

Isolation and Screening of *Aspergillus niger* Mutant for Improved Gluconic Acid Production

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Abstract: Gluconic acid (GA, C₆H₁₂O₇, or 2, 3, 4, 5, 6-pentahydroxyhexanoic acid) is a mild organic acid resulting from the oxidation of D-glucose. Gluconic acid has low toxicity, low corrosion and form complexes with metal ions giving it wide application in several industries. This study aimed to isolate and screen for *Aspergillus niger* mutant for improved gluconic acid production. A total of 204 *A. niger* were isolated from different fruit and soil samples. Ninety-two (45%) out of the 204 isolates showed organic acid production potentials using bromocresol purple plate and gluconic acid production potentials using calcium carbonate agar plate. Ten out of the ninety-two (92) isolates were selected for strain improvement using UV mutation technique. After exposure to ultraviolet (UV) light of 254 nm at different time intervals of 2, 4, 6, 8, 10, 12 minutes respectively, *A. niger* AEAJP-2.110(PX939154) had the highest strain improvement for gluconic acid than *A. niger* AEAJP-2(PX954262), its wild type was exposed to a two-step UV light for 2 minutes with a yield increase from 54.1g/ml to 259.7g/ml (4.8-fold increase over the wild strain). In conclusion, this study established that UV light is a potent tool for enhancing gluconic acid production in *A. niger*.

Key word: *A. niger*, Gluconic acid, Mutation, Ultraviolet light

INTRODUCTION

Gluconic acid (GA, C₆H₁₂O₇, 2, 3, 4, 5, 6-pentahydroxyhexanoic acid) is the oxidation product of glucose, naturally occurring in plants, fruits, wine (up to 0.25%), honey (up to 1%), rice, meat, vinegar and other natural sources (Anastassiadis and Morgunov, 2007). The physiological D-form of gluconic acid is usually formed by the microbial oxidation of glucose (Anastassiadis and Morgunov, 2007). It is a mild organic acid and a bio-based additive that has gained much interest as it has many industrial applications such as in the food, animal feed, medicine, textile, pharmaceutical and leather industries (Ramachandran *et al.*, 2017). It is an organic acid derived from the oxidation of glucose and has been explored as an additive in concrete production due to its chelating and pH-regulating properties. Gluconic acid is a versatile organic acid, which is non-corrosive, mildly acidic, less irritating, odourless, non-toxic, and easily degradable. Gluconic acid is an aldonic acid derived from β-D-glucose via a site-specific oxidation, in which the aldehyde group (-CHO) at C-1 is oxidized electrochemically or catalytically into a carboxyl group (-

COOH). It is readily soluble in water with pKa of 3.70 at 25°C (Ma *et al.*, 2022).

The biotechnological production of gluconic acid is primarily achieved using microbial fermentation, with filamentous fungi such as *Aspergillus* and *Penicillium* species playing significant roles (Anastassiadis and Morgunov, 2007). Although, many organic acids can be produced by living cells, only few are commercially manufactured via large-scale bioprocesses mostly employing fungi, such as citric, gluconic, itaconic, and lactic acid (Anastassiadis and Morgunov, 2007). Citric acid and gluconic acid are the largest commercially produced of fungal organic acids, from glucose or sucrose employing selected strains of *Aspergillus niger* (Anastassiadis and Morgunov, 2007).

A significant bottleneck remains in the production which is the low yield of wild-type strains, necessitating robust strain improvement strategies. The present study was thus, aimed at isolating and screening *Aspergillus niger* mutant with physical treatment (ultraviolet rays at different time intervals) for improved gluconic acid production and the characterization of the fermentation broth.

MATERIALS AND METHODS

Sample Collection: Soil samples were collected from University of Ibadan environs (Jaja, Gate, Research and Teaching farm, Botanical garden, Awo and Microbiology Department), Federal School of Statistics (FSS) environs, Plantain plantation at International Institute of Tropical Agriculture (IITA), Sugar plantation behind Odogbo barracks, Ojoo and Plantain plantation at Akinyele. All the sampling sites are within Ibadan in Oyo State, Nigeria. Fruit samples (both fresh and rotten) were collected from different areas within Ibadan metropolis (Moniya, Bodija, Ojoo, Agbowo and Ajibode) in Oyo State, Nigeria.

Isolation of *Aspergillus niger* from the Samples: Isolation of moulds from soil and fruit samples (Banana, apple, watermelon, pineapple and sugar cane) was done according to the methods of Singh *et al.* (1999) and Olutiola *et al.* (2000). From each sampling point, approximately 1 g of superficial soil (2 cm deep) was subjected to successive dilutions (1×10^{-1} - 1×10^{-5}) and 100 μ l aliquots was plated onto potato dextrose agar (PDA, LAB M) plates containing chloramphenicol (250 mg/l to suppress bacterial growth. The plates were incubated at 30°C and monitored daily for 14 days to isolate filamentous fungi. The isolated colonies were purified and plated on potato dextrose agar (PDA) and incubated at 30°C for seven days. These pure cultures were then stored in a refrigerator at 4°C during storage and after the procedure of Olutiola *et al.* (2000).

Morphological and Microscopic Characterization of the Isolated Fungi: Morphological identification and microscopic characteristics of the mould as carried out according to the method of Gautam and Bhadauria (2012). Macroscopic features like colony colour, texture and margins, as well as microscopic such as size of conidia and conidiophores and their arrangements were examined. A drop of 0.1% lactophenol cotton blue was placed on a clean slide. Then using strong sterile straight wire, a piece of actively growing

mould culture was removed and placed in the lactophenol stain carefully and slowly. It was covered with cover slip, taking care to remove air bubbles, and this was achieved by using the straight wire to slowly lower the cover slip. Then excess of stain flowing out the cover slip was removed with the help of a blotting and/or filter paper. The preparation was first examined under the low power of the microscope ($\times 40$) and then the high power ($\times 100$) according to the method of Ogbo (2005).

Screening of Isolates for Organic Acid Production: The fungi isolates were qualitatively screened for organic acid production under nitrogen-limited growth conditions in a medium with the following composition (g/l): Glucose 80 g, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g, KH_2PO_4 1.7, Na_2HPO_4 12, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4, CaCl_2 0.02, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.02, yeast extract 0.5, initial pH was 6.0, and sterile bromocresol blue (32 mg/l) was added post-autoclave as a pH indicator. This was cooled and inoculated with a disc of 8 mm diameter of the organism from 5 days old PDA culture plates using a sterile cup borer and incubated at 30°C for 48 hours. The diameters of the developed yellow zones around the fungal growth were measured and recorded. The change in colour of bromocresol purple from purple to bright yellow indicated organic acid activity. The fungal isolates with high yellow zones around their colonies, indicating acid production, were selected for gluconic acid screening (Sudarkodi *et al.*, 2012).

Primary Screening of Isolates for Gluconic Acid Production: The medium which was used for preparation of calcium carbonate plates contained, glucose, 10 g; $(\text{NH}_4)_2\text{HPO}_4$, 0.4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; KH_2PO_4 , 0.2 g; CaCO_3 , 50 g and agar 20 g dissolved in one litre of double distilled water and autoclaved at 121°C for 15 min. Calcium carbonate was sterilized separately and was added to medium at the time of plating. This was cooled and inoculated with a disc of 8 mm diameter of the organism from 5 days old PDA culture plates using a sterile cup

borer and incubated at 30°C for 5 days. Clear zones were formed around distinct colonies on the plates indicating the production of calcium gluconate. The *Aspergillus niger* producing clear zones were selected. The isolated strains were maintained on PDA slants at 4°C by subculturing at monthly interval (Singh *et al.*, 1999; Arotupin and Olaniyi, 2013).

Secondary Screening of Isolates for Gluconic Acid Production

(Spectrophotometric Method): The medium which was used for this preparation contained, glucose, 10 g; (NH₄)₂ HPO₄, 0.4 g; MgSO₄·7H₂O, 0.2 g; KH₂PO₄, 0.2 g; CaCO₃, 50 g dissolved in one litre of double distilled water and autoclaved at 15 lb for 15 minutes. Calcium carbonate was sterilized separately and was added to the medium at the time of plating. 10 ml of this medium was inoculated with a disc of 8 mm diameter of the organism from 5 days old PDA culture plates using a sterile cup borer and incubated at 30°C for 5 days (Singh *et al.*, 1999; Arotupin and Olaniyi, 2013).

Physical Treatment of the Selected *Aspergillus niger* for Gluconic Acid Production using Ultraviolet light Mutation Technique:

A two- step physical UV-ray mutagenesis was carried out by exposing the spore suspension of the selected filamentous fungi (10 ml 1×10⁹ spore/ml in Petri dish by UV illumination (2.5J/m²/s, 0.69 m) at different times ranging from 2 minutes to 12 minutes (Singh *et al.*, 2001).

Spectrophotometric Determination of Gluconic Acid:

A sample containing 0.05 to 0.5 millimole of gluconate ion was treated with 15 ml of 0.1 M copper sulfate solution. After addition of 10 ml of 2.5 N sodium hydroxide, the solution was boiled for 5 minutes, cooled to room temperature (25°C) and diluted to 50 ml. Part of this solution was centrifuged at about 2000 rpm for 15 to 20 minutes in stoppered centrifuge tubes. The absorbance of the mixture was measured at 660 nm using a spectrophotometer JENWAY: Model 6405, UK using 0.5 M sodium hydroxide solutions as the blank (Alt, 1955).

Characterization of the Gluconic Acid: The characterization of the gluconic acid was carried out using Fourier Transform Infrared (FTIR) Spectroscopy to confirm the presence of the gluconic acid in the fermentation broth (Worku, 2018).

Statistical Analysis: The statistical analysis of data obtained was carried out by taking the means of the triplicate and getting the standard deviation using SPSS 23.

RESULTS

Two- hundred and four (204) *Aspergillus niger* were isolated from the samples (soil and fruits) obtained for this research. One hundred and fifty-seven (157) of these organisms were obtained from soil samples from 5 different locations, while forty-seven (47) were isolated from fruit samples. The number of organisms from soil samples from the different locations are: IITA (22) UI (71) FSS (30), APP (20) and SCPO (14). The number of gluconic acid producing *Aspergillus niger* as compared with the total number of *Aspergillus niger* from these samples is shown in Figure 1. The % distributions of the gluconic acid producers from the different soil locations included 4(64%), 33(46%), 12(40%), 2(10%) and 9(64%). Also, 2(47%) out of the 47 *Aspergillus niger* isolated from the different fruit samples, had ability of producing gluconic acid. Figure 2 also shows the frequency of occurrence of gluconic acid producing organisms from samples obtained from different locations. The finding shows that most of the gluconic acid producing *Aspergillus niger* were obtained from UI soil (36%), followed by fruit samples (24%) and then least for APP soil sample (2%).

Plate 1. Shows isolation of fungi cultures on potatoes dextrose agar plates. Plate 2A shows the production of organic acid on mineral salt agar medium (with bromocresol blue), while Plate 2B shows the picture of uncultured mineral salt agar plate (with bromocresol blue). Plate 3. shows the clear zone formed by some of the wild-type and mutant *A. niger* strains on calcium carbonate agar plate, indicating gluconic acid

production. Figure 3. shows the screening of the selected *Aspergillus niger* isolates for organic acid and gluconic acid production. The quantities of the gluconic acid produced after screening for organic acid and gluconic acid production is shown in Fig 4. Isolate S₇A produced the highest gluconic acid of 100 g/l while isolate F₅B had lowest yield of gluconic acid of 49.1 g/l *Aspergillus niger* AEAJP-2 produced 54.1 g/l of gluconic acid. Figure 5 shows physical treatment of organisms with UV light to generate mutants of the wild type *Aspergillus niger*. The finding shows that *Aspergillus niger* AEAJP-2 exposed to UV light for 2 minutes had the highest gluconic acid yield of 131.4 g/l which was followed by isolate S₇A exposed to UV light for 8 minutes (123.1 g/l). The lowest yield of gluconic acid of 88.1 g/l as produced by S₅B exposed to UV light for 6 minutes. Figure 6 shows the finding of the second step exposure of the selected mutants from the first exposure that had higher gluconic acid production potentials.

The finding shows that *Aspergillus niger* AEAJP-2 after the second exposure at 2 minutes had the highest gluconic acid production of 259.7 g/l. This was followed by isolate F₅B which was exposed to second UV light at 6 minutes with a gluconic acid

value of 243.4 g/l. The lowest production was 177 g/l by isolate FSS₅ exposed to second UV light at 10 minutes.

The characteristics of the gluconic acid by the fungal isolates are shown in Figure 7 which the functional groups associated with gluconic acid. Table 1 shows the FTIR spectrum of purified fermentation broth with peak areas and functional groups.

The molecular characterization was carried out on the mutant with the highest gluconic acid improvement from the use of 2-step exposure of wild *Aspergillus niger* to Ultraviolet rays for 2 minutes at each step. Table 2 shows that the mutant is *Aspergillus niger* AEAJP-2.110 (PX939154), while it's corresponding wild *Aspergillus niger* is *Aspergillus niger* AEAJP-2(PX939154).

The finding for the phylogenetic relatedness of *Aspergillus niger* AEAJP-2 and *Aspergillus niger* AEAJP-2.110, *Aspergillus niger* AEAJP-2 and *Aspergillus niger* AEAJP-2.110 are shown in Figure 8-10.

Figure 9 and 10 shows the transmission electron microscopy of *Aspergillus niger* AEAJP-2 and *Aspergillus niger* AEAJP-2.110 with the highest gluconic acid production achieved with physical treatment (Ultraviolet ray at 2 minutes 2 step exposure process) at different wavelength of 20 µm, 50 µm and 100 µm respectively.

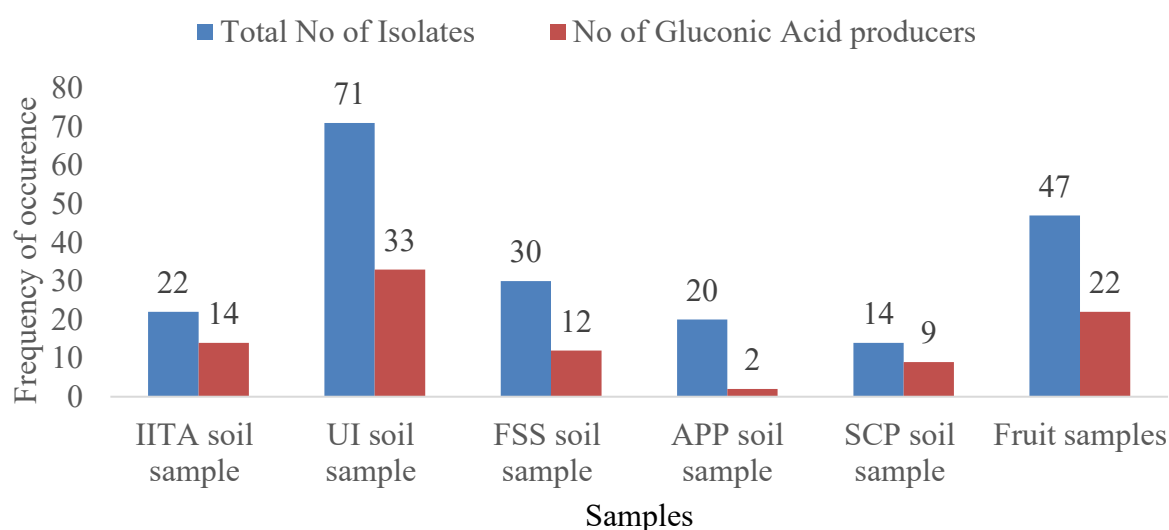


Figure 1: Distribution of Gluconic Acid Producers across Different Locations of Sample Collection

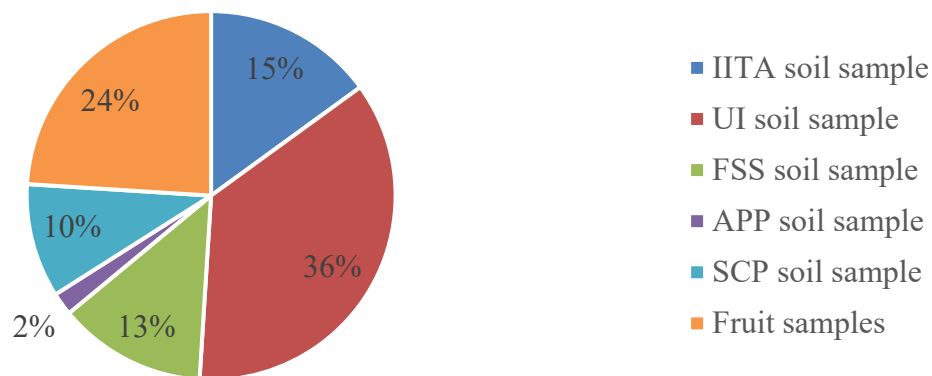


Figure 2: Frequency of Occurrence of Gluconic Acid Producing *Aspergillus niger* from Different Samples

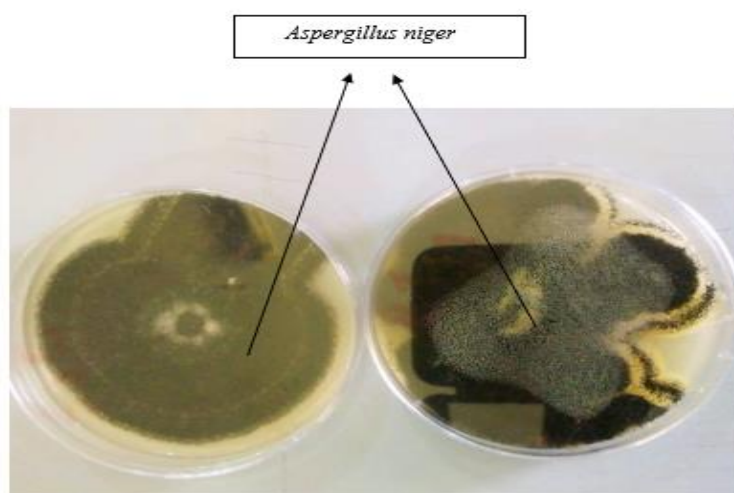
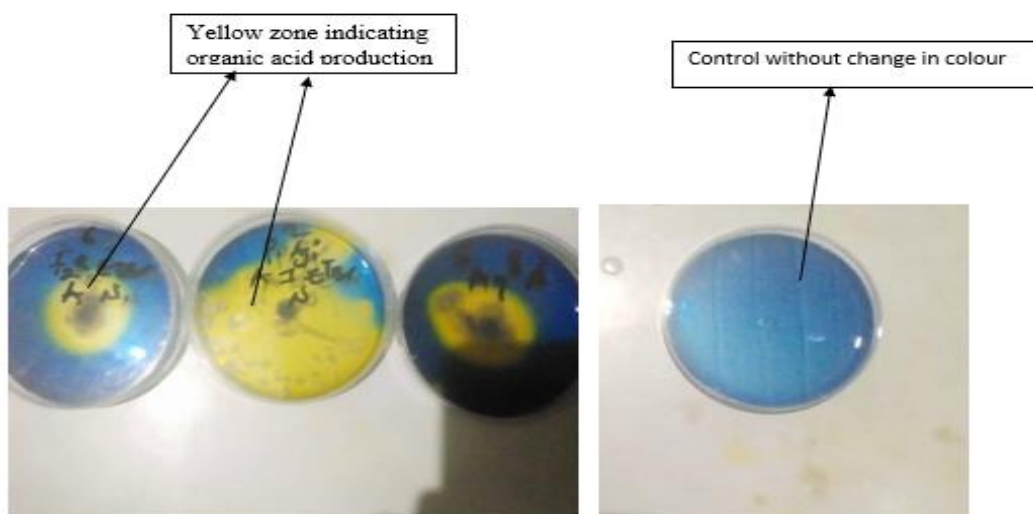


Plate 1. Culture of *Aspergillus niger* on Potato Dextrose Agar Plates (Magnification: 7.5)



A

B

Plate 2A: Yellow Zone Formation on Bromocresol Blue Mineral Salt Agar Medium Indicating Organic Acid Production, while Plate 2B Control (2A):4; Magnification (2B): 4.4)

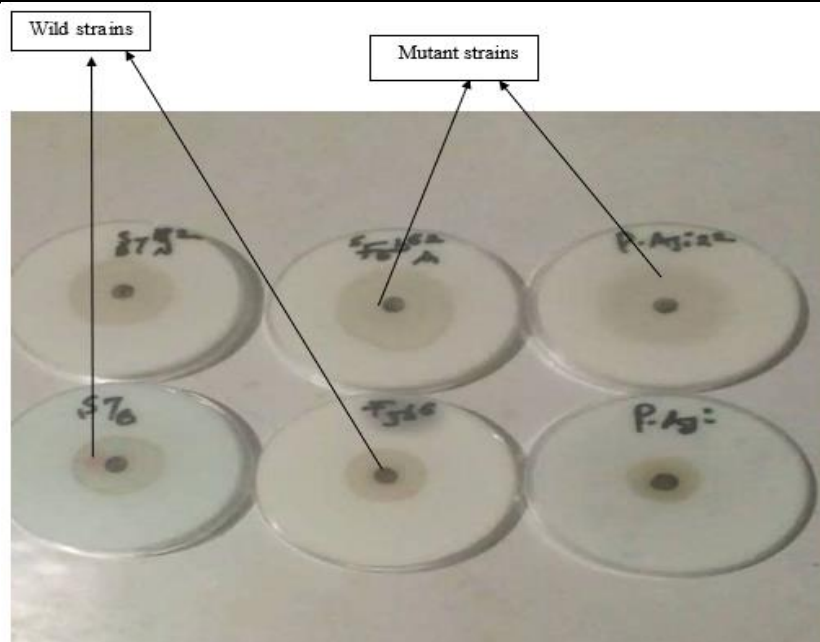


Plate 3: Clear Zone Formation by Wild-type and Mutant *A. niger* Strains on Calcium Carbonate Agar Plate, Indicating Gluconic Acid Production (Magnification: 6.6)

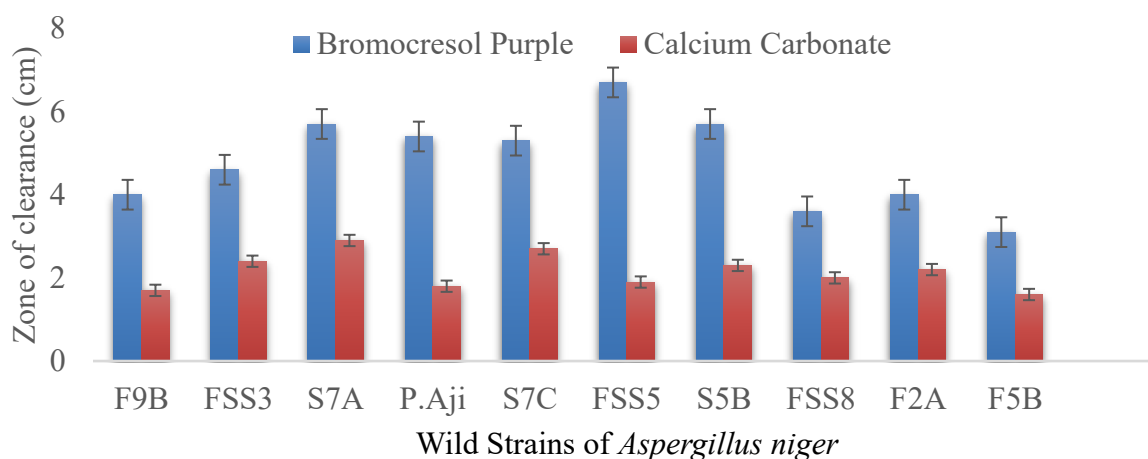


Figure 3: Wild Strains of *Aspergillus niger* Isolates for Organic Acid and Gluconic Acid Production

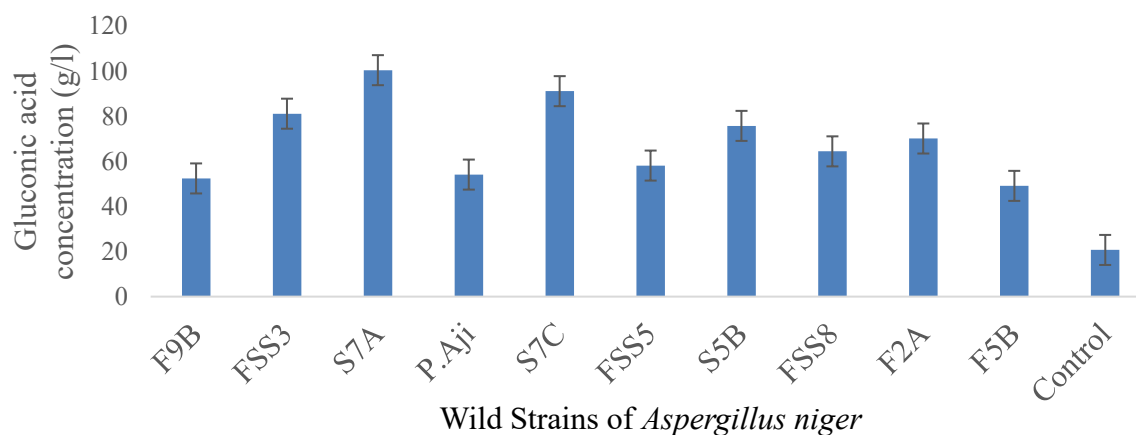


Figure 4: Wild Strains *Aspergillus niger* Based on the Quantity of Gluconic Acid Produced before Mutation

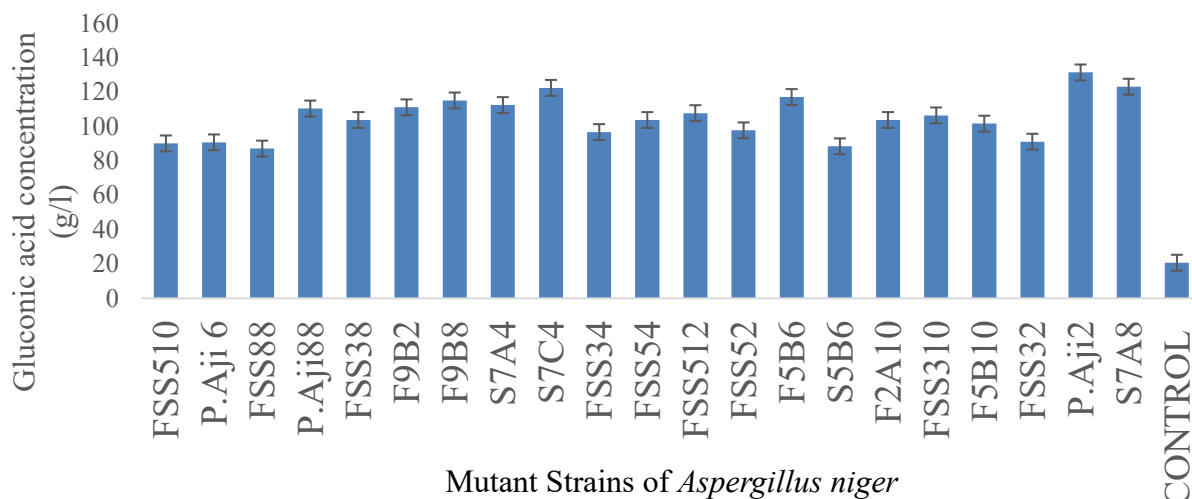


Figure 5: Selected Isolates after Exposure to Ultraviolet Radiation at Different Time Intervals (2, 4, 6, 8, 10 and 12 Minutes)

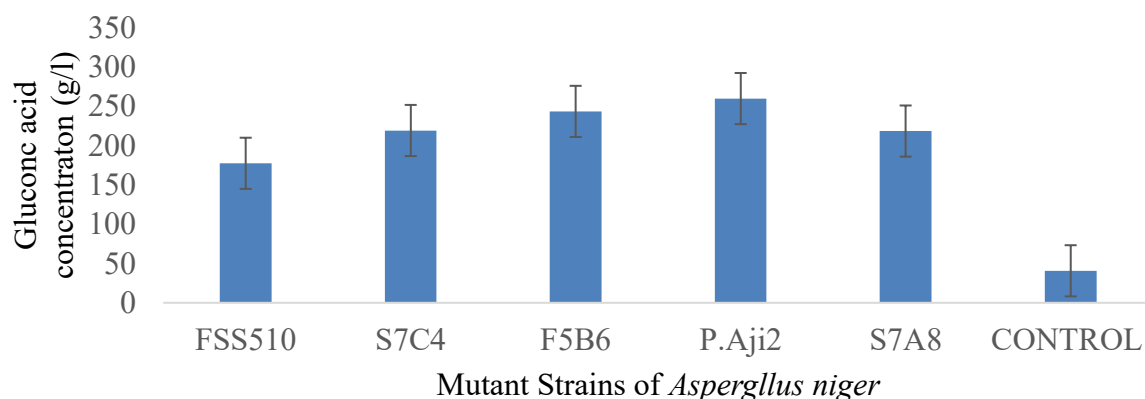


Figure 6: Mutant Strains of *Aspergillus niger* after 2-Step Exposure to Ultraviolet Radiation at Different Time Intervals (2, 4, 6, 8, 10 and 12 Minutes)

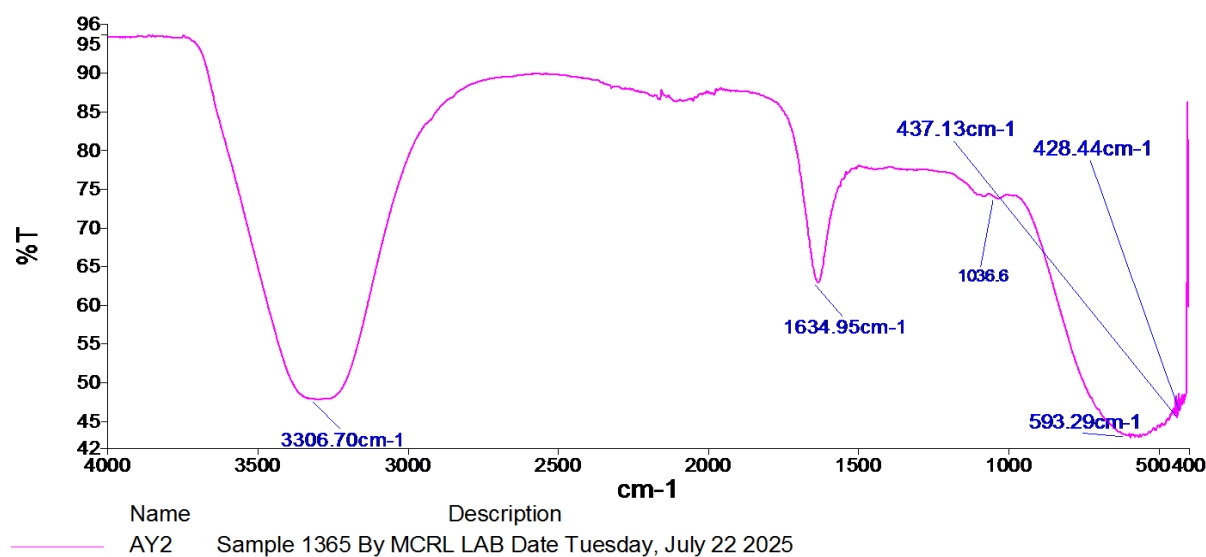


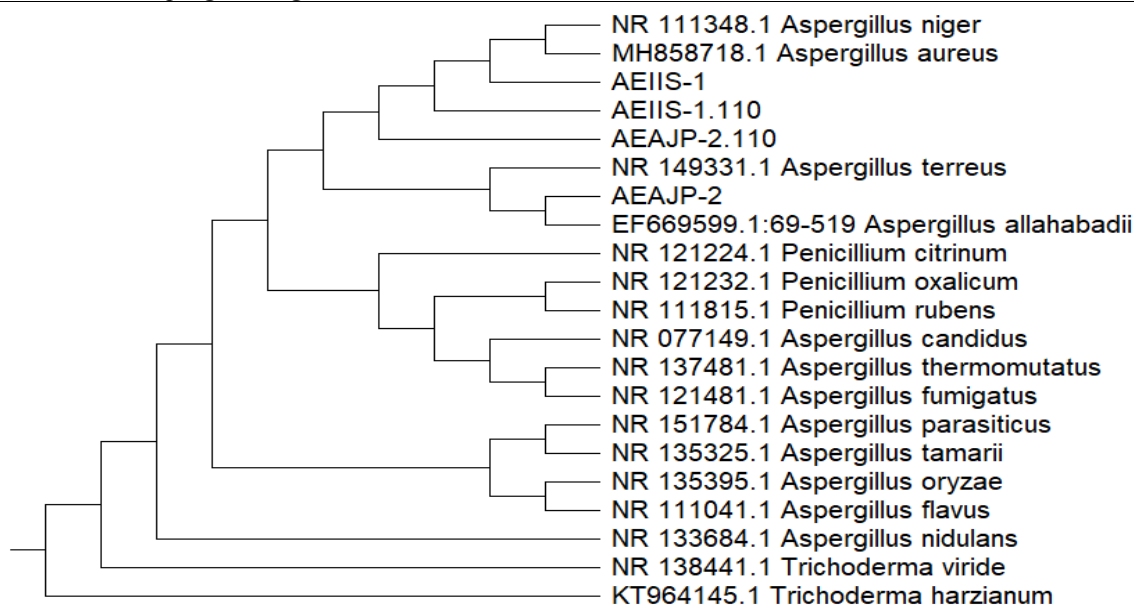
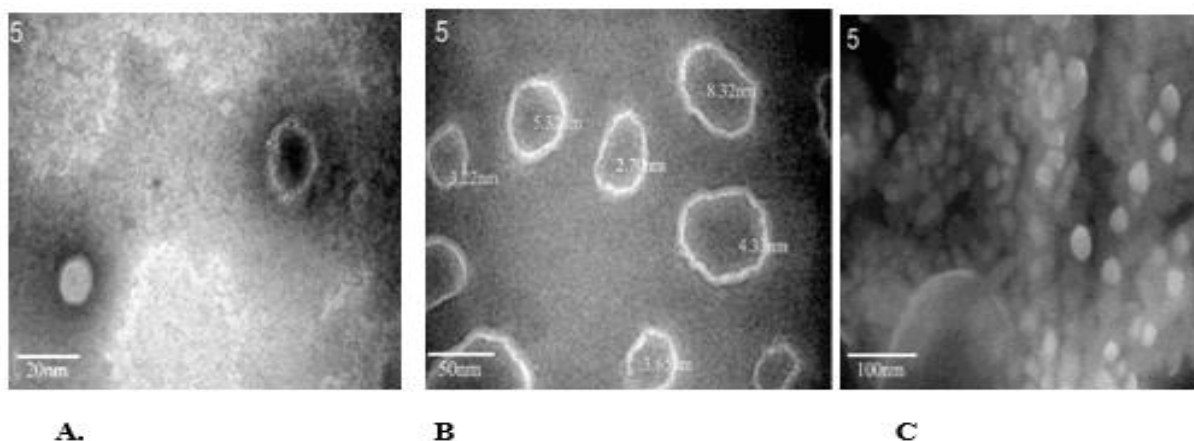
Figure 7: Spectra of Purified Fermentation Broth

Table 1: FTIR Spectrum of Purified Fermentation Broth Showing Peak Areas and Functional Groups

S/N	Peak (cm ⁻¹)	Functional group
1	3306.70	Carboxylic acid
2	1634.95	Alkanes
3	1036.80	Carboxyl compound
4	593.29	Halo compound
5	437.13	Cycloalkane
6	428.44	Cycloalkane

Table 2: Molecular Identities and Accession Numbers on GenBank of the Wild and Mutant *Aspergillus niger* Used for the Production of Gluconic Acid

Sample ID	Scientific name	Max score	Total score	Query cover	E-value	% identity	Accession
AEAJP-2	<i>Aspergillus niger</i>	1033	1033	100	0	100.00	PX954262
AEAJP-2.110	<i>Aspergillus niger</i>	915	915	99	0	97.41	PX939154

**Figure 8: Phylogenetic Relatedness of *Aspergillus niger* AEAJP-2 and *Aspergillus niger* AEAJP-2.110 (Scale: 1:2)****Figure 9: Transmission Electron Microscopy Micrograph of *Aspergillus niger* AEAJP-2 at 20nm(A), 50nm(B) and 100nm(C) Wavelength**

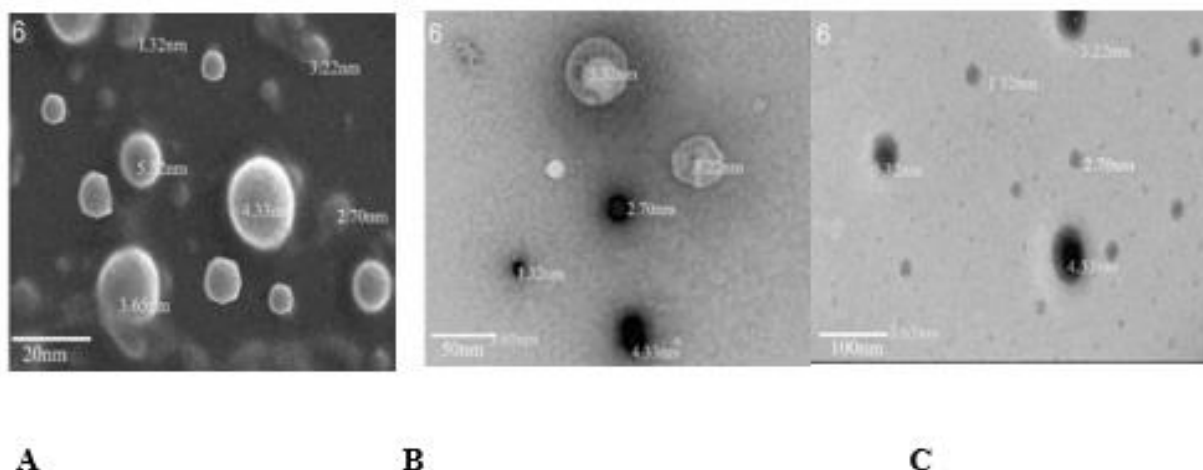


Figure 10: Transmission Electron Microscopy Micrograph of *Aspergillus niger* AEAJP-2.110 at (A) 20nm (B)50nm and (C) 100nm Represent the Various Wavelength

DISCUSSION

This study demonstrated the successful isolation, screening, and mutagenesis of *Aspergillus niger* strains to significantly enhance gluconic acid (GA) production. The wild (*Aspergillus niger* AEAJP-2 PX954262) and mutant (*Aspergillus niger* AEAJP-2.110 PX939154) were used in this research.

The isolation of 204 *Aspergillus niger* strains from diverse environmental samples, with 45% demonstrating organic acid-producing capability reflects the ubiquitous distribution of this species in soil and plant-associated habitats. The observation that soil fungal isolates from IITA and SCPO exhibited the highest proportion (64%) of gluconic acid producers is ecologically significant. This finding corroborates the work of Shindia *et al.* (2006), who reported that *Aspergillus niger* isolated from sugarcane waste samples demonstrated superior gluconic acid production compared to strains from other edaphic environments. The isolation made from fruit samples also revealed that 47% had gluconic acid production potentials. The selective enrichment of gluconogenic strains in agricultural soils, particularly those associated with plantain and sugarcane cultivation, suggests adaptive evolution toward efficient carbohydrate metabolism, likely mediated by prolonged exposure to plant-derived polysaccharides. This

ecological distribution also aligns with studies highlighting the role of nutrient-rich soils in enriching organic acid-producing fungi by (Zhang *et al.* 2024; Della Mónica, *et al.*, 2025).

The 204 isolates were subjected to screening for organic acid potentials and 10 of them with organic acid unitage values of greater than or equal 3.8 and gluconic acid unitage value of greater than or equal to 2.00 were selected. The calcium carbonate plate method exploited in this study leverages the differential solubility of calcium gluconate, which forms distinct zones of clearance proportional to gluconic acid concentration. This approach aligned with the selective isolation strategy advocated by Gromada *et al.* (1997), who demonstrated that resistance to sodium gluconate served as an effective selection marker for mutants with enhanced glucose oxidase production. Vijaysri *et al.* (2025) similarly reported that titratable acidity on glucose media was not predictive of gluconic acid yield, reinforcing the necessity of calcium carbonate plate selectivity.

The yield of gluconic acid (54.1g/l) prior to mutation from *Aspergillus niger* AEAJP-2 in this study was slightly lower than the 72.13g/l gluconic acid produced by identified strain VDF 11 as reported by Vijaysri *et al.* (2025), who worked with potato peel waste in India. The reason for this could be as a result of the fact that the

Indian potato waste may contain pre-adapted, nutritionally enriched *A. niger* populations Vijaysri *et al.* (2025).

In this study, 240 mutants were generated with the UV light, Ethidium bromide and their combinations with the most efficient mutants for Gluconic acid production obtained through sequential UV exposure. *Aspergillus niger* AEAJP-2.110, generated through two successive 2-minute UV irradiation cycles, achieved 259.7 g/l gluconic acid, representing a 4.8-fold increase over the parental strain, AEAJP-2 (54.1 g/l). This quantum leap in productivity substantially exceeds the 2.2- to 2.4-fold enhancement in glucose oxidase activity, the rate-limiting enzyme in gluconic acid biosynthesis reported by Gromada *et al.* (1997) following ethidium bromide and sodium gluconate selection. Furthermore, this yield surpasses the 80.60 g/l (87% higher gluconic acid yield) reported by Singh *et al.* (2001) for a mutant, *A. niger* ORS-4.410 (produced by two step exposure to UV) under optimized submerged fermentation.

The stepwise improvement pattern from 54.1 g/l (wild-type) to 131.4 g/l (first exposure) to 259.7 g/l second exposure) demonstrates cumulative mutagenic effect. This phenomenon is mechanistically attributable to progressive nucleotide dimerization events, particularly thymine-thymine cyclobutane pyrimidine dimers, which accumulate during sequential UV treatments. Such lesions, when imperfectly repaired by nucleotide excision repair pathways, introduce transition mutations that may constitutively upregulate the glucose oxidase (*gox*) gene or disrupt negative regulatory elements. This interpretation is consistent with the works of Raksha *et al.* (2012) and Ma *et al.* (2022), who reviewed that mutagen-induced promoter modifications can enhance *gox* expression by 3- to 5-fold in high-yielding industrial strains.

The molecular studies of the wild type (*A. niger* AEAJP-2) and mutant (*A. niger* AEAJP-2.110) provided evidence through

the accession numbers and identity percentages that mutation took place when the wild types of *Aspergillus niger* were exposed to UV light and treated with ethidium bromide. The mutant strains show lower "Percentage Identity" scores compared to their wild-type parents when BLAST was carried out against the GenBank database. *Aspergillus niger* AEAJP-2 has 100.00% identity to a reference sequence, suggesting it is a very typical or canonical strain. Its mutant, *A. niger* AEAJP-2.110, has 97.41% identity. The ~2.6% difference strongly suggests that mutations have been introduced, altering its sequence enough that it no longer matches the database reference perfectly. The wild type (*A. niger* AEAJP-2) has a unique GenBank accession number (PX939151) distinct from the mutant (*A. niger* AEAJP-2.110) with accession number, PX939152. This scenario suggested that these are unique genetic entities with novel sequences.

Transmission electron microscopy revealed distinct ultrastructural adaptations UV-generated mutant AEAJP-2.110. The UV mutant exhibited pronounced cell wall thickening, attributable to enhanced chitin and β -glucan deposition, accompanied by enlarged vesicular compartments within the cytoplasm. These morphological features are functionally linked to gluconic acid hyperproduction through the thickened cell walls which provide structural support for membrane-bound glucose oxidase thereby preventing enzyme shedding into the medium (Zhang *et al.* 2022).

The FTIR spectra revealed the presence of gluconic acid through its characteristic peaks with the carboxylic acid stretching vibrations found within the regions of 3338.0 cm^{-1} . This matches standard gluconic acid spectra and is similar to the findings of NIST (2023) and Shcherbakova-Sandu *et al.* (2025). Also, the spectra showed the presence of other functional groups such as carboxyl group, alkene, carboxyl stretching towards the hydroxyl group and the halo compounds.

CONCLUSION

This research confirmed that UV mutation is an effective strategy or potential tool for enhancing gluconic acid production in *Aspergillus niger* obtained from sugarcane soil and pineapple peels. The distinct morphological changes observed in the

mutants provided a deeper understanding of the cellular adaptations that contribute to increased productivity. In conclusion, UV light is an effective tool for increasing gluconic acid production in *Aspergillus niger*.

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