

## Research article

**Molecular analysis of acid-stable alpha-amylase (*asAA*) gene in *Aspergillus niger* using PCR-RFLP**Majid Zarrin<sup>1,2</sup>, Zahra Shialy<sup>2</sup><sup>1</sup>Infectious and Tropical Diseases Research Center, Health Research Institute, <sup>2</sup>Department of Medical Mycology, Medical School, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

(Received: April 2022      Revised: August 2022      Accepted: September 2022)

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**ABSTRACT**

**Introduction and Aim:** Amylase is an important enzyme with vast applications in various industries such as food and therapeutic industries. *Aspergillus niger* is commercially engaged in the making of alpha-amylase. Acid-stable alpha -amylase is mostly produced with microorganisms such as *Bacillus* and *Aspergillus*. The aim of this research was the molecular investigation of the acid-stable alpha-amylase (*asAA*) gene in *A. niger*.

**Materials and Methods:** Sixty-three *A. niger* isolates were evaluated in this study. PCR method was performed for amplification of a 347 bp DNA band of the *asAA* gene. The *Hpa* II Restriction endonuclease was used for the digestion of PCR fragments.

**Results:** A 347 bp DNA fragment was recovered from 49 out of 63 (78%) isolates. After cutting the PCR products with the *Hpa*II enzyme, 81.6% of isolates showed the expected band and 18.4% presented different restriction endonuclease patterns.

**Conclusion:** The results demonstrated the PCR-RFLP technique performed in this research was a valuable tool for analysis of the *asAA* gene in *A. niger* isolates.

**Keywords:** Acid-stable alpha-amylase (alphasAA) gene; *Aspergillus niger*; PCR-RFLP.

**INTRODUCTION**

**A**mylase is an important enzyme that has been greatly investigated and has massive applications in various industries such as food, textile, and therapeutic industries (1).  $\alpha$ -Amylases can catalyze the hydrolysis of 1,4-glycosidic bonds in starch for creating molecules like glucose and maltose (1,2).

Nowadays, many microbial amylases are accessible commercially and they have approximately entirely replaced starch hydrolysis with the chemical process in the industry. The microorganism's amylases have a wide range of industrial applications because they are more stable as compared to  $\alpha$ -amylases in animal and plant amylases (3). The correlations between amylase activity and fungal virulence have been reported (4,5).

Amylase activity in yeast, molds, and bacteria has been described (6-8). The amylases from the fungi were discovered to be more stable than the enzyme in bacteria (9). *Aspergillus niger* is commercially employed to produce  $\alpha$ -amylase (10). This fungus produces the highest level of amylase among molds. *A. niger* is an opportunistic fungus that causes human infections, and it is frequently responsible for otomycosis (11,12).

Amylases have an optimal pH for action, and they are divided into acidic, neutral, or alkaline. Amylases have

a pH between 2 and 12. Most amylases have activity in the neutral pH. The range of acid-stable  $\alpha$ -amylase is pH 4 to 5. The amylase exhibits higher activity in acidic conditions which can simplify the process of liquefaction and saccharification and reduce the cost of starch processing (13).

Industrial acid-stable  $\alpha$ -amylase is generally produced with microorganisms such as *Bacillus* and *Aspergillus* (13). Studies have demonstrated that the acid-stable  $\alpha$ -amylase produced by *A. niger* shows good acid stability (14).

In the current study, molecular analysis of the acid-stable  $\alpha$ -amylase (*asAA*) gene of *A. niger* has been investigated with the PCR-RFLP method.

**MATERIALS AND METHODS*****A. niger* isolates**

Sixty-three *A. niger* were tested throughout the current investigation consisting of environmental, clinical isolates, and reference strains. Environmental isolates of *A. niger* were recovered from air and soil samples.

*A. niger* was cultured on Sabouraud dextrose agar (Merck) and kept at a temperature of 37°C. The isolates were recognized by morphological traits and sub-cultured thrice to attain a pure culture. For all isolates, wet mount slides with lactophenol aniline blue were prepared and morphological features such as

conidiophore, vesicle, philiade and conidial arrangement were studied using a microscope.

### Extraction of genomic DNA

One ml dense spore suspension of any *A. niger* isolate was moved into a 100 ml YEPD medium and kept for 48 h at 37°C with 200 rpm to permit the mycelium to grow. The grown mycelium was collected, rinsed using 0.5 M EDTA and distilled water, and then with a pestle and mortar applying cold nitrogen, the mycelium was grounded into a fine powder. The powder (100 mg) was moved in an Eppendorf carrying 400  $\mu$ l of lysis solution. The tubes were stored for 20 min at 100°C, then 3 M CH<sub>3</sub>CO<sub>2</sub>K solution (150  $\mu$ l) was supplied to the tubes. The obtained combination was stored in the freezer for 10 min, and spun at 12000 rpm for 10 min. The upper aqueous phase was moved to a microtube, subsequently, 250  $\mu$ l of phenol/chloroform/isoamyl alcohol (25:24:1) was supplied into the microtube. This combination was mixed by vortexing in brief and spun at 12000 rpm for 10 min. Following transfer the upper layer to an Eppendorf, 250 $\mu$ l chloroform/ isoamyl alcohol (24:1) was supplied into the microtube. The microtubes were shortly shaken and processed by centrifugation at 12000 rpm for 10 min at 4°C. The upper layer was relocated to a 1.5 ml Eppendorf, and after adding a similar amount of isopropanol, the solution was stored for 10 min at -20°C and spun at 12000 rpm for 10 min. The pellet was rinsed with 300  $\mu$ l of ethyl alcohol 70 %. The DNA pellet was resuspended in 50  $\mu$ l sterile distilled water.

### PCR of *asAA* gene

The primers, Amy-ac1 (5'- GAC CTC AAG TCC CTC TCA-3') and Amy-ac2 (5'-TCG GCT ACC CAG TCA TAC-3') were employed for creating a 347 bp band of *asAA* gene. The reactions were provided in the amount of 50  $\mu$ l, containing 5  $\mu$ l PCR buffer, 2.2  $\mu$ l MgCl<sub>2</sub>, 1  $\mu$ l dNTP, 0.5  $\mu$ l Taq enzyme (CinnaGen, Iran), 50 pmol of each primer, and 50 ng DNA. The PCR was firstly operated at 95°C for 5 min and continued for 35 cycles (95°C for 1 min, 60°C for 1 min, 68°C for 2 min).

### RFLP analysis of amplicons

Restriction enzyme mapping of the 347 bp DNA fragment of *asAA* gene was expected. *Hpa*II (Thermo) was selected as a suitable enzyme for the analysis of the fragments. Restriction enzyme reactions were provided in the amount of 20  $\mu$ l consisting of 10 U *Hpa*II, 2  $\mu$ l restriction enzyme buffer, 8  $\mu$ l amplicon, and ultrapure water (CinnaGen, Iran). The digested amplicons were electrophoresed on 1.5% agarose gel.

### Sequencing of PCR products

A few PCR products of the *asAA* gene were sequenced (Bioneer Corporation, Daejeon, South Korea). Each

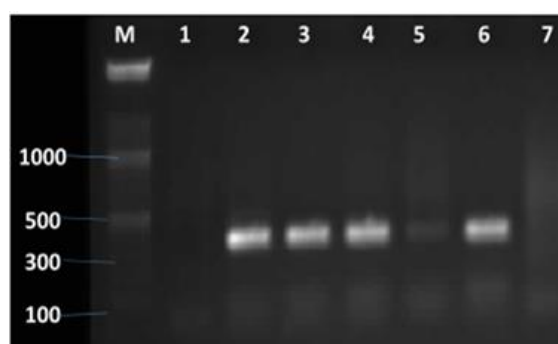
sequence was investigated in the NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The sequences showed 100% homology with *A. niger asAA* gene conserved in GenBank. The MEGA5 software was applied for DNA analysis.

## RESULTS

### PCR amplification of *asAA* gene

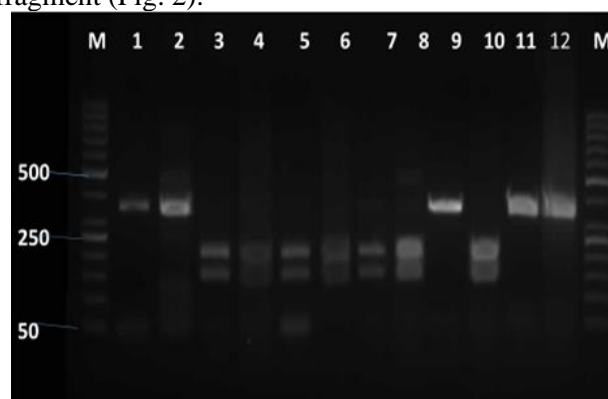
The PCR of the *asAA* gene with oligonucleotides Amy-ac1 and Amy-ac2 created a 347 bp fragment for 49 out of 63 *A. niger* isolates (78%) (Fig. 1). The 347 bp fragment of the *asAA* gene was sequenced for several isolates and aligned with reference alignments in the NCBI. A Basic Local Alignment Search Tool (BLAST) search displayed 100 % similarity with the reference alignments in the GenBank database.



**Fig.1:** PCR amplification of 347 bp *asAA* gene of *A. niger* strains using oligonucleotides Amy-ac1 and Amy-ac2. M: 1Kbp ladder; 1: M21/1; 2: M21/2; 3: M21/3; 4: M22; 5: M23/1; 6: M23/2; 7: No template control.

### Restriction endonuclease maps of the *A. niger* isolates

Cutting the *asAA* gene PCR products with *Hpa*II created the two bands restriction enzyme pattern of 202 and 140 bp for 40 (81.6%) *A. niger* isolates. Nine (18.4%) isolates including 1 clinical and 8 environmental isolates had no *Hpa*II site for the fragment (Fig. 2).



**Fig. 2:** Restriction enzyme analysis of 347 bp *A. niger asAA* gene band cut with *Hpa*II. M: 50 bp ladder; 1: M13; 2: M14; 3: M15; 4: M17; 5: M18; 6: M19; 7: M20; 8: M21/2; 9: M21/3; 10: M22; 11: M23/2; 12: M30.

## DISCUSSION

A broad range of amylolytic enzymes is produced by various microorganisms.  $\alpha$ -amylase enzymes are extensively used as biocatalysts in the saccharification of starch, detergent, alcohol, baking, de-sizing, and textile industries (15).  $\alpha$ -amylases are synthesized by a variety of microbes such as bacteria, filamentous fungi, and yeasts.

$\alpha$ -1,3-glucan in the yeast phases of fungal pathogens acts as a pathogenicity factor (5). In dimorphic pathogen *H. capsulatum*,  $\alpha$ -(1,4)-amylase gene (*AMY1*) is necessary for  $\alpha$ -(1-3)-glucan synthesis, hence associated with virulence. An orthologous gene to *AMY1* gene of *H. capsulatum* has been recognized in the fungus *P. brasiliensis*. Transcriptional levels of this gene have been developed in the yeast phase and interact with  $\alpha$ -1,3-glucan (5).

One of the amylases is acid-stable alpha-amylase which has very significant application in industries. Several acid-stable  $\alpha$ -amylases were refined and described in various microorganisms (3). The *asAA* gene has been identified in *A. niger*. Different studies have purified the acid-stable  $\alpha$ -amylase of *A. niger* (14,16). The *A. niger* acidic  $\alpha$ -amylase shows low molecular weight compared to acidic  $\alpha$ -amylase of other filamentous fungi. In the current study, the acid-stable  $\alpha$ -amylase (*asAA*) gene was amplified with PCR of *A. niger* genomic DNA as a template. The Primer set, Amy-ac1 and Amy-ac2 resulted in a 347 bp product for 49 out of 63 isolates (78%) including clinical, environmental isolates, and reference strain. Based on the result from RFLP, the *HpaII* enzyme generated two expected fragments with sizes 202 and 140 bp for 40 (81.6%) isolates. Nine (18.4%) isolates including 1 clinical isolate obtained from otomycosis and 8 environmental isolates had no *HpaII* site for the 347 bp fragment of *asAA* gene.

## CONCLUSION

The result of this study demonstrated that various sources of *A. niger* including clinical and environmental isolates have acid-stable  $\alpha$ -amylase (*asAA*) gene. Moreover, the RFLP method can detect different genotypes based on the *asAA* gene in *A. niger*.

## ACKNOWLEDGMENT

This study was based on an MSc thesis by Zahra Shialy, which was supported by the Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant no. 93154).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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