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Full Length Article

Genetic Diversity in Hyper Glucose Oxidase Producing *Aspergillus niger* UAF Mutants by using Molecular Markers

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Abstract

Glucose oxidase (β-D glucose: oxygen-oxidoreductase, GOD) is of prime importance in food and health industries for the production of gluconic acid, maintaining flavor and color stability and quantification of glucose in blood and urine etc. *Aspergillus niger* is one of the key producers of GOD. We report the genetic diversity assessment of *A. niger* University of Agriculture Faisalabad (UAF) wild type strain and its seventeen mutants. The mutants developed via four mutagens viz., UV rays, gamma rays, ethidium bromide and MNNG for the hyper production of Glucose Oxidase. Molecular typing methods have been used for the detection of polymorphism within the members of any fungal genera as well as species. However, this is the pioneering attempt which focuses on detection of polymorphism in *A. niger* UAF parent strain and its mutants using RAPD and SSRs. Out of 26 RAPD primers used in the study, 12 produced polymorphic banding pattern amplifying 125 bands with an average of 10.41 fragments per primer. The most diverse mutants were UV-180-A and UV-180-B. All the mutants and a wild type strain were divided into six different groups except the mutant EMS-F which appeared unclustered. For SSR analysis, six different microsatellites were applied (AN01-06), which amplified 2-3 alleles in the mutants ranging from 100-450 bp size. The results revealed the existence of high genetic variability among the mutant strains and a wild type strain.

Keywords: *Aspergillus* sp.; PCR; RAPD; SSR

Introduction

The genus *Aspergillus*, comprising of more than 180 species, is economically the most important group of filamentous fungi. While some of the members of this group are food spoilage, disease causing fungi (Sobiya e*t al.*, 2010; Saleemi *et al*., 2012; Ahmad *et al*., 2012), the others, especially *A. niger* are rendered safe for food and drug administration (Raclavasky *et al.*, 2006). *A. niger* is of special interest to modern biotechnologists owing to its exploration for secondary metabolites, aflatoxins, mycotoxins, plant growth regulators, citric acid, heterologous and homologous proteins and various commercial enzyme production (Mirhendi *et al*., 2009).

Glucose oxidase (β-D glucose: oxygen-oxidoreductase, GOD) produced by *A. niger*; a flavoenzyme synthesized inter and intracellular, is of prime importance in food and beverage industries (El-Sherbeny *et al*., 2005). In diagnostics it is used for the development of glucose dissecting kits for estimating glucose levels in hyperglycemia and hypoglycemia patients (Zia, 2007; Zia *et al*., 2010).

Enzymes can also be over expressed by mutations with certain mutagens such as gamma and ultra violet (UV) radiation, ethidium bromide and MNNG (N-methyl-N-nitro-N-nitrosoguanidine) etc. The mutagenic effects of these mutagens induce changes in the genome at gene or allele level. Genome level analyses are more efficient than physical and chemical detection of hyper producing mutants, in understanding the factors involved in hyper production of the GOD enzyme. It may help in detection of duplications, deletions and repetition of some specific sequences as of gene(s) transcribing GOD (Abed, 2008). In this context, various molecular techniques especially RAPDs and SSR are extensively employed (Devalk *et al.*, 2007) in DNA fingerprinting studies within the members of a particular fungal genera as well as species (Asano *et al*., 2011). However, their exploitation in genetic diversity assessment among the mutants of a single strain is another diverse area which is yet to be explored.

The present study is the pioneering attempt in this regard. We report here the detection of polymorphism in hyper Glucose Oxidase producing *A. niger* UAF (University of Agriculture Faisalabad) parent strain and its mutants using two molecular marker techniques, Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSRs). The results might be used for mapping and identification of genes responsible for GOD hyper production.

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Table 1: Percent increase in GOD activity in *A. niger* UAF and its mutants used in the study

Name	Increase in GOD activity (%)
Wild type	100
G-80-A	459
SA-150-F	431
M-120-B	326
M-120-C	112
EB-150-B	174
EB-150-C	227
EB-150-D	248
EB-150-E	223
EMS-A	260
EMS-C	260
EMS-D	186
EMS-E	211
EMS-F	193
UV-180-A	224
UV-180-B	199
UV-180-D	233
UV-180-E	245

Table 2: Composition of PDA and Vogel's medium

Components	PDA	Vogel's medium
	Concentration (g/100 mL)	Concentration (g/100 mL)
Glucose	2	50% W/V
Agar	2	-
Starch	2	-
Urea	3	-
KH_2PO_4	0.008	0.5
$MgSO_4.7H_2O$	0.05	0.02
$ZnSO_4.7H_2O$	0.001	-
KCl	0.015	-
NH_4NO_3	-	0.2
$(NH_4)_2SO_4$	-	0.4
Peptone	-	0.1
Trisodium citrate	-	0.5
Yeast extract	-	0.2

Table 3: List of RAPD primers and their sequences used in the study

Primer Name	Sequence	NPB*
GLB-05	TGCGCCCTTC	09
GLB-12	CCTTGACGCA	12
GLB-15	GGAGGGTGTT	10
GLK-08	GAACACTGGG	10
GLK-16	GAGCGTCGAA	8
GLK-19	CACAGGCGGA	8
GLK-20	GTGTCGCGAG	7
GLL-04	GACTGCACAC	11
GLL-05	ACGCAGGCAC	13
GLL-07	AGGCGGGAAC	09
GLL-08	AGCAGGTGGA	08
GLL-12	GGGCGGTACT	10

*Number of Polymorphic Bands

Materials and Methods

Microorganisms and Culture Maintenance

Seventeen mutants and the wild type strain of *A. niger* (Table 1) were used in the present study. The fungus was maintained on Potato Dextrose Agar (PDA), sub cultured after one month and stored at 4°C. One day after preparation, test tubes containing 8-10 mL of PDA slants were inoculated with *A. niger* spores in Biosafety Cabinet (BSE II, Model, 4A1, Italy), maintained at 30°C in dark. Fourteen days after culture, a loop of black conidial spores from PDA slants was inoculated into 30 mL Vogel's medium in a 100 mL Erlenmeyer flask (Table 2). Flasks were incubated in thermal incubator at 250 rpm in dark at 30°C, for 28 to 36 h. Mycelia were harvested and dried on whatman's filter paper No. 1, transferred to 50 mL falcon tubes and stored at -20°C.

DNA Fingerprinting Analyses

The genomic DNA was extracted from 17 mutants and a wild type strain of *A. niger* by modified CTAB method (Sobiya *et al*., 2010), purified by incubating the re-suspended sample at 37ºC for 30 min with RNase (Boehringer, Mannheim). DNA concentration was determined by NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware).

RAPD-PCR Analysis

Twelve primers (Table 3) were selected out of the 26 random primers on the basis of the polymorphic amplification for RAPD analysis of *A. niger* UAF strain and its mutants. Reaction mixture (25 µL) contained 2.5 µL template DNA, 0.2 µL taq DNA polymerase; 4.0 µL dNTPs, 3.5 µL $MgCl_2$ (25 mM), 2.5 µL PCR buffer (10X), and 2.0 µL RAPD decamer (10 p mole) and 8.3 µL d_3H_2O. Amplification was carried out in DNA thermal cycler (Peqlab Germany) comprising of 40 cycles each of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min with 5 min of initial denaturation at 94ºC and one cycle at 72ºC for 10 min for final extension.

Agarose Gel Electrophoresis

The electrophoresis was performed using Scie-plask, UK, gel electrophoresis apparatus. Agarose gel (2.5%) was prepared in 0.5X TBE buffer and a total sample volume of 8 µL (5 µL of PCR product and 3 µL of 6x loading dye) was loaded in each well. The gel was electrophoresed in 80 V for 1.5 h and then stained with ethidium bromide solution (10 mg/mL) for 15 min. DNA was visualized on a UV gel documentation system, Bio-Rad UV gel documentation system (Universal hood II Bio-Rad, Italy).

Microsatellite Analysis

Six polymorphic SSR primers (Table 4) of *A. niger* were used as reported by Esteban *et al.* (2005). PCR amplification was performed in a 20 mL volume containing 1.5 µL $MgCl_2$, 2 µL of 10x Taq-DNA buffer, 6.4 µL dNTPs, 1 µL each of forward and reverse primers, 0.2 U of *Taq* DNA polymerase, 2 µL of DNA template and 5.8 µL of d_3H_2O. Amplifications were carried out with denaturation

Table 4: List of microsatellite primers and their sequences used in the study as reported by Esteban *et al.* (2007)

Primer	Locus	Sequence (5'-3')	Size range (bp)	Gene Bank Accession no.
AN-1	ACNM 1	F= TCTCGACTCTGGCTCCTACC R= GTTTGCTTACTCACCGACTGGAAAA	464–509	AY081845
AN-2	ACNM 2	F= TGCCCTTACTCTGCCTCTCT R= GTTTCCATTATTCACCCTCCCTTCT	409–446	AX952973
AN-3	ACNM 3	F= TAACTTGCCTCCGTGGTTGT R= GTTTGAGACCGGAAACATTGGAGTAG	177–215	BE759201
AN-4	ACNM5	F= CGTTTTCTCGGAAGGTTTGA R= GTTTGTGCGTGTTGGGGACTATCT	163–204	ANAJ5117
AN-5	ACNM 6	F= CGACAGCCGCATCATAGTT R= GTTTCCTGCTCTTTTTGCCTTCTTT	429–458	AY081847
AN-6	ACNM 7	F= TGAGGGAAGGGGGTTTTATT R= GTTTGATCTACGGGGGTGTTTGTC	378–468	ANI278532

for 5 min at 94°C, 30 times cycles of 1 min at 94°C, 1 min at 60°C (primer annealing), and 1 min at 72°C and a final extension step at 72°C for 10 min. The PCR products were diluted 1/10 in water, and 3 mL of each was loaded in respective wells of PAGE gel. The gel was prepared by adding 63.75 mL of 0.5X TBE, 30% 29:1 acrylamide:bis acrylamide and 700 µL of 10% ammonium per sulfate (APS) to a homogenous solution. A 50 bp DNA ladder was loaded in the first well of the gel and samples were electrophoresed at 100V for 3h. The PAGE gel was stained in ethidium bromide solution (10 mg/mL) in the tray and the gel shaken at 150 rpm for 15 min. Gel documentation was performed using Bio-Rad UV gel documentation system (Model Universal hood II Bio-Rad). Bands scoring were "1" for presence of band and "0" for absence.

Statistical Analysis

A similarity matrix was developed using PopGen 32 software, version 1.44 (Nie, 1972) based on Nei's Un-weighted Paired Group of Arithmetic Means Average (UPGMA) to estimate genetic distance and relatedness of *A. niger* mutants and a wild type strain. Dendrogram was drawn for measurement of genetic diversity. Data were collected and aligned for cluster analysis using POP Gen version 1.32 for measurement of genetic distance among mutants.

Results

Genetic Diversity Based on RAPD Analysis

Twelve out if 26 Radom primers produced polymorphic banding pattern in seventeen mutants and a wild type strain. In total 125 fragments were produced with an average of 10.41 fragments per primer. The number of polymorphic bands produced in all the seventeen mutants and a wild type strain ranged from 5 to 12. The primer GLB-12 produced the highest number of polymorphic bands. i.e., 12 and the primer GLB-5 produced only five polymorphic bands. All the mutants showed a varying degree of genetic divergence based on their amplification profile. Reactions were repeated thrice to check the consistency of amplified RAPD products. The number of bands amplified by each mutant and a wild type strain of *A. niger* is presented in Fig. 1. Maximum number of bands was amplified by primer GLK-16 as against GLB-06 with the minimum number of amplified, observed in mutant EMS-C and EB-150-D, respectively. The maximum number of amplifications in wild type *A. niger* strain were produced by primer K-16. All the mutants were genetically diverse with wild type strain. The maximum similarity (78.63%) with wild type strain was found in G-80-A. On the other hand, the most closely related mutants were UV-180-A and UV-180-B with the genetic similarity of 61.60% (Fig. 2).

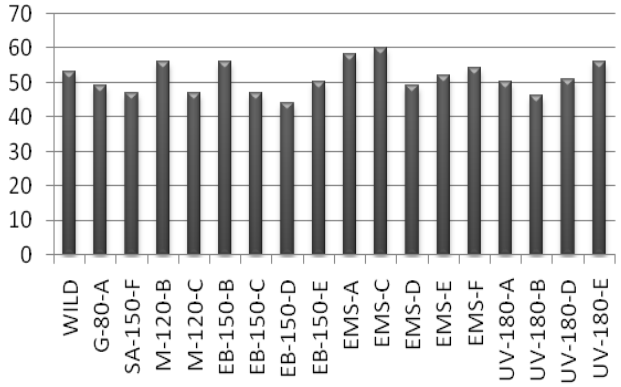

Fig. 1: No. of bands produced in each *A. niger* mutant and a wild type strain

Cluster analysis (Fig. 3) divided the mutants and a wild type strain into two main groups distinctly; group A and B. Group A consisted of EMS-C and UV-180-D with 78.63% similarity. While, group B comprised of all other mutants and a wild type strain. In group B wild type strain and mutant G-80-A were grouped together showing 61.60% similarity. EB-150-E clustered with EMS-A (74.40%), UV-180-A paired with UV-180-B (61.60%), EB-15-B clustered with UV-180-E (73.60%), M-120-C grouped with EB-150-C (72%), EMS-D clustered with EMS-E (72.80%) and SA-150-F clustered with M-120-B (70.40%).

Genetic Similarity among the Mutants

The highest genetic similarity was found in G-80-A (73.60%) with wild type strain of *A. niger*. However,

mutants EMS-E and UV-180-E had equal genetic similarity (58.40%) with wild type. Primer G-80-A exhibited maximum genetic similarity with wild type strain, followed by EB-150-E (69.6%), EMS-150-E (68%), EB-150-B (67.2%), EMS-D (66.4%), EMS-A (65.6%), EMS-C, EMS-F (64.8%), UV-180-A (64%), EMS-E (61.6%), EMS-E, SA-150-F (60.80%), M-120-C (58.45) and UV-180-B (53.6%) (Fig. 3).

Genetic Distance among the Mutants

The highest genetic distance of mutants from wild type strain of *A. niger* was found in UV-180-D (62.36%), while minimum genetic distance was observed in G-80-A (30.65%). Genetic distance of other mutants from wild type strain of *A. niger* ranged between the maximum and minimum values.

Genetic Diversity Analysis using SSRs

Microsatellites are considered very efficient molecular markers for the strain identification, screening and genetic diversity. The study employed six polymorphic SSR primers. The SSR primer AN-04 amplified the alleles ranging in size from 100 bp to 150 bp. The profile depicted three alleles (1st allele with almost 105 bp, 2nd with 120 bp and 3rd was about 140 bp size). AN-04 amplified bands of almost equal sized alleles. Hence, no variation in band size was found among the mutants and a wild type *A. niger*. The SSR primer AN-05 amplified a single allele ranging in size from 250 to 300 bp. The bands of almost equal sized alleles were amplified and no sharp differences were recorded. The results obtained by this primer in the present study were different from that of size of alleles given by Bart-Delabesse *et al.* (1998) and Esteban *et al.* (2008) for genetic diversity studies of *A. niger*. The Primer AN-06 when applied for SSR analysis of genomic DNA of *A. niger* mutants and wild type strain, amplified the allele ranging in size from 250 to 300 bp. The SSR profile presented two alleles (1st allele with almost 260 bp and 2nd with 290 bp). AN-06 amplified bands of almost equal sized alleles with no sharp differences.

Discussion

Mean number of bands produced per primer obtained in this study were found best in comparison to number of bands achieved by RAPD-PCR in *A. niger* and *A. flavus* and is in line with the early reports by Mirhendi *et al.* (2009), Sobiya *et al.* (2010) and Abed (2008). The RAPD analysis revealed ample polymorphism present in the study is found satisfactory. The high similarity values were due to the reason that all the mutants originated from single strain of *A. niger*. Sobiya *et al.* (2010) determined the genetic diversity present in *A. niger* mutants and found similar results of banding pattern, however band size varied from that of obtained by her. Abed (2008) differentiated *A. niger* strain with RPAD-PCR and found lower number of bands as compared to obtained in our study.

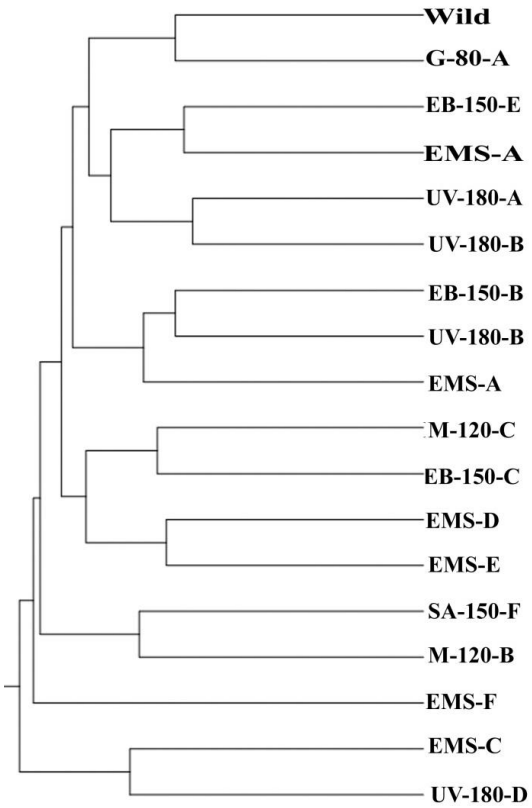

Fig. 2: Dendrogram showing 17 *A. niger* mutants and a wild type strain based on Nei's (1972) original measures

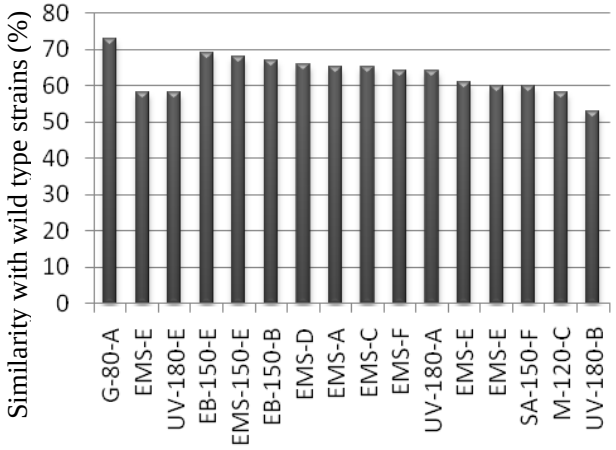

Fig. 3: Percent similarity of *A. niger* mutatns with wild type strain

Molecular evaluation of mutants is a good technique as compared to phenotypic or biochemical characterization and identification as discussed by Batista *et al.* (2008), Esteban *et al.* (2008), Herman *et al.* (2007) and Devalk *et al.* (2007). Other reports concerning the genetic diversity estimation using RAPD conducted by Nahid (2006), Elizabeth *et al.* (2006) and Raclavasky *et al.* (2006) described RAPD analysis to be a powerful tool with a

number of advantages i.e., easy generation of data and no requirement of previous knowledge of the genome. Genetic diversity among different species of the same genera as well as among the members of different genera reflects a rich history of strain identification and confirmation. The reason behind this is the fact that nucleotide diversity is the base of phenotypic diversity. The mutants with a most diverse genetic base were found to be UV-180-A and UV-180-A with a GOD % is 224 and 199, respectively. This range of GOD % is also detected in other mutants but all other mutants showed some varying degree of divergence among each other. At this stage it will be very difficult to correlate the degree of divergence among the mutants with the respective GOD % because, being random in nature RAPD will detect the variation throughout the genome. The variations found in the mutants from wild type strain may be attributed to mutagenesis, induced by different mutagens. Previously different techniques were applied for differentiation such as biochemical assays or phenotypic characterization, which cannot detect such minor changes at DNA level. This is only possible by using molecular identification techniques as presented in this study.

Aspergillus niger is one of the potent producer of α- and β-Galactosidases. The mutant-derivatives of *A. niger* produced two-fold higher α- and β-galactosidases. For testing genetic variability and its relationship with phenotypic properties of the two organisms, DNA samples of the mutant and wild type strain of *A. niger* were amplified with 12 deca-nucleotide synthetic primers. RAPD analysis showed significantly different pattern between parental and mutant cultures. The mutant derivatives yielded homogeneous, while parental strain formed heterogeneous amplification patterns. Seven primers identified 42.9% polymorphism in the amplification products, indicating that these primers determined some genetic variability between the wild and mutant strains of *A. niger*. Results indicated that RAPD can be effectively used to differentiate mutant strain from the parental strain based on their RAPD patterns.

Current study produced promising results in relation to genetic diversity among mutants and a wild type *A. niger*. Since, this is the pioneer study on evaluating diversity among mutants of a single species; it needs further exploration as far as genetic diversity estimates are concerned. Preferably by employing large number of primers, which might amplify the mutated region of the allele and in turn lead to more variations among the samples of *A. niger*.

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