British Microbiology Research Journal
12(5): 1-19, 2016, Article no.BMRJ.22953
ISSN: 2231-0886, NLM ID: 101608140

SCIENCEDOMAIN *international*
www.sciencedomain.org

Strain Development of *Aspergillus brasiliensis* Using Physical and Chemicals Mutagenesis for Possible Overproduction of Xylanase, Amylase, Protease and Cellulase under Submerged Fermentation (SmF)

Hooi Ling Ho[1*] and Muna Hassan Abduljubar[1]

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

Authors' contributions

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

Article Information

DOI: 10.9734/BMRJ/2016/22953
Editor(s):
(1) Giuseppe Blaiotta, Department of Food Science, Via Università, Italy.
Reviewers:
(1) Anonymous, Universiti Sains Malaysia, Malaysia.
(2) Hsion-Wen Kuo, Tunghai University, Taiwan.
(3) Otero Deborah Murowaniecki, Instituto Federal Farroupilha, Brazil.
Complete Peer review History: http://sciencedomain.org/review-history/13041

Original Research Article

Received 6th November 2015
Accepted 15th December 2015
Published 21st January 2016

ABSTRACT

Aims: Xylanase is commonly involved in the complete hydrolysis of xylan which consisted of hemicelluloses and celluloses as important components of plant cell wall. This enzyme is essential in wide range of applications in various industries along with other hydrolytic enzymes such as amylase, protease and cellulase. Microbial enzymes including xylanase possess several advantages over the use of conventional chemical catalysts due to greater catalytic activity, larger amount of production, higher degree of substrate specificity, higher economical viability and better biodegradable ability without destructive threat to the environment. Global enzymes market is expected to rise to USD millions by 2020. Due to the potential and bright future prospects of these microbial enzymes in biotechnological applications, random mutagenesis that is one of the potential approaches to produce enzymes with positive motivating characteristics is proposed and

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

implemented. Therefore, the present study was aimed to create mutants of *Aspergillus brasiliensis* using physical and chemical random mutagenesis and to elucidate possible overproduction of xylanase, amylase, protease and cellulase by mutants of *A. brasiliensis* under submerged fermentation (SmF).

Methodology: In this study, *A. brasiliensis* that has been classified as GRAS (generally regarded as safe) was subjected to physical mutagenesis of ultraviolet (UV) irradiation and chemical mutagenesis of nitrous acid (NA) and ethyl methane sulfonate (EMS) before screened for the possible overproduction of xylanase, amylase, protease and cellulase in SmF using wheat bran (agro-industrial residual) and yeast extract as the carbon and nitrogen source at the initial medium pH of 6.50 under the optimised growth conditions at 150 rpm at 30℃ for prolonged 168 h of fermentation.

Results: Based on the results, xylanase possessed as the highest maximum production of enzyme was notably observed at the optimum fermentation period of 72 h produced by EMS-mutated mutant of *A. brasiliensis.* Interestingly, 2 mg/mL of EMS was exerted as the optimum chemical mutagen followed by UV irradiation of 254 nm from a distance of 20 cm and lastly 0.17 mg/mL of NA. In fact, the maximum xylanase activity of 11.325±0.881 U/mL was attained after *A. brasiliensis* exposed to EMS for 45 minutes. On the other hand, the UV treated mutant of 30 minutes produced 10.052±0.987 U/mL of xylanase activity. Lastly, 9.127±0.410 U/mL of xylanase activity was obtained from NA treated mutant for 15 minutes compared to the wild type with 7.821±1.460 U/mL. The xylanase activity in mutants exposed to EMS, UV and NA was notably increased by 45%, 29% and 17% compared to the wild type. On the other hand, the enhancement in amylase activity was also observed in two mutants after UV exposure for 25 minutes and EMS treatment for 60 minutes with the production of 0.481±0.007 U/mL and 0.304±0.040 U/mL compared to wild type with 0.232±0.021 U/mL, respectively. Notably, there were increment of 107% and 31% in activity in mutants compared to wild type. Nevertheless, no possible overproduction of protease and cellulase was detected after the physical and chemical mutagenesis in this study.

Conclusion: In a nutshell, the mutants of *A. brasiliensis* with enhanced production of xylanase and amylase were anticipated to fulfill the industrial demand in more economical approach using agro-residual waste of wheat bran under SmF.

Keywords: Ultraviolet (UV) irradiation; nitrous acid (NA); ethyl methane sulfonate (EMS); xylanase; amylase; protease; cellulose; Aspergillus brasiliensis; submerged fermentation (SmF).

1. INTRODUCTION

According to Grand View Research, general global enzymes market is expected to reach USD 7,652 million by 2020. In textile industry, in particular, the global sales of enzymes attained USD 182.7 million in 2002. With the combined techniques of strain improvement and rational protein bioengineering, it would ultimately lead to expansion in global supply of enzymes. In fact, microorganism strains are improved by common yet worthy approaches involving mutagenesis. Mutagenesis might either occur spontaneously and randomly in nature through the exposure to UV irradiation and chemical mutagens or application of the latest method known as site-directed mutagenesis. Nonetheless, random mutagenesis is the easier and more productive approach to generate random mutation of a specific protein. The genetic materials of living microorganisms are altered due to random mutation. Mutation might produce mutant proteins with some interesting properties. It is one of the processes commonly used to possibly enhance and improve microorganisms in yielding larger production of commercial enzymes including xylanase, amylase, protease and cellulase. Most of wild type strains, which possess potential use as enzyme producers in industrial fermentation process are always subjected to strain improvement to produce more economical fermentation process [1]. In order to create mutants, UV irradiation is one of the important inducers used [2]. In fact, in most cases, UV mutation is harmful but sometimes it may lead to better adaptation of microorganisms especially fungi to their environments with improvements in biocatalytic performances, which would probably promoted mycelium growth and subsequently enhanced greater secretion of enzymes from the mycelium cells [2]. For example, possible overproduction of microbial cellulase enzyme could be obtained by inducing mutagenesis [3]. The effect of UV irradiation is

resulted from the possible changes in the promoter zones of the coding genes for enzymes. UV irradiation may deregulate the transcription of the mRNA corresponding to the enzymes leading to increment in production [4]. Javed et al. [5] found in their study that wild type of *Humicola insolens* produced 1.00 U/mL of carboxymethyl-cellulase (CMCase), 0.43 U/mL of filter paperase activity (FPase) and 0.30 U/mL of β-glucosidase. Interestingly, in their comparative study, enhancement of these enzymes production was achieved by UV mutants that possessed relatively higher cellulase activity of 1.21 U/mL of CMCase, 0.53 U/mL of FPase and 0.37 U/mL of β-glucosidase compared to their wild type strain. Prabakaran et al. [4] also investigated three most commercial and widely used enzymes including amylase, cellulase and lipase from selected fungal strains of *Aspergillus fumigattus, Penicillium chrysogenum and Verticillium terrestre.* After subjecting them to UV irradiation of 254 nm to induce mutation, they found that enhancement of these enzymes activity was observed in all mutant strains in different manner. Sambrook et al. [6] attributed such result to the production of thymine dimer after UV exposure. On the other hand, chemical mutagens may also induce random mutation within DNA sequences [7]. Notably, nitrous acid (NA) encourages deamination of cytosine to uracil and adenine to hypoxanthine [8]. As a result, it changes the structure of DNA [9]. Besides that, ethyl methanesulfonate (EMS) is also a strong mutagen used for improvement of enzymes production by inducing lesion in the DNA sequence. By adding ethyl group mainly in guanine bases, it leads to mispairing. As a result, alkylated guanine is paired with thymine instead of cytosine, resulting in guanine / cytosine to adenine / thymine transitions [10,11]. Furthermore, EMS may cause base-pair insertions, deletions, various constituents of the gene mutation including transcription and protein expression [12]. Subsequently, the mutation would possibly enhance protein production where it produces mutant proteins with motivating properties or novel proteins with commercial values. Therefore, random mutagenesis is the one of the common and easier processes recommended for strain improvement. It is still remained as the most valuable process for enhancement of enzymes productivity and profitability besides minimizing cost and time of production. Hence, this study is embarked on the following objectives to produce the mutants of *A. brasiliensis* using UV and chemicals random mutagenesis of nitrous acid (NA) and ethyl methane sulfonate (EMS) and to determine possible overproduction of xylanase, amylase, protease and cellulase from the mutants of *A. brasiliensis.*

2. MATERIALS AND METHODS

2.1 *Aspergillus brasiliensis* ATCC 16404

Aspergillus brasiliensis ATCC 16404 was used in this study. The strain was inoculated and streaked on potato dextrose agar (PDA) before incubated at 30℃ for 5 days until spores formation developed.

2.2 Preparation of Standard Inoculum

Sterile distilled water was aseptically used to harvest the spores of *A. brasiliensis* from PDA. The spore suspension was obtained by scrapping the agar surface with sterile hockey glass stick. Then, the spores were collected and transferred into a sterile falcon test tube. Thereafter, the spore suspension was serially diluted with sterile distilled water to obtain the standard inoculum by counting the spores using Hemocytometer [13]. One mL of 3×10^6 spores was used as the standard inoculum under SmF in this study.

2.3 Enzymes Production under Submerged Fermentation (SmF)

A. brasiliensis ATCC 16404 was grown in the medium with the formulation consisted of (g/L): pre-treated wheat bran, 10.0; yeast extract, 2.0; KH_2PO_4, 1.52; $MgSO_4.7H_2O$, 0.52 and KCl, 0.52. The pre-treated wheat bran was used as the primary carbon source under SmF in this study. The wheat bran was collected from Soon Soon Oilmills Sdn. Bhd., Malaysia. In order to treat the wheat bran, it was first being homogenized using a food grinder to obtain smaller and smoother structure before dried in an oven at 65℃ until constant weight was achieved. Then, the pre-treated wheat bran was mixed with other medium components except for yeast extract in 150 mL distilled water before adjusted its initial pH to 6.5. On the other hand, yeast extract as the nitrogen source was dissolved in 100 mL distilled water and likewise, its initial pH was adjusted to 6.5. Thereafter, both the carbon and nitrogen sources were autoclaved at 121℃ for 15 minutes separately to prevent Maillard reaction. Then, both the medium were mixed well after cooling down at room temperature to obtain the standard

working volume of 250 mL in a 500 mL Erlenmeyer flask. Subsequently, the standard inoculum was inoculated into the culture medium. Then, the flask was placed inside an orbital shaker and grown under the optimised condition of SmF at 30℃ at 150 rpm [13]. The fermentation was run for 168 h and samples were collected every 24 h for enzyme activity assays of xylanase, amylase, protease and cellulase, protein concentration, spore count and medium pH before recorded and calculated as mean value.

2.4 Physical Mutagenesis Using Ultraviolet (UV) Irradiation for Possible Overproduction of Enzymes by *Aspergillus brasiliensis*

The physical mutagenesis using UV irradiation was applied according to Irfan et al. [2] with modification. To begin, spore suspension of *A. brasiliensis* was prepared with strict aseptic condition in a laminar flow. Spores were scraped by sterile hockey stick and collected as stock of spore suspension. Then, the spore suspension was exposed to UV irradiation of 254 nm from a fixed distance of 20 cm away from the center of germicidal lamp for 30 minutes at a regular 5 minutes interval of 5, 10, 15, 20, 25, 30 minutes, respectively. The control of the experiment was conducted with non-treated wild type of *A. brasiliensis* without the exposure of UV irradiation. Next, 1 mL of the UV treated spore suspension was transferred into a new test tube and kept in dark for an hour to avoid photo-activation and to stabilize the thymine dimer that might occur. After an hour, a volume of 100 µL of UV treated spore suspension was subcultured on PDA before incubated at 30℃ for 5 days. Subsequently, spores of *A. brasiliensis* were harvested from PDA with sterilized sodium phosphate buffer, pH 7.0. One mL of inoculum size was standardized to 3×10^6 spores before inoculated into 250 mL prepared culture medium with adjusted initial medium pH of 6.5 in 500 mL Erlenmeyer flask. The flasks were incubated into an orbital shaker incubator at 30℃ at 150 rpm for 168 h [13].

Sampling analysis was performed at regular interval of 24 h. All the analysis was also conducted on the non-treated wild type used as the control of the experiment. The UV mutagenesis was performed in triplicate and the mean value of the analysis was quantified to investigate the possible overproduction of enzymes by UV treated mutants of *A. brasiliensis* after mutagenesis.

2.5 Chemical Mutagenesis Using Nitrous Acid (NA) for Possible Overproduction of Enzymes by *Aspergillus brasiliensis*

In this study, NA was used as chemical mutagen to trigger random mutagenesis according to Kabera et al. [14] with slight modification. Sterile distilled water was poured onto the PDA with *A. brasiliensis*. Spores were scraped by sterile hockey stick and collected as the spore suspension stock. One mL spore suspension of *A. brasiliensis* was added into 99 mL of 0.1 M sodium acetate buffer, pH 4.0 to form the final volume of 100 mL mixture before treated with 0.15 mL of 0.17 mg/mL sodium nitrite. Then, the treated mixture was incubated at 30℃ in a water bath for 60 minutes at regular 15 minutes interval starting from 15, 30, 45 and 60 minutes, respectively. To stop the reaction, 10 mL of the treated mixture was withdrawn and added into a test tube. Subsequently, 20 mL of 0.1 M sodium phosphate buffer, pH 7.0 was added and mixed in the test tube. Then, the test tube was placed in melting ice for 30 minutes. As the pH went down to 6.0, nitrite was converted to nitrous acid which was a strong chemical mutagen. Next, the test tube was centrifuged at 3000 rpm for 10 minutes at 4℃. Then, the supernatant was discarded and the spores were resuspended and washed 3 times with sterilized sodium phosphate buffer, pH 7.0. Thereafter, the spores were resuspended with 1 mL of the sodium phosphate buffer, pH 7.0. A volume of 100 µL of treated spore suspension was subcultured onto PDA before incubated at 30℃ for 5 days. One mL of inoculum size was standardized to 3×10^6 spores before inoculated into 250 mL culture medium with initial medium pH of 6.5 in 500 mL Erlenmeyer flask. The flasks were incubated in the orbital shaker incubator at 30℃ under 150 rpm for 168 h [13].

Enzymes activity assays were carried out every 24 h interval on the mutants of NA. All the analysis was also conducted on the non-treated *A. brasiliensis* as the control of the experiment. The NA mutagenesis was performed in triplicate to obtain the mean value of the analysis that was used to investigate the possible overproduction of enzymes by NA treated mutants of *A. brasiliensis* after mutagenesis.

2.6 Chemical Mutagenesis Using Ethyl Methane Sulfonate (EMS) for Possible Overproduction of Enzymes by *Aspergillus brasiliensis*

The development of mutants of *A. brasiliensis* with EMS treatment was conducted according to the protocol described by Radha et al. [15]. The spore suspension was prepared in the same approach as NA treated experiment. Then, one mL of spore suspension of *A. brasiliensis* was added to 2 mg/mL of EMS in test tube before incubated in a water bath at 30℃. The test tubes were incubated for 60 minutes at the regular time of 15 minutes interval consisting 15, 30, 45 and 60 minutes, respectively. After the EMS treatment, each test tube was centrifuged at 3000 rpm for 10 minutes at 4℃ and washed 3 times with sterilized sodium phosphate buffer, pH 7.0. Then, the spores were resuspended with 1 mL of the sodium phosphate buffer, pH 7.0. A volume of 100 µL of treated spore suspension was subcultured onto PDA before incubated at 30℃ for 5 days. Again, 1 mL of inoculum size was standardized to 3×10^6 spores before inoculated into 250 mL culture medium with initial medium pH of 6.5 in 500 mL Erlenmeyer flask. The flasks were incubated in the shaker at 30℃ at 150 rpm for 168 h [13].

Assays including enzymes activity were performed at regular 24 h interval on the mutants of EMS. All the analysis was also conducted on the non-treated *A. brasiliensis* as the control of the experiment. The EMS mutagenesis was performed in triplicate to obtain the mean value of the analysis that was used to investigate the possible enzymes overproduction by EMS treated mutant of *A. brasiliensis* after mutagenesis.

2.7 Sampling and Data Analysis

In this study, all experiments were conducted in triplicate. 10 mL samples of SmF were collected aseptically at regular interval of 24 h. The spore count and pH of the medium were analysed from the collected samples of SmF. Then, the samples were centrifuged at 9000 rpm for 20 minutes at 4℃. Subsequently, the supernatant was collected and used for the quantification of protein concentration and soluble enzymes activity based on their specific substrates. The samples that were collected for analysis were recorded as mean value. The standard error was also calculated and represented in Figures in this study.

2.8 Xylanase Activity Assay

Xylanase activity was assayed according to the method by Bailey et al. [16] with slight modification. Xylanase activity was carried out by mixing 0.1 mL of supernatant with 0.9 mL of 1% beechwood xylan in 0.05 M sodium phosphate buffer, pH 5.3. The enzyme mixture was then incubated at 50℃ for 30 minutes. In order to stop the reaction, 3, 5-dinitrosalicylic acid (DNS) was used to measure the concentration of xylose as the reducing sugar [17]. Total of 1.5 mL of 1% DNS was added into the enzyme mixture and incubated at 90℃ for 5 minutes. To stabilize the color, 0.5 mL of 40% potassium sodium tartrate was added. The test tube was then left in cold water for 15 minutes. Next, the absorbance was recorded at 575 nm using spectrophotometer (Secoman-Aias, France) to determine the amount of xylose present. In this assay, xylanase activity was catalyzed by the hydrolysis of xylan to liberate xylose. Then, the reaction was arrested by adding DNS that formed a red-brown complex with xylose. The color was stabilized by adding potassium sodium tartrate. To quantify the activity of xylanase, one unit (U) of xylanase activity is defined as the amount of enzyme releasing 1 µmoL of reducing sugar of xylose per minute under the assay conditions. The xylanase activity was measured according to the standard curve of xylose.

2.9 Amylase Activity Assay

Amylase activity was assayed according to Bhatanagar et al. [18] with slight modification. Amylase activity was carried out by adding 0.25 mL of supernatant to the reaction mixture containing 1.25 mL of 1% soluble starch in 0.5 mL of 0.1 M sodium acetate buffer, pH 5.0. After 10 minutes of incubation at 50^oC, maltose as the reducing sugar was liberated and estimated by DNS method [17]. The developed color was recorded at the wavelength of 575 nm using spectrophotometer. To quantify the activity of amylase, one unit (U) of amylase is defined as the amount of enzyme releasing 1 µmoL maltose equivalent per minute under the assay conditions. The amylase activity was measured according to the standard curve of maltose.

2.10 Protease Activity Assay

The protease activity was determined by monitoring the amount of tyrosine released from casein according to Shieh et al. [19] with slight modification. Protease activity was performed by adding 0.5 mL of supernatant to 2.5 mL of 1.2% casein prepared in 0.05 M sodium phosphate buffer, pH 6.0 before incubated at room temperature for 10 minutes. Then, 3 mL of 0.44 M trichloroacetic acid (TCA) was added and incubated for 30 minutes at room temperature. Next, the mixture was centrifuged at 6000 rpm for 10 minutes. Subsequently, 1 mL of supernatant was transferred to a new test tube containing 2 mL of 0.5 M NaOH with 0.1 mL of phenol reagent (Folin-Ciocateu's reagent: water = 1 : 2). The mixture was then incubated at 30^{o}C for 15 minutes and the absorbance was recorded at 660 nm. To quantify the activity of protease, one unit (U) of protease activity is defined as the amount of enzyme releasing 1 µmoL of amino acid equivalent to tyrosine per minute under the assay conditions. The activity of protease was measured according to the standard curve of tyrosine.

2.11 Cellulase Activity Assay

Cellulase activity was assayed according to Chinedu et al. [20] with slight modification. Cellulase activity assay was conducted by adding 0.1 mL of supernatant with 2 mL of 0.1% carboxymethyl-cellulose (CMC) prepared in 0.1 M sodium acetate buffer, pH 5.0. The mixture was then incubated at 30℃ for 30 minutes before quantified by DNS method [17]. The absorbance reading was measured at the wavelength of 575 nm to determine the concentration of glucose. To quantify the activity of cellulase, one unit (U) of cellulase activity is defined as the amount of enzyme releasing 1 µmoL of reducing sugar equivalent to glucose per minute under the assay conditions. The activity of cellulase was measured according to the glucose standard curve.

2.12 Quantification of Protein Concentration

Lowry method was used to quantify the protein concentration of the supernatants from the fermentation samples according to the absorbance reading at the wavelength of 750 nm [21]. Soluble protein concentration of the supernatants was measured using the standard curve of bovine serum albumin (BSA).

2.13 Spore Count of *Aspergillus brasiliensis*

The spore count from samples of fermentation was measured using a counting chamber of Hemocytometer [13].

2.14 Measurement of Medium pH

The medium pH of sample of fermentation was measured by using a pH meter.

3. RESULTS AND DISCUSSION

3.1 Production of Xylanase by UV Exposed Mutant of *Aspergillus brasiliensis*

Based on the results in the present study, the highest maximum xylanase production of 10.052±0.0001 U/mL was obtained at 72 h of SmF produced by the mutant of *A. brasiliensis* that was exposed to UV for 30 minutes as shown in Fig. 1A. In fact, the maximum xylanase activity was produced by 2.61±0.34 × 10^5 spores/mL of the UV treated mutant with the total soluble protein production of 0.443±0.057 mg/mL obtained at medium pH 5.37. The xylanase activity produced by this optimised mutant increased with the time course of the study until it reached the maximum activity at 72 h of SmF with 10.052±0.0001 U/mL as a result of rapid hydrolysis of susceptible hemicelluloses of xylan in wheat bran to convert to xylose as the reducing sugar. Nonetheless, the xylanase activity dropped thereafter due to the depletion of nutrients, presence of growth inhibitors and indigestion of susceptible portion of xylan molecules in the fermentation medium [22]. In contrary, the highest xylanase activity by the non-treated wild type control of *A. brasiliensis* was found to be only 7.821±1.468 U/mL obtained from 13±2.55 × 10^5 spores/mL at 72 h of SmF with the total protein production of 2.064±0.050 mg/mL at medium pH 5.65. Interestingly, there was an increment of 28.52% in xylanase activity produced by the optimised mutant compared with its wild type in the present study. Indeed, the specific activity of xylanase exerted by the mutant was found to be 22.690 U/mg where it was approximately 5.98-fold in increment in contrary to the wild type with only 3.789 U/mg at 72 h of SmF.

On the other hand, Dhiman et al. [23] reported fungal xylanase production was more favorable at acidic pH ranging from 4 to 6. The optimum

medium pH of 6.5 provides the most suitable condition for growth and xylanase production. Hence, the initial medium pH of 6.5 was selected and adjusted for the production of xylanase by *A. brasiliensis* in SmF in this study [13]. The profile of the medium pH in the present study remained unchanged between pH 5 to 6 as the fermentation time prolonged.

Random mutagenesis of UV irradiation was found to be one of the best approaches for strain improvement of *Aspergillus* spp for maximum production of various enzymes [24]. Based on Nicolas-Santiago et al. [3], two mutant strains that generated after UV exposure possessed significantly increase in their levels of xylanase activity, producing 403 U/L and 314 U/L compared to their wild type of 65 U/L. Indeed, there were drastic increment of 520% and 383% in xylanase activity as compared to their parent strain. Such results are also supported by the study of Irfan et al. [2]. They reported the maximum xylanase activity of 21.5±1.1 U/L was attained by the mutant of *Aspergillus niger* that was exposed to UV for 25 minutes compared to parental strain of 17.5±1.02 U/L. According to Irfan et al. [2], the overproduction of xylanase activity was possibly due to the changes occurred in the promoter zones of the gene coding for xylanase after UV mutagenesis. In fact, they also suggested that the UV irradiation might enhance the transcription of the mRNA corresponding to xylanase which led to the increase in enzyme production. According to Ali et al. [25], the structural alteration in DNA was associated with the activity of enzyme. Mutagenesis especially UV irradiation possibly changed structure of DNA by photolysis which encouraged the formation of pyrimidine dimer. This dimer would probably promote mycelium growth and subsequently enzyme activity which resulted in greater secretion of enzyme from mycelium cells. Mutants might able to produce enzyme with advanced properties for auto-tolerated during the rising of the pH and accumulation of toxic materials and waste products. Subsequently, the biomass was preserved as much as possible from degradation and thus, involved extensively in the production of metabolites including enzymes.

3.2 Production of Amylase by UV Exposed Mutant of *Aspergillus brasiliensis*

Fig. 1B shows the mutant of *A. brasiliensis* which was exposed to UV for 25 minutes produced the highest maximum amylase activity of 0.481 ± 0.007 U/mL with the total protein concentration of 0.529±0.003 mg/mL by 6.57×10^5 spores/mL at the medium pH of 5.79. There was 107.32% higher in activity in mutant as compared to the amylase activity of 0.232±0.021 U/mL produced by $1.19 \pm 0.378 \times 10^6$ spores/mL of non-treated wild type of *A. brasiliensis* with the total protein production of 2.313±0.029 mg/mL at medium pH 5.27. In fact, this result was confirmed by a study of Prabakaran et al. [4] where the overproduction of amylase activity was also occurred in the UV mutant strains. They attributed this increment in amylase activity to possible changes in DNA due to the formation of thymine dimer that the DNA pyrimidines of thymine and cytosine were the most sensitive to modification due to the UV rays absorption. This thymine dimer distorted the DNA helix and blocked further replications [6]. As a result, in this case, mutation occurred and led to better adaptation of microorganism to its environment with much improved biocatalytic performance including amylase enzyme production. The observation of Roheena et al. [9] also came in the same line as the present study that the mutant of *A. oryzae* which exposed to UV for 45 minutes gave the maximum alpha-amylase activity of 160±2 U/mL. They stated that the structural alteration in DNA was associated with activity of alpha-amylase. Enhancement in amylase production was obtained by UV mutagenesis [26]. The mutants of *A. fumigatus* showed the maximum amylase activity of 616.0 U/mL at medium pH 6.0. The UV mutant possessed an increment of approximately 44.52% in amylase activity over wild strain. In this study, the maximum amylase activity of 0.481±0.007 U/mL was attained by mutant of *A. brasiliensis* that exposed to UV for 25 minutes compared to other mutants as well as the non-treated wild type of *A. brasiliensis* as shown in Fig. 1B.

3.3 Production of Protease by UV Exposed Mutant of *Aspergillus brasiliensis*

Fig. 1C displays the optimum protease activity produced by UV mutant and wild type of non-treated control of *A. brasiliensis* at the maximum peak of 72 h and 96 h of SmF, respectively. Unfortunately, the non-treated wild type of *A. brasiliensis* produced the highest protease activity of 0.056±0.001 U/mL as compared to other UV exposed mutants of *A. brasiliensis.* The second maximum protease activity of 0.011±0.0001 U/mL was produced by 1.81±1.10

$\times 10^5$ spores/mL of the mutant that was exposed to UV for 15 minutes with total soluble protein production of 0.647±0.031 mg/mL at medium pH of 5.80. There was 80.8% less in activity in mutant as compared to the non-treated control of *A. brasiliensis* that displayed the highest protease activity of 0.056±0.001 U/mL produced by 1.280±0.539 $\times 10^6$ spores/mL with total soluble protein production of 2.167±0.001 mg/mL at medium pH of 5.57. Based on Berg et al. [27], the UV mutation could be harmful due to formation of pyrimidine dimer which might not fit into a DNA double helix. As a result, it blocked the DNA replication and gene expression until the lesion of DNA removed. Consequently, the decrease in protease activity obtained in the present study as a result of mutant strain was attributed to the possible changes due to the thymine dimer formation that distorted the DNA encoding gene of protease enzyme. Unfortunately, the UV mutation was harmful and blocked the gene expression encoding for protease enzymes. In the contrary, in another study, enhancement of protease production was observed through the UV mutant strain of 541.42±1.13 U/gds as compared to wild type according to Yousaf et al. [28].

3.4 Production of Cellulase by UV Exposed Mutant of *Aspergillus brasiliensis*

Based on the results as shown in Fig. 1D, the maximum cellulase activity of 0.030±0.007 U/mL was obtained at 96 h of SmF by 2.24±1.17 $\times 10^5$ spores/mL of the mutant of *A. brasiliensis* which had been exposed to UV for 30 minutes with total soluble protein production of 0.538 ± 0.007 mg/mL at medium pH of 5.87. However, the maximum cellulase activity produced by non-treated control strain was found to be higher, producing 0.052 ± 0.002 U/mL at 120 h of SmF. There was about 42.30% decrement in activity in mutant compared to the control strain of *A. brasiliensis* that showed the highest cellulase activity of 0.052±0.002 U/mL obtained by 1.19±0.38 $\times 10^6$ spores/mL with total soluble protein concentration of 2.313±0.029 mg/mL at medium pH of 5.27. Nicolas-Santiago et al. [3] stated that the UV ray was tend to affect mainly the hydrogen bonds of pyrimidic bases, the most vulnerable regulatory sequences that contained the highest concentration of cytosine and thymine. Based on their observation, UV irradiation enhanced cellulase activity of 363 U/L and 260 U/L in two mutant strains of *A. niger* as compared to wild type of 98 U/L. Unfortunately, in the present study, UV irradiation did not end up in yielding mutant strain of *A. brasiliensis* with high activity, instead with lower cellulase activity as compared to the parental strain that produced 0.052±0.002 U/mL. As a result, we suggested that the decrease in cellulase activity in mutants strain attributed to the down regulation of enzyme expression due to possible changes in the encoding gene for cellulase enzyme under the control of such vulnerable regulatory sequences that were easily affected by UV exposure.

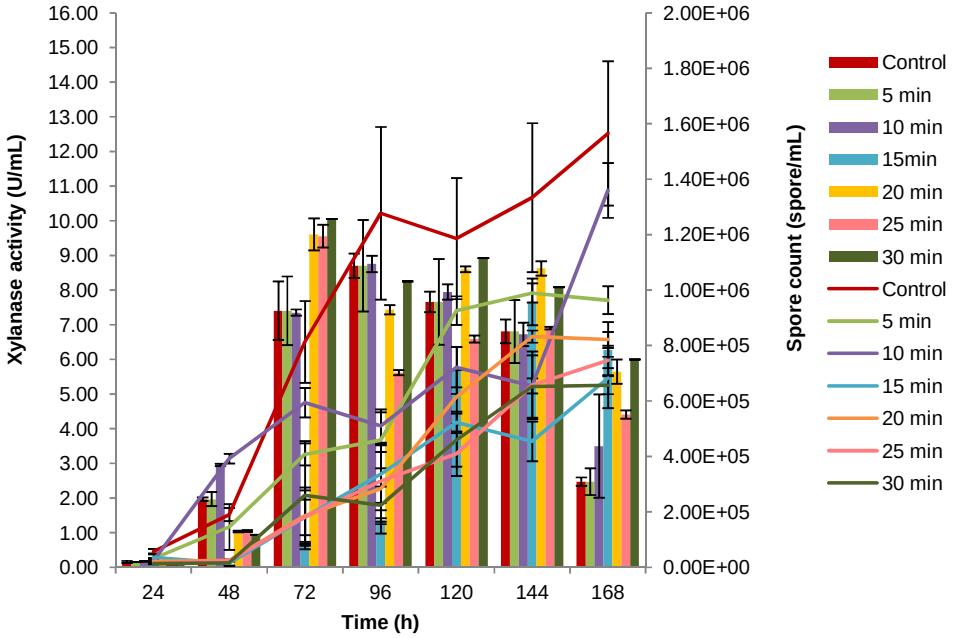
3.5 Production of Xylanase by NA Treated Mutant of *Aspergillus brasiliensis*

Based on Fig. 2A, the highest maximum xylanase activity of 9.127±0.409 U/mL at 72 h of SmF by the mutant of *A. brasiliensis* was obtained after it was being exposed to NA for 15 minutes. Total of 5.03±0.25 $\times 10^5$ spores/mL of the mutant produced the highest maximum xylanase production with the total soluble protein production of 0.4690±0.0079 mg/mL at pH of the medium 5.83. In fact, there was approximately 16.69% higher in maximum xylanase activity obtained from the mutant compared to the wild type. In contrast, 8.13±25.54 $\times 10^5$ spores/mL of the non-treated wild type of *A. brasiliensis* possessed lower maximum xylanase activity of 7.821±1.461 U/mL with the total soluble protein concentration of 2.064±0.012 mg/mL at the medium pH of 5.65. On the other hand, specific activity of xylanase obtained by the mutant was elucidated to be 19.460 U/mg. Notably, there was 5.136-fold more in specific activity from the mutant compared to the wild type of 3.789 U/mg. According to Zimmerman [8], NA was found to cause the deamination of cytosine to uracil and adenine to hypoxanthine. The effect of this mutagen was remarkable if it positively affected the driving gene of any encoding enzymes [14]. Therefore, the increase in xylanase activity in mutant of *A. brasiliensis* in present study might attribute to the possible changes in the driving genes encoding for xylanase enzyme. A study was conducted by Mishra et al. [1] where they noticed that all UV mutants and some of NA mutants possessed their stability in xylanase production. Considerable increment in xylanase activity of 150% to 200% and 130% to 180% until twelfth generation of mutants was attained compared to wild type in their study. They performed double mutation by subjecting the NA mutants to UV irradiation to further enhance their stability in xylanase production. Even though,

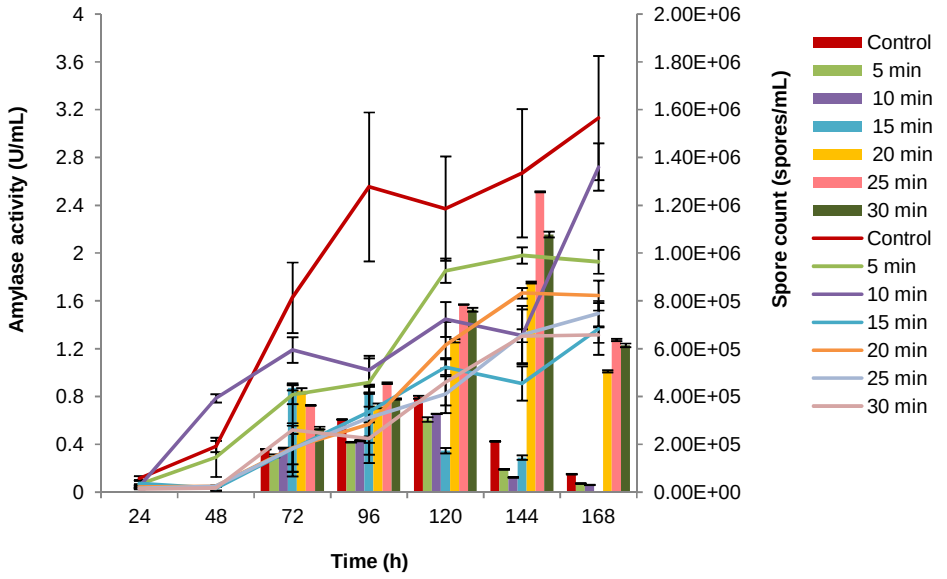
they found that the single treatment of UV irradiation was also an effective approach for enhancement of xylanase production as compared to further treatment with NA.

Besides that, Shah et al. [29] stated the hydrogen ion concentration also possessed a possible influence on enzyme production. The effect of pH on enzyme stability might influence the production of enzyme [30]. The study of Uhlig [31] suggested that the optimal medium pH of commercial xylanase from *A. brasiliensis* was determined to be between the range of 4 and 5. Thus, in the present study, the optimum pH of the medium of 5.83 gave the maximum xylanase production of 9.127±0.410 U/mL by *A. brasiliensis* that exposed to NA for 15 minutes at 72 h of SmF.

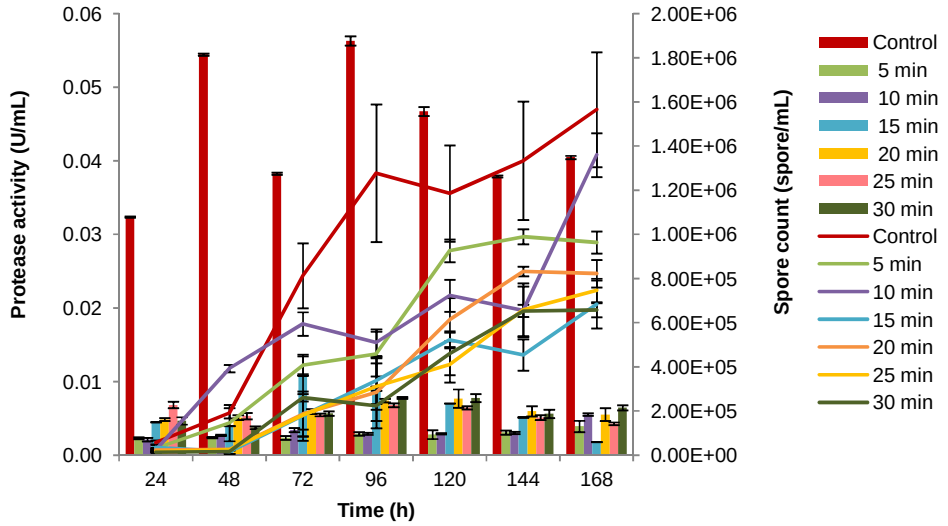
A



B



C



D

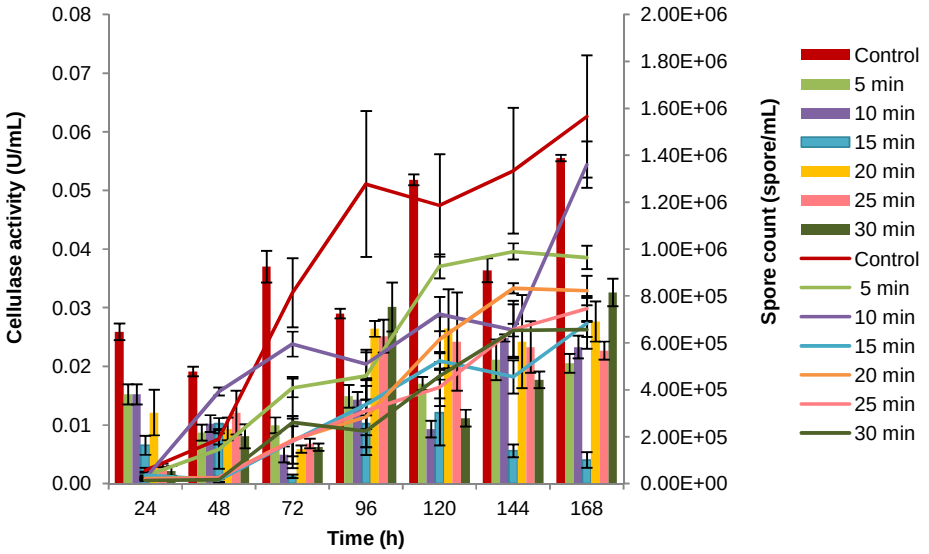


Fig. 1. Enzymes production of xylanase (A), amylase (B), protease (C) and cellulase (D) from wild type and mutants of *A. brasiliensis* under submerged fermentation (SmF) after UV mutagenesis

Enzymes activity is presented as column chart while spore count is presented as line graph

3.6 Production of Amylase by NA Treated Mutant of *Aspergillus brasiliensis*

Fig. 2B displays the profile of amylase activity obtained from the mutants and wild type of *A. brasiliensis* under SmF for 168 h after NA mutagenesis. In the present study, unfortunately, the highest maximum amylase activity of 0.232±0.020 U/mL was obtained at 96 h of fermentation by wild type compared to the mutant strain of 0.216±0.036 U/mL after NA treatment for 30 minutes. There was 7.41% more in amylase activity observed from 1.19±0.38 × 10^6 spores/mL of the control strain with total soluble protein production of 2.313±0.029 mg/mL at pH of the medium 5.27 when compared to

1.11±0.06 × 10^6 spores/mL of the mutant *A. brasiliensis.* In contrast, a study finding of Roheena et al. [32] stated that the mutant of *A. oryzae* which exposed to NA gave higher alpha-amylase activity of 285±0.9 U/mL as compared to its wild strain. Based on their observation, NA caused the alteration in the structure of DNA by replacing the amino group with hydroxyl group at the carbon number 6 of the encoding gene bases for the amylase. Nevertheless, in the present study, the NA treatment did not end up in yielding higher activity of amylase from the mutant strain of *A. brasiliensis,* in fact, the amylase production by NA mutant gave lesser amylase activity of 0.216±0.036 U/mL as compared to the parent strain that achieved 0.232±0.020 U/mL. The decrease in amylase activity was due to the alteration in DNA structure of the amylase encoding gene that resulted in negative production of the enzyme after NA exposure [32].

3.7 Production of Protease by NA Treated Mutant of *Aspergillus brasiliensis*

Fig. 2C shows the maximum protease activity by mutants and non-treated wild type control strain of *A. brasiliensis* displayed at 96 h and 120 h of SmF, respectively. Unfortunately, the highest maximum protease activity of 0.056 ± 0.001 U/mL was obtained by non-treated wild type of *A. brasiliensis*. Much lower maximum protease activity of 0.008±0.002 U/mL was harvested by 7.18±4.2 × 10^5 spores/mL of the mutant of *A. brasiliensis* that was exposed to NA for 60 minutes with total soluble protein production of 0.4972 ± 0.0062 mg/mL at the medium pH of 5.61. Notably, there was 85.79% lower in activity in mutant compared to the maximum protease activity of 0.056 ± 0.001 U/mL obtained by 1.28±0.53 × 10^6 spores/mL of non-treated wild type with total protein amount of 2.167±0.001 mg/mL at the medium pH of 5.27. According to Devehand and Gwynne [33], the primary action of NA as mutagenic agent was to induce a lesion to provoke modification of the base sequence of DNA molecule. A mutation would appear when this lesion is remained unrepaired. As a result, protein that resulted from the expression of this mutated gene would be defected. Therefore, the decrease in protease activity in mutant of *A. brasiliensis* in the present study was suggested to cause by the possible changes in the driving genes encoding for protease enzyme as a result of permanent lesion in the modification of cytosine and adenine residues after the mutagenesis of NA.

3.8 Production of Cellulase by NA Treated Mutant of *Aspergillus brasiliensis*

In this study, the non-treated wild type control of *A. brasiliensis* displays the highest maximum cellulase activity at 168 h of SmF compared to the mutants with treated NA as shown in Fig. 2D. The mutant of *A. brasiliensis* that was exposed to NA for 30 minutes displayed lower maximum activity of cellulase at 120 h of SmF. Indeed, the highest maximum cellulase activity of 0.052±0.002 U/mL was obtained by control strain of wild type compared to lower activity of 0.035±0.009 U/mL obtained by the mutant strain that was exposed to NA for 30 minutes with total soluble protein production of 0.4783±0.0002 mg/mL at medium pH of 5.54. There was 32.23% lesser in cellulase activity in mutant as compared to control strain that displayed the highest cellulase activity of 0.052±0.002 U/mL obtained by 1.19±0.38 × 10^6 spores/mL with total protein concentration of 2.313±0.029 mg/mL at pH of the medium of 5.27. In contrary, a study by Javed [5] proved the overproduction of enzymes exerted by the NA treated mutants. Notably, 1.18 U/mL of the hyper-cellulolytic activity of CMCase, 0.46 U/mL of FPase and 0.79 U/mL of β-glucosidase were generated from the wild type in their study. Interestingly, increment of 15.25%, 6.52% and 62.02% in activity in mutants were observed compared to its wild type, respectively [5]. Drazic and Delac [34] stated that NA might enhance the enzyme activity. NA caused the alkylation of guanine which formed the permanent lesion within the structure of DNA and thus generated mutation with positive improvement instead. Nonetheless, in the present study, NA treatment was not seem to end up in yielding high amount of enzymes producing mutant strain of *A. brasiliensis,* in contrary, the cellulase production by NA treated mutant gave lesser cellulase activity as compared to the parental strain that produced 0.052±0.002 U/mL.

3.9 Production of Xylanase by EMS Treated Mutant of *Aspergillus brasiliensis*

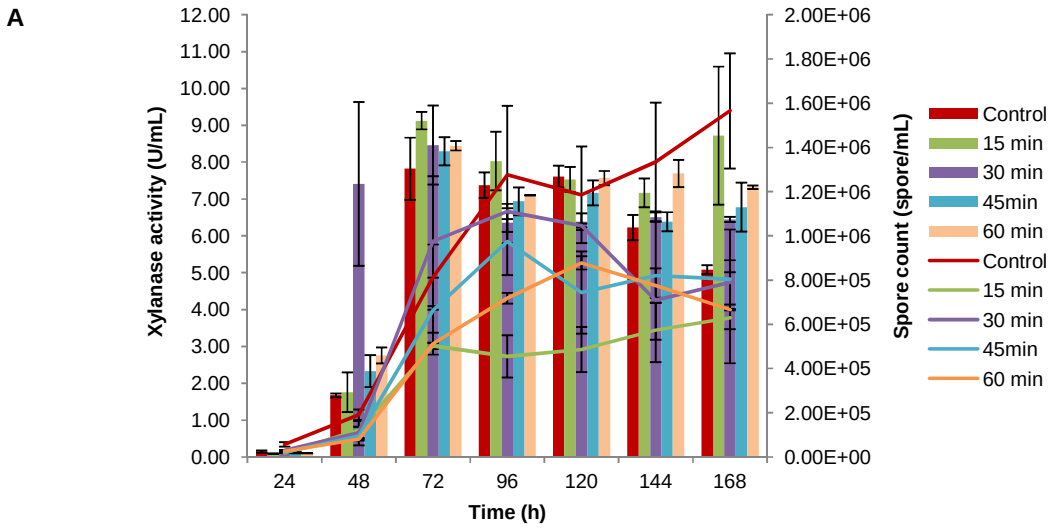
In this study, the production of xylanase activity by *A. brasiliensis* was exposed to 2 mg/mL of EMS. Fig. 3A shows both of the control and mutants of *A. brasiliensis* displayed their maximum xylanase production at 72 h of SmF. Of all the mutants investigated, the highest maximum xylanase production of 11.325±0.881 U/mL was obtained by the mutant of *A. brasiliensis* that exposed to EMS for 45

minutes. In this study, the mutant showed the optimised xylanase activity of 11.325±0.881 U/mL that reached the maximum peak at 72 h of SmF by 5.17±1.66 × 10^5 spores/mL with total soluble protein production of 0.471±0.070 mg/mL at medium pH of 5.66. Indeed, there was 44.80% more in activity was anticipated from the mutant as compared to the maximum activity of 7.821±1.460 U/mL obtained by 8.13±2.55 × 10^5 spores/mL of wild type with the total protein concentration of 2.064±0.050 mg/mL at the medium pH of 5.65. Indeed, similar finding was also exhibited by Reyes and Noyola [35]. Based on their study, the hyperxylanolytic activity of 33.5 U/mL was attained by EMS mutant with 2.7 times higher in activity compared to its parental strain. Alkylating agents such as EMS possesses several effects on DNA [36]. The main action of EMS is to induce lesion in the DNA sequence by addition of ethyl group to the bases which lead to mispairing. EMS alkylates guanine to pair with thymine instead of cytosine [11]. As a result, in this case, fortunately, mutation with motivating result was observed. Hence, the increase in xylanase activity in mutant of *A. brasiliensis* in present study was anticipated after the exposure of EMS. Additionally, higher specific activity of xylanase of 24.044 U/mg was also obtained by the EMS mutant. There was 6.345-fold increment in specific activity in mutant was attained compared to wild type with 3.789 U/mg. This study was also relevant to the study of Shivanna et al. [37] where EMS mutated *A. niger* was able to produce higher amount of protein with improved specific activity of 1.26 U/mg as compared to the parental strain. After the alteration of genes in mutant strain, it retained its ability in possessing high protein yield.

On the other hand, the optimal medium pH at 5.66 was anticipated to encourage the growth of the mutant and subsequently to produce the optimised xylanase activity of 11.325 ± 0.881 U/mL at 72 h. The overall medium pH in the study was seemed to be in constant. *A. brasiliensis* required slight acidic pH for growth and enzyme activity of xylanase [38]. Likewise, Ikram et al. [39] exerted similar results to the present study that showed the maximum xylanolytic activity of 270.53 U/mL with 1.5-fold increment at pH of medium of 5.5 obtained from the mutant strain of *A. niger* was achieved.

3.10 Production of Xylanase by Mutants of *Aspergillus brasiliensis* after Random Mutagenesis of UV, NA and EMS

In a nutshell, the maximum xylanase activity of 11.325±0.881 U/mL was attained after treatment of EMS for 45 minutes followed by 10.052±0.987 U/mL from mutant after exposure of UV for 30 minutes and lastly 9.127±0.410 U/mL from mutant with treated NA for 15 minutes with the increment of 1.5, 1.3 and 1.2-fold as compared to their parental wild type as shown in Fig. 4.



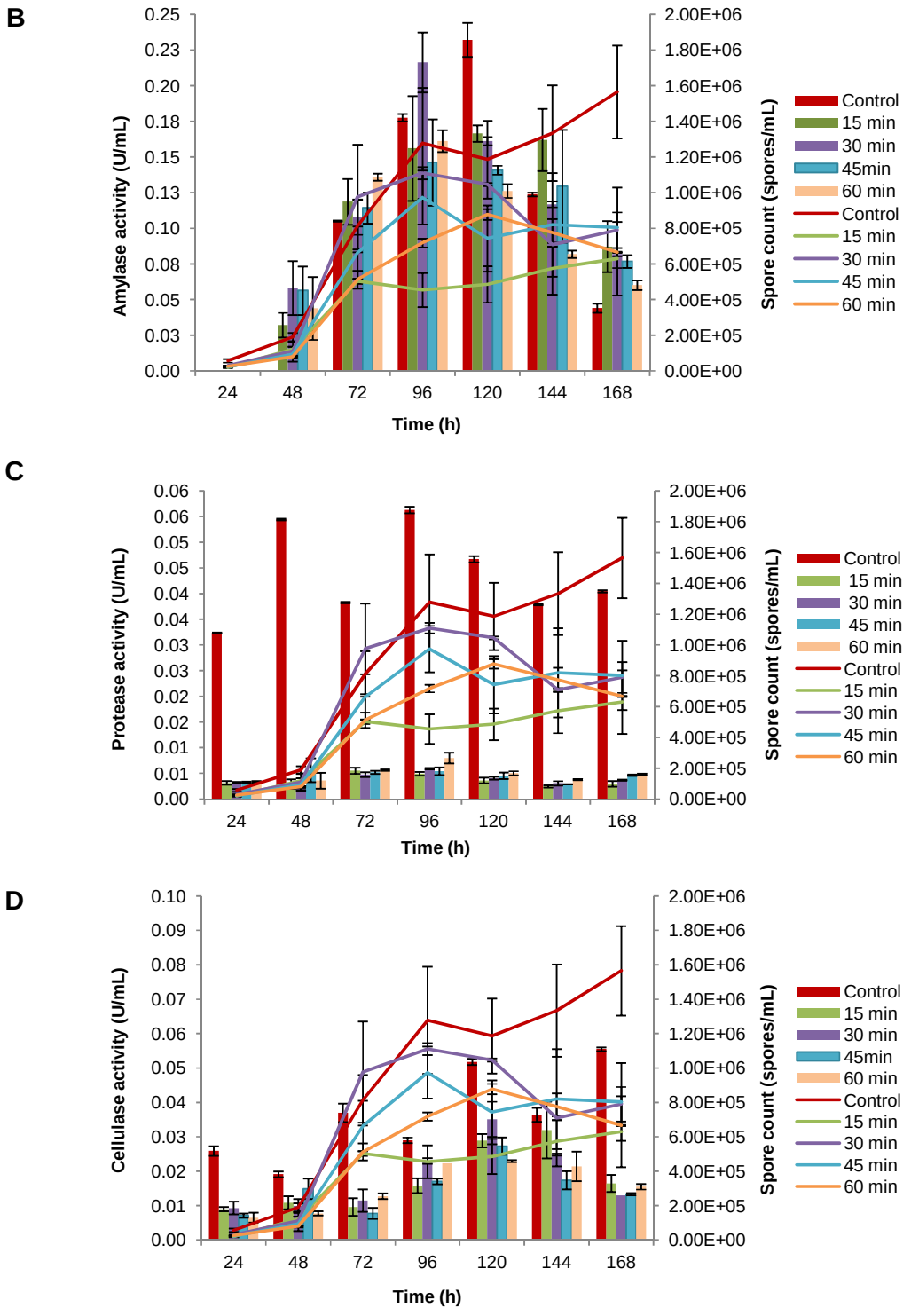


Fig. 2. Enzymes production of xylanase (A), amylase (B), protease (C) and cellulase (D) from wild type and mutants of *A. brasiliensis* under submerged fermentation (SmF) after NA mutagenesis

Enzymes activity is presented as column chart while spore count is presented as line graph

3.11 Production of Amylase by EMS Treated Mutant of *Aspergillus brasiliensis*

Based on Fig. 3B, almost all EMS treated strains displayed hyper amylase activity as compared to non-treated wild type. The optimised amylase activity of 0.304±0.040 U/mL was achieved by the mutant of *A. brasiliensis* that was exposed to EMS for 60 minutes at 144 h of SmF compared to non-treated control of *A. brasiliensis*. Indeed, the optimised amylase activity was produced by 6.57×10^5 spores/mL at medium pH of 5.79 with the total protein production of 0.529 ± mg/mL. In contrast, relatively less amylase activity of 0.232±0.021 U/mL was produced by non-treated control strain at 120 h of SmF. Notably, there was as high as 31.03% increment in activity in mutant as compared to the maximum amylase production of 0.232±0.021 U/mL by 1.19×10^6 spores/mL of wild type *A. brasiliensis* with total protein of 2.313±0.029 mg/mL at pH medium 5.27 in the present study. Moreover, this study also presented similar results to the study obtained from Roheena et al. [32] where the improvement and enhancement of amylase production were anticipated using EMS mutagenesis. They found that the mutants of EMS exhibited the highest amylase activity of 280±0.9 U/mL with 2.1-fold increment in activity over the parental strain. Indeed, they anticipated the increment in amylase activity occurred as a result of the mutation on DNA where the addition of ethyl group to the bases resulted in mispairing of bases enhanced positive enzyme production after EMS mutagenesis.

3.12 Production of Protease by EMS Treated Mutant of *Aspergillus brasiliensis*

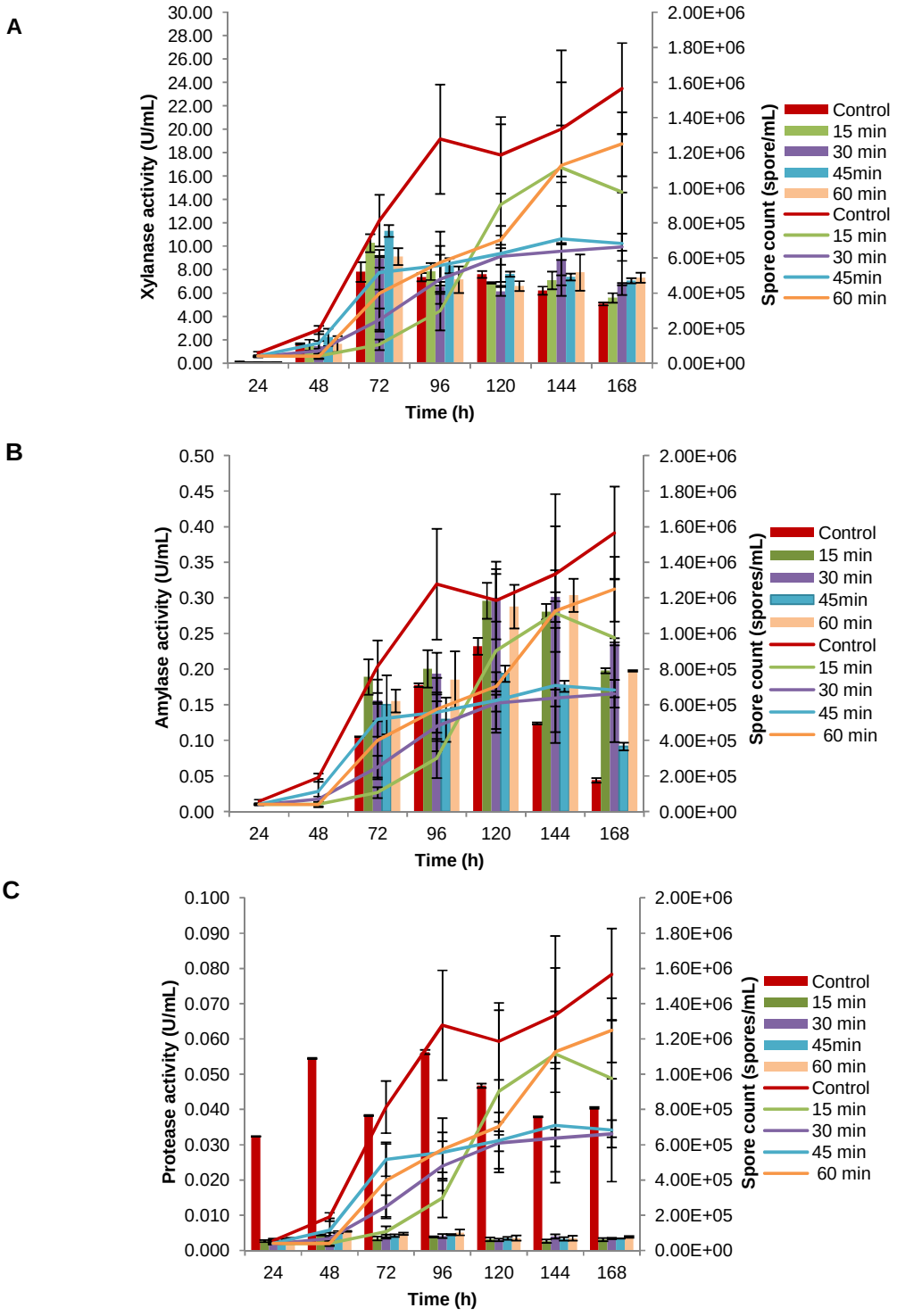
The non-treated wild type of *A. brasiliensis* produced the highest maximum protease activity of 0.056±0.001 U/mL as compared to the mutants of *A. brasiliensis* even though, relatively lower maximum protease activity of 0.005±0.0003 U/mL was obtained by the mutant that was exposed to EMS for 60 minutes. Fig. 3C displays the profile of the protease activity produced by the non-treated control and EMS treated mutants in SmF. In this study, the lesser maximum protease activity was obtained by $4.00±2.60 \times 10^5$ spores/mL of the mutant of *A. brasiliensis* which was exposed to EMS for 60 minutes with the presence of total soluble protein concentration of 0.449±0.062 mg/mL at medium pH of 6.00. There was 90.39% decrement in activity in mutant was observed as compared to the non-treated *A. brasiliensis* that unfortunately possessed much higher maximum protease activity of 0.056±0.001 U/mL from $1.280±0.539 \times 10^6$ spores/mL with the total protein concentration of 2.167±0.001 mg/mL attained at medium pH 5.57.

According to Sega [12], EMS treatment causes either base-pair insertions or deletions. Indeed, EMS induces permanent lesion leads to modification of the base sequence of DNA [33]. In the present study, EMS treatment did not encourage yielding of high protease activity from the mutant strain of *A. brasiliensis*, instead, the protease production by the EMS mutant gave lesser activity of 0.005±0.0003 U/mL as compared to the wild strain that produced 0.056±0.001 U/mL. However, in some cases, positive results of overproduction from EMS treated mutants were observed instead. The increase in protease activity in mutant strain occurs as a result of the modification in the driving gene sequences encoding for protease enzyme that enhances the gene expression after being exposed to EMS mutagen. In a study employed by Radha et al. [15], the maximum protease activity of 7.12 U/mL was displayed by mutant of *A. niger* that was exposed to EMS. In fact, identical result also reported by Yousaf et al. [28] where the maximum yield of protease activity achieved by EMS mutant of *A. oryzae* was detected to be 662.61±0.36 U/gds.

3.13 Production of Cellulase by EMS Treated Mutant of *Aspergillus brasiliensis*

In this study, the highest maximum cellulase activity of 0.052±0.002 U/mL was obtained by the non-treated control at medium pH 5. Unfortunately, relatively lower maximum cellulase activity of 0.024±0.013 U/mL with the total soluble protein concentration of 0.424±0.024 mg/mL at medium pH 5.74 was produced by two mutants that were exposed to EMS for 15 and 45 minutes, respectively. Fig. 3D shows the profile of cellulase activity produced by EMS mutants and wild type that reached their maximum activity peak at 96 h and 120 h, respectively. There was approximately 53.86% decrement in activity in the mutants as compared to the wild type of *A. brasiliensis*. Rajoka et al. [40] stated that mutagenesis was capable to degrade operator gene of cellulase. In this study, the decrease in cellulase activity in the mutants was the consequence of the mutation that led to

degradation of operator gene and presence of the repressors after exposure of EMS. In contrast, according to Chandra et al. [41], the increase in cellulolytic activity was detected after they developed a mutant strain of *T. citrinoviride* using EMS mutagen. Based on their result findings, the mutant produced 0.63 U/mL of endoglucanase, 3.12 U/mL of β-glucosidase and 1.94 U/mL of cellobiase, respectively. Notably, there were 2.14, 2.10 and



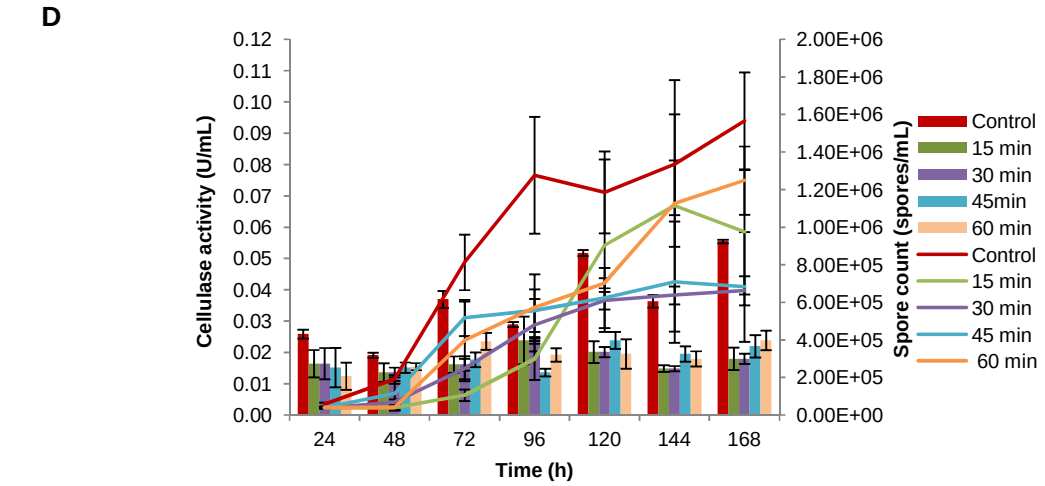


Fig. 3. Enzymes production of xylanase (A), amylase (B), protease (C) and cellulase (D) from wild type and mutants of *A. brasiliensis* under submerged fermentation (SmF) after EMS mutagenesis

Enzymes activity is presented as column chart while spore count is presented as line graph

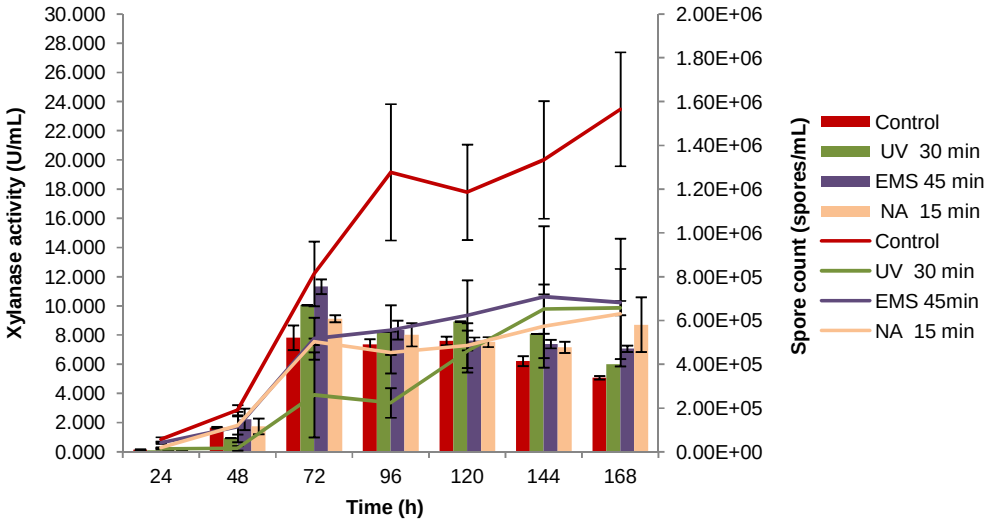


Fig. 4. Profile production of xylanase by wild type and mutants of *A. brasiliensis* after exposed to UV, EMS and NA

Xylanase activity is represented in a column chart while spore count is represented in a line chart for 7 days. The maximum xylanase activity was achieved by mutants of A. brasiliensis that exposed to EMS for 45 minutes followed by UV for 30 minutes and NA for 15 minutes

4.09-fold of increment in enzymes activity were attained by mutant strain compared to the parental strain. Furthermore, another study by Elakkiya and Muralikrishnan [42] reported that EMS was a strong mutagenic agent that induced permanent changes in DNA structure via frame shift mutation. Mutant strains of EMS showed higher cellulase activity of 1461.24 U/g as compared to wild type of 1350 U/g.

4. CONCLUSION

Based on the results obtained, among the enzymes, xylanase was elucidated to possess the highest activity produced at 72 h of SmF. Indeed, 2 mg/mL EMS of the chemical mutagen possessed the best inducer of mutation for xylanase activity followed by UV irradiation at 254 nm from a distance of 20 cm and lastly using

treatment of 0.17 mg/mL NA. Notably, the maximum xylanase activity of 11.325±0.881 U/mL was attained by the mutant after treatment of EMS for 45 minutes followed by 10.052±0.987 U/mL from the mutant after exposure of UV for 30 minutes and lastly 9.127±0.410 U/mL from the mutant with treated NA for 15 minutes. There were increment of 1.5, 1.3 and 1.2-fold in activity for EMS treatment of 45 minutes, UV for 30 minutes and NA for 15 minutes compared to 7.821±1.460 U/mL from non-treated wild type at 72 h of SmF. Furthermore, there were as high as 76.5, 67.9 and 61.6-fold increment in xylanase activity obtained as compared to 0.148±0.054 U/mL from wild type at 24 h of SmF. Interestingly, the xylanase activity of mutants and wild type showed their maximum peak at 72 h of SmF. Nonetheless, the fermentation period beyond 72 h exerted decrease in xylanase production. Besides that, enhancement in amylase activity was also observed in two different mutants of *A. brasiliensis* after exposure to UV for 25 minutes, producing 0.481±0.007 U/mL and treatment of EMS for 60 minutes, producing 0.304±0.040 U/mL as compared to wild type of 0.232±0.021 U/mL. In fact, there were drastic increment by 107% and 31% in activity as compared to wild type. Unfortunately, no overproduction of protease and cellulase was detected after the physical and chemical mutagenesis in this study. On the other hand, the overall medium pH was observed to be remained unchanged as the fermentation prolonged. pH of medium was constant in the range of 5 to 6. Besides that, when the microorganisms reached their maximum biomass concentration, it increased sporulation until the death phase of growth cycle was achieved. The spore count decreased thereafter due to nutrient degradation as fermentation progressed. Furthermore, we also concluded that 72 h of SmF was the optimum time of fermentation to attain the maximum spore count that possessed the optimised xylanase production. In this study, 5.17×10^5 spores/mL of mutant that exposed to EMS for 45 minutes followed by 5.03×10^5 spores/mL of the mutant after the treatment of NA for 15 minutes and 2.61×10^5 spores/mL of the mutant with exposure of UV for 30 minutes were produced to accomplish the maximum xylanase production in this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mishra L, Madhavi VM, Harmalkar M. Effect of UV and nitrous acid treatment on production of xylanase enzyme by *Acinetobacter* sp. Int J Curr Microbiol App Sci. 2014;3(1):45-53.
2. Irfan M, Javed J, Ouratulain S. UV mutagenesis of *Aspergillus niger* for enzyme production in submerged fermentation. Pak J Biochem Mol Biol. 2011;44(4):137-140.
3. Nicolas-Santiago DS, Regalado-González C, García-Almendárez B, Fernández FJ, Téllez-Jurado A, Huerta-Ochoa S. Physiological, morphological, and mannanase production studies on *Aspergillus niger* uam-gs1 mutants. Elec J Biotechnol. 2006;9(1):50-60.
4. Prabakaran M, Thennarasu V, Ayeswariya R, Mangala R, Chandrakala N, Mohan N. Comparative studies on the enzyme activities of wild and mutant fungal strains isolated from sugarcane field. Ind J Sci Technol. 2009;2(11):46-49.
5. Javed MM, Ikram ULH, Irfana M. Multiple mutagenesis for the over-expression of cellulase in *Humicola insolens*. Pak J Bot. 2011;43(1):669-677.
6. Sambrook J, MacCallum PP. Molecular cloning: A laboratory manual, 3rd edition; 2000.
   Available:http://hum-molgen.org/literature/12-2000/
7. Zia MA, Ur-Rahman K, Sheikh MA, Khan IA. Chemically treated strain improvement of *Aspergillus niger* for enhanced production of glucose oxidase. Int J Agric Biol. 2010;3:964-966.
8. Zimmerman FK. Genetic effects of nitrous acid. Mutation Res. 1977;39:127-148.
9. Roheena A. Studies on the production of alpha amylase by *Aspergillus oryzae* using submerged fermentation. Dissertation, University Lahore; 2005.
10. Demirkan E. Production, purification, and characterization of α-amylase by *Bacillus subtilis* and its mutant derivates. Turk J Biol. 2011;35:705-712.
11. Bhat R, Upadhyaya N, Chaudhury A, Raghavan C, Qiu F, Wang H, Wu J, McNally K, Leung H, Till B. Chemical and irradiation induced mutants and tilling, In: Upadhyaya NM, Ed., Rice Functional Genomics: Challenges, Progress and

Prospects, Springer, New York. 2007; 148-180.
DOI: 10.1007/0-387-48914-2_8148-180

12. Sega GA. A review of the genetic effects of ethyl methane sulfonate. Mutation Res. 1984;134(2-3):113-142.
13. Ho HL, Jamila SH. Optimisation of medium formulation and growth conditions for xylanase production by *Aspergillus brasiliensis* in submerged fermentation (SmF). J Biodivers Biopros Dev. 2014;1:1.
14. Kabera J, Viateur U, Prudentienne M. Use of nitrous acid mutant of *Aspergillus niger* for citric acid production from local cane-molasses. Afr J Microbiol Res. 2010;4(13):1446-1452.
15. Radha S, Himakiran RB, Sridevi A, Prasad NBL Narasimha G. Development of mutant fungal strains of *Aspergillus niger* for enhanced production of acid protease in submerged and solid state fermentation. Eur J Exp Biol. 2012;2(5):1517-1528.
16. Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol. 1992;23(3):257-270.
17. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem. 1959;31(3): 426-428.
18. Bhatnagar D, Joseph L, Raj RP. Amylase and acid protease production by solid state fermentation using *Aspergillus niger* from mangrove swamp. Ind J Fisheries. 2010;57(1):45-51
19. Shieh CJ, Phan Thi LA, Shih IL. Milk-clotting enzymes produced by culture of *Bacillus subtilis* natto. Biochem Eng J. 2009;43(1):85-91.
20. Chinedu NS, Nwinyi OC, Okochi V. Growth and cellulose activity of wild type *Aspergillus niger* AN301 in different carbon sources. SENRA Academic Publishers, Burnaby, British Columbia. 2008;2(2): 357-362.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193(1):265-275.
22. Reese E, Lolad T, Rarrish JE. Modified substrates and modified products as inducers of carbohydrates. J Biotechnol. 1969;100:1151-1154.
23. Dhiman SS, Sharma J, Battan B. Industrial application and prospects of microbial xylanases. A review. J Biores. 2008;3(4):1377-1402.
24. Kang SW, Ko EH, Lee JS, Kim SW. Overproduction of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. Biotechnol Let. 1999;21:647-650.
25. Ali S, Haq I, Qadeer MA. Novel technique for microbial production of 3, 4-dihydroxy phenyl L-alanine by a mutant strain of *Aspergillus oryzae*. Proc Biotechnol. 2002;5:119-124.
26. Singh S, Singh S, Bali V, Sharma L, Mangla J. Production of fungal amylases using cheap, readily available agriresidues for potential application in textile industry. BioMed Res Int; 2014.
Available:http://dx.doi.org/10.1155/2014/215748
27. Berg JM, Tymoczko JL, Stryer L. Mutation Involve Changes in the Base Sequence of DNA. Biochemistry, 5th edition, New York, W H Freeman; 2002.
Available:http://www.ncbi.nlm.nih.gov/books/NBK22525/ (Accessed 13 Jan 2015)
28. Yousaf M, Irfan M, Zia ulla K, Syed QA, Baig S, Amber I. Enhanced production of protease by mutagensized strain of *Aspergillus oryzae* in solid submerged fermentation of rice bran. Sci Int Lahore. 2010;22(2):119-123.
29. Shah IJ, Gami PN, Shukla RM, Acharya DK. Optimization for α-amylase production by *Aspergillus oryzae* using submerged fermentation technology. Basic Res J Microbiol. 2014;1(4):01-10.
30. Kalra MK, Sandhu DK. Optimal production of cellulolytic enzymes and their location in *Trichoderma pseudokoningii*. Acta Biotechnologica. 1986;6(2):161-166
31. Uhlig H. Industrial enzymes and their applications, New York: John Wiley and Sons, Incorporated. 1998;142-143.
32. Roheena A, Ikram ULH, Tehreema I., Zahidali B, Manzoor IK. Random mutagenesis for enhanced production of alpha-amylase by *Aspergillus oryzae* IIB-30. Pak J Bot. 2013;45(1):269-274.
33. Devehand M, Gwynne DI. Expression of heterologous proteins in *Aspergillus.* J Biotechnol. 1991;17:3-10.
34. Drazic M, Delac V. Mutagenic action of N-methyl N-nitro N-nitroso guanidine. J Exp Biol. 1970;11:141.
35. Reyes LM, Noyola TP. Isolation of a hyperxylanolytic *Cellulomonas flavigene* mutant growing on continuous culture on sugar cane bagasse. Biotechnol Let. 1998;20(5):443-446.

36. Talebi AB, Talebi AB, Shahrokhifar B. Ethyl methane sulphonate (EMS) induced mutagenesis in Malaysian rice (cv. MR219) for lethal dose determination. American J Plant Sci. 2012;3:1661-1665.
37. Shivanna GB, Govindarajulu V. Screening of asporogenic mutants of phytase-producing *Aspergillus niger* CFR 335 strain. Microbial Ecol Health Disease. 2009;21:57-63.
38. Gomes DJ, Gomes J, Steiner W. Production of highly thermostable xylanase by a wild strain of thermophilic fungus *Thermoascus aurantiacus* and partial characterization of the enzyme. J Biotechnol. 1994;37:11-22.
39. Ikram ULH, Hussain R, Hameed U, Javed M. Selection of *Aspergillus niger* mutant using antimetabolite 2-deoxy D-glucose after n-methyl n-nitroso n-guandine (MNNG) treatment. Pak J Bot. 2008;40(6):2613-2623.
40. Rajoka MI, Bashir A, Malik KA. Mutagenesis of *Cellulomonas biazotea* for enhanced production of xylanases. Biores Technol. 1997;62:99-108.
41. Chandra M, Kalra A, Sangwan NS, Gaurav SS, Darokar MP, Sangwan RS. Development of a mutant of *Trichoderma citrinoviride* for enhanced production of cellulase. Biores Technol. 2009;100(4): 1659-1662.
42. Elakkiya P, Muralikrishnan V. Cellulase production and purification of mutant strain *Trichoderma viride.* Int J Curr Microbiol App Sci. 2014;3(9):720-727.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13041