Food Science and Quality Management
ISSN 2224-6088 (Paper) ISSN 2225-0557 (Online)
Vol.35, 2015

Phenotypic and Genotyping Study of Aspergillus Niger: Molecular Detection of Calmodulin, 18srRNA and Pepsin like Protease Genes Based on Multiplex PCR

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Abstract
Aims: Aspergillosis can be diagnosed using PCR. For this purpose, the genes encoding the 18S rRNA, Calmodulin and Pepsin-like protease genes of *Aspergillus niger*, were elucidated. Genus specific sequences could be identified in region of 18S rRNA.
Methodology: 24 fungal strains were isolated from different localities of Al-Hillah city. Isolates were screened for glucose oxidase production using submerged fermentation and molecular techniques like 18S rRNA. DNA was isolated and amplified using PCR. Gene sequencing was done and homology analysis was studied. Rate of glucose oxidase production was also analyzed.
Results: The method described in this study represents a rapid and reliable procedure to assess the presence in food products of two ochratoxigenic species of section Nigri. Glucose oxidase hyper producing isolate was identified as *A. niger* strain. The F17 strain gave best reproducible results (87. 5±0.05U/g of cell mass) after 72 h. of fermentation at 30ºC and at a medium pH of 7.2.The 18srDNA was used to detect *A. niger* was very affective. The identification and isolation of tannase gene from *A. niger* which is considered as an important bioreactor and industrial fungus were reported
Conclusion: Our results revealed that Glucose oxidase was produced naturally by *A. niger* in large quantity instead of using other manipulation techniques of genetic. The PCR technique we have used appears to be adequate to study a large group of microorganisms (fungi) and it help to identify risk of pathogenicity of aspergillosis.
Keywords:*Aspergillus niger*, Calmodulin, Pepsin-like protease, 18S rRNA, aspergillosis, Tannase.

Introduction

Aspergillus species belonging to section *Nigri* are distributed worldwide. Many species of them cause food spoilage, and several are used in the fermentation industry (**1**), or candidate in the biotechnology industries, such as *A. niger* which has been granted the GRAS (Generally Regarded As Safe) status by the Food and Drug Administration of the US Government. Recently, the significance of these species, commonly known as "black aspergilli" has completely changed since they were identified as the main fungi responsible for the ochratoxin A (OTA) accumulation in grapes and wine (**2, 3**). OTA is a potent nephrotoxin diffusely distributed in food and feed products such as grains, legumes, coffee, dried fruits, beer, wine, and meats. Since *A. tubingensis* and *A. niger*, together with *A. carbonarius*, are known to produce OTA, their exact identification within the *A. niger* aggregate group of species is very important to avoid overestimation of contamination and toxicological risk (**4**). In spite of this, the identification of species within the *A. niger* "aggregate", a group of species within section *Nigri*, is still controversial. Molecular studies reduced the number of synonym names of described species within the black aspergilli and supported the division of this "aggregate" in two to four morphologically indistinguishable species: *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* (**5**). These four species are very difficult to differentiate by classical morphological criteria, such as conidial shape, colour and size (**6**). However, effectiveness in the estimation of OTA contamination is dependent on the association between species and OTA production, hence the use of primers identified on genes involved in the metabolic pathway would be more helpful. Although two primers were recently developed to detect OTA producers by Polymerase Chain Reaction (PCR) (**7,8**), genes involved in steps of OTA biosynthesis have not yet been identified. A PCR-based identification and detection of species could be a useful tool for identifying potential ochratoxigenic *Aspergillus* spp. The calmodulin gene has proved to be highly useful in discrimination of species belonging to section *Nigri*, since it contains some species specific diagnostic traits, suitable for diagnostic purposes. Molecular differences were exploited to set up a qualitative PCR assay for *A. carbonarius* and *A. japonicus*/*A. aculeatus* detection (**8**). Moreover a quantitative real-time PCR assay using TaqMan chemistry was recently set up for *A. carbonarius* DNA quantification on grapes (**9,10**). The objective of this work was to set up a species-specific PCR assay based on differences in sequences found in the calmodulin gene for an accurate identification of *A. niger* and *A.*

tubingensis in pure cultures.

Aspergillus niger fermentation is generally recognized as safe by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act (**11, 12**) so the metabolites produced by *A. niger* can be used in food and medical industries without any objection. β-D-glucose: oxygen oxidoreductase commonly known as glucose oxidase is the enzyme which has the capacity to oxidize glucose. It is an important enzyme due to a vast number of applications in various fields. Most important application of glucose oxidase is in diagnostics, as it is utilized on commercial scale in colorimetric diagnostic kits for the determination of glucose in blood, serum or plasma. It is used for the removal of glucose or oxygen to improve flavour, colour, texture and shelf life of various products in the food industry as well. Recently, glucose oxidase has also been used in biofuel cells and for the purpose of preservation of food (**13, 14**).

The genus Aspergillus is a ubiquitous saprophytic soil fungus which colonizes the respiratory tract in humans (**15**) and is responsible for opportunistic infection in immunocompromised patients (**16**). Indeed, invasive aspergillosis is responsible for up to 41% of the deaths of patients with acute leukemia, and despite the severity and high mortality attributable to this mycosis, there has been little progress in accurately diagnosing infection antemortem (**17**). Recent studies have described PCR methods for detecting Aspergillus fumigatus and Aspergillus flavus in clinical material from immunosuppressed patients (**18**). However, other species can be involved (6), and so it seemed important to us to ascertain that a patient had infection with any Aspergillus organism. We therefore elucidated the complete sequences of 18S rRNA of several species of Aspergillus and related fungi and aligned them with sequences from other sources (**19**).

Tannin acyl hydrolase (tannase), commonly referred to as tannases, hydrolyses the ‘ester’ bond (galloyl ester of an alcohol moiety) and the ‘depside’ bond (galloyl ester of gallic acid) in substrates such as tannic acid, methylgallate and m-digallic acid (**20, 21**).Gallic acid is the product of acidic or enzymatic hydrolysis of tannic acid and is taken into account as an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry. Aspergillus niger has been used as an important bioreactor to prepare different type of industrial enzymes and materials. A. niger also releases large amounts of citric acid into the environment, causing it to become acidic. To adapt to this acidic environment, some of the enzymes of A. niger such as tannase, amylase, protease, cellulase, and hemicellulase, are more acid stable than the same enzymes secreted by other bacterial and fungal species. Moreover optimum temperature for the A. niger tannase activity (70°C) was considerably higher than those of tannases produced in other experiment (**22**). The purified tannase so far from different sources have been shown to have a molecular mass of 90kDa to over 300 **(23)**.

Proteolytic degradation affects mainly heterologous proteins evidenced by improvement of heterologous protein production upon deletion of the protease genes (**24**). Four extracellular proteases with acid pH optima (an aspartic protease, PEPA, a glutamic protease, PEPB and two serine carboxypeptidases, PEPF and PEPG) have previously been characterized (**24, 25**). A fifth protease gene, encoding an extracellular subtilisin-type serine protease, PEPD, has been cloned based on conserved amino acid sequences within subtilisins (**26**). Furthermore, three proteases that are homologous to yeast vacuolar proteases have been cloned from A. niger; these are PEPE, a pepsin-type aspartyl endoprotease that is the homologue of the vacuolar pep4 gene product in yeast, PEPC, a subtilisintype serine endoprotease and CPY, a serine carboxypeptidase (**27**).

Material and Methods

Fungal strains were grown in shaken cultures (150 rpm) in PDA medium. 50 mg of filtered, frozen mycelium from each strain were used for total DNA extraction using the Promega DNA Miniprep Kit (USA). DNA purity and quantity were determined by biodrop and electrophoresis, (100 bp DNA ladder, Bioneer, Korea). Total DNA extracted from all strains was used for PCR amplification of **688** bp fragments of the calmodulin gene with **CL1** and **CL2A** primers (**28**). Amplifications of the partial calmodulin gene were set up in 100 µl reaction mixtures containing 2.5 U of Taq premix DNA polymerase (Bioneer, Korea), 30 pmol of each primer, 20 nmol of each deoxynucleoside triphosphate, and approximately 10 ng of fungal template DNA. PCR reactions were performed using the following conditions: denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples.

Analysis of the 18S rRNA genes. Specific primers of Aspergillus organisms included the 18S rRNA sequence of A. fumigatus described by Barns and associates (**29**) and the 18S rRNAs of A. flavus, A. nidulans, A. niger, and A. terreus which were sequenced by us. The complete 18S rDNA was amplified by PCR using the general eukaryotic 5' and 3' 18S rRNA primers (**30**). PCR was performed with a 100 µl reaction mixture containing 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 1.4 mM MgCl2, 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 0.1% Triton X-100, 50 pmol of each of the two primers, 0.2 U of SuperTaq DNA polymerase (premix master mix, Bioneer, Korea), and 0.5 p.g of DNA from each Aspergillus isolate which had been denatured at 94°C for 5 min. Next, 30 cycles of amplification were performed by denaturing for 1 min at 94°C, annealing the primer for 1 min at 42°C, and allowing elongation for 3 min at 72°C.

Primers for tannase gene were designed from highly conserved regions of amino acids from an alignment of the translated sequences of tannase gene from several sources. The process of PCR degenerate was performed as follows: 3min 94ºC x1, 1min 94ºC, 1min 48ºC, 3mins 72ºC x 29, 1min 94ºC, 1min 48ºC, 7mins 72ºC x1. PCR with above conditions produced a clear product of ca. **950** bp with A. niger genomic DNA which was the size of product predicted.

2.5 ml O-dianisidine, 0.3 ml glucose and 0.1 ml peroxidase solution (1 mg/ml) were added to the cuvette with the help of a micropipette and allowed to equilibrate at 25ºC and was zeroed at 436 nm. Then 0.1 mL of sample extract was added to the cuvette and mixed the reaction mixture thoroughly. The absorbance per minute was determined using 7 cycles of 00.30 seconds each, using spectrophotometer (OPTIMA, JAPAN). ΔA/min over liner portion of reaction was measured.

Activity of glucose oxidase = ΔOD/min X TV x dilution factor /8.3 X SV X cell mass (g)

Results & Discussion

The data advises that locally isolated strain of *Aspergillus niger* is producing the enzyme glucose oxidase in large amount as compared to other isolated wild strains of *Aspergillus niger* reported in literature. This wild strain can be very beneficial in mass production of the particular enzyme at the industrial level.

Twenty two fungal isolates were morphologically identified as Aspergillus niger and were screened for their capability of producing glucose oxidase (Table 1) using submerged fermentation with modified M-8 medium. Many isolates showed significant biomass and maximum glucose oxidase production as compared to other isolates. These isolates were selected and recultured repeatedly under same conditions and it was found that showed reproducible results with maximum production of GOD i.e. 127.4 ±0.102 U/g of cell mass.

Enzyme of glucose oxidase producing *by Aspergillus niger* in large amount, these strains can be very beneficial in mass production of the particular enzyme at the industrial level.

Table 1. Distribution of of *A. niger* strains used in present study

Strain	Glucose oxidase activity (U/g of cell mass)	The mass cell (g/25ml of medium)
F1	1.58±0.07	55.7±0.3
F2	1.09±0.038	41.98±0.11
F3	1.62±0.01	47.8±0.2
F4	2.2±0.1	58.4±0.2
F5	0.91±0.08	73.5±0.1
F6	0.94±0.07	37.9±0.02
F7	1.09±0.09	67.04±0.2
F8	1.17±0.06	64.67±0.3
F9	0.65±0.1	39.09±0.4
F10	0.69±0.02	16.41±0.2
F11	0.96±0.06	51.33±0.3
F12	0.31±0.002	32. 1±0.2
F13	2.42±0.2	73.21±0.4
F14	1.61±0.18	33.67±0.4
F15	1.05±0.087	32.01±0.1
F16	1.29±0.18	48.4±0.2
F17	1.94±0.13	87. 5±0.05
F18	1.09±0.17	55.6±0.3
F19	0.074±0.01	NIL
F20	1.09±0.08	64.04±0.1
F21	0.87±0.07	59.6±0.3
F22	0.052±0.03	36.57±0.1

The genetic results of specific genes used in our study was revealed in figure1. *Aspergillus* genus Sensitivity of the PCR, Serial 10-fold dilutions of purified *A. niger* nucleic acids isolated from the same sample were tested by PCR, a sensitivity of 10 pg of nucleic acid was obtained (on the basis of the DNA content), as detected by gel electrophoresis. However, when the rRNA was first transcribed into cDNA, 10 fg of nucleic acids was detected on the gel. rRNA has been used as the target for the development of species- or genus-specific PCR assays for several different microorganisms, such as mycobacteria and mycoplasmas (**19**).

Throughout last year's tannase enzyme have been isolated, purificated and characterizated from Aspergillus species (A. *oryzae, A. kawachii, A. avuleatus, A. niger, A. awamori*) and described (**31, 32**). Tannase

from A. niger and the β-glucosidase from A. kawachii are partially intracellular when produced by liquid fermentation, extracellular under solid culture conditions (**31**) and also thermostable and unspecific. Such bifunctionality may be a survival way for micro-organisms such as Lactobacillus plantarum and A. niger during natural selection period to survive in extreme environments or to efficiently degrade cellulose and tannins, abundant in decaying plant material (**32**).

It is unknown whether the two functions (esterase for the tannase and hydrolase for the ß-glucosidase) are carried out by one or two domains within the protein structure (**34**). However there is a low chance that a single-domain protein, highly homologous to a known enzyme, has a different function. In the present study, we cloned the partial sequence of gene the encoding tannase in A. niger and analyzed the sequence (**35**).

Aspergillus niger and A. tubingensis, species belonging to section Nigri, are commonly found in plant products and processed food, such as grapes, cereals, coffee and derived products. These two species are very difficult to differentiate by classical morphological criteria and some isolates are known to produce ochratoxin A. A PCR-based identification and detection assay was developed as a tool to identify A. niger and A. tubingensis, using molecular differences obtained by sequencing the calmodulin gene.

Specific primers described in this work have been designed on the basis of calmodulin gene sequence comparisons of several strains of Aspergillus species and taking into consideration the phylogenetic and taxonomic analysis made in previous publications (**8, 9**). In accordance with previous studies (**12, 37**) we demonstrate that the calmodulin-based PCR method has high degree of specificity for the identification of Aspergillus at species level within the black aspergilli. in contrast, ITS regions showed low degree of specificity consisting in variation of only three different nucleotides between these species (**36, 38, 39**).

ACKNOWLEDGMENT

This research was supported by the GENETIC and MOLECULAR laboratory, University of Babylon, College of Science for women. We thank all colleagues for critical suggestions in study coordination.

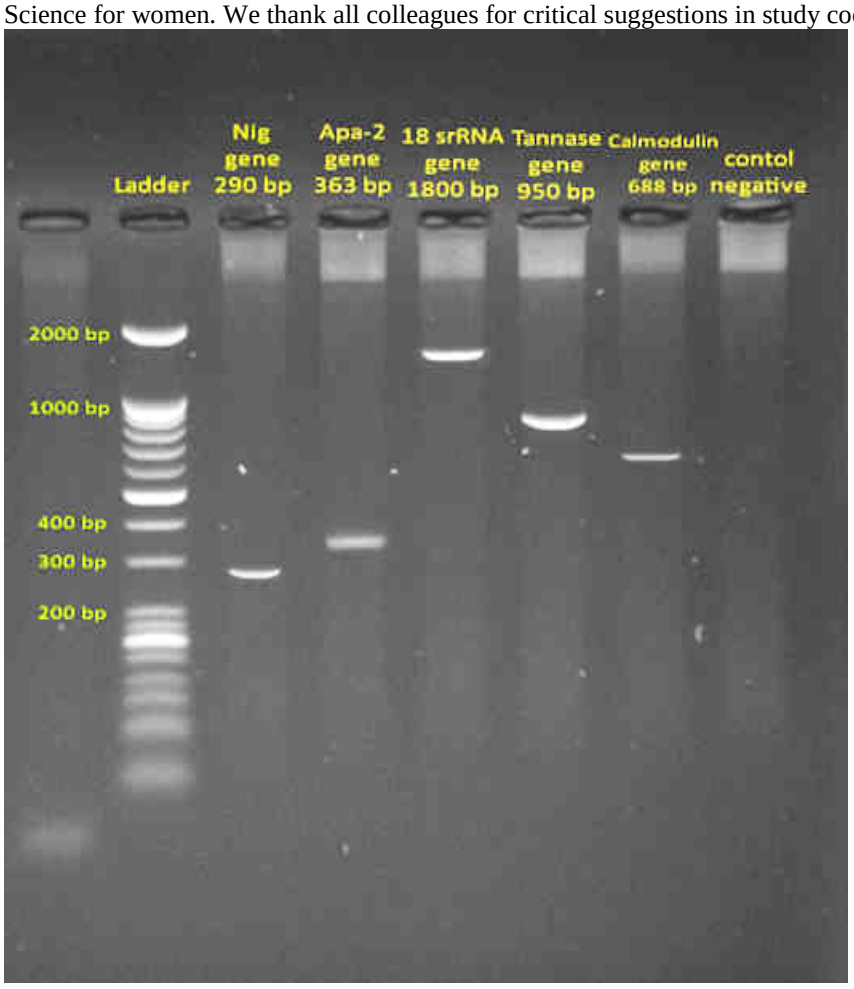

Figure 1
The genetic results of specific genes used in our study

***Nig* gene**: 290 bp.
***Apa-2* gene**: 363 bp.
***18srRNA*:** 1800 bp.
***Tannase* gene**: 950 bp.
***Calmodulin* gene**: 688 bp.

Table 2. Primers of specific genes used in present study

No.	GENE		PRIMER	bp.
1	NIG1	F	5'-GATTTCGACAGCATTT(CT/TC)CAGAA-3'	290
		R	5'-AAAGTCAATCACAATCCAGCCC-3'	
2	Apa-2	F	5'-TAT CTC CCC CCG GGC ATC TCC CGG-3'	363
		R	5'-CCG TCA GAC AGC CAC TGG ACA CGG-3'	
3	18S rDNA	F	5'-CCTGGTTGATCCTGCCAGTA-3'	1800
		R	5'-GCTTGATCCTTCTGCAGGTT-3'	
4	Tannase gene	F	5'-TTCTGCTCTGGATCGCAATCTG-3'	950
		R	5'-ACTAGTGATTGATGGGGGAGAGG-3'	

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