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Effect of chemical and physical mutagens on amylase producing potentiality of *Aspergillus flavus* NSH9

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ABSTRACT

Aspergillus flavus NSH9 is a potential source of α-amylase and glucoamylases. Between two of its glucoamylases, one can digest the raw starch granules, while both being pH and thermostable. The purpose of the study was to enhance the production of amylases (α-amylase and Glucoamylase) from *A. flavus* NSH9 by random mutagenesis using Ultraviolet (UV) irradiation and Ethidium bromide (EtBr) treatment. Glucoamylase and α-amylase activity were evaluated by 3, 5-dinitrosalicyclic acid (DNS) method and starch-iodine method, respectively. Mutated *A. flavus* NSH9 by EtBr treatment (10µg/ml) in PDA plate produced highest amount of both crude glucoamylases (GA) (1.47 ± 0.087 U/mL/min) and raw starch degrading glucoamylase (RSD-GA) (0.839 ± 0.036 U/mL/Hour). The highest value was 1.6 and 1.5 times higher compared to control for GA and RSD-GA, respectively. Here as, UV induced radiation produced about 1.3 and 1.4 times more GA and RSD-GA compared to control, respectively. The activity of α-amylase was about 1.7 times higher in the treatment group with 5µg/ml EtBr in sublethal condition than without the treatment group. In conclusion, both UV and EtBr treatment increased the amylases production from *A. flavus* NSH9. As the single mutation process of *A. flavus* NSH9 enhanced all three enzymes, the strains could be used for the commercial production of amylase.

INTRODUCTION

The amylases (α-amylases, β-amylases, and glucoamylases) are among the most valuable enzymes and are important for biotechnology, representing a class of industrial enzymes with approximately 30% of the world enzyme market [1-2]. Alpha amylase (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) is a family of endo amylases that randomly cleaves the α-1,4 linkages throughout of the starch particles, generating glucose, maltose, dextrin, and oligosaccharides [3]. Whereas, glucoamylase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3, GA) is an exo-acting enzyme that produces β-D-glucose from the nonreducing ends of raw or soluble starches and associated polysaccharide chains by hydrolyzing α-1, 4 linkages [4]. Both amylases have a considerable role in starch processing in the food industries, for instance, for the glucose and fructose syrup derived from liquefied starch with both amylase and glucoamylase action [5-6]. Amylase is also used in different food processing, beverage, various fermented foods, pharmaceuticals, textile, and bioethanol industries [6-10]. Though, the sources of amylases are many such as plants, animals, bacteria and fungi, yet amylases of fungal origin are generally recognized as safe [9]. That is also the reason, for a significant percentage of fungi are continuously screened for α-amylase or glucoamylase production with properties that are better suited to specific industrial applications [11-13]. *A. flavus* has previously been recorded to be an active amylase enzyme producer [13-14]. We have previously purified and sequences one noble α-amylase gene; and two nobles thermostable glucoamylase were also sequences and expressed from *A. favus* NSH9 [15- 17]. One of its glucoamylase had starch binding domain (SBD) at C terminus and can degrade the raw starch granules [17].

Microorganisms can be easily controlled by means of genetic manipulation or other methods. These can be subject to strain development, mutations, genetic engineering, and other modifications that can increase the production of enzymes [18-19]. The techniques used for strain improvement can be applied either separately or in different combinations [20]. Random mutagenesis is widely used in the food industry for the classical strain development reasons [21]. The mutagens are of chemical or physical in nature. Chemical mutagenesis includes the use of Ethidium bromides (EtBr), ethyl methyl sulphonate (EMS), nitrous acid, and N-methyl N'nitro-N nitroguanidine (NTG) [22]. Physical mutagenesis encompasses UV rays, X-ray, and gamma rays. Among physical agents, UV treatment is commonly used in industries because it is very efficient and does not need any instruments [22]. The natural ability of microorganisms to create amylase has indeed been enhanced by mutational therapy, where both chemical and UV radiation mutagenesis have been shown to increase amylase activity [22-24]. Irradiation and chemical mutagens are mostly employed mutagens for mutations using random mutagenesis and some of them have considerably increased the characteristics of amylases in order to satisfy one or all of the 3 E's i.e. Energy, Environment and Economy [25-27]. The purpose of the study was to improve the production of amylases (α-amylase and two glucoamylases) from *A. flavus* NSH9 through random mutagenesis by using UV radiation and EtBr at different concentration at sublethal condition.

MATERIALS AND METHODS

Culture media for enzyme production

A. flavus NSH9 has been used as an inoculum to produce amylase (α-amylase and two glucoamylases). So far as the enzyme production is concerned, active-growing fungal mycelium has been transferred from the potato dextrose agar (PDA) plate to the minimum salt culture medium (MSM) containing (g / L): 4 g of yeast extract, 1 g $(NH_4)_2SO_4$, 20 g raw sago starch, 3 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ [18, 26]. The pH was set to pH 5.0 prior to autoclaving. Two pieces (approximately 5 mm in diameter of each cutted piece) of a 7-day-old PDA fungal crop were used for fermentation in 250 mL of a conical flask containing 50 mL of MSM medium containing 2% (w / v) of raw sago starch. The incubation was conducted at room temperature for 5 days on a rotary shaker at 150 rpm. [17,28]. Details of sample preparation was discussed in the previous study [28].

Chemical and physical treatment for mutation

For chemical treatment in sublethal condition with EtBr at different concentration from 1µg/mL and 5µg/mL were added in the MNS culture media; and method is considered as treatment group one. The induction/fermentation procedure was also followed as discussed above.

Other chemical treatment with EtBr in PDA plate: In this method EtBr at concentration of 10µg/mL were added in potato dextrose agar (PDA) plate, and 100 µL of fungal spore suspension were spread onto the PDA plate. The process is considered as treatment group two. After that, the PDA plates were incubated for 7 days for growing mutated *A. flavus* NSH9. Two pieces of 7-day-old mutated fungal culture grown in PDA were then used for fermentation of 50 mL MSM medium. The incubation was carried out, as discussed above.

UV irradiation treatment was used as physical treatment for mutation and considered as treatment group three. Actively growing fungal mycelium was subjected to UV irradiation. First the fungal cultures grown actively for 5 days were suspended in 0.1M sodium acetate buffer (pH 5.0) and then were centrifuged for 10 min at 6000 rpm; this cell suspension was utilized for the mutation process. The mutation was induced by treatment with 10 mL of suspension which was aseptically pipetted into sterile flat-bottomed petri dishes. The sterile petri dishes containing the suspension were then exposed to UV light in a laminar airflow cabinet fitted with a germicidal lamp, according to Kumar et al. [29]. The samples were exposed for 30 min. The mutated fungal cell suspensions (150 µL/plate) were spread onto PDA medium for 7 days. Afterward, two pieces of 7-day-old mutated fungal culture grown in PDA were used for fermentation of 50 mL MSM medium. The incubation was carried out, as discussed above.

Glucoamylase assay

Glucoamylase activity was evaluated according to the technique used earlier by Karim et al. [15] and released glucose was measured using 3, 5-dinitrosalicyclic acid (DNS) method [30]. The absorbance measurements were performed at

absorbance of 540 nm using a spectrophotometer. The enzyme activities were estimated using a calibration curve prepared with D-glucose as standard (Figure 1). One unit of glucoamylase activity is characterized as the amount of enzyme that releases 1 μ mole of glucose equivalent per minute from soluble starch under the test condition (at 55°C and pH 5.0) [15].

Raw starch degrading ability (RSDA) of glucoamylase

The degrading capacity of crude glucoamylase preparation to raw starch was determined by combining 0.5 mL of enzyme preparation with 0.5 mL of 1 percent (w / v) raw sago starch in 0.1 M sodium acetate buffer, pH 5.0. After 24 hours of incubation at 37 ° C with shaking at 150 rpm, the supernatant was collected for enzyme testing [28]. The reaction mixture was assayed as described in above GA assay using standard curve of glucose (Figure 1). One unit of the RSDA was defined as the amount of enzyme required to release one µ mole of glucose per hour under the assay conditions.

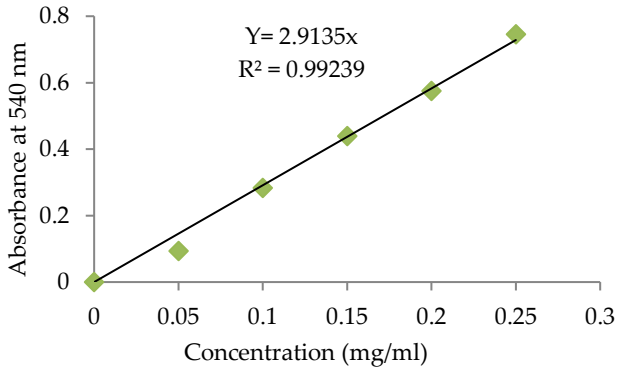

Figure 1. Glucose standard curve generated with DNS method at 540 nm. The concentration of glucose used in this study was ranging from 0.05 mg/mL to 0.25 mg/mL.

Alpha amylase estimation

α-amylase activity has been calculated using starch-iodine method according to Xiao et al. [31] with minor modifications as discussed in previous study [16]. A standard curve of the starch-iodine complex was prepared using a different amount of starch in 400 µL samples containing 50–400 µg of starch (Figure 2), and the absorbance was measured at 580 nm. α-amylase activity unit (U) was defined in the starch-iodine assay as the disappearance in the assay reaction of an average of 1 mg of iodine binding starch material per min per mL.

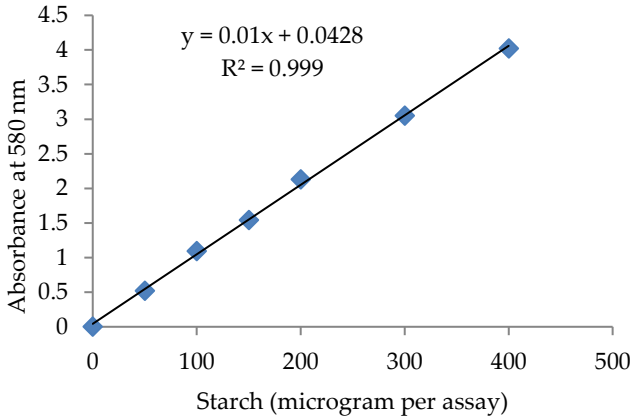

Figure 2. Absorbance of the starch-iodine complex standard curve generated with soluble starch at 580nm. The amount of starch used in the standard curve was ranging from 50 to 400 microgram. Calculated, Y = 10.0428 Absorbance (starch-iodine complex) at 580 nm for 1 mg of starch.

RESULTS

Effect on crude glucoamylase

In this study, UV radiation and EtBr at different concentrations in sublethal condition and in PDA plate were used to improve the production of GA, RSD-GA, and α-amylase. The crude GA production was comparatively higher in different treatment groups compared to control (without treatment) group (0.913 ± 0.09), and the production was significantly ($p \leq 0.05$) different between the groups as shown by one-way ANOVA (Figure 3). The highest GA activity (1.47 ± 0.087 U/mL/min) was observed in the culture media of the treatment group two with 10µg/ml EtBr in PDA plate method (Figure 3), and the value was about 1.6 times higher compared to without treatment group. This value was significantly higher from all groups (treatment and control) except the treatment group with 5 µg/ml EtBr (1.387 ± 0.101) in sublethal condition ($p \leq 0.05$, by Tukey test).

Effect on raw starch degrading glucoamylase

Like GA, RSD-GA production also gradually expanded along with increasing concentration of EtBr at sublethal state (Figure 4). As compared to control, all the treatment groups produced significantly higher RSD-GA ($p \leq 0.05$). The maximum RSD-GA activity (0.839 ± 0.036 U/mL/Hour) was observed in the culture media of the treatment group two with 10µg/ml EtBr in PDA plate method (Figure 4). The highest value was about 1.5 times higher than control, which was remarkably close to treatment groups with UV and

EtBr at 5µg/ml concentration (Figure 4). Meanwhile, UV induced radiation also produced about 1.4 times more RSD-GA as compared to control (Figure 4).

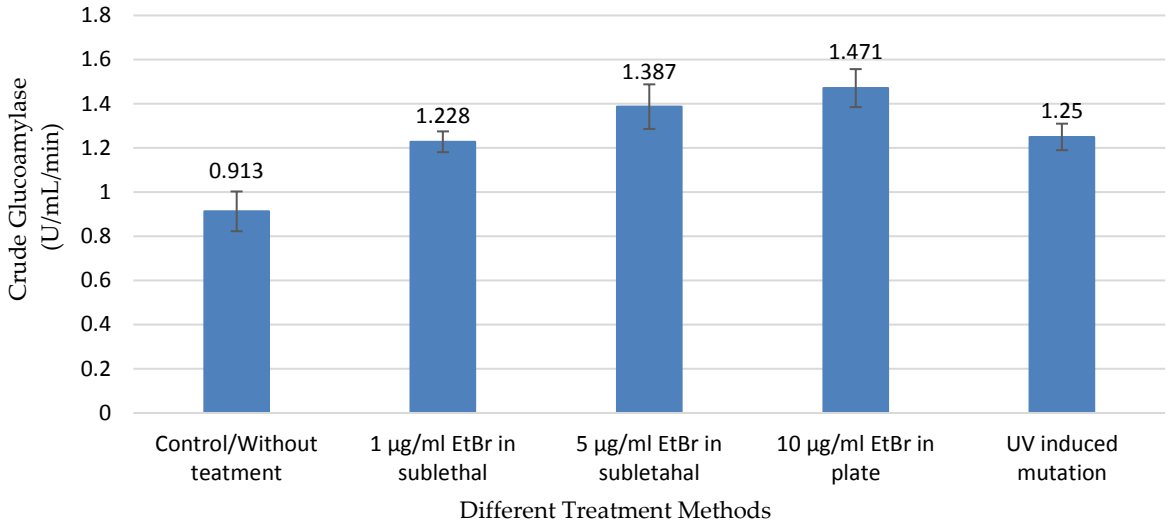

Figure 3. Crude glucoamylase activity in different treatment by chemical and physical mutagens. The highest activity was recorded from mutated *A. flavus* NSH9 by EtBr in PDA plate (10µg/mL). Error bars show standard deviation among three independent observations.

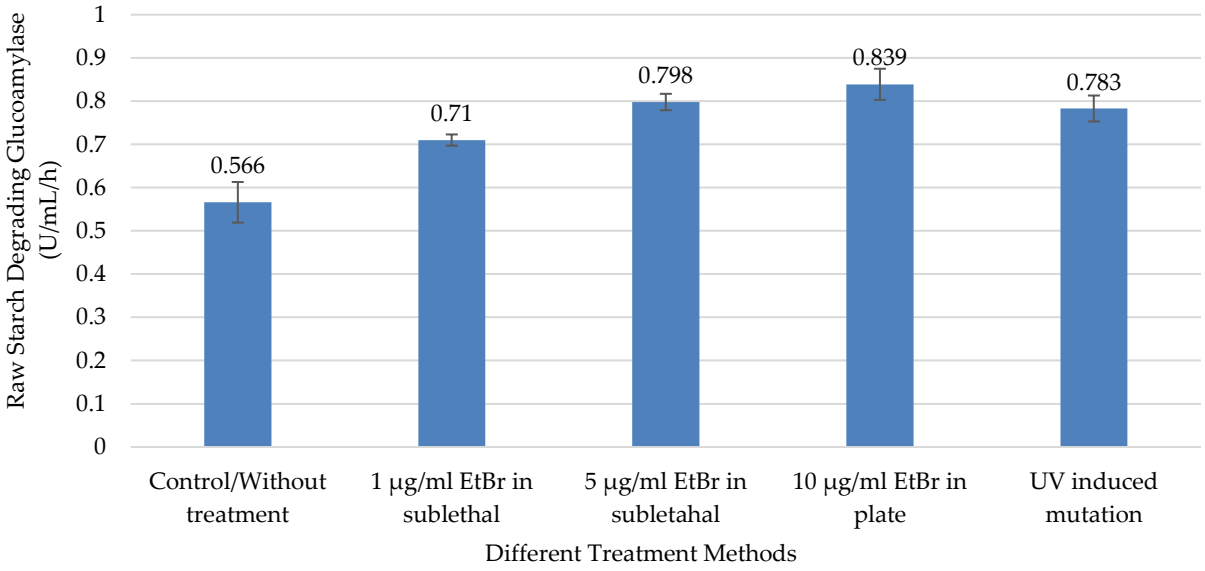

Figure 4. Raw Starch Degrading glucoamylase (RSDG) activity in different treatment by chemical and physical mutagens. The highest activity was recorded from mutated NSH9 by EtBr in PDA plate (10µg/mL). Error bars show standard deviation among three independent observations.

Effect on α-amylase

Like crude GA and RSD-GA, α-amylase production also differed significantly in various groups as found by one-way ANOVA ($p \leq 0.05$). Compared to control, all the treatment groups produced significantly higher ($p \leq 0.05$) α-amylase except the one with EtBr at low concentration (1µg/ml EtBr in sublethal condition).

The highest α-amylase activity (0.162 ± 0.011 U/mL/min) was observed with 5µg/ml EtBr in sublethal condition (Figure 5), and that was about 1.7 times higher than the control group. Meanwhile, UV treatment also produced about 1.63 times more α-amylase as compared to control (Figure 5). This highest value was significantly higher from only the control and treatment group with 1 µg/ml EtBr (0.108 ± 0.017) in sublethal condition ($p \leq 0.05$, by Tukey test).

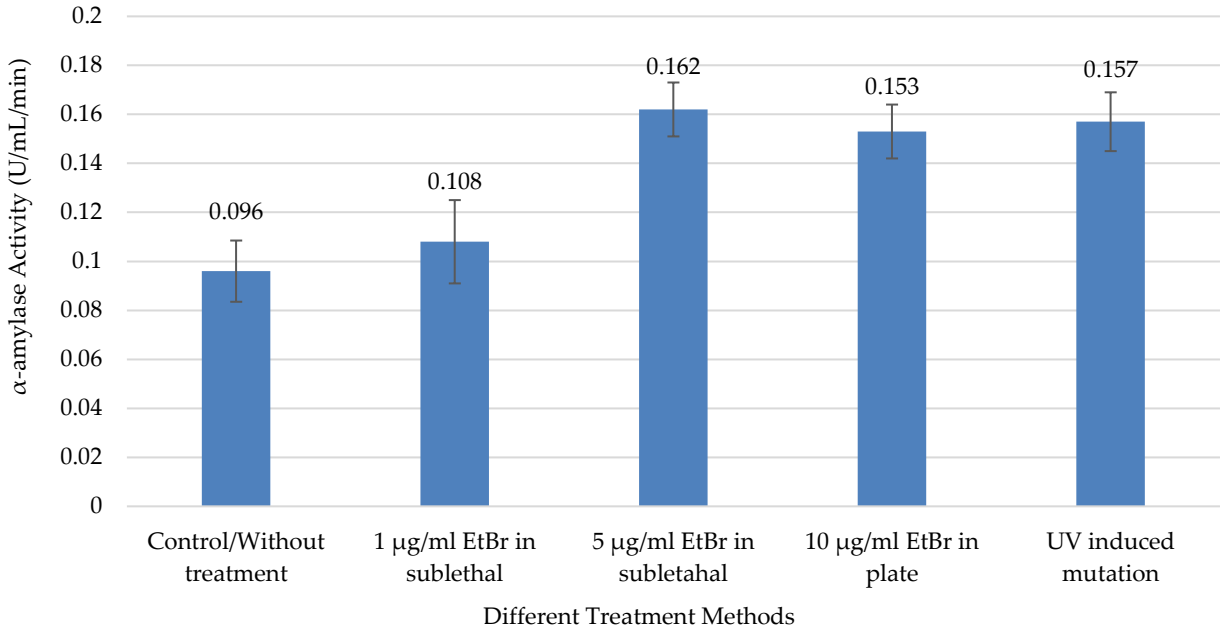

Figure 5. α-amylase activity in different treatment by chemical and physical mutagens. The highest activity was recorded from *A. flavus* NSH9 by EtBr at sublethal condition in the culture (5 µg/mL). Error bars show standard deviation among three independent observations.

DISCUSSION

Previous studies demonstrated that *A. flavus* NSH9 is a potential candidate for α-amylase, GA, and RSD-GA [10, 15-17, 28]. So, it's improvement through chemical and physical mutagens will be more industrial significance for amylases production [19]. This study shows the impact of chemical and physical mutagens on the ability of amylase production from *A. flavus* NSH9. Like previous studies, this study exhibited a significant increase in amylases (all three enzymes) production by mutagens EtBr and UV rays [25-26, 32-35]. The production of amylase (glucoamylase and amylase) by EtBr was higher compared to the UV radiation method in this study. A previous study observed that chemical mutagens (EtBr) are more capable of production glucoamylase from *Aspergillus niger* than physical mutagen (UV), which is similar to this study [32]. They also reported that combination of both the EtBr and EMS can give the best result for glucoamylase production [32]. About 1.5 times higher RSD-GA/RSDE activity was observed in this study with the EtBr treatment at 10µg/mL in PDA plate compared to wild strains, whereas a previous study recorded twofold higher RSDE from *Aspergillus* sp by combine treatments of γ-irradiation of Co^{60}, UV and NTG [34]. The higher amount of α-amylase from the mutated *A. flavus* NSH9 by EtBr at 5µg/mL in sublethal condition was found in the study, which is comparable to the previous research in sublethal cellulose production [36]. Like the previous study, the chemically mutated strain produced a higher amount of α-amylase compare to UV mutated strains [26].

The research has few drawbacks, as the study used only one stage mutation technique, whereas the combination mutation method or the sequential mutation technique would have been better suited to the selection of potent mutants for the hyper-production of the desired enzyme. The research did not examine the nucleotide sequence of the intended mutated enzyme (amylases) to know the exact mutation point of the gene sequence. The study did not measure the toxic level of EtBr while using enzyme production at sublethal concentration.

CONCLUSION

The higher production of both glucoamylases was recorded from mutated *A. flavus* NSH9 by EtBr in the PDA plate. Whereas the highest α-amylase production was recorded from the same fungi by EtBr at a concentration of 5µg/ml in the culture media. Although chemical treatment appears to have been more effective in improving the production of all amylases by fungal strain testing, UV radiation also

increased the production of all enzymes. As the single mutation process of *A. flavus* NSH9 enhanced all three amylase enzymes, the strains could be used in the industrial development of amylase, and thus could be potential sources of starch processing.

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AUTHOR CONTRIBUTIONS

KMRK proposed the original idea and reviewed the scientific contents described in the manuscript. KMRK and AR performed the experiments and analyzed the data. Both KMRK and AR wrote the initial draft and reviewed the finial manuscripts. The authors received no financial support for the research, authorship, and publication of this manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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