The Journal of Animal & Plant Sciences, 24(5): 2014, Page: 1438-1443
ISSN: 1018-7081

ISOLATION, IDENTIFICATION AND ENZYME PRODUCTION PROFILE OF *A. NIGER*

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ABSTRACT

Black *Aspergilli* (*Aspergillus* section *Nigri*) have agricultural and industrial importance. Several species of this group are recognized human pathogens and some are mycotoxin producers. Despite of their wide use in teaching and research, taxonomy of this group remains unclear due to very close morphological features. Present study is aimed to distinguish *Aspergillus niger* strains that were isolated from fruits and vegetables by utilizing morphological, physiological and molecular approaches. Results demonstrated that micro- and macro-morphological characters of *A. niger* isolates were similar and strains were very close to each other on the basis of rDNA spacer sequence also. The most interesting finding of this study was that the production and activity of catalase, pectinase, lipase and urease enzymes by different isolates that were similar morphologically as well as genetically, was very different.

Key words Taxonomy, morphology, enzyme, molecular characterization, Internal transcribe spacer.

INTRODUCTION

Aspergilli have always been present in human environment, but prior to the development of microscope they were identified as white, yellow, green, red or black molds without serious attempt of interpretation of other features. Micheli (1729) was first to distinguish stalks and spore heads in *Aspergillus*. Taxonomy of *Aspergillus* is complex and ever evolving and genus is easily identified by its typical morphological characteristic with the help of several available taxonomic keys and guides (Hawksworth, 2011). Up till now, anamorphic genus *Aspergillus* has been documented to comprise between 260-837 species (Geiser *et al.*, 2007, Samson and Varga, 2009, Hawksworth, 2011), 10 different teleomorphic genera, and some mitosporic states (Raper and Fennell, 1965; Klich and Pitt, 1988; Geiser, 2009). The genera within genus are further divided into sections *Fumigati, Circumdati, Flavi and Nigri* and section *Nigri* (black aspergilli) is regarded as the most important one (Samson *et al*., 2006).

A. niger is the most frequently and commonly reported species in this section, while contribute significant role in industry and many fields of applied research (Schuster *et al*., 2002). The history of safe use for *A. niger* comes primarily from its use in the food industry and for the production of amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (Ward, 1989; Bennett and Klich 1992; Andersen *et al.*, 2011). Besides, beneficial role of genus *A. niger* in human life, its ill effects are also well documented (Powell *et al*., 1994). This fungus is well known as biodeteriogen and also causes economic losses due to spoilage of bakery, fruit and vegetable products, damages wood, cotton fibers and many other materials (Gravesen *et al*., 1999). *A. niger* has also been reported as postharvest pathogen of citrus, onion and of cherry, maize and peanuts (Palencia, 2010; Thomidis and Exadaktylou, 2012; Sibi *et al*., 2012).

Many taxonomists investigated taxonomy of black *Aspergilli* using morphological, biochemical and molecular approaches. Mosseray (1934) described black aspergilli within 35 species and species number squeezed within their *A. niger* group to 12 by Raper and Fennell (1965). Al-Musallam (1980) revised the taxonomy of the *A. niger* group, primarily based on morphological features and Abarca *et al*. (2004) investigated taxonomical features based on molecular studies. In addition, Samson *et al*. (2004, 2006) identified *A. niger* group using polyphasic approach. For present study, recent taxonomic approach was used to group and detect any unusual isolate(s) that have same morphological and genetic features.

MATERIALS AND METHODS

Isolation and purification of fungal strains: Rotten fruits and vegetables were collected from different areas of Lahore and stored at 4 °C in pre-sterilized sealed polythene bags until further study. For each sample, substrate, date, and site of sampling was recorded. Isolation and cultivation of black spores of *A. niger* from the specimens was carried out on 2% Malt Extract agar medium (MEA) (Raper and Fennell, 1965; Samson and Pitt, 2000; Klich, 2002). Spores from the infected parts of fruits or vegetables were transferred onto the MEA medium aseptically. Inoculated Petri plates were incubated at 25 °C for 3-4 days. Cultures were purified by sub-culturing the spores from actively growing colony of black *Aspergillus* on fresh MEA medium. Again the inoculated plates were incubated at 25 °C for 6-7 days

(until full plate growth). All isolates were preserved for identification and future reference at 4 °C.

Species identification and differentiation: Isolates were identified morphologically (Raper and Fennell, 1965) and differentiated on the basis of biochemical tests. Further characterization was carried out on the basis of sequencing of ITS region, amplified by PCR.

a. Morphological and cultural studies: Comprehensive description of each isolate, based on morphological and cultural results was prepared. Colony color, size, zonation was observed by naked eye while type of the conidial heads, presence of exudates, aerial and submerged mycelium, sclerotia and sexual state (if found) were studied under stereo light microscope. Under the compound microscope shape, size, ornamentation of conidia, vesicle, conidiophores, foot cell and striation was observed (Raper and Fennell, 1965). Identification of all isolates was confirmed by comparing this data with published authentic literature. For record and reference, cultural and microphotography was also carried out.

b. Biochemical studies: The variability among the isolates was studied on the basis of production and activity of lipase, catalase, pectinase and urease as described below.

Lipase activity test: Lipase activities of the individual *A. niger* strain was determined following the method of Haba *et al.* (2000). The composition of the medium used to assay the lipase activity was (all components amount is shown in w/v ratio) peptone 2%, NaCl 1%, $CaCl_2.2H_2O$ 2% and agar 2%. pH of the medium was adjusted to 6.0. Tween 80 (1% v/v) was added as lipase substrate to the sterilized molten medium before its pouring into the Petri plates. A disc of 3 mm diameter from the actively growing fungal culture was placed in the centre of the medium and Petri plates were incubated at 25 °C for 5-6 days. Lipase activity was assessed by the presence of opaque halo around the inoculum.

Catalase activity test: The principle of catalase activity is based on the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. Catalase activity of *A. niger* isolates was determined using the method described by Bailey and Scott (1994). In this method, a disc of 3 mm diameter from the actively growing culture of isolate was transferred aseptically into 3% hydrogen peroxide. Emergence of oxygen bubbles around the fungal disc was used as an indicator of catalase activity of each isolate.

Pectinase activity test: Method described by Kobayashi and coworkers (1999) was used to study the pectinase activity of the *A. niger* isolates. For determining the pectinase activity, fungal growth medium was prepared by adding 0.1% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% pectin and 2% agar. After autoclaving, medium was inoculated by a 3 mm disc from actively growing culture and incubated for 5-6 days at 25 °C. Enzyme activity was evaluated by the radius of no growth zone.

Urease activity test: The method for this assay was the phenol red rapid urease test (Finegold and Baron, 1986). 0.001% phenol red was added to MEA growth medium and after sterilization; medium petriplates were inoculated with 3 mm disc of fungal inoculums. Inoculated Petriplates were incubated at 25 °C for 5-6 days. Appearance of pink color in the culture was used as a marker of positive urease activity.

c. Molecular analysis: Finally molecular characterization was used to screen the genetic differences of morphologically similar isolates. For molecular analysis, amplification and sequencing of ITS region of fungal genome was carried out.

Fungal DNA isolation: For genomic DNA isolation, fungal spores harvested in saline Tween 80 (0.9% NaCl, 0.1% Tween 80) were used to inoculate 100 ml of 2% Malt Extract broth. Cells were grown overnight at room temperature in an orbital shaker at 100 rpm, harvested by ethanol sterilised muslin cloth and washed with cold sterilized distilled water. About 300 mg of these cells were ground to fine powder in liquid nitrogen in a sterile pester and mortar. The powdered cells were incubated at 37 ºC for 30 min in 2 mL Nucleon Reagent B (400 mM Tris pH 8, 120 mM EDTA, 150 mM NaCl and 1% (w/v) SDS) with 0.5 µL of 10 mg/ml RNase A. 0.5 mL of 5 M sodium perchlorate was added and mixed thoroughly by inverting the tube several times. An aliquot of 2 mL of ice chilled chloroform (at -20 ºC) was added and mixed vigorously. The tube was then centrifuged at 4000 rpm for 3 min. The supernatant was carefully transferred to new sterile tube and 2 mL of ice cold 96 % ethanol was added. Precipitated DNA was collected and washed with 70% ethanol. Air dried DNA pellet was suspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and incubated at 65 ºC for 15 min to inhibit potential DNase activity.

Polymerase chain reaction (PCR): The internal transcribed coding regions of genome were amplified using the genomic DNA as template. Taq polymerase with appropriate buffer was used for amplification in a 25 µl PCR reaction mixture. (1 µL of DNA, 0.2 mM dNTPs, 0.5 µM of each primer, ITS1 forward (5′- TCC GTA GGT GAA CCT GCG G -3′) and ITS4 reverse primer (5′- TCC TCC GCT TAT TGA TAT GC-3′) and 0.5 u of Taq polymerase). The PCR reaction was carried out according to the following programme; one cycle at 95 ºC for 90 sec followed by 30 cycles each of denaturation at 95 ºC for 10 sec, annealing at 60 ºC for 20 second elongation at 60 ºC for 10 sec. The PCR product was run on 1% agarose gel and visualized by UV light and sent for DNA sequencing.

RESULTS AND DISCUSSION

Isolates identified: Five strains of *A. niger* were isolated from different fruits and vegetables. Each isolate was given an accession number that was used throughout this study to represent the results of that particular strain. Detail of these isolates is summarized in Table 1.

Morphological characters of isolates: All the isolates of *A. niger* showed similar morphological characters and obviously identified as the same species of black *Aspergillus*. Colonies on malt extract agar grow more rapidly, 4-5 cm in 5-7 days at room temperature. Heavily sporulated with dark black color conidia, slight odor, lacking the exudates; reverse colorless. *Condial heads;* large and globose. *Conidiophores* long, wide, thick-walled, mostly black, commonly 15-20 µm wide with smooth and thick walls, usually colorless. *Vesicle*; globose 50-80 µm diameter; *Striation*; sterigmata in two series, dark brown in color, primary sterigmata 20-25 x 8-10 µm in size. Secondary sterigmata bottle shaped, 6-10 x 2.5-3.5 µm;
Conidia globose, rough echinulated walls, 3.0-5.0 µm in size (Figure 1a, b, c).

Enzymes activities: *A. niger* is well known for the production of many commercial enzymes (Hurst *et al.,* 1977; Smith *et al.,* 1993; Liu *et al.,* 2001; Monga *et al.,* 2011). In this study, *A. niger* isolates from different sources were assessed for their ability to produce five different enzymes. The variability among the isolates for the production of these enzymes was used as an indicator of their genetic difference (if any).

Enzyme production is helpful when distinguishing the closely related species. A great variation of same enzyme production by different strains was recorded. Results demonstrated that catalase and urease were produced by all isolates. However, the ability of producing these enzymes was not similar in all strains. Results demonstrated that An 02 and An 05 have high, An 01 and An 04 moderate and An 03 has low level catalytic activity (Figure 3). Results of the study conducted by Buckova *et al.* (2005) on catalase production by *A. niger* strains suggested that catalytic activity by this fungus depends on the substrate from where this fungus was isolated as well as the growth medium. Similarly strains were found to be different with each other with respect to their urease production ability. An 01 and An 04 showed elevated, An 05 average while An 02 and An 03 had little urea catalysis potential. Ghasemi *et al.* (2004) screened thirteen strains of *A. niger* and observed different levels of urease production. Two of the strains showed highest enzymatic activity among all isolates and activity was almost one third less in certain strains as compare to the highest level.

Aspergillus and *Penicillium* species are the commercial source of pectinase production (Said *et al.,* 1991). Significant difference was recorded for lipase and pectinase production of strains where some strains were totally unable to produce these enzymes and others had high potential of producing these bio-molecules (Table 2). Adham and Ahmad (2009) studied the ability of *A. niger* strains for lipase production and concluded from the results that each strain was different from the other strain for their lipase production ability and also dependent on the nature of growth medium.

Molecular analysis: Identification of the black aspergilli on the basis of morphological structures can be helpful but that particularly the strains related to *A. niger* are difficult to distinguish. Sequencing of ITS region was applied to establish the clear and authentic identification in this study (Henry *et al.,* 2000). Using the IT1 and IT4 (ITS1 forward and ITS4 reverse) primers and total genomic DNA as template, an approximately 650 bp internal transcribed spacer (ITS) region, ITS1–5.8S–ITS2 DNA was amplified (Figure 4). The sequence of these isolates was compared with the other *A. niger* strains in GenBank and also with each other. Genetically all these five strains were more than 99.5% similar with each other as determined by their nucleotide alignment using NCBI, bioinformatics tools. These strains were deposited in First Fungal Culture Bank of Pakistan (FCBP) and their ITS region sequences were deposited in GenBank. The details of submissions are summarized in Table 3.

Table 1. Detail of the isolates used in the present study.

Strain No.	Infected host plant	Infected part of plant
An 01	*Abelmoschus esculentus*	Seed
An 02	*Mangifera indica*	Fruit
An 03	*Zea mays*	Seed
An 04	*Allium cepa*	Bulb
An 05	*Pyrus malus* (*Malus domestica*)	Fruit

Table 2. Enzymatic activity of the *A. niger* strains.

Accession no.	Enzymatic activity			
	Lipase	Catalase	Pectinase	Urease
An 01	+++	++	-	+++
An 02	-	+++	-	+
An 03	+++	+	++	+
An 04	++	++	-	+++
An 05	-	+++	++	++

Enzymatic activity is shown on a scale where '+++' represents the high, '++' is for moderate, '+' denotes poor but still tangible and '-' denotes no enzymatic activity. A representation of these growth levels is shown in Figure 2

Table 3. Details of the accession numbers allocated to the *A. niger* strains by FCBP and GenBank.

Strain	FCBP accession no.	GenBank accession no.
An 01	FCBP416	KF496078
An 02	FCBP469	KF496079
An 03	FCBP518	KF496080
An 04	FCBP648	KF496081
An 05	FCBP684	KF496082

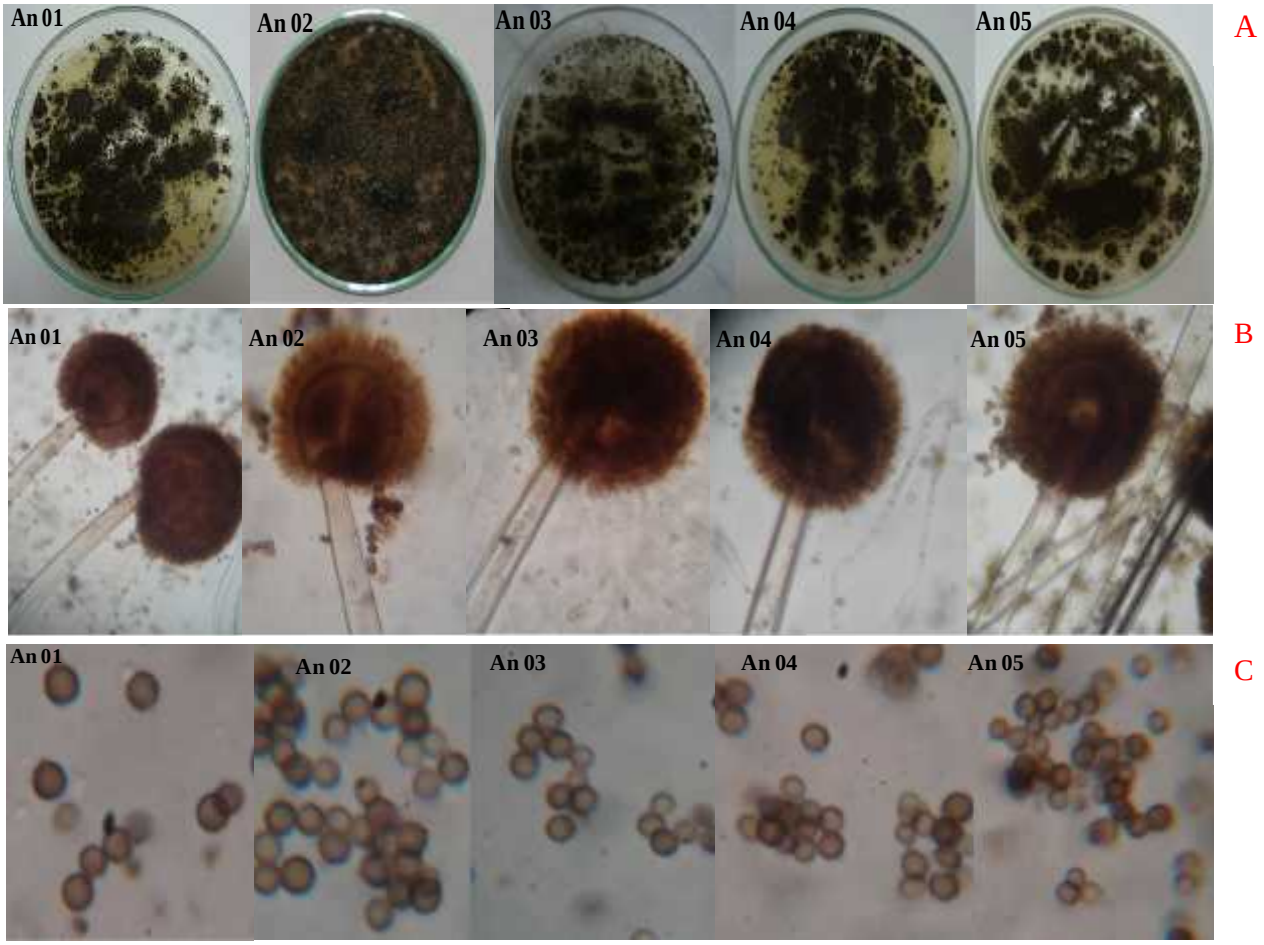

Figure 1. Morphology of *A. niger* isolates. A: Colonies grown in MEA at 25 ºC for 7 days. B: Conidial heads showing vesicles and conidiophores. C: Dark brown echinulated spores.

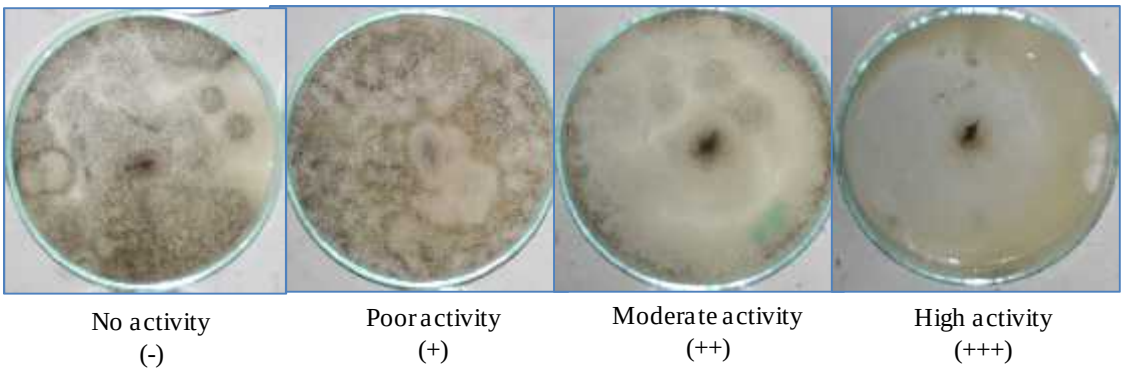

Figure 2. Representative growth showing levels of lipase or pectinase activity.

Figure 3. Catalytic activity of various isolates of *A. niger*. Gas bubbles are the indicative of catalysis of hydrogen peroxide into water and oxygen.

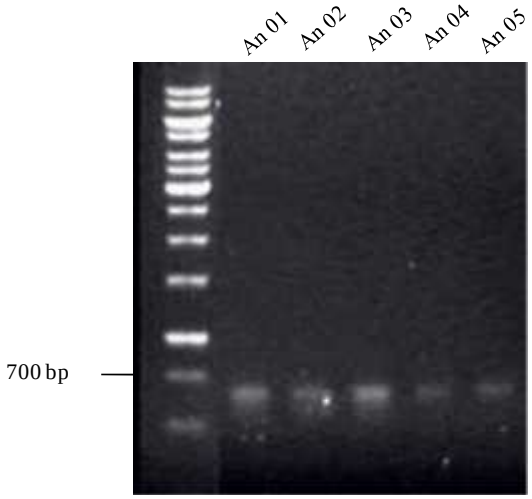

Figure 4. PCR amplification of ITS regions of *A. niger* isolates on agarose gel along with 1kb DNA ladder.

Conclusions: The overall objective of the work presented here was to develop deeper insight into the taxonomic differentiation of industrially and agriculturally important fungal species, *A. niger*. It is concluded from the present study that although all strains were morphologically as well as genetically similar but still have differential enzymes production profiles.

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