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Proteolytic Activity of *Aspergillus niger* Strains Isolated from Soil

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A B S T R A C T

This study was proposed to determine the recovery rate of *Aspergillus* species in Ankara, Turkey, from soil and their identification as well as to evaluate the proteolytic activity of the isolated strains. 20 different soil samples were collected and searched for the presence of the fungal species after selective plating and internal transcribed spacer (ITS) gene region sequencing of cPCR fragments. Also it was the first report to screen the biologically active peptides production from the recovered *Aspergillus* isolates by plate assay method using two agar media including Calcium Caseinate and Skim Milk Agar with different pH values. In addition to the activity of *Aspergillus* protease as crude extracted enzyme obtained by fermentation was determined using agar well diffusion method. Overall, 9 (45%) samples were morphologically proposed as positive fungal isolates and only six of them (66,7%) were sequenced and identified as *Aspergillus niger*with recovery rate of 30% (6/20). These six *A. niger* isolates were allocated in the NCBI GenBank Database and assigned GQ229076.1, EU314995.1, GQ229076.1, GQ229076.1, GQ229076.1 and GQ229076.1 accession numbers. The proteolytic activity of the extracted enzyme on Calcium Caseinate Agar of *A. niger* isolates was high and showed wide clearance zone of 10, 16 and 20 mm diameter at pH 5, 6.6 and 7.3 respectively in compared to that of a reference *A. niger* strain ATCC 16404 which was only 8, 12 and 14 mm at same pH.The current proteolytic activity showed qualitative documented results at different PH. Consequently, the acid and alkaline protease activities of *A. niger* isolates were confirmed. Using Calcium Caseinate and Skim Milk Agar allowed the efficient trial for *Aspergillus* peptides production and activity creating large scale production of it.

Introduction

Proteases are one of the most important groups of enzymes that play an important role in various industries (60% share) such as food, textiles, leather, de-hairing, photos, cheese-making, detergents, pharmaceutical and feed (Zambare *et al*., 2011 and Nirmal *et*

al., 2011). Proteases occur in all organisms in both Prokaryotes and eukaryotes. Bacteria secrete proteases to hydrolyse the peptide bonds in proteins and therefore break the proteins down into their constituent amino acids. Bacterial and fungal proteases are particularly important to the global carbon and nitrogen cycles in the recycling of proteins, and such activity tends to be regulated by nutritional signals in these organisms (Sims, 2006). Fungal proteases have attracted the attention of environmental biotechnologists because fungi can grow on low cost substrates and secrete large amount of enzymes into culture medium which could ease downstream processing (Anitha and Palanivelu, 2013). In recent years, fungal protease production has gained significance and different fungal strains can secret various extracellular hydrolytic enzymes such as amylases, lipases and proteases (Nirmal *et al.,* 2011 and Souza *et al.,* 2015). Proteases have been classified on the basis of their acid/base properties into three groups acidic, neutral and alkaline proteases. The acidic proteases are those which have their pH optimum in the range of 2.0 to 5.0 and have mainly fungal origin. The proteases having their pH optimum at 7.1 or around are called neutral. The alkaline proteases have their pH optimum in the range of 8 to 11(Rao *et al.,* 1998). Fungal proteases have a wide pH rang 4-11 (Rao *et al.,* 1998). For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases (Nirmal *et al.,* 2011).Proteases produced commonly by filamentous fungi belong to *Aspergillus*, *Rhizopus* and *Penicillum* genera (Souza *et al.,* 2015). The ability of many species of *Aspergillus* and other genera to produce proteases is well known, therefore, this study was subjected for detection of the fungal recovery rate of soil origin in Golbasi city and Gazi University, Ankara Governorate, Turkeyand for molecularly identification of the recovered isolates. In addition to the proteolytic activity of the isolated *Aspergillus* strains was evaluated by different method using two types of agar media at same time as a good trial.

Materials and Methods

Sample collection and fungal strain isolation

Twenty soil samples were collected in a sterile container from different districts of Golbasi city and Gazi University in Ankara Governorate. For fungal isolation, the collected soil samples were subjected for double fold serially dilutions in sterile distilled water and plated onto Potato Dextrose Agar (PDA) (Himedia M096) plates and the plates were incubated at 30^{o}C for 7 days(Raja *et al.,* 2017) then they sub-cultured on Sabouraud-2 % Dextrose Agar(Oxoid CM0041) plates. The fungal growth were stained by Lactophenol Blue and Safranin and directly examined under the light microscope (Raja *et al.,* 2017). The *Aspergillus* isolates were morphologically identified based on microscopical examination of conidia and conidiphore, pigment properties of the colonies on PDA and the typical characteristic morphology that was previously proposed according to Pictorial Atlas of Soil and Seed Fungi (Tsuneo Watanabe, 2002).Then those suspected identified fungal isolates by various morphological characteristics were subjected for molecular identification followed by genus-species confirmation.

Molecular identification of the fungal isolates

For molecular identification of the suspected fungal isolates, they were grown on Malt Extract Agar (MEA)(Merck 1.05398) for 7 days at 25 °C. Genomic DNA were extracted from culture using a microbial DNA extraction kit (Ultraclean Microbial DNA Isolation Kit, Mobio) following the

manufacturer's protocols, and extracted DNA was stored at -20 °C. The internal transcribed spacer (ITS) regions of the fungal rDNA genes were amplified by conventional PCR according to Visagie *et al.,* (2014) using the Genus specific primer pairs of V9G-F, TT ACGTCCCTGCCCTTTGTA; LS266-R, GC ATTCCCAAACAACTCGACTC (Samson *et al.,* 2010) and a Veriti® 96-Well Thermal Cycler (Applied Biosystems®). Cycling condition reaction was carried at95°C/3 min for primary denaturation followed by 40 cycles of 95°C/1min, 58°C/30 sec and 72 °C/1min for denaturation, annealing, elongation respectively. PCR products were separated by agarose gel electrophoresis (1% w/v in 1X TAE), visualized by Gel Red staining, cleaned using EXOSAP-IT (Affymetrix, Santa Clara, CA, USA) and used for sequencing. For species confirmation, PCR fragments containing ITS locus were sequenced using a primer pair of ITS1, TCCGTAGGTGAACCTGCGG and ITS4, TCCTCCGCTTATTGATATGC (White *et al.,* 1990). Sequencing reactions were performed with the CEQ™ DTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) using the CEQ 8000 Genetic Analysis System. The sequences were allocated GenBank accession numbers and compared with those deposited in the NCBI GenBank Database.

Screening of *Aspergillus* isolates proteolytic activity by plate assay method

For screening the biologically active peptides production by the isolated *Aspergillus* strains, it was carried using two types of agar media as a first application trial (first time). The all identified *Aspergillus* isolates were spotted onto two agar media including Calcium Caseinate Agar (Merek 1.05409) medium at different pH of 4.2, 5.0, 6.6, 7.3 & 8.7 and Skim Milk Agar (Himedia M763) medium at pH 7 for evaluation their protease-producing ability then they were incubated at 30^{o} C for 48 hrs. The clearance zone diameter of the used fungal isolates was measured.The protease producing ability of the isolated strains was selected based on the zone of clearance compared with that of the used known protease producing reference *A. niger* ATCC 16404 strain.

Extraction and detection of *Aspergillus* proteolytic enzymes

Obtaining of crude enzyme by semi solid fermentation

The PDA slants of 7 days old cultures of all isolated *Aspergillus* strains were separately wetted by 0.1% Tween-80. The spores were scratched by sterile glass wood to obtain homogenous spore suspension of 1x 10^{7} spore/ml. Ten g of whole-wheat flour were added to 250 mL Erlenmeyer flask (repeat two times). One group of them were moistened with 15 ml of distilled water and other were moistened with 15 ml of salted solution [ammonium nitrate 0.5, potassium dihydrogen phosphate 0.2, sodium chloride 0.1 and magnesium sulphate 0.1 (g/100ml)]. The flasks were sterilized, at 121^{o}C, cooled, inoculated by a one ml of spore suspension and incubated at 30^{o}C for 96 hrs. After incubation, 75ml of distilled water was added to the flasks and shaken on rotary shaker for one hour at 180 rpm. (Ikram-ul-Haq, 2003). After filteration and centrifugation 9000 g at 4^{o}C for 20 min the supernatant was collected and used as crude enzyme (two types, one moistened with distilled water and the other with salted solution) (Nagamani *et al.,* 2012).

Detection of enzyme proteolytic activity by well diffusion method

The proteolytic activity of each isolated *A. niger* strains was qualitatively determined using fungal crude prepared enzyme which obtained after fermentation procedure. The

well diffusion method was also carried onto two different agar media including Calcium Caseinate Agar at different pH of 4.2, 5, 6.6, 7.3, and 8.7 and solidified for one hour as well as Skim Milk Agar at pH 7 using two different holes which were punched for each plate. After that, two concentrations of 50 and 100µL of each crude enzyme type which prepared previously from all isolated *A. niger* strains were separately loaded into the holes. The plates were incubated for 24 hrs at 37^{o}C. The enzyme proteolytic activity extracted from all strains was determined based on the zone diameter of clearance against two enzyme concentrations (each type)onto agar medium.

Results and Discussion

Morphological identification

Cultural characters

On PDA and SDA medium, the typical characteristic morphology of *Aspergillus* showed white initial growth of colonies that becoming black later (salt and pepper appearance) in six fungal isolates with reverse turning pale yellow colony colour(Fig 1a) while the other three obtained isolates showed greenish colonies and dark greyish-brown

Microscopic examination of fungal isolates

The direct microscopical examination of six fungal isolates showed spherical vesicles; smooth and colorless uniseriate conidiophores as shown in Fig (1b) while the rest of the three obtained isolates showed branching or simple conidiophores supporting phialides in brush-like clusters known as penicillium in one isolate and rhizoids with non-sepetated hyphae (sporangiophore is unbranched) suspected to be *Rhizopus* spp. in two isolates. After cultural and direct microscopical examination of soil samples, nine samples (45 %; 9/20) were positive and showed different fungal colonial appearance. Six of these (66.7%; 6/9) positive isolates were suspected to be giving rise to *Aspergillus* with recovery rate of 30% (6/20); one isolate was suspected to be Penicillum (5%; 1/20) and the rest two isolates (10%; 2/20) showed colonial and cultural characters of Rhizopus.

Molecular Identification of Fungal isolates

After molecular identification of the isolated fungal isolates, all of them showed 1500bp fragment (Fig 2) of the ITS regions of the fungal genus specific rDNA genes. After sequencing, the amplified fragments were allocated in GenBank and compared with those deposited in the NCBI GenBank Database giving rise similarity more than 94% to *A. niger* in six isolated strains only gave GenBank accession numbers of GQ229076.1, EU314995.1, GQ229076.1, GQ229076.1, GQ229076.1 and GQ229076.1 representing fungal isolates numbers 1-6 respectively.

Screening of proteolytic activity of fungal strains

In this study, the clearance of proteolysis was qualitative observed after spotting of strains on two new agar media of calcium caseinate agar and skim milk agar used at first time within different pH range. The clearance diameter zone range of all *A. niger* strains isolated from soil on calcium caseinate agar was 3.4- 20mm at pH 7.3 and on skim milk agar was 10-16mm at pH 7. The highest clearance zone at pH 5.0, 6.6, 7.3 & 8.7 on calcium caseinate agar was for *A. niger* strain code no. 2 with diameters 10, 16, 20 & 14 mm respectively (Table 1) while that of reference *A. niger* strain ATCC 16404 at same pH on same agar medium was 8, 12, 14, 15 mm respectively (Figure3 A& B).

The proteolytic activity of *Aspergillus niger* strains

The proteolysis of crude enzymes that extracted from *A. niger* strains on calcium caseinate agar plates by two concentrations at pH 5 or 6 (moistened with distilled water and salted solution in the fermentation medium) was qualitatively and separately determined. The detected clearance proteolysis zone diameter range of 100µL crude enzyme moistened with salted solution prepared from *A. niger* strain; isolate code no. 2 was 10-14and 1-2mmat Ph 5 and 6 respectively and it was wider than that of the reference *A. niger* ATCC strain16404 which was determined to be 1-2 mm at pH 6. And the enzyme proteolysis of the reference *A. niger* ATCC strain 16404 at pH 5 was not showed at all (Fig 4). While the detected clearance zone diameter of enzyme moistened with distilled water as well as 50 µL crude enzyme concentration proteolysis was negligible and not increase than 1 mm around the well.

Table.1 The clearance zone diameters of isolated *Aspergillus niger* strains on calcium caseinate and skim milk agar

Code number *of A. niger* strains	Clearance zone (mm) on					
	Calcium caseinate agar at different pH*					Skim milk agar at pH*
	4.2	5.0	6.6	7.3	8.7	7
**Reference strain ATCC 16404	-	8	12	14	15	12
1	-	4	6	8	4	10
2	-	10	16	20	14	16
3	-	4	4.4	6	-	12
4	-	3.2	2.6	4	-	14
5	-	3.3	2.7	4.2	-	10
6	-	3.0	2.4	3.4	3.5	12

*Means of triplicate readings
**Mean growth pattern of known protease producing strain of *A. niger*
Means not followed by the same superscripts are significantly different ($p < 0.05$).

Fig.1 *Aspergillus niger* isolate code no.2. Colony morphology on PDA (a) and microscopic examination of conidia and conidiphore stained by Lactophenol Blue (b)

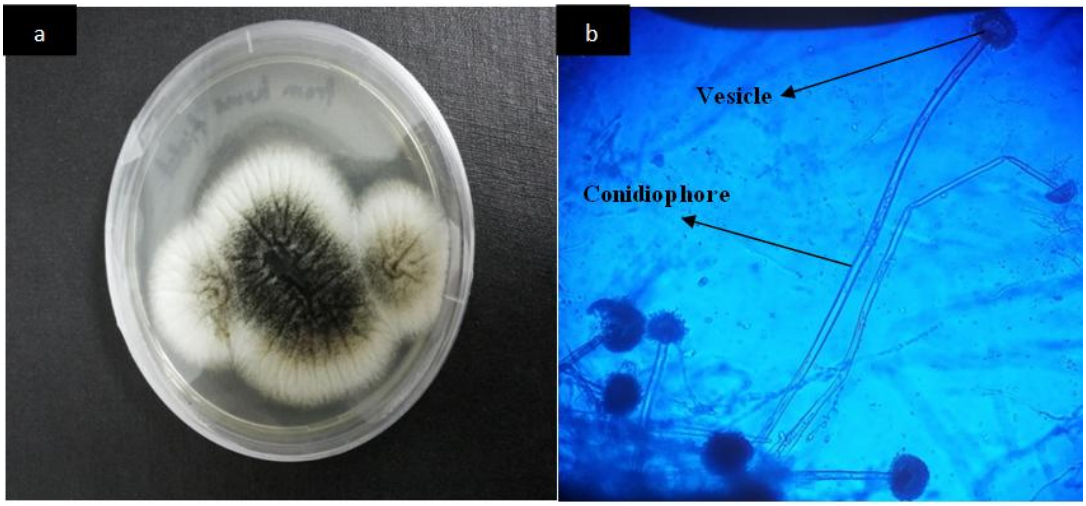


Fig.2 Agarose gel electrophoresis showing typical amplification product of 1500 bp rDNA gene fragments of ITS regions in nine fungal isolates. M: 300-10000 bp Ladder

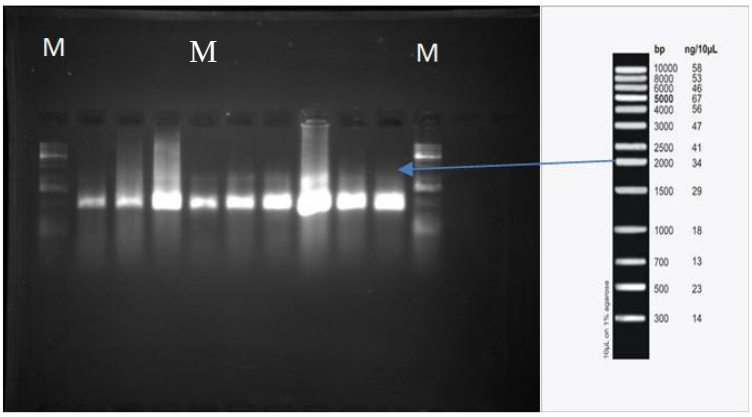
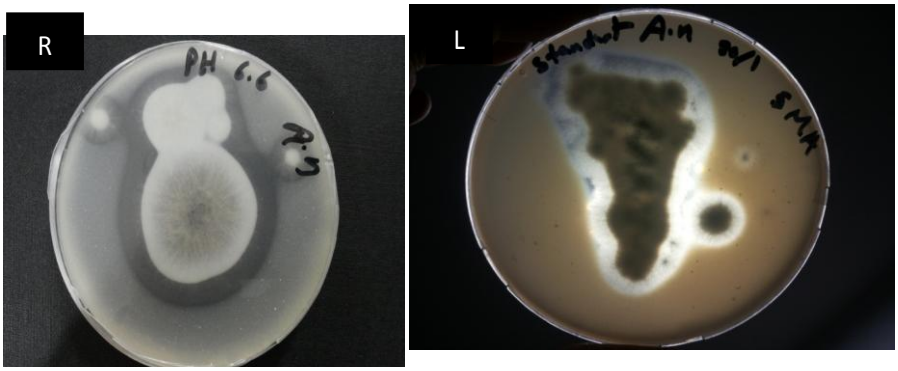


Fig.3 The proteolytic activity of*A. niiger* strains

A) A refrence *A. niiger* ATCC strain 16404; showed the clearance zone on Calcium Caseinate Agar at pH 6.6 (R) on Skim Milk Agar at pH 7 (L)



B) The isolated strain of *A. niiger* code no.2 showed the clearance zone on Calcium Caseinate Agar at different pH (7.3, 8.7, 6.6) (R) and on skim milk agar at pH 7 (L).

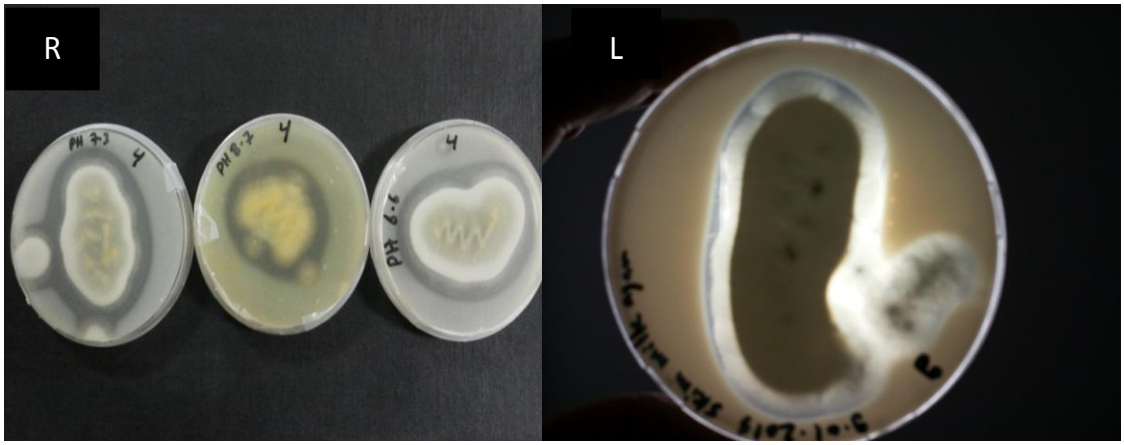
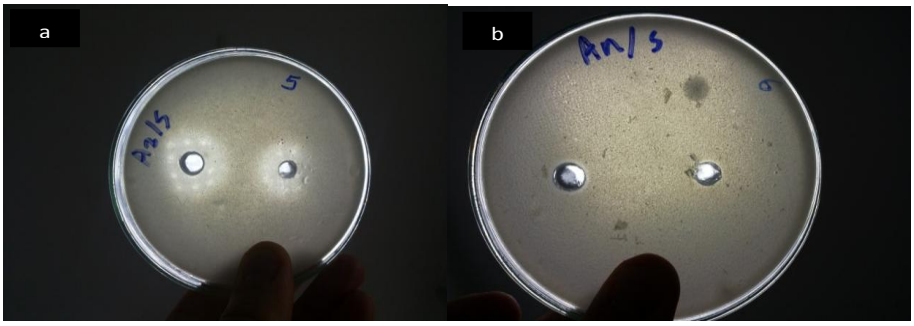


Fig.4 The proteolysis activity of *Aspergillus niger* crude enzyme 100 µL on Calcium Caseinate Agar moistened with salted solution in the fermentation medium. The clearance zone was 10-14 mm at pH 5 for *A. niger* strain code no. 2 (a) and 1-2 mm at pH 6 for reference *A. niger* ATCC 16404 (b)



Realy, it is important to note that the morphology of colony, microscopic examination, conidia and conidiophore characteristic were selected as basis for morphologically identification of the fungal strains. Herein, the present study isolated strains belonging to the genus *Aspergillus* were identified aswhite initial growth of colonies that becoming black later on SDA, dark-brown to black conidia, with uniseriate colorless conidiophores, spherical vesicles and white mycelium, these characteristics were typically identified by Silva *et al.,* (2011). Similar various reports have been published which used the morphological characters as key identifying factors such as Watanabe, (2002); Morya and Yadav (2009) and Bandh, *et al.,* (2012). Other finding proved that during the investigation of 105 fungal colonies, 6 *Aspergillus* species (*A. sparsusi, A. janthinellum, A.ochraceous, A. flavus, A. terreus* and *A. niger*) were observed. The maximum fungal species belongs to Deuteromycotina and *Aspergillus* was dominant among most isolated genera (Sudarkodi *et al.*, 2015).

Molecularly, of nine identified fungal strains showed 1500bp fragment of the ITS regions of the fungal genus specific rDNA genes, six (66.7%) were identified as *A. niger* strains after sequencing with recovery rate of 30% (6/20). Similar molecular results by Visagie *et al.,* (2014) and Alsohaili and Bani-Hasan (2018) showed that fungal species were isolated and identified at the species level using rDNA ITS sequences comparison and analysis. The eight isolated fungal species were belong to four classes and five of them were *Aspergillus* as the following: Eurotiomycetes (*A. niger, A. tubingensis,* and *P. citrinum*); Dothideomycetes (*A. alternate, A. gaisen,* and *A. tenuissima*); Sordariomycetes (*F. oxysporum*) and Mucoromycotina (*R. stolonifer*) (Alsohailiand Bani-Hasan 2018).

In the present work, production of proteolytic enzymes by fungal isolates was screened by the Plate assay methodusing two types of agar media (including calcium caseinate agar medium at different pH of 4.2, 5.0, 6.6, 7.3 & 8.7 and skim milk agar medium at pH 7, then they were incubated at 30^{o} C for 48 hrs) as protease substrate good application trial that giving good results. Similarly, the Plate assay method was used for detection of proteolytic activity of fungal isolates by Hankin and Anagnostakis (1975) using gelatin agar medium instead of our used media, in which gelatin is the protein source of that growth medium. The fungal isolates were spot

inoculated in petridishes with nutrient agar medium supplemented with 1% gelatin (Peptone, 5g; Beef extract, 3g; NaCl, 5g; Agar, 15g; Distilled water, 1 liter, pH 6). Then they were incubated at 28 ± 1°C for 3 days. After a week of incubation, gelatin degradation was observed as a clearing zone around fungal colonies. Previously, the clearance zone produced on the agar plate contain casein was related to the amount of protease produced by the fungus (Alane B Vermelho *et al.*, 1996). Recently, the same method as a simple method for qualitative determinations of protease activity but with bromocresol green dye was used by Ponnuswamy Vijayaraghavan *et al.*, (2013).

Herein, the highest protease production was for *A. niger* strain code no. 2 with clearance zone diameters 10, 16, 20 & 14 mm at pH 5.0, 6.6, 7.3 & 8.7, respectively on calcium caseinate agar comparing with that of the used reference strain. Nearly to our finding, two fungal species i.e. *Aspergillus flavus* and *Aspergillus niger* out of the total six isolates which showed highest proteolysis zone around the colony were proved by Sudarkodi *et al*,. (2015). On other hand, of the eight, *A. niger* AM07 was considered to be the best amylase producing strain on starch containing nutrient agar but with zone diameter of 7.0 mm clearing which consider lower than that of our detected diameter (Omemu *et al.*, 2005).

Hence, it seems that *A. niger* strains of soil origin produced acid, neutral and alkalin proteases but the standard *A. niger* strain ATCC 16404 produced neutral and alkaline proteases only. Because the high proteolytic activity upon spot directly on calcium caseinate agar was showed with *Aspergillus niger* strain code no. 2 at pH 5.0, 6.6, 7.3, 8.7 while with *A. niger* strain ATCC 16404 at pH 6.6, 7.3, 8.7.

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