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Mutation of *Aspergillus* sp. using Ultraviolet Light and Nitrous Acid for Amylase Production from Banana Peels

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Abstract

Strain improvement through mutagenesis is important in the commercial development of microbial fermentation. This study aimed to produce amylase from banana (*Musa sapientum*) peels by using mutant *Aspergillus* sp. strains obtained via ultraviolet (UV) radiation and nitrous acid-based mutagenesis. Results revealed that banana peels pretreated with 0.8 N HCl had a biomass yield of 3.02 g/L and amylase activity of 2.81 U/L. Of the UV mutants, strain AUV1 had the highest biomass and amylase activity of 4.50 ± 0.21 g/L and 3.46 ± 0.14 U/L, respectively. Subsequently, HNO_2 mutants showed that strain ANA6 had 30.64% and 60.85% higher amylase activity than AUV1 and the wild strain, respectively. This study showed that banana peels can be utilized for amylase production. In addition, UV and HNO_2 are effective mutagens for *Aspergillus* sp. strain improvement to enhance amylase productivity.

Keywords: amylase, banana, fermentation, nitrous acid, mutation

Introduction

Banana (*Musa sapientum*) fruit is utilized by different industries for different industrial products, and the peels are discarded into the environment as waste [1, 2]. However, these discarded peels can cause pollution and affect the environment; they can also cause various infectious diseases by supporting the growth of pathogens [3]. Banana peels have greatly increased in amount with the use of improved cultivation techniques, and the waste generated has increased by approximately 20%–40% in high banana production regions of Africa [4]. This percentage poses serious challenges in effective management and waste disposal. These wastes are rich in organic matter that can serve as natural substrates for microorganisms [5]. Utilization of this available substrate to produce appropriate enzymes can help minimize the pollution caused by the waste [1].

Enzymes are naturally occurring proteins that act as biological catalysts for certain biochemical events and are typically produced by living cells as a component of their metabolic processes [6, 7]. The use of enzymes can help overcome the limitations of using chemical catalysts. Enzymes are highly specific, catalyze reactions faster than chemical catalysts, and work under mild conditions [8]. Enzymes, such as amylase, are widely used in several industries, including the food, brewing, detergent, distilling, textile, paper, pharmaceutical, and solid waste bioconversion industries [1, 7, 9, 10]. To meet the industrial demand, low-cost medium is required for the microbial production of amylase. The use of microorganisms for amylase production is economical because microbes are easy to manipulate to obtain enzymes of desirable characteristics [11]. Numerous studies have explored fungi that can produce amylase [12, 13, 14]. Fungi typically excrete amylase extracellularly, which make the extraction of this enzyme from fermentation media easy, and *Aspergillus* species are frequently exploited as amylase sources [15, 16]. The synthesis of amylase by the fungus *Aspergillus niger* has attracted considerable attention because of its ubiquitous nature and non-fastidious nutritional requirements [17, 18]. The amylase production capacity of *A. niger* is due to its tolerance to acidic pH that discourages bacterial contamination [18]. In addition, the temperature stability of amylase from fungi is desirable for the commercialization of enzymatic processes [19].

The demand for amylase in various biotechnological application processes has significantly increased [2]. Thus, amylase production needs to be quantitatively and qualitatively enhanced. Enzyme production can be optimized through strain improvement [20]. The

commercialization of microbial fermentation depends heavily on strain improvement through mutagenesis [10, 21]. A crucial step in the development of an efficient technology for amylase production on an industrial scale is the induction of mutation by using ultraviolet (UV) and chemical mutagenic agents [19].

UV radiation can alter the structure of DNA's pyrimidine bases, which triggers the formation of pyrimidine dimers. The few fungi that survive UV mutation can improve enzyme yield by several folds than their parental strains [22, 23]. Through duplication and deletion, chemical mutagens, such as nitrous acid (NA), also enhance the efficiency of some genes within DNA molecules. These mechanisms could upregulate the expression of genes linked to improved enzyme production in fungi [20, 24]. These two mutagens have various modes of action, all of which invariably increase the synthesis of microbial enzymes [25]. Sharma *et al.* [26] reported that a UV light mutant derivative of *A. niger* produces more lipase than the parental strain. Therefore, improving the *A. niger* strain through mutagenesis would offer a simple and effective means of producing amylase to satisfy the enormously growing demand of its industrial application [10, 26, 27]. The present study aimed to produce amylase from banana peels by using a mutant strain of *Aspergillus* sp. via ultraviolet radiation and HNO_2 treatment.

Materials and Methods

Sample collection and preparation of fermentation medium. Peels of banana of the variety Pisang Raja were obtained from a major open stall market in Benin City, Nigeria. The peels were washed several times in running tap water and then in sterile, distilled water before being sun dried. The banana peels were completely immersed in varied HCl concentrations (0.6, 0.8, 1.0, 1.2, and 1.4 N) in a ratio of 1:4 (substrate: solution) for 60 min at a 28 ± 2 °C and then washed with sterile distilled water to remove the chemicals. The pretreated banana peels were dried at 60 °C for 12 h in a hot air oven and ground into a fine powder with a blender. The ground banana peel suspension (25% w/v) was prepared using sterile distilled water. The treated substrate was filtered through muslin cloth to trap solids left in the broth. Then, 100 mL of the banana peel media with various acid pretreatments was transferred into 250 mL Erlenmeyer flasks and autoclaved at 121 °C for 15 min. Samples were prepared in triplicate and designated according to the acid concentration.

***Aspergillus* sp. isolation and inoculum preparation.** An isolate of *Aspergillus* sp. from a spoiled onion was employed. Based on the cultural characteristics and microscopic morphology, the fungal isolate was identified as previously described by Barnett and Hunter [28] and Larone [29]. The identified *Aspergillus* sp. isolate was maintained on potato dextrose agar (PDA) slants and then stored at 4 °C. A five-day-old culture of *Aspergillus* sp. was flooded with 10 mL of sterile 1% $^{v}/_{v}$ Tween 80 solution to loosen the spores from the hyphae. The spore solution was filtered through a muslin cloth to remove any hyphal pieces that might have been present [30]. A hemocymeter was used to count the quantity of spores, and 10^6 spore/mL of inoculum was utilized to inoculate the media.

Exposure of *Aspergillus* sp. spores to UV irradiation. A spore suspension of *Aspergillus* sp. from a 5-day-old PDA culture was prepared in 0.1 M phosphate buffer, and 10 mL portions of the spore suspension were transferred into sterile flat bottom 80 mm Petri dishes. The exposure to UV irradiation was carried out in a "Dispensing–Cabinet" equipped with a TUP 40W Germicidal lamp, which emits 90% of its radiation at 2540–2550 Å [31]. With intermittent shaking, the exposure was carried out at 20 cm distance away from the germicidal lamp (UV light source) center. Exposure times ranged from 0, 30, 60, 90, 120, 150, and 180 min. Each UV-exposed spore suspension was stored in the dark for an entire night to prevent photoreactivation. It was then serially diluted in phosphate buffer and plated on PDA medium. The plates were incubated for 5 days at 28 °C, and the numbers of colonies in each plate were counted. From the plates that showed less than 1% survival rate, seven colonies (designated as AUV1–AUV7) were selected randomly and tested for amylase activity.

Mutation of *Aspergillus* sp. spores with HNO_2. The mutant strain (UV treated) with the highest amylase-producing ability was further exposed to HNO_2 following the method described by Karanam and Medicherla [32] with modifications. Then, 1 mL of sterile stock solution of 0.01 M sodium nitrate was transferred to 9 mL of 10^6 dilution of UV-treated *Aspergillus* sp. spore suspension. From the aliquots, 1 mL was withdrawn after 15, 30, 60, and 90 min of chemical treatment. Phosphate buffer (0.5 mL) was added to each sample, and 0.1 M NaOH was used to neutralize the NA. The exposed suspension (0.1 mL) was plated on PDA medium, and the suspension was uniformly distributed using a sterile spreader. Each HNO_2 exposed spore suspension was stored overnight to avoid photo reactivation. The plates were incubated at 28 °C for 5 days. A total of 10 colonies (designated as ANA1–ANA10) were selected randomly from the plates with less than 1% survival rate. The mutant strains selected based on their morphology, size, and shape were further streaked on PDA plates and then incubated at 28 °C for 5 days. The treated spores (HNO_2) were used for fermentation and assayed for amylase activity.

Fermentation. Submerged fermentation was carried out at 28 ± 2 °C on an orbital shaker at a speed of 120 rpm using the banana peel media with various acid pretreatments. Fermentation was carried out using the acid pretreated BP media for 10 days. The medium in each flask was inoculated with 500 µL of *Aspergillus* sp.

inoculum (10^6 spores/mL). All media were placed on an orbital shaker set to ferment at 28 ± 2 °C and 120 rpm. Fungal biomass and amylase production were determined every 2 days for 10 days.

The mutant strains produced by the mutagenic chemicals were examined using the medium with the highest amylase yield. The same fermentation process was carried out, but fungal biomass and amylase production were determined after 8 days of fermentation.

Analytical methods. Fungal biomass and amylase production were determined every 2 days for 10 days. At the interval of 2 days, the fermenting broth was pasteurized in a water bath at 65 °C for 30 min. The fungal mycelia were collected through filtration using a pre-weighed Whatman No 1 Filter paper and washed twice with 50 mL of sterile distilled water. A Genlab hot air oven (YIA 110 model, England) was used to dry the collected fungal biomass on the filter paper at 90 °C to a constant weight [33].

In accordance with the method described by Ramakirshna *et al*. [34], amylase activity was assayed by measuring the amount of glucose released from starch using a reaction mixture comprising 1 mL of crude enzyme, 1 mL of 1% starch solution, and 0.1 mL of citrate buffer solution (pH 4.5). Incubation was performed at 60 °C for 1 h, and the reaction was stopped by immersing the reaction tube in water at 100 °C for 2 min. The liberated reducing sugars were evaluated using the 3,5-Dinitrosalicylic acid (DNS) method [35]. One unit of amylase activity (U) was defined as the amount of enzyme that liberated 1.0 mole of D-glucose from starch in a 1.0 L reaction mixture under the assay conditions.

All assays were carried out in triplicates, and means and standard deviations were determined using SPSS version 23. Amylase production data from the different media were compared using t-test.

Results

Fungal biomass of *Aspergillus* sp. among the various concentrations of hydrochloric acid (HCl) is shown in Figure 1 and peaked after 8 days of fermentation. The banana peels pretreated with 0.8 and 1.4 N HCl had the highest and least biomass of 3.02 ± 0.07 and 2.45 ± 0.07 g/L, respectively. A significant difference in fungal biomass was found between the 0.8 N HCl pretreated medium and the other media ($p < 0.05$).

The amylase activities of the banana peel media with different concentrations of HCl pretreatment are shown in Figure 2. The highest activity was obtained on day 8 of fermentation. The amylase activity from the different concentrations of HCl acid pretreatment methods revealed that 0.8 N induced the highest activity, followed by 0.6 N, and the least was induced by 1.4 N HCl acid, with values of 2.81 ± 0.07, 2.62 ± 0.09, and 2.02 ± 0.17 U/mL, respectively, on day 8. A significant difference in amylase activity was found between the 0.8 N HCl pretreated medium and the other media ($p < 0.05$).

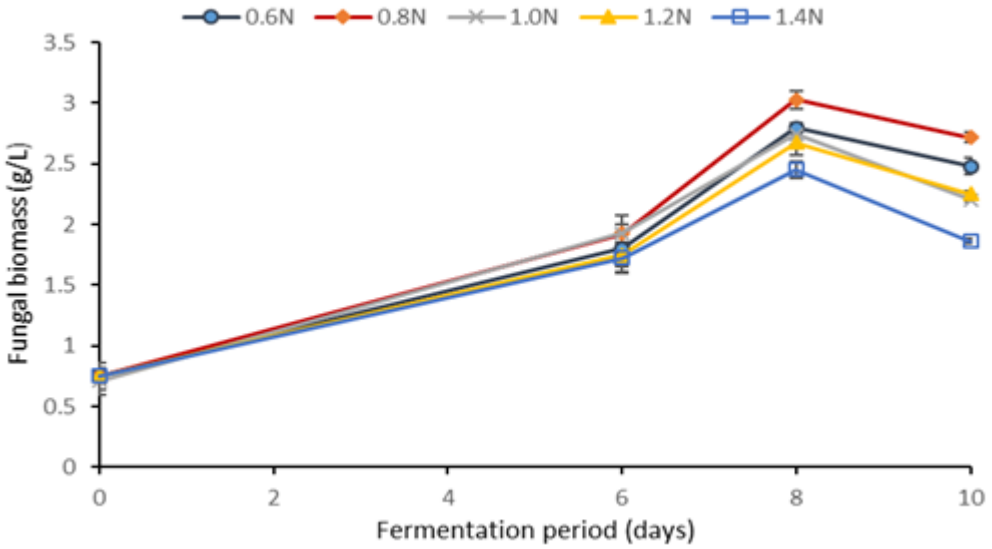

Figure 1. Fungal Biomass of *Aspergillus* sp. using Different Concentrations of Acid Pretreated Banana Peel Media in Submerged Fermentation at 120 rpm on a Time Course Basis. Values are the Mean of Triplicate Experiments, and Error Bars Represent the Standard Deviation

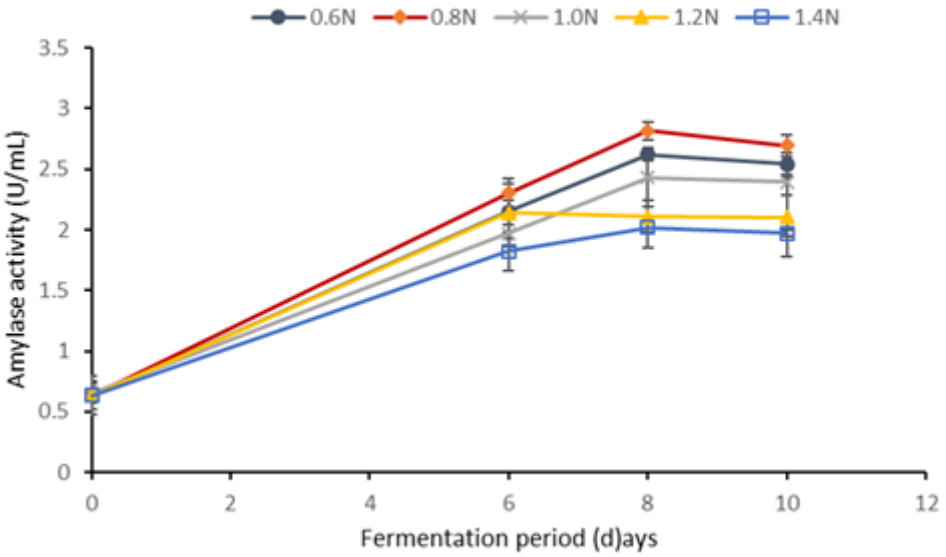

Figure 2. Amylase Yield of *Aspergillus* sp. using Different Concentrations of Acid Pretreated Banana Peel Media in Submerged Fermentation at 120 rpm on a Time Course Basis. Values are the Mean of Triplicate Experiments, and Error Bars Represent the Standard Deviation

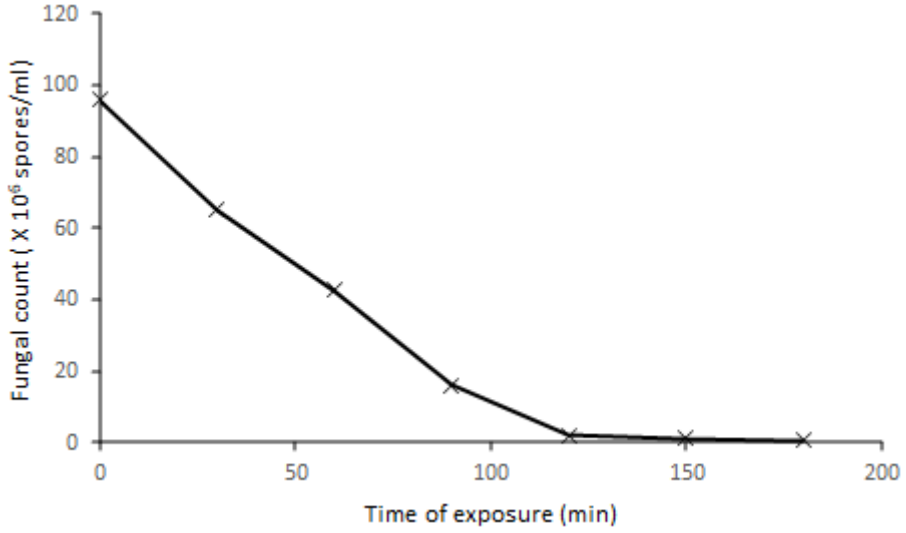

Figure 3. Kill Curve of *Aspergillus* sp. Spores Exposed to UV Irradiation

Table 1. Fungal Biomass and Amylase Yield of UV-exposed *Aspergillus* sp. in Banana Peel Media Pretreated with 0.8 N HCl in Submerged Fermentation at 120 rpm for 8 Days. Values are the Mean and Standard Deviation of Triplicate Experiments

Mutant strain	Fungal biomass (g/L)	Amylase activity (U/mL)
AUV1	4.50 ± 0.21	3.46 ± 0.14
AUV2	4.36 ± 0.04	3.16 ± 0.08
AUV3	4.15 ± 0.12	3.08 ± 0.11
AUV4	4.12 ± 0.23	3.04 ± 0.11
AUV5	4.06 ± 0.02	3.00 ± 0.12
AUV6	4.19 ± 0.01	3.11 ± 0.11
AUV7	4.35 ± 0.07	3.13 ± 0.17
Wild type	3.03 ± 0.01	2.81 ± 0.21

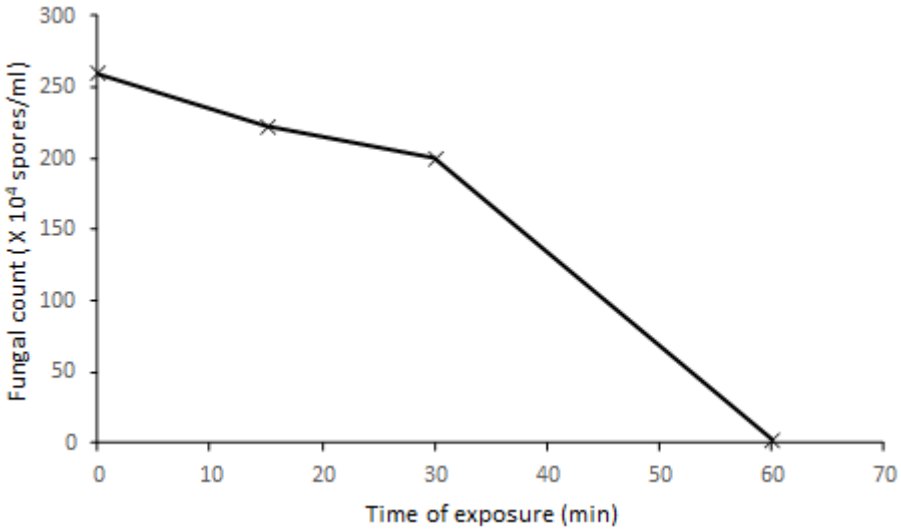

Figure 4. Kill Curve of *Aspergillus* sp. UV Mutant Strain (AUV1) Exposed to HNO_2 Mutagen

Table 2. Fungal Biomass and Amylase Yield of UV + HNO_2-exposed *Aspergillus* sp. in Banana Peel Media Pretreated with 0.8 N HCl in Submerged Fermentation at 120 rpm for 8 days. Values are the Mean and Standard Deviation of Triplicate Experiments

Mutant strain	Fungal biomass (g/L)	Amylase activity (U/mL)
ANA1	5.95 ± 0.20	4.36 ± 0.18
ANA2	5.90 ± 0.29	3.56 ± 0.30
ANA3	5.56 ± 0.25	4.01 ± 0.28
ANA4	5.39 ± 0.34	3.98 ± 0.34
ANA5	5.31 ± 0.37	3.46 ±0.26
ANA6	6.38 ± 0.14	4.52 ± 0.19
ANA7	5.88 ± 0.27	4.26 ± 0.20
ANA8	5.73 ± 0.04	3.55 ± 0.21
ANA9	5.45 ± 0.43	3.96 ± 0.33
ANA10	5.35 ± 0.51	3.95 ± 0.27
AUV1	4.50 ± 0.21	3.46 ± 0.14

The survival rate during the *Aspergillus* sp. spore exposure to UV radiation is shown in Figure 3. A survival rate of less than 1% was obtained after 180 min of exposure. From the plate, seven mutant strains were randomly selected and labeled as *Aspergillus* sp. UV mutant strains (AUV1–AUV7), fermented, and then analyzed for their fungal biomass and amylase activity.

The 0.8 N HCl pretreated medium was further used in the fermentation of the various mutant strains. Th fungal biomass and amylase activity of UV *Aspergillus* sp. mutant strains (AUV1–AUV7) and wild type are presented in Table 1. Among the UV mutant strains, AUV1 had the highest fungal biomass and amylase activity of 4.50 ± 0.21 g/L and 3.46 ± 0.14 U/mL, respectively, after 8 days of fermentation. The fungal biomass and amylase activity of the AUV1 mutant strain were significantly different ($p < 0.05$) from those of the wild type.

The *Aspergillus* sp. UV mutant strain (AUV1) with the highest yield of biomass and amylase was further subjected to HNO_2 treatment. The survival rate during the exposure time is shown in Figure 4. The less than 1% survival rate of AUV1 treated with HNO_2 was obtained after 60 min. From the plate, 10 mutant strains were randomly selected and labeled as *Aspergillus* sp. UV + HNO_2 mutant strains (ANA1–ANA10).

Table 2 shows the fungal biomass and amylase activity of *Aspergillus* sp. UV + HNO_2 mutant strains (ANA1–ANA10) and UV-exposed *Aspergillus* sp. (AUV1). Among the mutant strains, ANA6 had the highest fungal biomass and amylase activity of 6.38 ± 0.14 g/L and 4.52 ± 0.19 U/mL, respectively, after 8 days of fermentation. The fungal biomass and amylase activity of ANA6 were statistically significantly different ($p < 0.05$) from those of AUV1.

Discussions

Banana peels were selected for this research as a potential substrate for amylase production. Banana peel is a good substrate for microbial growth and amylase production because of its nutritional composition, such as carbohydrate and protein [5]. Pretreatment with acid is a crucial step in the microbial fermentation of lignocellulosic materials for the synthesis of enzymes [6]. The varied number of fermentable sugars generated after various pretreatments may be the cause of the variance in fungal growth in the banana peel media treated with different concentrations of acid [5]. Fermentation is dependent on the ability of *Aspergillus* sp. cell sugar transporters to transport various sugars across the cell membrane from the medium [36]. As a result, the fungal biomass and amylase activity were highest in the banana peels pretreated with 0.8 N HCl. The results of this experiment demonstrated that banana peel fermentation with 0.8 N HCl successfully produced large quantities of fermentable sugars for amylase synthesis. This is due to the fact that the fungal cells in the acid pretreated media have comparatively high favorable conditions for effectively using the available sugars for enzyme productivity.

The highest fungal biomass and amylase activity in the study peaked on day 8 of fermentation and then decreased. Fermentation duration is a critical factor during amylase production [37]. Fungal biomass and enzyme production increase as the growth time progresses and then decrease after the optimum duration because of nutrient depletion in the medium [8]. Our results agreed with the report of Singh *et al*. [19] that *Aspergillus fumigatus* NTCC1222 produces the highest yield of amylase after 6 days of fermentation. The difference in the high yield day of fungal biomass and amylase activity could be due to the inoculum size, growth medium source, and composition of the growth medium. The decline in biomass and amylase yield after 8 days of fermentation could be attributed to nutrient exhaustion, substrate inhibition, and enzyme denaturation due to possible interactions with other medium components used [19, 38].

The metabolic activity of microorganisms during fermentation is influenced by medium composition and microbial cell mutation invariably [38]. For maximum microorganism efficiency, the focus should be on strain improvement through the use of mutagenic agents [24]. Genetic variation is an ideal optimization process for metabolite production. The influence of UV on the survival of *Aspergillus* sp. exposed for different times was studied. A survival rate of less than 1% was obtained after 180 min of *Aspergillus* sp. exposure to UV radiation. The survival population of microbes after UV exposure varies depending on the strain under investigation [22].

Mutant strains from the UV treatment showed an improvement in fungal growth and enzyme production. Among the mutant strains, AUV1 produced the highest fungal biomass and amylase activity. UV radiation alters the DNA base (cytosine and thymine) structure, resulting in the formation of pyrimidine dimers. Dimers influence the pattern of the DNA double helix strands, which improves the biocatalytic performance [23]. When compared with the wild strain, AUV1 had a 23.13% higher amylase activity. This result is consistent with the findings of Singh *et al.* [19] that the UV mutant strain *A. fumigatus NTCC1222* has a 44.52% higher amylase activity than the parent strain. This finding suggests that UV treatment improves amylase production in isolates exposed to it. In the present study, AUV1 was chosen for further strain improvement using HNO_2.

Strain improvement via mutation using HNO_2 can induce the production of enzymes, such as amylase [20], and this is an optimization parameter for a cost-effective production of enzymes [39]. After 60 min exposure, the *Aspergillus* sp. spores with HNO_2 treatment had less than 1% survival rate of the isolate. A high yield of fungal growth was obtained using various strains; however, ANA6 had the highest growth on day 8 of fermentation. HNO_2 is thought to be a highly effective mutagen [20]. The HNO_2 treatment of the isolates improved the biomass yield and amylase activity more than the control (AUV1) strain. The mutant strains increased fungal biomass and amylase activity more than the control strain (AUV1). This result is due to the increased metabolic activity of the strains. This result implies that the mutants' ability to produce enzymes varies when exposed to different mutagens [24]. This pattern is expected to be observed in the amylase activities of the mutant strains.

Among the strains, ANA6 had the highest amylase activity. Inducing mutagenesis can increase fungal amylase production. This phenomenon can be ascribed to the fact that HNO_2 altered the structure of DNA. The structural alteration in DNA is associated with the activity of the enzyme, probably promoting mycelial growth [40]. Mutant strains with HNO_2 treatment showed an improvement in enzyme production. This result could be because the DNA structure was changed by replacing the amino group with the hydroxyl group. HNO_2 promotes the deamination of cytosine to uracil and adenine to hypoxanthine, thereby altering the DNA structure [20, 24]. Furthermore, ANA6 showed the highest biomass and amylase yield among the strains. The amylase activity of ANA6 was 30.64% higher than that of AUV1 and 60.85% higher than that of the wild type. Sharma *et al.* [20] found that mutant strains exposed to HNO_2 for 90 min have 116% higher amylase activity than the wild strain.

Conclusions

Banana peels can be used for amylase production. Improved strains can reduce costs and increase product yield. The combination of UV and HNO_2 mutation was the most effective mutagen for *Aspergillus* sp. strain improvement of amylase productivity enhancement. The high-yielding *Aspergillus* sp. mutant strain (ANA6) can be commercially exploited for large-scale amylase production. This study contributes to the development of an *Aspergillus* sp. mutant strain that can utilize banana peels to produce amylase, thereby reducing pollution hazards caused by this waste.

Conflict of Interest

The authors declare no conflict of interest.

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