MOLECULAR EXPRESSION PROFILE OF DIFFERENT CELLULOLYTIC ENZYME GENES IN *ASPERGILLUS NIGER* IN RESPONSE TO UV RADIATION AND CHEMICAL MUTAGENESIS

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Aspergillus niger has several different types of cellulolytic enzyme genes, which are expressed at different levels in response to mutagenesis by UV radiation and chemicals like ethyl methyl sulphonate (EMS). In the present research, the *A. niger* normal (N) strain was subjected to mutagenesis by UV radiation and EMS. The resultant three strains of *A. niger*, i.e. chemically mutated (CM), UV mutated (UM) and normal (N), were subjected to biochemical analysis in the first stage of the study and to cellulolytic enzyme gene expression profile analysis in the second stage. Total protein contents were found to be highest (12 mg/mL) in the chemically mutated form of *A. niger.* As for the enzyme activity, the greatest amylase and pectinase activity was observed in the UV mutated form of the fungus, while the maximum cellulase enzyme activity was observed in the chemically mutated form. This enzyme activity was measured by determining the diameter of the clear zones in plate assays.
For all three strains of *A. niger*, the comparative expression profiles of cellulolytic enzyme genes CbhB, Exo, eglA, eglB, eglC and eng1 were monitored by reverse transcriptase PCR (RT-PCR) with 0.5% carboxymethyl cellulose (CMC) and 0.3% Avicel in the growth medium. While there was no expression for the Eng1 gene transcript in any of the three strains, Egl-C was found to be expressed predominantly in the *A. niger* (UM) strain. The remaining transcripts of the cellulolytic enzyme genes were found to be at higher expression levels in *A. niger* (CM).

Keywords: RT-PCR, cellulolytic enzyme genes, *Aspergillus niger*, mutagenesis

INTRODUCTION

Aspergillus niger is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated as an opportunistic pathogen.[1] Amongst the filamentous fungi, *A. niger* has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase, hemicellulases, glucanases, acid proteinase, and catalase.[2]

Cellulose is the most abundant and renewable biopolymer on Earth.[3] Much of the cellulose in nature exists as waste paper.[4] The different cellulolytic enzymes that cleave the β-1,4-glucosidic bonds between glucosyl residues within the amorphous and crystalline regions of cellulose molecules involve the use of endo and exoglucanase, as well as cellobiase.[5] Cellulases

have a wide range of potential applications in the food, animal feed, textile, fuel, and chemical industries.[6] They have also been used by the paper and pulp industry,[7] the medical/pharmaceutical industry, for waste management, protoplast production, genetic engineering and as household absorbent material.[6]

The genes of endoglucanases (EglA, EglB, EglC and Egl1), exoglucanase (ExoR) and cellobiase (CbhB) are expressed in different fungi to variable extents, based on the genetic profile of the fungus, as well as the type of inducer used for these gene expressions.[8] There are reports of several types of inducers for cellulase genes,[9] but there is no evidence for the types of genes encoding for cellulolytic enzymes that are induced in local normal, as well as chemically and UV modified strains of *A. niger*. For this reason, the present study was designed as a means to ascertain the exact expression profile of different cellulolytic enzyme genes by RT-PCR in the three local strains of *A. niger*. The biochemical screening in terms of different enzymes, including urease, amylase, pectinase and cellulase, as well as the total proteins, was also carried out for these strains of *A. niger*, because of their potential for industrial exploitation.

EXPERIMENTAL

Chemical mutagenesis of *A. niger*

The normal strain of *A. niger* (N) was obtained from Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore. The purity of the fungal strain was determined using microscopic analysis. The normal *A. niger* strain was subjected to chemical mutagenesis using Ethyl Methyl Sulphonate (EMS) as described earlier.[10] Briefly 6 x 10^5 mL^{-1} conidial suspension in a 1% sterile NaCl solution containing 150 µg mL^{-1} EMS was subjected to incubation at 37 °C for 45 minutes. After incubation, the conidial suspension was washed thrice with the 1% sterile NaCl solution and was subjected to centrifugation at 8000 rpm for 2 minutes to remove traces of EMS. After these washings, the final volume of conidial suspension was adjusted to 0.4 mL and from this solution 0.05 mL of conidial suspension was spread on a potato dextrose agar (PDA) medium (potato extract 4.0 g, dextrose 20 g and agar 15 g per liter of the medium) plate, containing 0.1% Triton X-100 and L-sorbose as colony restrictors. After 5 days of incubation, the surviving conidia were screened on a PDA medium containing 0.5% CMC and 0.3% Avicel. The conidial colony showing the largest zone of clearance, in comparison with normal conidia, was picked and cultured for further biochemical and molecular analyses.

UV mutagenesis of *A. niger*

The conidia of the normal *A. niger* strain from the one week old PDA plate were subjected to UV mutagenesis after preparation of a 6 x 10^5 mL^{-1} conidial suspension in 1% sterile NaCl solution. Briefly, 0.2 mL of conidial suspension from the 6 x 10^5 mL^{-1} stock was spread on a PDA plate containing 0.1% Triton X-100 and L-sorbose under aseptic conditions. The plate was then exposed to 254 nm UV irradiation, for up to 50 minutes, with a 5 minute rest period for every 10 minutes of exposure under the UV lamp (UVGL-58, UVP upland, USA). After 5 days of incubation the survived conidia were screened on a PDA medium containing 0.5% CMC and 0.3% Avicel. The conidial colony showing the largest zone of clearance, in comparison with normal conidia, was picked and cultured for further biochemical and molecular analyses.

Biochemical screening of *Aspergillus niger* strains

The biochemical screenings of the three *A. niger* strains – namely, the chemically modified (CM), UV modified (UM) and normal (N) – were carried out by employing different tests on the total amount of soluble proteins[11] in 0.2 g of mycelium. The tests performed included a urease test,[12] a pectinase test,[13] an amylase test[14] and a cellulase test.[15]

Culturing of fungal strains for RNA isolation

The strains of *A. niger* were each grown in 100 mL of a CMC broth medium (potato extract 0.4 g and dextrose 2.0 g), containing 0.5% CMC and 0.3% Avicel as inducers for cellulolytic enzymes. The fungal strains in the broth medium were grown at 30 °C for 5 days before proceeding to RNA isolation from cultures.

RT-PCR analysis of different cellulolytic gene transcripts

Total RNA was isolated from all the *A. niger* strains by adopting the kit method (Favorgen, USA). The cDNA of the genes were synthesized by using (BioTeke Corporation) reverse transcriptase coupled with reverse gene specific primers (Table 1) at 42 °C for 1 hour. The synthesized cDNA of each gene was subjected to normal PCR by using combinations of forward and reverse primers (Table 1). The 50 µL PCR reaction mixture contained 1X PCR buffer 4 mM $MgCl_2$, 0.4 mM dNTPs, 100 pmol of each primer, 8 µL of cDNA and 5 units of taq polymerase. The melting temperatures – 55 °C and 60 °C – were checked in order to evaluate the expression profiles of different cellulolytic genes. The amplified gene products were analyzed on 1% agarose gel and the results were documented.

Table 1
Primers used for the amplification of different cellulolytic enzyme genes

Name	Sequence (5`-3`)	Length
cbhBF	GCAAGTGTACCCACTCACACA	21
cbhBR	AAGCGGTTGTACGTGCAAGA	20
ExoF	TGTGCTCTCGTTGCCCTCTTG	21
ExoR	AGTGCATTGGCGCCTTCCTC	20
eglAF	TCCCCGTGTCACTTGCTATG	20
eglAR	CAGTTCATAGTCGCCGCTAGA	21
eglBF	ATCTCAACCAAGCAGCCATT	20
eglBR	CCAGGATATCCAGCATACCC	20
eglCF	TGGTGTTACCGGTCTCTTCAAAAC CGA	27
eglCR	GCTATACCAGGGATAGACTTACACTGCA	30
eng1F	CGACTTGGTCAGTTTGATACC	21
eng1R	ATACCCGTGTAAGCAGTTCC	20

Table 2
Biochemical profiling of different strains of *A. niger*

Sr. No	Biochemical test	*Aspergillus niger* (CM)	*Aspergillus niger* (UM)	*Aspergillus niger* (N)
1	Total proteins	12±0.3 mg/ml	11±0.2 mg/ml	10±0.2 mg/ml
2	Urease	18±0.1 mm	18±0.05 mm	28±0.4 mm
3	Amylase	25±0.2 mm	32±0.6 mm	25±0.4 mm
4	Pectinase	6±0.1 mm	8±0.2 mm	6±0.1 mm
5	Cellulase	7±0.05 mm	5±0.1 mm	5±0.3 mm

CM (Chemically modified), UM (UV modified), N (Normal)
Total protein was isolated from 0.2 g of mycelium. Each experiment was repeated thrice and average values are provided for each test

RESULTS AND DISCUSSIONS

A. niger mutagenesis by UV irradiation and chemicals like EMS leads to significantly high production of cellulase enzyme, as compared to the normal *A. niger* strain. In this study, we attempted to identify the various cellulase genes that were stimulated in response to UV and chemical mutagenesis. Additionally, we studied the effects of chemical and UV mutagenesis on other enzymes in order to determine if their level of production was increased in comparison with the normal *A. niger* strain.

The chemically modified (CM), UV modified (UM) and normal (N) strains of *A. niger* were previously screened for the presence of different industrial enzymes: pectinase, amylase, urease and cellulase (Table 2). The resultant activity for each enzyme in the plate assays was determined by measuring the ratios of the clear zone diameter around the fungal disc of the mutagenic strains, relative to the clear zone around the (N) fungal strain on solid malt extract agar (MEA) medium (malt extract 20 g, peptone 1 g, dextrose 20 g, agar 15 g per liter of the medium). *A. niger* (CM) was found to be the most efficient regarding cellulase production, as the diameter of the clear zone in the plate assay was greater than those of the other two strains with a measurement of 0.7 cm (Table 2). *A. niger* (UM) was found to be the most efficient regarding amylase and pectinase enzyme production in the plate assays (Table 2). In the case of *A. niger* (N), the level of urease enzyme activity was quite high with regard to *A. niger* (UM) and (CM). The total soluble protein contents were found highest for each strain in 5 mL of a 20 mM Tris-Cl buffer solution (pH 8.0) with *A. niger* (CM) having a concentration of 12 mg/mL, *A. niger* (UM) with a concentration of 11 mg/mL, and *A. niger* (N) having a concentration of total soluble proteins of 10 mg/mL (Table 2). These results, which are also supported by previous research,[16,17] suggest that chemical mutation is particularly useful for the genetic improvement of *A. niger* regarding cellulase enzyme production and that UV mediated mutation is beneficial for an increase in amylase and pectinase enzyme production.

Table 3
RT-PCR analysis of different genes of hydrolytic enzymes in *A. niger*

Sr. No.	Genes induced	Accession numbers of genes targeted	Amplified product size (bp)	*Aspergillus niger* (CM)	*Aspergillus niger* (UM)	*Aspergillus niger* (N)
1	CbhB	XM_001389539	587	++	+	+
2	ExoR	XM_001389615	598	++	+	+
3	Egl-A	AJ224451	391	++	-	+
4	Egl-B	GQ292753	470	++	++	+
5	Egl-C	AY040839	515	+	++	+
6	Eng1	XM_001391932	996	-	-	-

CM (Chemically modified), UM (UV modified), N (Normal), ++ (Amplified DNA band intensity 60 ng or above), + (Amplified DNA band intensity less than 60 ng), - (No amplification observed)
Note: Inducer used while fungal growth in broth is 0.5% CMC and 0.3% Avicel. The experiment was repeated thrice

The molecular expression profiles of six cellulolytic enzyme genes – cbhB, ExoR, EglA, EglB, EglC and Eng1 – were determined by RT-PCR for *A. niger* (UM), (CM) and (N) (Table 3). The optimized melting temperature for all of the gene specific primers was found to be 55 °C. These results are consistent with the observations made in several experiments. The experimental data indicated that only one gene transcript, Eng1, was not expressed in any of the tested strains. In the case of *A. niger* (CM), all expressed gene transcripts, except for Eg1C, were expressed in relatively high amounts – i.e. 60 ng and above. EglC expression was comparatively less than all the other gene transcripts for this strain. In the case of *A. niger* (UV), EglA gene transcripts, along with the aforementioned Eng1 gene transcripts, were not induced (Table 3) however; EGLB and EGLC were expressed in relatively higher amounts, which were comparable to (CM) *A. niger* (Table 3). In *A. niger* normal control all expressed gene transcripts were in a comparatively lower amount with less than 60 ng being detected (Table 3).

These results suggest that the genes for different cellulolytic enzymes are expressed at differential rates in response to different mutagenic agents. This is further supported by the work of other researchers.[18] For the expression of the EglB gene, both chemical and UV mutagenesis of *A. niger* are useful, while for EglC gene expression UV mutagenic is the method of choice (Table 3). In the case of chemical mutagenesis, cbhB, ExoR and EglA gene transcripts are more efficiently expressed than for the UV and normal strains.

This study describes the effect of chemical and UV treatment on the expression of different types of cellulolytic enzyme genes which have wide industrial applications.

CONCLUSION

The chemical mutagenesis induced by ethyl methyl sulphonate (EMS) in *Aspergillus niger* resulted in enhanced expression of the cellulolytic enzyme genes CbhB, Exo, eglA, eglB and eglC, as determined by RT-PCR, as well as cellulase enzyme production in plate assays. Total protein contents were also found to be the highest (12 mg/mL) in the chemically mutated form of *Aspergillus niger*, as compared to UV and non-mutagenic forms of the fungus.

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