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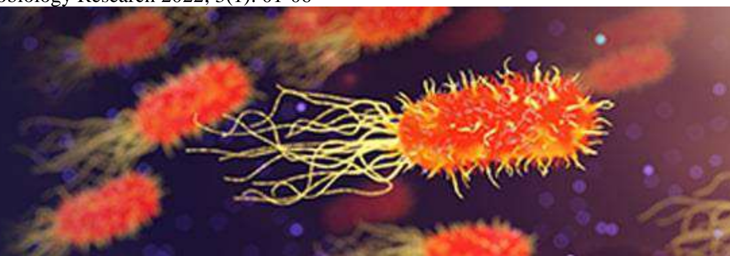

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Preliminary studies into transmogrification of *aspergillus* genome for over expression of cellulase

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Abstract
The aim of this investigation was achieved by exposing native *Aspergillus niger* (WldAN) to an ultraviolet (UV) germicidal lamp (256 nm) kept at a distance of 40 cm for a time interval ranging from 2 –14 minutes, in a total of 56 minutes and finally using the improved strain to synthesize a more stable cellulase. The results showed growth improvement of the modified isolate with a density of 3.54 g/ml at the 9th hour as compared to 3.36 g/ml density of the native strain at the 7th hour on the 3rd day. Cellulase produced by submerged fermentation at 25 °C for 4 days using the native *Aspergillus niger* (WldAn) and modulated *Aspergillus niger* (UVAn) respectively recorded activities of 3.50 U/ml at 60 °C and 2.85 U/ml at pH 4.0 as compared to 2.40 U/ml at 50 °C and 2.24 U/ml at pH 5.0 recorded for cellulase synthase from the native strain.

Keywords: Microbial modulation, growth density, submerged fermentation, biocatalyst

1. Introduction

The number of biotechnology and bioengineering applications has expanded in recent years, and this has created a growing demand for hyper active industrial microorganisms secreting biocatalyst with tolerance at extreme conditions. Microbial cellulase make up a large fraction of industrial enzymes with a growing demand for an improved catalytic performance in biotransformation processes applicable to the detergent, paper, textile, food processing and energy industries. Its functional property is required for the degradation of lignocellulosic polysaccharides into the simple monomeric sugars that are converted through fermentation processes [Zechel, 2000, Lorenz *et al*. 1951] [32, 17]. *Aspergillus niger* amongst others is widely known to be abundant and ubiquitous strain used to synthesize cellulase. Their prevalence can be attributed not only to their high diversity, exceptional evaluability and generation time but also to their robust mechanism of gene regulation for improved viability [Adrio and Demain, 2010] [3]. The main challenge of this isolate among other filamentous fungi is the interwoven network of filament structural growth affecting a liquid reaction such as submerged fermentation. Recent advances in our understanding of prokaryotes have provided the scientific community with an expanding molecular toolbox for altering and manipulating the genetic makeup of various species [Gopal and Kumar, 2013] [12]. The ability to engineer biological organisms by *in vitro* techniques for useful purposes has contributed to the development of synthetic biology and metabolic engineering. The evolution of medium formulations through screening of various temperature (°C), pH, substrate conditions, and their different combinations can significantly improve microbial growth, viability and overall yield of product during process development. [Rosano and Ceccarelli, 2014] [25] Reported that individual cells can quickly and efficiently adapt to new environment by redirecting their metabolic flux and adjusting their protein content in response to external stimuli. The main aim of this investigation was to ascertain the effect of ultra violet (UV) light as an external stimulus on *Aspergillus niger* expression.

2. Materials and Methods

2.1 *Aspergillus niger*

Aspergillus Niger was collected from a culture centre located at Latitude 6.465422, DMS 6° 27' 55.5192" N; Longitude 3.406448, DMS 3° 24' 23.2128" E in Nigeria, West Africa. The isolated was preserved at 4 °C after collection aseptically and finally used for the following activities highlighted below.

2.2 Morphological characteristic of *Aspergillus niger*
The isolate was sub-cultured using potato dextrose broth fortified with 25µg/100ml chloramphenicol prepared at 25 °C, pH 5.1±0.2 according to manufacturer's instruction. A dilution of 1ml microbial load was collected at 1hour interval and density was recorded using UV Spectrophotometer (MultiMode Reader Spectramax M3) at 600 wavelengths.
Furthermore, a sterile wire loop full of blue naphthalene solution was placed on a clean grease free glass slide and with the aid of the same sterile wire loop a small piece of culture specimen was smeared with the solution and finally a cover slip was applied gently over the mixture avoiding bubbles. The prepared specimen was observed under the fluorescence microscope at 40X magnification.

2.3 Molecular identification *Aspergillus niger*
Fungal growth was transferred from the surface of a single agar plate into a pre-cooled (20 ºC) sterile ceramic mortar, overlaid with liquid nitrogen, and ground with a sterile ceramic pestle into a fine powder. 2 ml of buffer G-2 (Genomic DNA buffer set; Qiagen, Valencia, Calif.) containing RNase (200mg/ml; Sigma Chemical Company, St. Louis, Mo.) was added to suspend the powder and the suspension was transferred into a clean test tube. 45 µl of proteinase K solution (20 mg/ml stock solution; Sigma) was added and the suspension was incubated with intermittent agitation for 3 h at 55 ºC. The suspension was centrifuged at 21,500xg for 10 min, then supernatant was transferred into a clean test tube, and DNA was extracted and purified using Genomic-tip 20/G columns (Qiagen) according to the manufacturer's instructions. 2 µl of glycogen solution (20 mg/ml; Gentra Systems, Minneapolis, Minn.) was added to the eluted DNA which was then precipitated by standard methods using isopropanol and ethanol. DNA was resuspended in 60ml of DNA rehydration buffer (PureGene kit, Gentra Systems) and stored at 20 ºC until used. Universal fungal primers ITS3 (5'GCA TCG ATGAAG AAC GCA GC) and ITS4 (5'TCC TCC GCTTAT TGA TATGC), directed to the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA), were employed to amplify DNA amplicons for sequencing. PCR amplification was conducted in a GeneAmp model 9700 thermal cycler (Perkin Elmer Applied Biosystems, Foster City, California). All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification kit (Qiagen). Purified amplicons were then sequenced using the same primers as described above and Big Dye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer Applied Biosystems). Products were analyzed on an automated capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer Applied Biosystems) according to the manufacturer's directions [Hinrikson *et al*. 2005, Raminez *et al*. 2009] [14].

2.4 UV mutagenesis on *Aspergillus niger*
The spore suspension containing 2.1 × 10^6 colony forming units (cfu)/ml was spread on the potato dextrose agar plates. The induction of modulation was carried out by exposure to UV germicidal lamp (256 nm) kept at a distance of 40 cm for time intervals ranging from 2–14 min, in a total of 56 minutes. A modification of [Chidananda *et al*. 2008] [7] was applied. After irradiation, the plates were wrapped in aluminum foil and kept overnight at 4 °C. Later the plates were incubated at 28 °C, for 5 days.

2.5 Screening of cellulase production
The isolates; native *Aspergillus niger* (WldAn) and modified *Aspergillus niger* (UVAn) were screened for the production of cellulase following the method of (Lakshmi *et al*. 2014). Potato Dextrose Agar medium used in this method contained (%): Yeast extract (0.01g), KH_2PO_4 (0.2g), $CaCl_2.H_2O$ (0.03g), MgSO4.7H_2O (0.0005g), $MnSO_4.H_2O$ (0.00016g), $ZnSO_4.7H_2O$ (0.0004g), Carboxymethyl Cellulose – 1%, pH of the medium was adjusted to 5.2. After autoclaving at 121 °C and 15psi pressure for 15 minutes the medium was poured into Petri dish plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 ml of fungal suspensions prepared using the strains mentioned above. The plates were incubated at room temperature (28 ± 2 °C) for three days. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates that were shaken at 50 rev/ min for 15 min. The Congo - Red staining solution was then discarded, 10 ml of 1 N NaOH was added to the plates and shaken again at 50 rev/min for 15 minutes. Finally, 1 N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of clear yellowish zones around the fungal spore inoculated wells indicating positive hydrolytic reactions by the strains. A modification of [Peciulyte *et al*. 2017] [20] was carried out for the test above.

2.6 Cellulase production by submerged fermentation
Synthase secretion using the native (WldAn) and modified (UVAn) strains by submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium. The composition of the medium contained the following (%): L-Glutamic acid, 0.03g; NH4NO2, 1.4; K2HPO4, 0.2g; CaCl2, 0.2g; MgSO4, 0.03g; FeSO4, 0.5g; MnSO4, 0.16g; ZnSO4, 0.14g; dried wheat straw, 3g. The medium in separate flasks was sterilized by autoclaving at 121 ºC for 15 min.
The flask was inoculated with 1ml of the above said inocula and incubated in a rotary shaker (120 rpm) at 30 ºC for 4 days. At the end of the fermentation the culture broth from submerged fermentation was centrifuged at 6000 rpm for 15 min and the supernatant was used as a source of extracellular enzyme.

2.7 Cellulase Assay
Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper. Endoglucanase (CMCase) activity was determined by incubating 0.5 ml of the supernatant with 0.5 ml of 2% carboxymethyl cellulose (low viscosity CMC, SIGMA) in 0.05 M sodium citrate buffer (pH 4.8) at 50 ºC for 30 min. Filter paper degrading activity (FPCase) was determined by incubating 1.0 ml of the supernatant with 1.0 ml 0.05 M of the sodium citrate buffer (pH 4.8) containing Whatman Nº 1 filter paper strip – 1.0 × 6.0 (= 50 mg). After incubation for 24 h at 50 ºC, the reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent to 1 ml of the reaction mixture. In these tests, reducing sugars were estimated colorimetrically with 3,5- dinitrosalicylic acid [Miller, 1959] [19] after Miller (1959) [19] and using glucose as standards. The enzymatic activity of total (FPcellulase) and endoglucanase (CM-cellulase) was defined in the International Units (IU) [Ghose, 2009] [10]. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per min.

2.8 Effect of pH and temperature on the activity of crude enzyme filtrates
Culture filtrates (0.5 ml) were added to 0.5 ml carboxyl methyl cellulose (2 % w/v) in 0.05 M citrate buffer, pH 4.8 in test tubes. Mixture was incubated in water bath at temperatures ranging between 30 to 100 °C for 2 h. Endocellulase activity was then determined following the DNSA method recommended by International Union of Pure and Applied Chemistry. The effect of pH values ranging from pH 2.0 to 11.0 on activity of enzymes was assayed in 0.05 M citrate buffer at pH 4.8 and in 0.05 M phosphate buffer at pH 7. All the tubes were incubated at 50 °C.

2.9 Protein estimation for cellulase

The protein concentration of the crude amylase samples was determined by [Lowry *et al*. 1951] [18], with bovine serum albumin (BSA) as standard. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table.

The final volume in each of the test tubes was 5 ml. The BSA range was 0.05 to 1 mg/ ml. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. The solutions were incubated at room 30 ºC for 10 mins. Then add 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660nm. The absorbance was recorded against protein concentration to get a standard calibration curve.

2.10 SDS-PAGE for cellulase

The crude cellulase samples were concentrated and loaded onto a 12.5% SDS-PAGE gel. The gel was left to run at an initial 50V, 70V and finally 100V. After the completion of gel run, it was flooded with commasie blue staining reagent for 1h, thereafter a destaining solution (400ml ethanol: 100ml glacial acetic acid:500ml distilled water) was applied to remove the commasie blue stain. Molecular weight of each sample was identified using a protein marker.

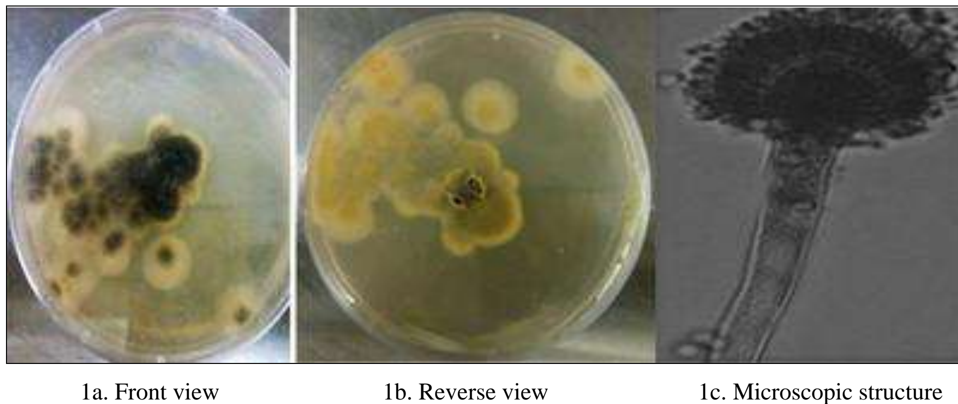
3. Results

Table 1: Morphological properties *Aspergillus niger.*

Macroscopic	Observation	Microscopic	Observation
Growth	Fast	Hyphae	Brached Septate
Elevation	Umbonate	Conidia size	45µm
Surface colour	Whitish wool to Dark brown	Philiades	Biseriate
Reverse colour	River pattern yellow	Cleistothecia	Present
Marginal	Entire	Visicle	Globose
		Length	300µm

Hyphae were septate and hyaline. Conidial heads were radiate initially, splitting into columns at maturity. The specie was biseriate (vesicles produced metulae that support the conidiogenous phialides). Conidiophores were long, smooth and hyaline, becoming darker at the apex, and terminate in a globose vesicle.

In this experiment, microbiological analysis recorded the properties shown in Table 1 above. These properties were similar to description recorded by [Abd-Elsalam *et al*. 2010] [1].



1a. Front view 1b. Reverse view 1c. Microscopic structure

Fig 1: (1a). Front view of growth appearance for *Aspergillus Niger* on potato dextrose agar (PDA), (1b). Reverse growth appearance of *Aspergillus Niger* observed on potato dextrose agar (PDA), (1c). Microscopic appearance of *Aspergillus Niger* obtained using a digital camera Canon Power Shot A550, 7.1 mega pixels attached to fluorescence microscope at X40 magnification. The specimen had been prepared by wet mount technique following the method of [Leck, 1999] [16].

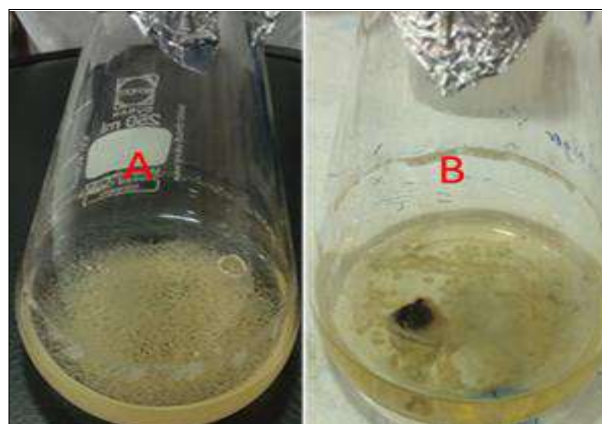


Fig 2: Ultraviolet (UV) mutagenesis of *Aspergillus Niger*. The image labelled (A) showed growth of native *Aspergillus Niger* (WldAn) while image labelled (B) showed growth of modified *Aspergillus Niger* (UVAn) after exposure to Ultraviolet (UV) light. From the images displayed the effect of UV mutagenesis on *Aspergillus Niger* showed faster growth as observed from the increase in biomass seen in Flask (B) after 72 hour

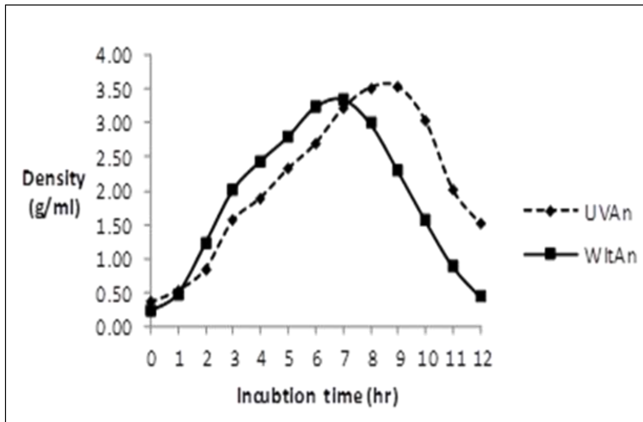


Fig 3: Growth curve of *Aspergillus* spp. Comparative growth curve between the native *Aspergillus Niger* (WldAn) and modified *Aspergillus Niger* (UVAn) was illustrated in the figure above. From the graph the effect of modulation using ultraviolet light as a mutagenic agent improved the viability of the wild strain and optimal growth density (g/ml) was observed at 7th hour and 9th hour respectively

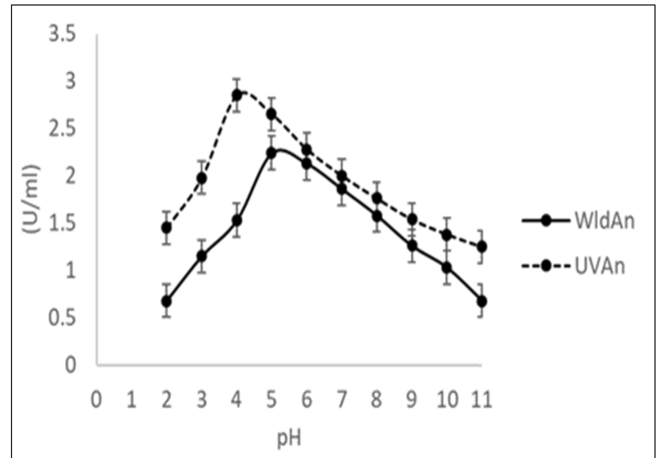


Fig 4: Effect of pH. The figure above is showing optimal activities of cellulase from native *Aspergillus Niger* (WltAn) and modulated *Aspergillus Niger* (UVAn)

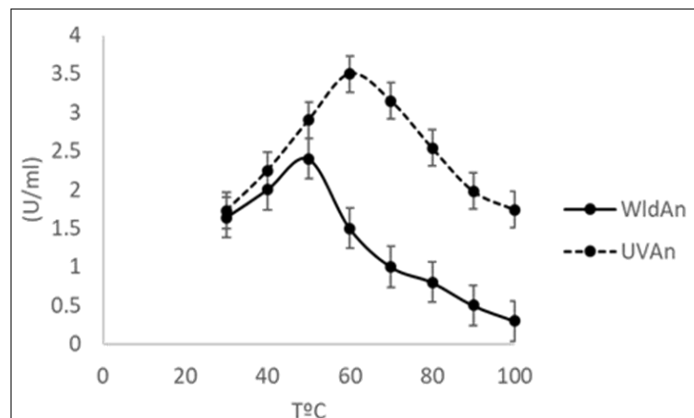


Fig 5: Effect of optimal temperature (°C). The figure above is showing optimal activities of cellulase from native *Aspergillus niger* (WltAn) and modulated *Aspergillus niger* (UVAn).

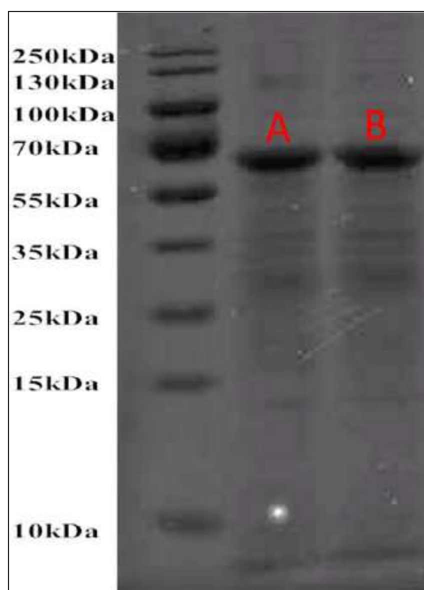


Fig 6: Cellulase molecular characterization. The figure above showed a 12.5% gel electrophoresis images of cellulase samples in kilodalton (KDa). Sample (A) is the expression of cellulase obtained from native *Aspergillus Niger* (WldAn) while sample (Bi) represents the expression of cellulase obtained from the modified *Aspergillus Niger* (UVAn). From the images it was observed a slight difference in molecular weight (KDa) and also concentration indicating positive effect of UV rays on cellular enhancement.

4. Discussion

One of the major prospect of biotechnology activities involved in various industries is the production of enzymes using various strains in fermentation system under suitable conditions. For commercial production of enzymes filamentous fungi are mostly preferred because of high efficiency in a process as compared to those obtained from yeast and bacteria [Bakri and Thonart, 2003] [5]. For efficient enzyme production, strains can be improved by mutagenesis which has become a successful technique. This process is mostly trial and error process involving laborious steps of procedures in performance [Iftikhar *et al*. 2010] [15]. For improved yield of cellulase production from different fungi treatment was done with chemicals as external stimulators such as chemicals ethyl methyl sulphonate (EMS), N-methyl–N–nitro–Nnitrosoguanidine (MNNG) and nitrous acid (HNO2). Sub-lethal concentration of mutagens in fungus has been used to improve growth condition and increase the rate of synthase stability [Rudravaram *et al*. 2003] [26]. The test strain, native *Aspergillus niger* (WldAn) had been re-isolated using potato dextrose agar (PDA) at 28 °C and its characteristics confirmed. The macroscopic and microscopic morphological properties were determined and recorded as shown in Table 1 and Figure 1 respectively. From the table, properties of microscopic properties of the native *Aspergillus niger* (WldAn) were recorded and with the aid of a fluorescence microscope, its cellular structure was captured at a 40X magnification. In this investigation ultraviolet light was used as an external stimulus to modulate the genome of the native *Aspergillus niger*

(WldAn) for the purpose of improved expressions. The strain was exposed to germicidal lamp at 256 nm wavelength for a total 56 minutes distributed between 2 – 14 minutes at a distance of 40cm. Figure 2 showed a comparable growth medium A for the native *Aspergillus niger* (WldAn) and medium B for the stimulated *Aspergillus niger* (UVAn) respectively at 72 hours. It was physically observed that stimulated strain grew faster than the native strain and the growth densities for each strain was illustrated in Figure 3. From the figure it was observed that growth density from medium B had increased with time (hr) recording a 3.54 g/ml at the 9th hour while medium A recorded 3.36 g/ml at 7th hour, both after 72 hours respectively. The results recorded was comparable to the investigation by [Varalakshmi *et al*. 2009] [31] involving the use of ultraviolet (UV) and gamma (γ) irradiations to obtain mutants yielding higher cellulase production from conidia of thermophilic fungi.

It was also similar to the investigation by [Prabakaran *et al*. 2009] [22] where results showed effect of UV mutation on three fungal strains from sugarcane field, and highest production of cellulases was observed by *Penicillium chrysogenum* with UV exposure time of 5 minutes. In another report by [Bapiraju *et al*. 2004] [6], *Rhizopus* sp. mutated by UV showed highest lipase activities as compared to parent strain. For strain mutation, UV rays are important inducers and the main effect of this light is to modify the structure of pyrimidine (cytosine and thyamine) causing the formation of thyamine dimmer which distort the structure of DNA heliex and block the further replication process [Sambrook and Russell, 2001] [27]. [Sandana *et al*. 2001] [28] had reported that *Aspergillus* strains can be treated with UV irradiations or chemicals such as N-methyl, N-nitro, N-nitrosoguanidine, dimethyl sulphate, EMS, ethidium bromide and nitrous acid to induce mutation for the improvement of amyloglucosidase production. In most cases UV mutation are very harmful but sometime at low intensity it may lead to a better adaptation of an organism to its environment. An improved biocatalytic performing UV mutagenic *Aspergillus niger* have the ability to produce 156% and above of synthase as compared to the wild strains. It was also reported by [Solebad *et al*. 2006] that UV treated *Aspergillus niger* UAM-GS1 increased the production of hemicellulolytic and cellulolytic activites. Both the native and mutagenic strains were used to produce cellulase synthase using 1 ml of microbial load separately inoculated into different submerged fermentation media. The isolates were used to produce cellulase at 28 °C for a period of 4 days but the reaction was terminated on the 3rd day. Crude samples were analyzed for cellulase production and optimal activities were determined at a temperature (°C) range 30-100 °C and pH 2 – 11 as shown in Figure 4 and 5 respectively. The assay carried out showed that synthase optimal activities from modified *Aspergillus niger* (UVAn) recorded 3.50 (U/ml) and 2.85 (U/ml) at 60 °C and pH 4.0 respectively while synthase from native *Aspergillus niger* (WldAn) recorded 2.40 U/ml and 2.24 (U/ml) at 50 °C and pH 5.0 respectively. The pH and temperature of a fermentation medium are vital factors that have deep influence on the yield and quality of a biosynthesized product [Hanif *et al*. 2004] [14]. The optimum temperature obtained from this study correlated with the finding of [Gilna and Khaleel, 2011] [11], who reported cellulase activity increased due to effect of mutation when an improved *Aspergillus* sp was cultured on selected lignocellulosic wastes under liquid state fermentation. Although as the temperature was further increased, there was a gradual reduction in the enzyme production.

This may be due to the fact that higher temperature denatures the enzymes or slows down microbial growth if the strain does not possess high thermophilic tolerance [Shazia *et al*. 2010] [29]. Many workers have reported different optimal temperatures for cellulase production either in shaker or in bioreactor studies using both native and mutagenic *Aspergillus* spp. suggesting that the optimum temperature for cellulases production also depends on the differences within the same genus of the same fungus [Akinyele *et al*. 2013a] [4]. Furthermore, in this investigation we had shown cellulase synthase activities of differential pH and the results correlated with report by [Phan *et al*. 2010, Acharya *et al*. 2008, Coral *et al*. 2002] [21, 2] stating that the activities cellulase from modified *Aspergillus spp* were stable at pH 4.0, 4.5 and 5.0. To further ascertain effect of the external stimulation, the sizes of synthases isolated from native *Aspergillus niger* (WldAn) and modulated *Aspergillus niger* (UVAn) were expressed on a gel. Their sizes in kilodalton (KDa) were determined to ascertain effect of the ultraviolet light stimulus and the corresponding weights were recorded as shown in Figure 6. From the Figure, it was observed that the cellulalse synthase from modulated strain had increased slightly up to 68-70 KDa indicating significant effect of cellular reaction to the stimulation as compared to the size of synthase from the native *Aspergillus niger* recording molecular weight up to 63-65 kDa. In addition, no comparative investigations have been published on the cellulases from these organisms but the differences recorded appeared as a result of differential properties in morphology between the species [Gautam *et al*. 2010] [9]. *Aspergillus* species are particularly important industrial filamentous fungi employed in the large-scale production of both homologous and heterologous boiocatalysts [Ramamoorthy *et al*. 2012] [23]. Also the application of UV for *Aspergillus niger* improvement did enhance the property of the strain and the cellulase produced gave higher activities which were observed to be more stale at increasing temperature (°C) and lower pH. The activities of these biocatalyst obtained from this investigation are in place with expected industrial requirements for new enzymes.

Conclusion

Microbial modulation for the purpose of improved overexpression of biocatalysts have proven to be effective in developing standard strains with specific traits as well as increasing the yield and stability in bioprocess reactions under extreme conditions. The successful completion of this investigation was achieved by modulating a native *Aspergillus niger* using ultraviolet light as an external stimulus and the effect of the external factor resulted to a faster growth which was compared to a wild strain. Cellulase synthass isolated by submerged fermentation was observed to be more stable at extreme conditions. Due to the superior capacities of hyper-producing strains and secreting proteins, filamentous fungi are extraordinary hosts for the overexpression of recombinant enzymes. This research investigation has contributed to industrial application by providing another technique for improving metabolic reactions and controlling contamination in a bioprocess system.

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