Bioresources. 2008;1377-1402.
7. Butt WA, Haq IU, Iqbat J. Biosynthesis of xylanase by UV treated mutant strain of *Aspergiluus niger* GCBMX-45. Journal of Biotechnology. 2002;10-14.
8. Haq IU, Hussain R, Hameed U, Javed M. Selection of *Aspergillus niger* mutant using anti-metabolite 2-deoxy D-glucose after N-Methyl N-Nitro N-Nitroso Guanidine (MNNG) treatment. Journal of Pak. J. Bot. 2008;2613-2623.
9. Tasneem M, Khan A, Ashraf H, Haq IU. Xylanase biosynthesis by chemically mutated strain of *Aspergillus niger*. Journal

of Food Technol. 2003;178-181.
10. Bailey MJ, Biely P, Poutanen K. Laboratory testing of methods for assay of xylanase activity. J Biotechnol. 1992;23:257-270.
11. Lowry OH, Rosebrough NJ, Farr AC, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951;193:265-275.
12. Rana K, Haq IU, Ashraf H. Production of amylosidase by UV irradiated strain of *Aspergillus niger* using solid state fermentation. Journal of Food Technol. 2005;68-71.
13. Lipika M, Madhavi V, Mugdha H. Effect of UV and nitrous acid treatment on production of xylanase enzyme by *Acinetobacter* sp. International Journal of Current Microbiology and Applied Sciences. 2014;3(1):45-53.
14. O'Donelle D, Wang LP, Xu JF, Ridgway D, Gu TY, Young MM. Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. Biochemical Engineering Journal. 2001;8(3):187-193.
15. Uhlig H. Industrial enzymes and their applications, New York: John Wiley & Sons, Inc; 1998.
16. Brakhage AA, Brulle JV. Use of reporter gene to identify recessive *trans*-acting mutations specifically involved in the regulations of *Aspergillus nidulans* penicilin biosynthesis genes. Journal of Bacteriology. 1995;177(10):2781-2788.
17. Hanim C, Yusiati LM, Cahyanto MN, Wibowo A. Mutagenic improvement of xylanase production from xylanolytic bacteria and its phylogenetic analysis. Microbiology Indonesia. 2013;56-57.
18. Jiang X, Geng A, He N, Li Q. New isolate of *Trichorderma viride* strain for enhanced cellulolytic enzyme complex production. Journal of Bioscience and Bioengineering. 2011;111(2):121-127.
19. Chusnul H, Lies MY, Nur MC, Ali W. Mutagenic improvement of xylanase production from xylanolytic bacteria and its phylogenetic analysis. Microbiology Indian. 2013;7:51-58.
20. Muller HJ. Types of cisible variation induced by X-rays in *Drosophilia.* Journal of Genetics. 1930;22:299-334.
21. Ezio R. Mutagenesis in *Arabidopsis.* Circadian Rhythns: Methods and Protocols; 2007.
22. Haughn G, Somerville CR. Selection for herbicide resistance at the whole-plant level. Application of Biotechnology to Agricultural Chemistry. 1987;98-107.
23. Suribabu K, Lalitha TG, Hemalatha KPJ. Strain improvement of *Brevibacillus borostelensis* R1 for optimization of amylase production by mutagens. Journal of Microbial Biochemistry Technology. 2014;6:123-127.
24. Haq I, Ali S, Saleem A, Javed MM. Mutagenesis of *Bacillus licheniformis* through ethyl methanesulfonate for alpha amylase production. Pakistan Journal of Botany. 2009;4(3):1489-1498.
25. Nadeem M, Syed Q, Liaquat A, Baig S, Kashmiri A. Study on biosynthesis of alkaline protease by mutagenized culture of *Bacillus pumilus.* Pakistan Journal of Food Science. 2010;20(1-4):24-30.
26. Pradeep MR, Narasimha G. Utilization of pea seed husk as a substrate for cellulase production by mutant *Aspergillus niger*. Insight Biotechnology. 2011;1(2):17-22.
27. Shivanna GB, Govindarajulu V. Screening of asporogenic mutants of phytase producing *Aspergillus niger* CFR 335 strain. Microbial Ecology in Health and Disease. 2009;(21):57-63.
28. Vu VH, Pham TA, Kim K. Fungal strain improvement for cellulase production using repeated and sequential mutagenesis. Journal of Mycobiology. 2009;267-271.
29. Gromada A, Fiedurek K. Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase production. Journal of Applied Microbiology. 1997;648-652.

*© 2015 Ho and Ho; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.*

*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://www.sciencedomain.org/review-history.php?iid=930&id=39&aid=9030*