Enhancement of cellulase activity from indigenous strain of *Aspergillus niger* through incorporation of physical, chemical and combined mutagenesis

Waqas Ahmad[1], Muddassar Zafar[1]* and Zahid Anwar[1]

[1]Department of Biochemistry and Biotechnology, University of Gujrat, Hafiz Hayat Campus, Gujrat, Pakistan

ABSTRACT

Cellulases are naturally occurring enzymes to degrade cellulose into simpler components which are used in wide industrial bio-products. The activity of Cellulase enzymes can be enhanced through various mutations and modifications. In this study, cellulase from *Aspergillus niger* was targeted for mutation by physical and chemical methods using UV radiations and ethyl methane sulfonate (EMS), respectively. During physical mutagenesis, the maximum enzyme activity (322 µmol/mL/min) was observed at 10 min UV irradiation as compared to the control (94 µmol/mL/min), while during chemical mutagenesis, the maximum enzyme activity (390 µmol/mL/min) was observed after treatment of microbial culture with 150 µg/mL concentration of EMS. The enzyme activity was further increased (428.5 µmol/mL/min) by combined mutagenesis including 10 min UV irradiation followed by treatment of culture with 150 µg/mL concentration of EMS. Hence, it was found that treating fungal cultures with physical, chemical and combined mutagenesis can significantly enhance their enzyme activities.

Key words: *Aspergillus niger*, Cellulase, Physical mutagenesis, Chemical mutagenesis, Combined mutagenesis

INTRODUCTION

Cellulose is a polysaccharide of glucose monomer which contains β 1-4 glycosidic linkage. Cellulase (EC 3.2.14) is enzyme known as natural biocatalyst which hydrolyzes cellulose to beneficial sugars. Following three types of cellulase based upon their mechanism of action such as based upon cleavage site of cellulose, 1,4-β-D-glucanase (endoglucanases), exo-1,4-β-D-glucanase (exocellulases), and β-glucosidase (processive endoglucanases) (Juturu and Wu, 2014).

Cellulases are potentially being used in industries like textile, paper production, agriculture, medical and food processing. Cellulases have gained further interest for agriculture, biotechnology and bioenergy uses (Trivedi *et al.*, 2016). Production of biofuel from biomass is extensively being studied by catalyzing biomass from cellulases (Sun and Cheng, 2002). In industrial process, sustained supply and product management are key to success and cellulases get benefit in it due to its synergistic action, yielding useful products. Industrial cellulases are derived from fungi and bacteria. As use of cellulase in industry is wide and there is need for better and efficient cellulase which is being studied by genetically modified cellulase generally known as mutant cellulases. These mutant of genetically engineered are aimed to meet following drawbacks; loss of cellulase, time of fermentation and energy consumption (Li *et al.,* 2017). Furthermore, mutant strains of bacteria and fungi are genetically prepared which contain mutant cellulase genes leading to enhanced cellulases production and activity overcoming the challenges. Whereas for successful production of cellulases, it is essential to know about cellulose binding domain, catalytic domain, type of structural motif and reaction mechanism which would ultimately enhance enzyme stability and enzyme kinetics (Graham *et al.,* 2011; Garvey *et al.,*

2013). Temperature tolerant and pH tolerant mutant strains are being targeted for better activity and stability which are derived from archaeal bacteria and fungi (Lewin *et al.,* 2017).

There are many apporaches to get mutant cellulase by following physical or chemical genetic mutation. In a fungus system like *A. niger* such approaches are feasible because of its tolerance to harsh conditions (Cortesao *et al.,* 2022). Site specific mutations in cellulase can be attained by gene specific nucleotide addition or deletion. Some random mutations are also studied for incearsing the catalytic activity, stability and yield of cellulase. *A. niger* FCBP-02 was subjected to solid state fermentation experiment and its cellulase was mutated by UV exposure for 5-40 min along with ethyl methane sulfonate (EMS) treatment. It was observed that mutant cellulase had more enzymatic activity than its wild type and the genetic mutations were analyzed by RAPD-PCR (Shafique *et al.,* 2009). Other approach is adaptive evolution to obtain cellulase mutant which is quite frequent approach. In a study, adaptive evolution based mutant strain showed 5 times higher enzyme activity than parental one. This mutant cellulase strain showed low expression of *noxR* which was further confirmed by *noxR* knock don model of *A. niger*. It was observed that noxR expression were involved in cellulase degradation (Phitsuwan *et al.*, 2013). Another studied approach is gene insertion of other species in *A. niger* and producing a genetically engineered *A. niger*. Such approach is reported by Jian *et al*. (2016) and Gao *et al*. (2017), where cellulase of *Thrichoderma* was inserted in *A. niger* and cellulase activity not only increased but also showed temperature and pH stability (Sohail *et al.,* 2009). Fermentation is also being applied for the production of broad spectrum antibiotics in addition to enzymes, in order to overcome the increased disease rate through controlled drug delivery (Muhammad *et al.,* 2018).

Many researchers have been trying to produce different cellulosic enzymes from various microbial sources, due to its significant industrial applications, through biochemical fermentation and molecular biology tools (Zahid et al., 2012, Mirza *et al.,* 2016, Imran *et al.,* 2017; Imran *et al.,* 2018, Muhammad *et al.,* 2018, Hamid *et al.,* 2019).

In this study, physical, chemical and combined mutagenesis techniques were applied to incorporate mutations in an indigenous strain of *A. niger* to improve its cellulytic capability.

MATERIALS AND METHODS

Microorganism

An indigenous strain of *Aspergillus niger* was obtained from Industrial Biotechnology Lab, Department of Biochemistry and Biotechnology, University of Gujrat. On a medium consisting of potato dextrose agar (PDA), colonies of *A. niger* were cultured.

Chemicals

Analytical grade chemicals from companies like Sigma-Aldrich (USA), Merck Group (Germany), and Bio-Rad (USA) were used in this study.

Sporulation Media

Using 0.1N solutions of sodium hydroxide (NaOH) and hydrochloric acid (HCl), the pH of the solution was adjusted to 5.0. Sporulation medium was autoclaved at 121 degrees Celsius for 15 min at a pressure of 15 pounds per square inch. The fungal culture was transferred to the sporulation medium slants in a manner that assured aseptic conditions were maintained while

working inside a laminar air flow hood. To encourage sporulation, the slants were placed in an incubator at 33 degrees Celsius for four days.

Molecular Identification of Fungal Strain

From the freshly made agar plate, a single, easily identifiable colony was carefully selected and sent off to a commercial facility for full-length 18S rRNA sequencing. There has been a public record of the nucleotide sequence of the 18S ribosomal RNA (rRNA) in the NCBI database. The Basic Local Alignment Search Tool (BLAST) was also used to find similarities in the 18S rRNA database using this sequence. Multiple sequence alignments (MSAs) were built by using the clustal omega algorithm to find sequences with similar characteristics. In the discipline of Phylogeny, a phylogenetic tree has been generated using the Multiple Sequence Alignment (MSA) and the maximum likelihood technique as reported by Dereeper *et al.* (2008).

Incorporation of Physical Mutagenesis

From the falcon tube, one milliliter of the spore suspension was poured onto each of three pre-sterilized, empty petri plates. UV radiation at 254nm was used, and the culture was kept at a distance of 15 cm from the lamp (Yamada *et al.,* 2017). The UV treatments lasted for 10, 20, and 40 min, respectively. The petri dishes were left exposed to the UV light. The spore suspension was exposed to ultraviolet light, and then the mutant culture was inoculated into an inoculum medium.

Incorporation of Chemical Mutagenesis

Random mutagenesis of *A. niger* was done with the treatment of different concentrations of Ethyl methane sulphonate (EMS) which was purchased from Sigma-Aldrich (America). To prepare

different concentrations of EMS, a stock solution of 300 µg/mL was prepared. Using stock solution, further dilutions of EMS (50, 100, 150, 200 and 250 µg/mL) were prepared. Nine eighty microliter of spore suspension prepared from PDA slant was transferred into 6 Eppendorf tubes. One tube was kept as a control with no EMS added, while in each of other 5 tubes 20 µL of above mentioned concentrations of EMS solution were added, preparing a total 1 mL mixture. Then the mixture consisting of spore suspension and EMS solutions were homogenized on vortexer and incubated for 24 hours in a rotary shaker (210 rpm) at 28 ^{o}C.

Incorporation of Combined Mutagenesis

The combined mutation was achieved through the application of a combination of UV and EMS mutation treatment method. In this experimental procedure, a spore suspension was subjected to UV radiation as the initial step, followed by treatment with EMS solution in order to induce mutant colonies of *A. niger*. 1 mL spore suspension was aseptically transferred onto sterilized petri plates and subjected to UV radiation for duration of 10 min. The UV lamp was positioned at a distance of 15 cm from the plate, ensuring controlled and sterile condition. The spore suspension, which had undergone UV treatment, was carefully transferred into Eppendorf tubes that already contained EMS dilution of 150 µg/mL. Subsequently, the spore suspension that had undergone treatment was subjected to an incubation period of 24 hours within a rotary shaker operating at a temperature of 28 ^{o} C.

Following 24-hour incubation period, a spore suspension that had been treated with UV (10min) and EMS (150 µg/mL) underwent centrifugation at a speed of 13000 rpm for a duration of 5 min. Following the spore sedimentation process, a volume of 100 µL was carefully transferred onto sterilized petri plates that were pre-filled with a medium consisting of carboxy methyl cellulose

(CMC) and potato dextrose agar (PDA). The spore suspension was evenly distributed onto the plates using a glass spreader. The petri plates underwent incubation for a duration of 7 days at a temperature of 28 ^{o}C. Following the initial culture, the transfer of the culture to the inoculum medium was conducted to facilitate growth and development.

Inoculum Medium

The inoculum medium was prepared in 250 mL Erlenmeyer flask. All the ingredients as mentioned in Table 1, were dissolved up to 100 mL of distilled water and autoclaved. Different flasks of inoculum media were prepared, one kept as control for the spore suspension that was not exposed to the UV radiations or treated with different concentrations of EMS while the other flasks were exposed to UV radiation and treated with EMS. In laminar flow hood, fungal spore suspensions were transferred to inoculum medium and incubated for 5 days at 28 °C.

Table 1: Chemical composition of Inoculum media for the growth of *A. niger*

Sr. #	Ingredient	Quantity (g/mL)
1	Glucose	2.00
2	Carboxymethyl cellulose	2.00
3	Sorbose	0.1
4	Triton	0.1
5	Ammonium Sulphate	0.02
6	Calcium chloride	0.05
7	Magnesium sulphate	0.05
8	Potassium hydrogen phosphate	0.02
9	Distilled water	up to100 mL

Screening of Mutant Colonies of A. niger

The Congo Red Assay was employed as a method for the systematic evaluation of mutants. The media, which was composed of a 2% CMC solution, was employed for the analysis of cellulase activity. In order to accomplish the intended objective, a 0.5% solution of Congo red solution was meticulously prepared by dissolving 0.5 grams of Congo red in 100 mL of distilled water. Similarly, a 2% solution of NaCl was meticulously prepared by dissolving 2 grams of NaCl in 100 mL of distilled water.

The Petri plates were subjected to staining using a solution of 0.5% Congo red dye. A few milliliters of a 0.5% Congo dye solution were added to each plate, ensuring that the growth layer of colonies was fully covered. The plates were then incubated for a duration of 30 min on a shaker. Following the incubation period, the dye solution was discarded and a thorough washing procedure was carried out. The colonies underwent a thorough cleansing process utilizing a 2% sodium chloride (NaCl) solution. The mutant colony exhibiting the largest clearance or halozone was selected based on the diameter of the halozone and subsequently utilized for inoculating subsequent cultures.

Cellulase Assay

The enzyme activity of cellulase was measured in all samples treated with physical, chemical and combined mutagenesis following the DNS method. The experiment used CMC as the substrate, and the higher cellulytic activity of the fungus resulted in a greater consumption of the CMC and the subsequent production of glucose. Glucose absorption was measured at 540 nm (Zhang *et al.*, 2009).

RESULTS AND DISSCUSSION

In the present research work, physical, chemical and combined mutagenesis were applied to enhance cellulase activity from indigenous strain of *A. niger.*

Vegetative Growth

The pure cultures of *A. niger* obtained from the industrial biochemistry lab, Department of Biochemistry and Biotechnology, University of Gujrat, Main campus Gujrat, as mentioned in the materials and method, were grown on the PDA slants containing nutritional medium at 28 °C, to get pure culture. After 5-6 days of incubation, the colonies of *A. niger* started appearing. The initial color of colonies of *A. niger* was green, which turned into blackish green after the formation of conidia. After getting pure culture on PDA slants, to observe the growth pattern further the *A. niger* was re-cultured on to PDA petri plates with and without carboxy methyl cellulose (CMC) which act as a substrate.

A. niger is the most commonly used soft rot fungus for commercial cellulase production via solid state fermentation (SSF) due to its ease of handling and increased control over environmental factors including temperature and pH. Theoretically, the SSF process is capable of producing a complete cellulase system, but in practice it produces incomplete or deficient cellulase components (Abdullah *et al.,* 2018). Industrial and domestic agro-waste generates substantial quantities of cellulase enzymes when hydrolyzed by cellulolytic microorganisms, such as *A. niger*, on PDA plates containing CMC as a substrate (Omojasola and Jilani, 2008). The fungus was morphologically confirmed using light microscope (Figure 1).

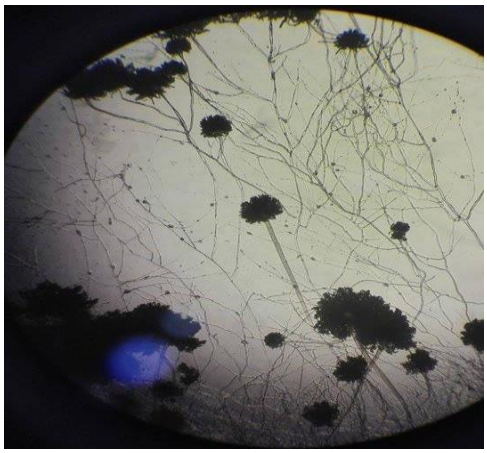

Figure 1: Morphological view of *A. niger* using light microscope

Inoculum Media

A. niger spore suspension were treated with 2g CMC concentrations and incubated for 5-7 days. CMC was added as a substrate because it enhanced the cellulase production. CMC based spore suspension showed different growth patterns and color intensity as compared to the control media that was without CMC.

Enzymes in fungi is related to transcriptional regulation by the presence of available carbon sources. In the presence of glucose, the cellulase genes transcription is being repressed while endoglucanase was induced by CMC substrate. They achieved the maximum growth of the fungus at 2% w/v of low viscosity CMC (Padmavathi *et al.,* 2012).

Molecular identification

Advanced Biosciences, Pakistan was contracted to sequence the whole 18S rRNA gene from a single unique colony. To facilitate the retrieval of similar sequences from the 18S rRNA database, I submitted the sequence into the NCBI nucleotide database. The 18s homologous database sequence has been shown in Figure 2.

GACCCGTGCCAGGGGTCTTAGTATAAGCACTTTATACTGTGAAACTGCGAATGGC
TCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGT
AATTCTAGAGCTAATACATGCTGAAAACCTCGACTTCGGAAGGGGTGTATTTATT
AGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGAATCATAATAACTTAACG
AATCGCATGGCCGGGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTT
TCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGG
TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCCCGGGAAGGCAG
CAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTG
ATACGGGGCTCTTTTGGGTCTCGTAATTGGAATGAGTACAATCTAAATCCCTTAA
CGAGGAACAATTGGAGGGCAAAACTGGTGCCAGCAGCCGCGGTAATTCCAGCTC
CAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTTTTAGTTGAACCTTGGGT
CTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCCCCT
GGGGAATCTCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAA
AAAATTAGAGTGGGAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATA
GAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAAT
AGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAAAACTTGGATTT
GCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGG
AACGACCAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATA
AACTATGCCGACTAGGGATCGGTCGGTGTTTCTATTATGACCCGTTCGGCACCTT
ACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGGAGTATGGTCGCAAGGCTGAAAC
TTAAAGAAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTA
ACTCATCACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATTGACAGATTGA
GAGCTCTTTCTTGATCTTTTGGATGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGT
GATTTGTCTGCTTAATTGCGATAACGAACGAGACCTCGGCCCTTAAATAGCCCGG
TCCGCATTTGCGGGCCGCTGGCTTCTTAGGGGGACTATCGGCTCAAGCCGATGGA
AGTGCGCGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGC
GCTACACTGACAGGGCCAGCGAGTACATCACCTTGGCCGAGAGGTCTGGGTAAT
CTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACG
AGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTT
TGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGAC
TGGCTCAGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAAGTTGGTCAAACCCG
GTCATTAGAGAAGTAAAAAGTTAGTACCCCG

Figure 2: 18s rRNA sequence of indigenous strain of *A. niger* for molecular identification

The obtained sequence was compared with other sequences of cellulase belonging to different microorganisms using nBlast. The result of nBlast showed 98.78% similarity index with *A. niger* CBS 55465 as shown in the Figure 3.

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus niger CBS 554.65 18S rRNA gene, partial sequence; from TYPE material	Aspergillus niger	3068	3068	100%	0.0	98.78%	1724	NG_065763.1
Aspergillus terreus ATCC 1012 18S rRNA gene, partial sequence; from TYPE material	Aspergillus terreus	2948	2948	98%	0.0	98.00%	1733	NG_064804.1
Aspergillus glaucus JCM 1575 18S rRNA gene, partial sequence; from TYPE material	Aspergillus glaucus	2942	2942	98%	0.0	97.94%	1733	NG_063391.1
Aspergillus cremeus NRRL 5081 18S rRNA gene, partial sequence; from TYPE material	Aspergillus cremeus	2937	2937	98%	0.0	97.88%	1751	NG_063231.1
Aspergillus neoflavipes CBS 260.73 18S rRNA gene, partial sequence; from TYPE material	Aspergillus neoflavipes	2931	2931	98%	0.0	97.82%	1771	NG_062800.1
Aspergillus flavipes NRRL 302 18S rRNA gene, partial sequence; from TYPE material	Aspergillus flavipes	2926	2926	98%	0.0	97.77%	1771	NG_063230.1
Aspergillus clavatus NRRL 1 18S rRNA gene, partial sequence; from TYPE material	Aspergillus clavatus	2920	2920	98%	0.0	97.71%	1776	NG_081374.1

Figure 3: nBlast comparison of 18S rRNA sequence of *A. niger*

Clustal Omega was used to recover homologous sequences, and an MSA was then built from those sequences. Following the procedures outlined in the preceding section, a phylogenetic tree was constructed in Phylogeny using maximum likelihood. Figure 4 shows that three *Aspergillus niger* species (BMB-18S rRNA, CBS-18S rRNA, and NRRL-18S rRNA) are closely related to one another in phylogenic trees.

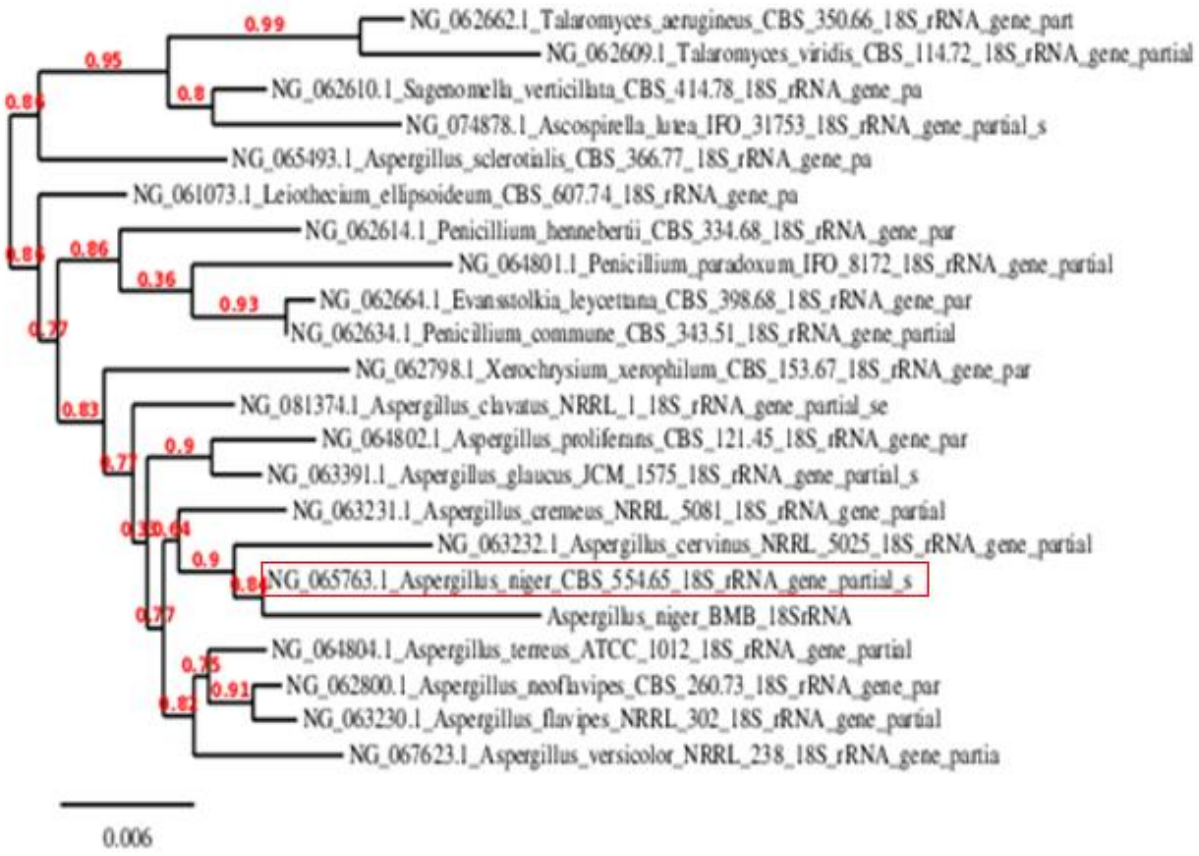

Figure 4: Phylogenetic tree of *A. niger* showing its relationship with other species

Phylogenetic analysis may be performed by comparing sequences of the small subunit of ribosomal RNA. I compared the 558 nucleotide stretches that make up the 18S rRNA gene to see how closely related 11 *Aspergillus species* and their close relatives are genetically. The 18S rRNA genes of *A. niger* and allied species have been sequenced using a guided PCR sequencing approach in order to learn more about their evolutionary connections (Nikkuni *et al.,* 1996).

In a study, Imran *et al.* (2019) examined the phylogenetic relationships in *Aspergillus*. Cellulase-producing *Aspergillus* species use glucose as a carbon source in SSF by hydrolyzing cellulose into it (Peterson, 2008). According to the quantity of corn stover substrate, the cellulose content gradually reduced up until the fourth day of incubation. After the fourth day, the cellulose

content dramatically reduced and the production of the cellulase complex enzyme by microorganisms increased (Vega *et al.,* 2012). Three *Aspergillus* species were identified as having strong cellulolytic activity and being suitable for solid-state cellulase synthesis (Imran *et al.,* 2019). All fungal isolates showed a decrease in decreasing sugar concentration between the first and second day of incubation. According to Jafari *et al.* (2017), *Aspergillus terreus* displayed a high cellulase yield (50.33 U/mL) at pH 5.5, and these reducing sugars were created by cellulases after acting on cellulose (Shahriarinour *et al.,* 2011). Additionally, during the fourth and sixth days of incubation, cellulolytic activity was seen. According to Mahmood *et al.* (2014), all fungi showed cellulase activity after 24 hours. A cellulosic substrate must be present at the site of cellulase action in order to stimulate synthesis. Cellulase synthesis was triggered by cell mechanisms when insoluble and soluble cellulosic and hemicellulosic fibers came into contact with the fungal isolates' exterior layer. It is thought that soluble fibers stimulate the synthesis of enzymes. The soluble byproduct of cellulase enzymatic breakdown of cellulose is hemicellulose. Hemicellulose is a powerful inducer of cellulase synthesis, according to numerous studies (Chinedu *et al.,* 2011).

Physical Mutagenesis of A. niger

The physical mutagenesis was performed by UV irradiation to spore suspension of *A. niger*. UV light has been known to produce wide spectrum of mutational modifications including transitions and transversions in the base sequence of DNA molecule.

A. niger spore suspensions were subjected to ultraviolet light for 10, 20, and 40 min and injected into inoculum media. The findings of UV mutagenesis demonstrated that a sample subjected to UV radiations for 40 minutes resulted in a 90% spore death rate. After inoculating the inoculum medium with the spore suspension from this culture, a very thin mycelium growth was seen.

After 5 days of incubation in liquid inoculums culture, the spore suspension that had been subjected to UV radiations for 20 min had produced extremely thin mycelium development, indicating that 70% of the spores had been killed by the UV radiations.

Enzyme activity in *A. niger* was 94 µmol/mL/min in a sample that had not been exposed to UV light (Control). It served as a kind of regulation. The comparison of cellulase activity before and after UV mutagenesis has been shown in Figure 5.

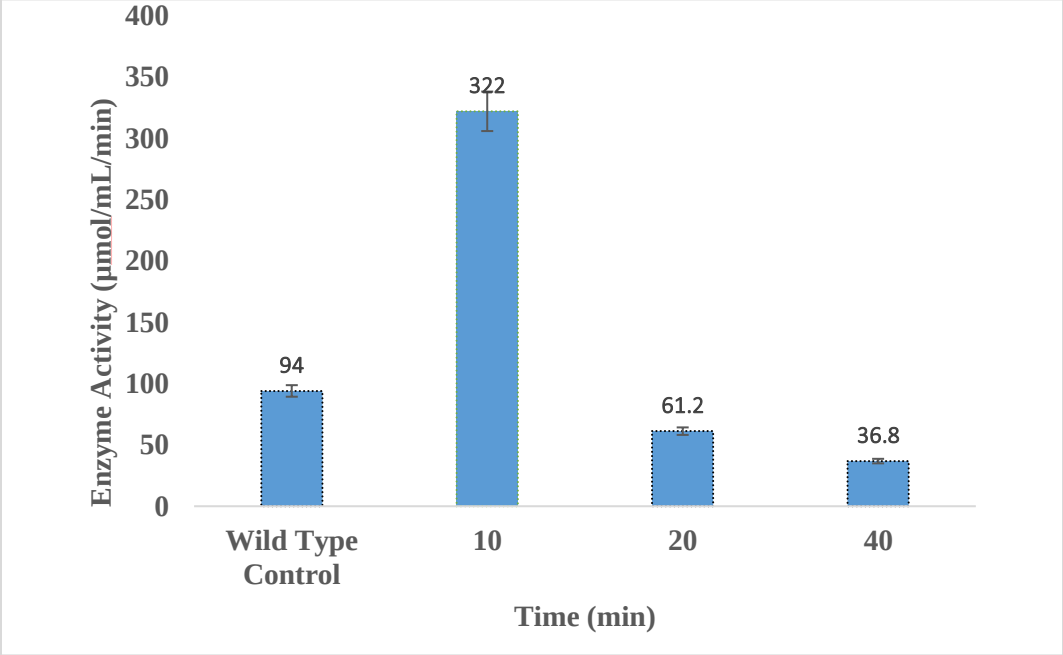

Figure 5: Comparison of cellulase activity after physical (UV) treatment of *A. niger* at different time intervals (10, 20 & 40 min). The maximum cellulase activity (322 µmol/mL/min) was observed at 10 min UV irradiation.

Based on regression analysis of glucose absorbance data, the enzyme test indicated that the sample exposed to UV radiations for 10 min had the highest enzyme activity, at 322 µmol/mL/min. Enzyme activity was measured, and found to be 61.2 µmol/mL/min for those

exposed for 20 min, and 36.8 µmol/mL/min for those treated for 40 min. Irradiating *A. niger* with ultraviolet (UV) light was employed to induce mutation in the fungus in order to obtain a mutant strain that was optimal for cellulase overproduction. According to Jafari *et al.,* 2017, Response Surface Methodology (RSM) was successfully used to examine the influence of the mutation conditions, namely the amount of time the strain was exposed to UV light and distance from the source of UV light, on the clear zone area that was produced surrounding the strains (Jafari *et al.,* 2017).

Incorporation of Chemical Mutagenesis

The chemical mutagenesis was performed by the treatment of Ethyl Methane Sulfonate to spore suspension of *A. niger*. EMS mutagen was used to mutate the *A. niger* for higher cellulase production. To produce an improved cellulase producing strains, 50,100,150, 200 and 250 µg/mL concentrations of EMS were used.

All the samples of spore suspension treated with EMS concentrations (50 to 250 µg/mL) were transferred into the petri plates of potato dextrose agar media containing CMC as a substrate for the cellulase enzyme production by mutant *A. niger.* L-Sorbose that was used as a colony restrictor, allowed the growth of mutants only. The samples of EMS- treated spore suspension showed different growth patterns on PDA media as compared to the control that was not treated with EMS, as the mutagenic treatment affected the sizes and shapes of mutant colonies. The spore suspension of *A. niger* treated with EMS concentration of 150 µg/mL showed maximum enzyme activity and significant morphological changes in the growth pattern were also observed.

Numerous distinct cellulolytic enzyme genes in *A. niger* are expressed at various levels in response to UV radiation and compounds like EMS that cause mutation. UV light and EMS were used to cause mutagenesis in the *A. niger* normal (N) strain. The chemically altered strain of *A. niger* had the highest level of total protein (12 mg/mL). The chemically modified variant of the enzyme showed the highest levels of cellulase enzyme activity (Mahmood *et al.,* 2014). Abdullah et al., 2018 have also reported the use of three mutagens UV, EMS and H_2O_2 from *Penicillium echinulatum* which showed the 1.5 fold more cellulase production by developed *T. reesei* mutant strains by chemical mutation and observed that 2 fold enhanced endoglucanase activity (Abdullah *et al.,* 2018).

Screening of Mutants by Congo red Assay

Cultures of EMS treated spore suspension were screened through Congo Red Assay. Those cultures were analyzed on ability of generating halozones after staining with dye. Different concentrations of EMS-treated cultures showed different levels of clearing zones. The culture which was treated with 150 µg /mL EMS concentration showed clearest halozones showing the ability that the *A. niger* had the ability to hydrolyze the polysaccharide substrate (Carboxymethyl cellulase) into their monomer units by producing hydrolysis enzyme which is cellulase. The mycelium growth in the sample treated with 50 and 100 µg /mL EMS concentration was also clear but sample of *A. niger* sample treated with 250 µg /mL EMS concentration was very thin and not very clear. The results of mycelium growth are shown in Figure 6.

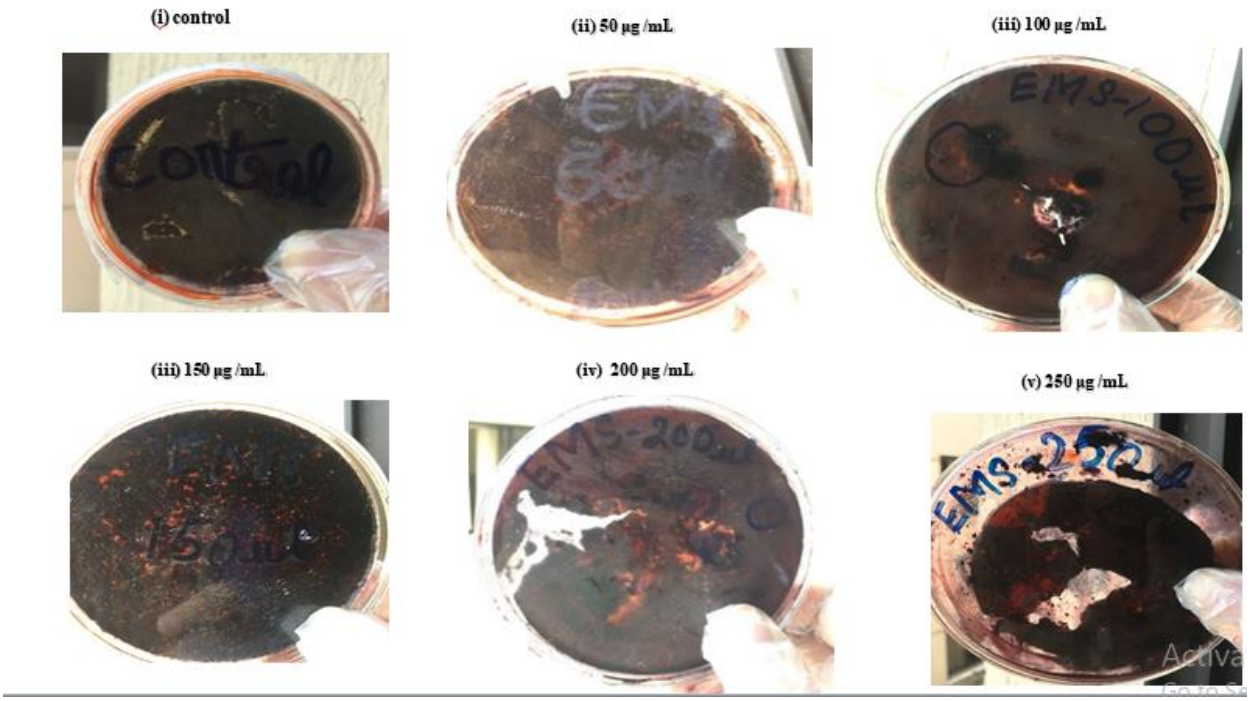

Figure 6: Screening of mutants by Congo red assay after chemical (EMS) treatment of *A. niger*

The maximum enzyme activity (390 µmol/mL/min) was obtained for the sample that was treated with 150 µg/mL EMS concentration. The enzyme of activity 131.2 µmol/mL/min, 183.1 µmol/mL/min and 104 µmol/mL/min were observed for the samples treated with 50, 150 and 200 µg/mL EMS concentration respectively, that was more than the enzyme activity of the control (94 µmol/mL). The enzyme activity obtained for the sample treated with 250 µg /mL EMS concentration was 53.7 µmol/mL/min which was very less as compared to the enzyme activity of control.

There by resulting that, to achieve the chemical mutagenesis in spores of *A. niger*, the most appropriate concentration of EMS was 150 µg/mL. The comparison of cellulase activity after EMS treatment of *A. niger* at different concentrations is shown in the above Figure 7.

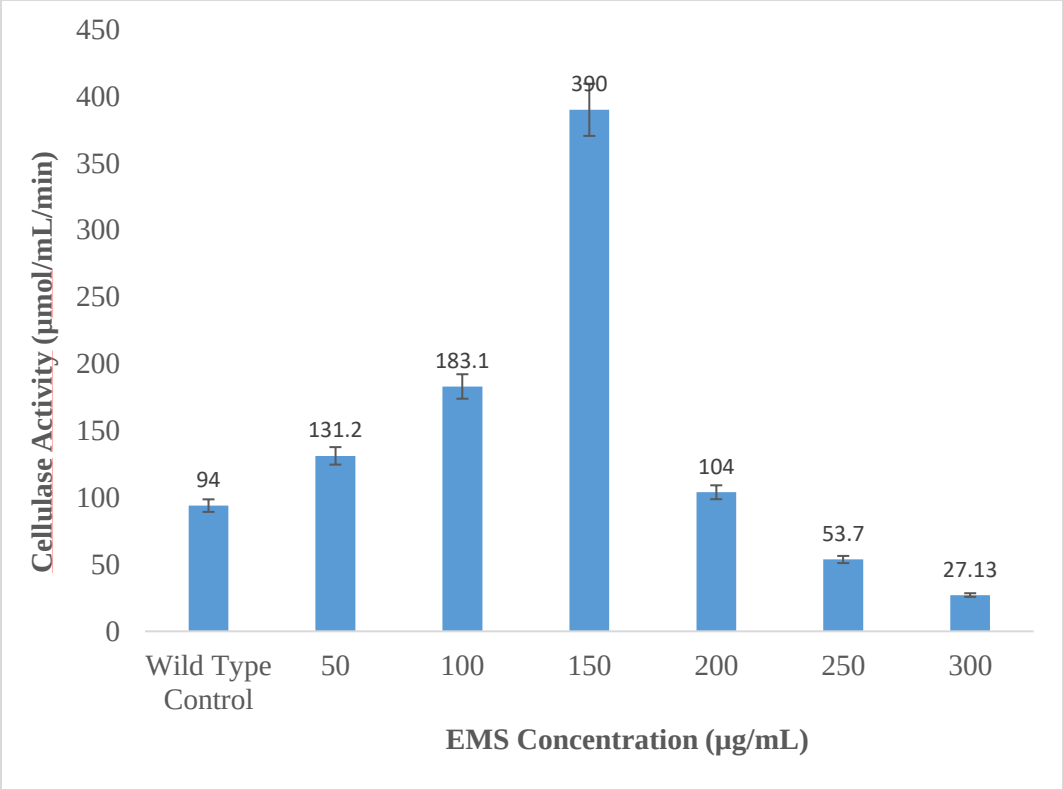

Figure 7: Comparison of cellulase activity after chemical (ethyl methane sulfonate) treatment of *A. niger* at different concentrations. The maximum cellulase activity (390 µmol/mL/min) was observed after treatment with 150 µg /mL concentration of EMS.

Cellulase enzymes degrade insoluble cellulose to soluble sugars. Shafique *et al.* (2009) also discussed about *A. niger,* a filamentous fungus, produced extracellular cellulases in SSF and SmF. To create hyperactive cellulase enzyme makers, UV irradiation (5-40 min) and EMS treatment (50-300 g/mL) were used. The cellulase activity of mutant A-Ch-5.5 (96 Units/mL) and A-UV-5.6 (71 Units/mL) is much higher than that of the wild-type *A. niger* (53.7 Units). Because of their superior genetic make-up, mutants outperformed the wild type. According to RT-PCR results and cellulase enzyme production in plate assays as reported by Mahmood *et al.* (2014), the chemical mutagenesis caused by ethyl methyl sulphonate (EMS) in *A. niger* resulted in increased

expression of the cellulolytic enzyme genes endoglucanase A, endoglucanase B, and endoglucanase C. Comparing the chemically treated, UV irridiated and non-mutagenic forms of *A. niger*, the greatest total protein levels were also discovered (12 mg/mL).

Incorporation of Combined Mutagenesis

The combination of mutagenesis was performed by fungal spore suspension exposure to 10 min UV and 150 µg/mL EMS concentration, as they showed maximum cellulase enzyme activity during physical and chemical mutagenesis. The culture showed very clear and thick mycelium growth. It was observed that the enzyme activity was maximum for combined mutagenesis including UV exposure (10 min) and EMS treatment (150 µg/mL conc.) as compared to the enzyme activity of individual UV exposure and EMS treatment. The results of enzyme activity after combined (both EMS and UV) treatments have been shown in the Figure 8. In the previous studies, enhanced enzyme production has been reported after combined treatment with EtBr, EMS and then UV to the species of *Bacillus subtilis* strain which showed 2 times more activities of β-glucosidase (Cubero *et al.,* 1999; Mahmood *et al.,* 2014).

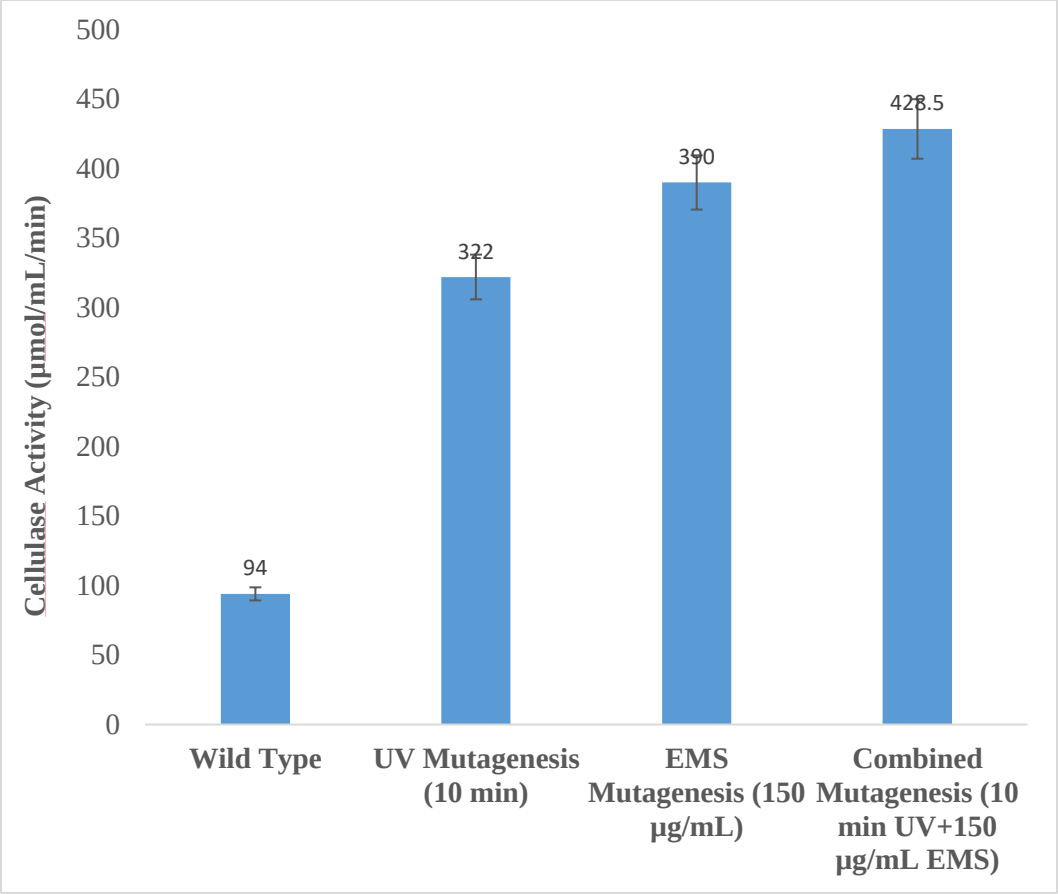

Figure 8: Comparison of cellulase activity from *A. niger* after physical, chemical and combined mutagenesis. The maximum enzyme activity (428.5 µmol/mL/min) was observed after combined treatment with 10 min UV exposure followed by 150 µg/mL EMS concentration as compared to individual physical and chemical mutations.

The mutant treated with combined mutagenesis i.e. UV exposure of 10 min and EMS concentrations of 150 µg/mL, showed the maximum enzyme activity (428.5 µmol/mL/min). Mutagenic substances have ability to create stable hyper-producers of cellulase mutants. Stable UV and chemical mutants of *A. niger* have been reported to produce two and three fold more enzymes than the wild-type organism (Shafique *et al.*, 2009). These durable and efficient mutants had 60–84% molecular genetic similarity. Chemical mutants had substantial genetic variation

from wild-type strains and this could be the reason for enhancement of cellulase activity in our mutant of *A. niger* treated using strategy of combined mutagenesis.

CONCLUSION

The physical, chemical and combined mutations were incorporated to an indigenous strain of *A. niger.* For physical mutation, fungus was treated with UV radiations at different intervals and for chemical mutation, fungus was treated with ethyl methane sulphonate (EMS) using different concentrations. However, the strategy of combined mutagenesis maximally enhanced activity of cellulase (428.5 µmol/mL/min) from *A. niger* as compared to both individual physical and chemical mutagenesis treatments yielding activities of 322 µmol/mL/min and 390 µmol/mL/min, respectively.

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