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Full Length Article

Molecular and Biochemical Screening of Local *Aspergillus niger* Strains Efficient in Catalase and Laccase Enzyme Production

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Abstract

Ten local *Aspergillus niger* isolates were subjected to RAPD analysis and out of 20 random oligonucleotide primers, primer P2 (5′-ACGGCGTATG-3′) was optimized to screen all ten isolates of *A. niger*. Results showed amplifications of different sizes in range of 150-1500 bp and isolates 658 and 880 showed 100% genomic similarity while isolate 764 showed least genomic homology (22.54%). Based on RAPD analysis representative isolates from each cluster were short listed for further downstream molecular and biochemical analysis. These isolates included 880, 764, 506, 1005 and 744. In ribotyping hyper variable region V4 of 18S rRNA gene was amplified in five isolates which were found to be genomically diverse in RAPD analysis. Results showed that amplified sequence of 18S rRNA gene was highly conserved and 100% similar with each other and to the already reported sequence (Accession No JX393054.1). In catalase test on all isolates of *A. niger* indicated that isolates 744, 506, 764 and 880 were the most efficient in the production of catalase enzyme, while remaining isolates except 1109 showed intermediate catalase activity. In laccase assay isolate 744 was found to be most efficient and isolate 840 was found to be least efficient, while the remaining isolates were found to be at same level in laccase assay. Results suggested that isolate 744 can be exploited further for the production of catalase and laccase enzyme at industrial scale.

Keywords: *Aspergillus niger*; RAPD; Ribotyping; Catalase; Laccase

Introduction

Members of *Aspergillus* section *nigri* are regarded as soil fungi, frequently colonizing plant debris and decaying agricultural crops (Varga *et al*., 2004). Many species cause food spoilage and several are used in the fermentation industry (Yuan *et al*., 2006). Moreover, some strains of *Aspergillus* cause aspergillosis in human beings (Kami *et al*., 2001). Different species of *Aspergillus* reported in literature are good sources of industrial important enzymes like catalases, cellulases, amylases, laccases and several other enzymes (Wheeldon *et al*., 2008). Sixteen fungal enzymes were approved for use in food of which 13 were obtained from *Aspegillus* (Archer, 2000; Ahmad and Butt, 2013).

Catalases (EC1.11.1.6) are the class of enzymes, which catalyze the decomposition of hydrogen peroxide (H_2O_2) to oxygen and water (Halliwell, 1990). This function is essential to control H_2O_2 levels in living systems which is toxic to cells and by product of mitochondrial electron transport, β-oxidation of fatty acids, and photorespiration (Montavon *et al*., 2007). Catalase is used in the textile industry, removing H_2O_2 from fabrics to make sure the material is peroxide free (Goodsell, 2004) as well as in the food industry for disposing of H_2O_2 used in pasteurizing milk prior to cheese-making (Chu *et al*., 1975). Laccase is one of the few oxido-reductases commercialized as industrial catalyst and can be used for textile dyeing/finishing, wine cork making, tooth whitening and many other industrial, environmental, diagnostic and synthetic uses (Xu-Feng, 2005). Laccase production was reported from some soil ascomycete species including *Aspergillus* and *Penicillium* (Scherer and Fischer, 1998) which contained laccase genes whose products known to oxidize syringaldazine (Lyons *et al*., 2003).

Different molecular techniques are frequently being used to evaluate genomic diversity in fungal isolates. RAPD markers are very useful for the determination of genetic diversity, taxonomic identifications and paternity analysis (Nesbitt *et al*., 1995). RAPD markers typically have high overall variability and so can be useful for detecting genetic differences within species as well (Sunnucks, 2000; Nawaz *et al*., 2013). Due to highly conserved flanking region of 18S rRNA gene, it is dependable marker to screen environmental biodiversity among species (Woese *et al*., 1990; Hanif *et al*., 2012). Amplification of rRNA gene in ribotyping and analysis of SNPs has become important molecular aspect to differentiate fungi into different strains even within the same species (Balajee *et al*., 2008).

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The current study was carried out to evaluate most efficient strains of *A. niger* for the production of catalase and laccase enzymes by using enzymatic assays as well as molecular approaches including RAPD and amplification of rRNA gene with subsequent genetic sequencing to find most efficient in enzymes production.

Materials and Methods

Ten isolates of *A. niger* were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. List of these isolates and their substrates are described in the Table 1.

A. niger

A. niger strains were subcultured on potato dextrose agar (PDA) medium by method as described by Dhingra and Sinclair (1995) by inoculating the spores of actively growing colony on the culture media Petri plates. The plates were incubated at 25°C for seven days and then preserved at 4°C for subsequent molecular and biochemical analysis.

Fungal DNA

For DNA isolation cultures were inoculated on the PD broth medium and flasks were put on the shaker at 25˚C for 6 days. Culture media were filtered and mycelia were used for genomic DNA extraction by using genomic DNA reagent kit (Geneaid Biotech Ltd., Taiwan, Cat # GR100). Genomic DNA was isolated from 0.5 g mycelium. The purified DNA samples were stored at -20˚C for subsequent downstream studies.

RAPD

RAPD analysis of ten different strains of *A. niger* was carried out by adopting the method as described earlier Ranganathan *et al.* (2002). Twenty different oligonucleotide primers (Elim Biopharmaceuticals, Inc) were initially screened to evaluate genomic diversity. The conditions of RAPD were optimized by adjusting different melting temperatures (25, 30 and 35˚C) and different $MgCl_2$ concentrations (1.5-3.0 mM). The standard reagents supplied by Enzynomics Inc. Korea were used in RAPD amplifications. The 50 µL RAPD mixture contained PCR buffer (1x), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), primer (25 pmol/µL), taq polymerase (2.5 U), genomic DNA (1.0 µg). PCR tubes with 50 µL reaction mixture were placed in the PCR thermocycler (Gene Amp Biosysytem-9700, Applied Bio-system) and following temperature cycling conditions were programmed; initial denaturation at 94˚C for 5 min, followed by 40 cycle denaturation at 94°C for 1 min, annealing at (35°C) for 1 min, primer extension at 72˚C for 1 min and final extension temperature 72˚C for 5 min. Amplification products were subjected to electrophoresis at 70 volts for 30 min by using 1% agarose gel stained with ethidium bromide and photographed by using UV transilluminator (UVipro-gold, UV-tech). Amplification profiles of different isolates of *A. niger* were compared with each other by using MINITAB software.

Ribotyping

A. niger strains (880, 764, 506, 1005 and 744) found genomically diverse in RAPD analysis were selected and hypervariable V4 domain of 18S rRNA gene was amplified by employing previously reported primers (Machouart-Dubach *et al*., 2001). The primer sequences were 5′ GTAATTCCAGCTCCAATAGCG 3′ for FaL and 5′ GTATCTGATCGTCTTCGATC 3′ for FaR (5′ ends at positions 577 and 1007 respectively, according to the18S DNA sequence Z75578 of *Saccharomycese cerevisiae*, James *et al*., 1997). The conditions for 18S rRNA gene amplification were optimized by adjusting different melting temperatures (50˚C, 55˚C and 60˚C) and $MgCl_2$ concentration (1.5 to 3.0 mM). The reaction mixture (50 µL) composition was same as described for RAPD analysis. The PCR cycling condition involved initial denaturation at 94˚C for 3 min, followed by 35 cycles and each cycle has denaturation at 94˚C for 1 min, annealing at 55°C for 1 min, primer extension at 72°C for 1 min and final extension temperature 72°C for 5 min followed by 1% agarose gel analysis. The amplified fragments were purified by following the protocol of Wizard SV Gel and PCR Clean-Up system (Promega) and sequenced in both directions from Core Sequencing Facility, at University of Illinois, Urbana, IL, 61801. The derived sequences were analyzed by Chromas software and sequence comparison was performed with BLAST network services at NCBI and subsequently aligned with 18S rRNA V4 domain of reported sequence (Accession No. JX393054.1) by using Clustal W software.

Enzymatic

For catalase assay 3% H_2O_2 solution was prepared and 5 mL of this solution was poured in a test tube. 0.1 g fungal biomass of each strain of *A. niger* was dipped carefully with straight wire/loop and was looked for bubbling pattern observed within 10 sec. Experiment was repeated thrice to plot mean values.

The laccase assay reaction mixture contained 0.85 mL of 0.1 M sodium citrate (pH 5.0), syring aldazine 0.1 mL (0.1 mM) and 0.05 mL of the culture filtrate. The enzyme activity was determined at 525 nm by using UV spectrophotometer (UV 1800 SHIMADZU). One unit of enzyme activity (U) was defined as the amount of enzyme that causes an increase of 1.0 in the absorbance per minute under the assay condition as described earlier (Yesilada and Sam, 1998). Experiment was repeated thrice to plot mean values.

Table 1: *Aspergillus niger* strains and their sources

Accession no.	Substrate	Location
0002	Garden soil	University of the Punjab, Lahore
0074	Air	University of the Punjab, Lahore
506	Air	Khanaspur
658	Sea sand	Karachi
744	Skeleton of crane	Zoology museum, University of the Punjab, Lahore
764	Blotting paper	Lahore
840	Filter paper	Lahore
880	Air	Ruhtas fort, Jhelum
1005	*Aleo vera* gel	Lahore
1109	Rhizosphere of guava plant	Lahore

Results

In the current research total ten isolates of *A. niger* were initially subjected to RAPD analysis to evaluate the genomic diversity prevailing among these isolates before proceeding towards ribotyping and enzymatic evaluations. The genomic DNA purified from all the 10 *A. niger* isolates was subjected to RAPD analysis by using 20 different primers after quantification by spectroscopic method by taking absorbance at 260 nm. The quantity of purified genomic DNA from all the strains was found to be in range of 0.1-0.5 µg/5 µL of sample and found suitable for downstream molecular analysis. In all the ten samples genomic DNA band of 15-23 kb size was observed indicating good quality intact DNA.

In RAPD analysis out of 20 random oligonucleotide primers, primer P2 (5′-ACGGCGTATG-3′) was optimized to screen all ten isolates of *A. niger* and showed amplifications of different sizes in range of 150-1500 bp (Fig. 1; Table 2). Based on amplification patterns the ten fungal isolates were divided into five groups in dandrogram analysis (Fig. 2). Group I contained isolates 658 and 880 (100%), group II have isolates 0074, 840, 1005 and 1109 (68.38%), group III and V included isolates 506 (55.28%) and 764 (22.54%) respectively, while group VI contained isolates 0002 and 744 (36.76%) based on genomic similarity percentages (Fig. 2). Based on RAPD analysis representative isolate from each cluster was short listed for further downstream molecular and biochemical analysis and these isolates included 880, 764, 506, 1005 and 744.

For ribotyping which involved amplification of hyper variable region V4 the primer set FaL and FaR were used as described in Fig. 3. In ribotyping the targeted region generated a fragment of approximately 431 bp. The derived sequences were analyzed by Chromas software and sequences were aligned to NCBI data base using BLAST and subsequently aligned with reported sequence (Accession No JX393054.1) by using ClustalW software (Fig. 4). Then sequences of AN880, AN1005, AN506, AN744 and AN764 were submitted to EMBL database under accession numbers KF257918, KF257919, KF257920, KF257921 and KF257922, respectively.

Catalase test was performed for all ten isolates of *A. niger* and isolates 744, 506, 764 and 880 were found to be most efficient regarding production of catalase enzyme (Table 3), while remaining isolate showed intermediate catalase activity except 1109, which showed least catalase activity (Table 3). In laccase assay isolate 744 was found to be most efficient, while isolate 840 was found to be least efficient (Fig. 5). The remaining isolates were found to be at same level in laccase assay (Fig. 5).

Table 2: Amplicons sizes obtained in RAPD analyses of *A. niger* strains amplified by Primer P2

Strains	Resolved Bands	Fragment size (bp)
744	7	200, 300, 350, 500, 600, 900, 1000
1109	2	200, 300
880	5	150, 200, 300, 350, 400
658	4	150, 200, 300, 350, 400
0074	5	200, 300, 350,
840	4	200, 300, 400,
0002	7	200, 300, 350, 400, 500, 600, 1200
506	1	1200
1005	1	200
764	7	250, 350, 500, 600, 800, 900, 1500

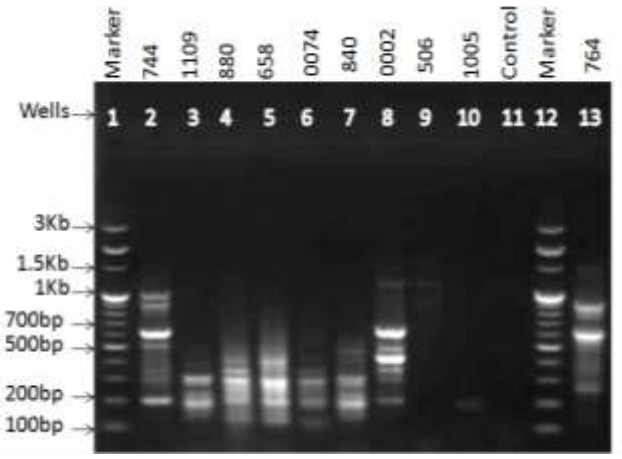

Fig. 1: RAPD analysis of *A. niger* isolates with Primer P2.**1, 12**: DNA ladder (Enzynomics, Cat# DM001), **11**: Negative control, **2-10, 13**: Experimental samples

Discussion

Fungi are effective producers of different commercially important enzymes including catalases and laccases

Table 3: Catalase test for *A. niger* strains

Strains	+++	++	+
0002		✓	
0074		✓	
506*	✓		
658		✓	
744*	✓		
764*	✓		
840		✓	
880*	✓		
1005*		✓	
1109			✓

Key: *: Samples subjected to genetic sequencing, +++: Spontaneous bubble formation, ++: Start slowly but gradually speed up, +: Very slow

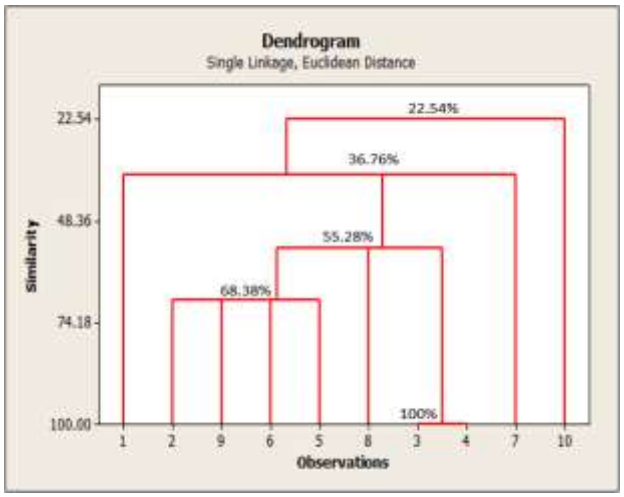

Fig. 2: Dandrogram analysis of *A. niger* isolates based on RAPD results

Key: **1**: 774, **2**: 1109, **3**: 880, **4**: 658, **5**: 0074, **6**: 840, **7**: 0002, **8**: 506, **9**: 1005, **10**: 764

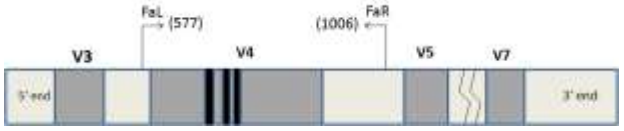

Fig. 3: Gene map 18S rRNA gene. **V3-V7**: Variable regions of the gene and black area of **V4** indicated hyper variable region. FaL and FaR primers were used to sequence the V4 domain of *A. niger* strains

(Isobe *et al.*, 2006). Catalases deactivate reactive oxygen species (ROS) like H_2O_2, superoxide and hydroxyl radicals, as a result of which their levels are maintained at safe concentrations (Calera *et al.*, 2000). Fungal laccases have higher redox potential as compare to plants or bacterial laccases and have several biotechnological applications (Thurston, 1994). Ability of fungal laccases to oxidize phenolic and nonphenolic aromatic compounds has increased interest in the application of these enzymes for various industrial applications, including food, pulping, textile, waste water treatment, and bioremediation is growing (Couto and Herrera, 2006).

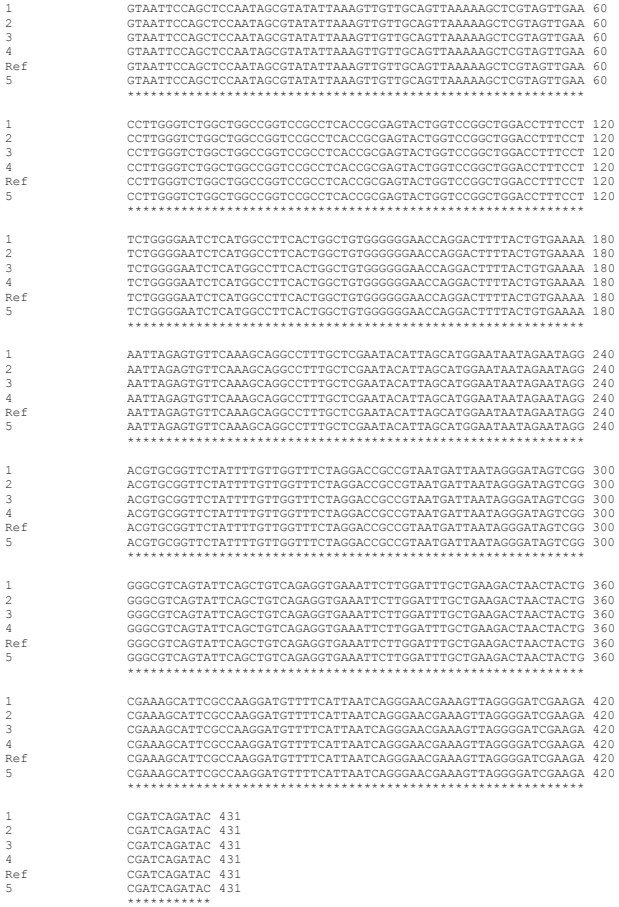

Fig. 4: Multiple alignment of hyper variable region V4 of 18S rRNA gene of *A. niger* strains found unique in RAPD analysis. **Ref**: Reference sequence JX393054.1 of *A. niger*; 1: 506; 2: 744; 3: 764; 4: 880; 5: 1005

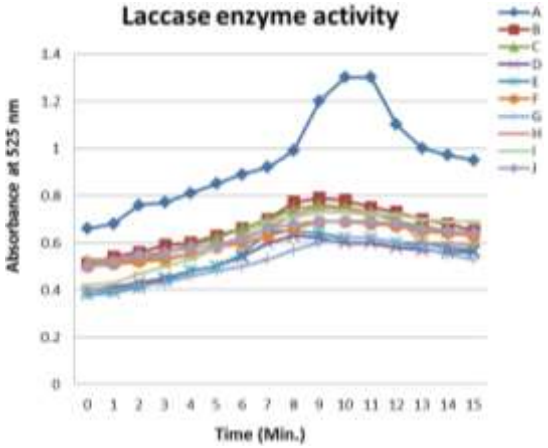

Fig. 5: Comparative laccase assay for ten strains of *A. niger*

A: 744, **B**: 880, **C**: 0002, **D**: 1005, **E**: 764, **F**: 505, **G**: 658, **H**: 1109, **I**: 0074, **J**: 840

In the current research total ten isolates of *A. niger*

were initially subjected to RAPD analysis to evaluate the genomic diversity prevailing among these isolates before proceeding towards ribotyping and enzymatic evaluations. For genomic diversity analysis RAPD is most frequently studied technique and also reported by other workers as well for genomic diversity analysis in fungi isolated from diverse sources (Bennett and Klich, 1992). The genomic DNA purified from all 10 *A. niger* isolates was subjected to RAPD analysis by using 20 different primers. Out of 20 random oligonucleotide primers, primer P2 (5′-ACGGCGTATG-3′) was optimized to screen all ten isolates of *A. niger* and showed amplifications of different sizes. Based on amplification patterns the ten fungal isolates were divided into five groups in dandrogram analysis. Group I contained isolates 658 and 880 (100%), group II have isolates 0074, 840, 1005 and 1109 (68.38%), group III has 506 (55.28%) and IV contained isolates 0002 and 744 (36.76%) and group V included 764 (22.54%) based on genomic similarity percentages. The results are in agreement with Abed (2008) who also reported the genomic diversity in *A. niger* isolates obtained from different environmental conditions.

All 10 *A. niger* isolates were evaluated for the catalase and laccase enzyme activity. Regarding catalase enzyme production strains 744, 506, 764 and 880 were found to be most efficient, while strain 1109 was found to be least efficient. However, remaining strains were at same level for this enzyme production. In laccase enzyme production again strain 744 was found to be most efficient while strain 840 was least efficient for laccase enzyme production. The current diversity in enzymatic profile of *A. niger* isolates may be attributed to diversity in environments from which these isolates were collected as described earlier Schuster *et al*. (2002). By evaluating the comparative enzymatic profile, *A. niger* strain 744 was found to be most efficient for catalase and laccase enzyme production and has commercial utilization as catalase enzyme from *A. niger* strain is relatively thermostable as described earlier by Nishikawa *et al*. (1993).

In order to determine the variation in 18S rRNA gene of local *A. niger* strains induced by different environments, hyper variable region V4 was amplified and genetically sequenced. The V4 domain of the 18S ribosomal gene was targeted because of its high degree of polymorphism among fungal families and species (Kappe *et al*., 1996). The ribotyping results suggested that sequences of V4 domain are 100% similar within the same species of *A. niger* revealing that 18S rRNA sequences are highly conserved and are being used in phylogenetic analyses of fungi of higher taxonomic ranks (Swann and Taylor, 1993; Wilmotte *et al*., 1993). These results suggested that the local strains of *A. niger* are potent source of different enzyme production and can be further exploited for commercial production of catalase and laccase enzymes.

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