Isolation and Identification of Alkaline Protease Producing *Aspergills niger* from Iraqi Soils

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Abstract. Twenty purified isolates were obtained by using different soil sources, only twelve isolates belonging to Aspergillus genera depending on cultural and morphological characterization. The isolates were used as alkaline protease producer. The highest proteolytic, enzymatic activity (95.83U/ml) was obtained from *Aspergillus* sp. ZE isolate. This isolate was identified by 5.8 rRNA gene sequencing as *Aspergillus niger* (accuracy of 99%), which was matched with the sequence of *Aspergillus niger* strain GM775228 recorded in Gene bank under the ID: GM 775228.1.

1. Introduction

Proteolytic enzymes are most important groups in industrial applications and one of the largest diverse families of enzymes known, and alkaline proteases have great application in the detergent and leather industry and are involved in every aspect of organism’s function [1];[2]. Fungi are important sources of proteolytic enzymes because of their ability to grow at different temperatures, pH and use a variety of substrates as nutrients [3]. A variety of microorganisms like bacteria, fungi, yeast and actinomycetes are known to produce protease enzymes, one of the main hydrolytic enzymes secrete from different Filamentous fungi, and are known to produce acid, neutral, alkaline and metalloproteases and single organism can secrete more than one type of protease [4].

Many species of *Aspergillus* have ability to produce proteases, such as *Aspergillus oryzae* which produces acid, neutral and alkaline proteases. This study aim was alkaline protease production from local fungal isolates *Aspergillus* sp.

2. Materials and Methods

2.1. Mold isolates sources.

Mold isolates were collected from different types of soils in Baghdad.

2.2. Culture media

2.2.1. Potato Dextrose Agar (PDA).

The PDA media was prepared as described by the manufacturing company. The pH media was adjusted to 7 and 9 by NaOH (0.1N) and autoclaved at 121°C and 15 pound/inch² for 15 min. The prepared media was preserved by adding chloramphenicol (0.025%).

2.2.2. Potato Dextrose broth (PDB).

The media was prepared as described by the manufacturing company. The media was autoclaved and used for fungi isolated growth.

2.2.3. Skim milk agar.

The media was prepared according to [5]; [6]. Fifteen gm of agar was dissolved in 800 ml distilled water and autoclaved at 121°C and 15 pound/inch² for 15 min, 10g of skim milk powder was dissolved in 200 ml distilled water and autoclaved at 121°C and 15 pound/inch² for 5 min, and mixed with sterilized agar. The mixture (media) was cooled to 50°C and poured in petri dish for primary screening depending on Clear Zone area.
2.2.4. Yeast extract casein medium for quantitative screening (Production media).
The production media was prepared according to [7], contained Glucose 10, Casein 5, KH2PO4 2, K2HPO4 2, MgSO4.7H2O 1 were dissolved in 1000ml distilled water and pH was adjusted to 7 and divided in 250ml Erlenmeyer flasks(100ml/flask) and autoclaved at 121o C and 15 pound/inch² for 15 min.

2.3. Isolating and purifying.
Many soil samples were collected from various locations and sources. The samples were transferred to the laboratory in clean polyethylene bags prior to isolating processes. A pour plate method was used as described by [8] for isolating processes. After preparing the decimal dilutions for the samples, 1 ml of each dilution was poured in two dishes containing PDA media (pH 7 and 9). The dishes were incubated at 30øC for 5 days. The purification process of isolates was carried out by sequential transferring into PDA media and incubation at 30øC for 5 days.

2.4. Identification of molds isolates
The cultural and morphological characteristics of mold isolates on PDA media were carried out by using lactophenol dye and examined under microscope field. The isolates which not belonging to the genus *Aspergillus* (according to the taxonomic keys) were discarded [9]; [10].

2.5. Qualitative screening
The fungal isolates were spot inoculated in Petri dishes with skim milk agar. Following inoculation, the Petri dishes were incubated at 30øC for 5 days. Then, casein degradation was observed as a clear zone around fungal colonies.

2.6. Spore suspension preparation.
The spore suspension of 12 isolates was prepared as described by [11]. The spores were counted according to [11], using hemocytometer slide and the number of spores for all isolates was adjusted to 10⁷ spores/ml.

2.7. Enzyme Production medium (Yeast extract casein medium).
The selected five fungal isolates were inoculated in sterile 100 ml of protease specific fermentation broth containing (% w/v): yeast extract 0.5, MgSO4 0.1, glucose 1.0, K2HPO4 0.2, KH2PO4 0.2, Casein 0.5, pH 9.0. Flasks were inoculated and incubated at 28˚C for 5 days in shaker incubator [7]. The fermentation media were filtered through Whatmann filter paper No.1 and then the filtrates were centrifuged at 8,000 rpm at 4˚C for 10 minutes. The clear supernatant was used as source of protease enzyme [13].

2.8. Measurement of enzyme activity.
Protease activity was determined according to the method of [14] by using casein as substrate. A reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M carbonate–bicarbonate buffer (pH 9.5) and 1 ml crude enzyme and incubated at 40˚C for 5 min. The reaction was stopped by the addition of 3 ml of 10% TCA (Trichloroacetic acid). The solution was filtered using Whatmann No.1 filter paper. 5 ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent was added to 1ml of filtrate. The absorbance was measured at 660 nm against the enzyme blank using UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min under standard assay conditions.

2.9. Molecular identification

2.9.1. DNA extraction.
The local *Aspergillus* sp. isolate (ZE) was cultured on PDB using shaker incubator of 150 rpm at 30øC for 5 days. 3 ml of the broth media culture was centrifuged at 13000 xg for 1 min. (the supernatant was ignored). 100-500 mg of precipitated biomass cells was taken and smashed under liquid nitrogen using a mortar. The smashed sample was transferred to a 1.5 ml Eppendorf tube. A molecular biology kit (from bio basic Inc, Canada) was used to extract the DNA from the mold isolate according to the kit extraction method (EZ-10 Spin Column Fungal Genomic DNA Mini-Preps, no. FT82012). DNA purification was estimated by Nano drop.
2.9.2. Polymerase chain reaction (PCR).
PCR was used to amplify the Internal Transcribed Spacer (ITS) of the rRNA 5.8s ribosomal gene in order to confirm the selected isolate type which includes the two zones ITS1 and ITS2 (using the primers in table (1) as described by [15]. The concentration of the primer was 100 picomole and the numbers of nitrogen bases were 17 in forward primer and 19 in the reverse one. The amplification was done in 20 µl which then added to the master mix that was supplied by the Bioneer Company as shown in Table (2).

Table 1. The sequence of used primers

| Primer               | Sequence                  | GC%  | Temp. C° |
|----------------------|---------------------------|------|----------|
| Forward (5.8s)       | CGC TGC GTT CTT CAT CG     | 58.5 | 55.7     |
| Reverse (ITS1F)      | TCC GTA GGT GAA CCT GCG G  | 63.2 | 60.7     |

Table 2. The compounds in the master mix

| Compounds in the master mix         | Vol.(µl) |
|-------------------------------------|----------|
| 10 P mole Forward primer            | 2        |
| 10 P mole Reverse primer            | 2        |
| DNA extract                         | 4        |
| Deionized water                     | 12       |
| Total volume                        | 20       |

The master mixture was mixed for few seconds using vortex. The tube was placed in PCR thermo cycler. The device was programmed according to table (3) and the amplification was taken place to amplify the extracted DNA. In the end of the reaction time, 5 µl of 5.8S rRNA amplifying product was withdrawn for electrophoresis assay.

Table 3. Conditions of the PCR master mixture.

| No. | Step            | Temperature | Time  | Cycles       |
|-----|-----------------|-------------|-------|--------------|
| 1   | Initial Denaturation | 94 °C       | 5 min.| One cycle    |
| 2   | Denaturation    | 94 °C       | 30 sec.|              |
| 3   | Annealing       | 53 °C       | 40 sec.|              |
| 4   | Extension-1     | 72 °C       | 40 sec.| 35 cycle    |
| 5   | Extension -2    | 72 °C       | 5 min.|              |
| 6   | Holding         | 4 °C        | ∞     | One cycle    |
2.9.3. The Electrophoresis of DNA amplification products on agarose gel.
The PCR products were loaded on 1.5% agarose gel using a horizontal electrophoresis. 5 µl of PCR products and 2 µl of loading buffer for every each 5 µl of DNA extract. The mixture was mixed well and located into the sample gel wells. The sample was subjected to electrophoresis assay for 1 hour, 5 volt/cm and 70 milliamps in order to initiate the movement toward negative and positive poles. The DNA bands were detected by UV light Tran's illuminator device.

2.9.4. Determination of nitrogen bases sequence.
The gene amplifying products have been sent to Korean Macrogen Company in order to determine the nitrogen bases (DNA sequencing) for the pure isolate ITS zone.
The sequencing was compared with the available information on that gene with NCBI website using BLAST Nucleotides software to identify the species of the chosen isolates.

3. Results and Discussion
3.1. isolation.
Twenty fungal isolates were obtained from different types of soils in Baghdad. Twelve isolates were identified as *Aspergillus* according to the cultural and morphological characteristics (Table 4). It was observed that the fungal isolate formed black to gray colonies on potato dextrose agar media. It was distinguished by their heavy growth when it was incubated at 30°C for 5 days. In the optical microscope field, a septate mycelium was observed [9]; [10]. According to previous observations, the isolate was identified as *Aspergillus*.

[16] reported that the isolation process for any microorganism need to know the most important characteristic which represents the main target of this process. Generally, the desired characteristic is a restricted factor to isolate the microorganism from its natural media.

| Characteristic          | Observations                                      |
|-------------------------|---------------------------------------------------|
| Colonies                | Black, heavy growth on PDA at 30°C after 5 days   |
| Mycelium                | Septate                                           |
| Conidial heads          | Black                                             |
| Vesicle                 | Spherical shape                                   |
| Conidia                 | The mature is large, immature is small            |

3.2. Qualitative screening by using skim milk agar.
All twelve *Aspergillus* sp. isolates were spot inoculated in the center of skimmed milk agar plates and incubated at 30°C for 5 days. Eight isolates which showed highest zone around the colony (Table 5), were selected for this investigation.

3.3. quantitative screening.
Quantitative screening is defined as an additional confirmatory test for the capabilities required in order to obtain knowledge about the microorganisms that are isolated in this study. It has provided a variety of information necessary to assess the ability of microorganisms in industrial uses [17].

The eight isolates that were obtained from qualitative screening were subjected to quantitative screening.
As shown in Table (6), all the eight isolates had enzymatic activity, despite that the isolate varied in their capabilities to produce the alkaline protease, isolate *Aspergillus* sp. ZE was the most distinguished one among others by giving the enzymatic activity with values reached 95.83U/ml. The *Aspergillus* sp. ZE was subjected to molecular identification.
Table 5. A clear zone around fungal colonies

| Isolates        | Clear zone diameter (mm) |
|-----------------|--------------------------|
| Aspergillus sp. ZE | 19.0                     |
| Aspergillus sp. AB | 17.5                     |
| Aspergillus sp. AC | 16.8                     |
| Aspergillus sp. AD | 16.2                     |
| Aspergillus sp. AF | 16.0                     |
| Aspergillus sp. AG | 15.7                     |
| Aspergillus sp. AH | 15.2                     |
| Aspergillus sp. AJ | 14.9                     |
| Aspergillus sp. AF | -                        |
| Aspergillus sp. AS | -                        |
| Aspergillus sp. AM | -                        |
| Aspergillus sp. AN | -                        |

Table 6. Enzymatic activity (U/ml) of the local Aspergillus sp. isolates

| Isolates        | Enzymatic activity (U/ml) |
|-----------------|---------------------------|
| Aspergillus sp. ZE | 95.83                     |
| Aspergillus sp. AB | 88.20                     |
| Aspergillus sp. AC | 83.73                     |
| Aspergillus sp. AD | 81.70                     |
| Aspergillus sp. AF | 80.70                     |
| Aspergillus sp. AG | 79.18                     |
| Aspergillus sp. AH | 76.60                     |
| Aspergillus sp. AJ | 75.18                     |

3.4. Molecular Identification.
3.4.1. DNA extraction.
The DNA was extracted from Aspergillus sp. ZE and the purity of DNA was examined by Nano Drop with a purity of 1.99 which is adequate for Polymerase Chain Reaction (PCR) process. [18] reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On the other hand, an adequate quantity of DNA may reduce the accuracy.
3.4.2. Polymerase Chain Reaction (PCR).
A PCR for the local *Aspergillus sp.* ZE for the internal transcribed spacer (ITS) which were represented by ITS1 and ITS4 that were located within the ribosomal gene (5.8S ribosomal RNA-rRNA) was carried out. The electrophoresis on 1.5% agarose show (by using U.V detector), that there was a clear band represents the genes amplifications (Figure 1). The molecular size of gene amplification band was 300bp comparing with ladder size at the same conditions, which refers to the prime binding to the complete sequence in DNA pattern. The interference space, ITS1& ITS4 ribosomal unit 5.8s rRNA was used to distinguished the various species of mold, with accuracy identification results.

![Figure 1. Electrophoresis for the local *Aspergillus sp.* ZE for the internal transcribed spacer (ITS) on agarose gel.](image)

3.4.3. Sequence analysis of amplification products.
The sequence of nitrogen bases, of the internal transcribed spacer ITS1, for the local mold isolate (*Aspergillus sp.* ZE) was studied by sending the amplification products to the Korean company Macrogen (Figure 2). The nitrogen bases sequence (240 base-pair) which was taken from the local isolate sequence (of the present study) is shown in Fig. (2). The PLAST program has been used to find out the similarity of gene with the bank information (NCBI). The results showed that there is a match between isolation and 99% with global isolation sequences global ID: MG775228.1 Registered on the NCBI website and registered in the United States of America which belong to *Aspergillus niger* strain MG775 228. (Fig.2)
Figure 2. Match the sequence of nitrogen bases for internal transcribed spacer (ITS) for the local Aspergillus sp. B1b within the ribosomal gene (5.8S ribosomal RNA-rRNA) with global isolate Aspergillus niger strain MG775228.

4. Conclusion.
In this study, The isolates of Aspergillus genera were used as alkaline protease producer. The highest proteolytic, enzymatic activity was obtained from Aspergillus sp. ZE isolate. This isolate was identified by 5.8 rRNA gene sequencing as Aspergillus niger (accuracy of 99%), which was matched with the sequence of Aspergillus niger strain GM775228 recorded in Gene bank under the ID: GM775228.1.

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