



Original article

Evaluation of *in vitro* activities of extracellular enzymes from *Aspergillus* species isolated from corneal ulcer/keratitis



Annanthode Balakrishnan Sangeetha^{a,1}, Ahmed Abdel-hadi^{b,c,1}, Anamangadan Shafeeq Hassan^d, Coimbatore Subramanian Shobana^a, Sreeram Suresh^a, Baskaran Abirami^a, Kanesan Panneer Selvam^e, Raid Saleem Al-Baradie^b, Saeed Banawas^b, Mohammed Alaidarous^b, Bader Alshehri^b, Abdul Aziz Bin Dukhyil^b, Sugapriya Dhanasekaran^f, Palanisamy Manikandan^{b,g,*}

^a Department of Microbiology, PSG College of Arts & Science, Coimbatore 641 014, India

^b Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Al Majmaah 11952, Saudi Arabia

^c Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut Branch, Egypt

^d Department of Microbiology, Dr. G.R. Damodaran College of Science, Coimbatore 641 014, India

^e Department of Microbiology, MR Government Arts College, Mannargudi 614 001, India

^f Department of Medical Laboratory Sciences (Pathology), College of Applied Medical Sciences, Prince Sattam Bin Abdulaziz University, Wadi-Al Dawaser Campus, Riyadh Province, Saudi Arabia

^g Greenlink Analytical and Research Laboratory India Private Limited, Coimbatore 641 014, India

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ABSTRACT

Mycotic/fungal keratitis is a suppurative, generally ulcerative infection of the cornea. The filamentous fungi, *Aspergillus* spp. are the second leading cause of mycotic keratitis, particularly in India. *Aspergillus* spp. produce a range of extracellular enzymes that are used to break down complex molecules and used for growth and reproduction, also for survival on/in host organism. The current study was designed with an objective to screen *in vitro* extracellular enzyme activity of *Fusarium* and *Aspergillus* isolates from mycotic keratitis patients and to correlate the same as a putative virulence factor. Extracellular enzymes viz., deoxyribonuclease (DNase), protease, lipase, elastase, keratinase, etc., produced by *Aspergillus* have key role in keratomycosis and hence their (n = 85) *in vitro* activities were investigated. It was found that, the majority of the *Aspergillus* isolates produced protease (n = 75; 88% of 85) followed by lipase (n = 59; 69% of 85), DNase (n = 35; 41% of 85), elastase (n = 26; 31% of 85) and keratinase (n = 13; 15% of 85). The enzyme activity indices (EAI) for DNase, elastase, protease and lipase ranged between 1.01 and 1.98, whereas elastase EAI varied between 1.26 and 1.92. DNase, protease and lipase showed a maximum EAI of 1.98 and lowest EAI value of 1.01, respectively. Extracellular enzymes of *Aspergillus* spp. may have potential role in the onset and progression of keratitis.

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1. Introduction

Mycotic keratitis/keratomycosis is the infection of the cornea due to pathogenic fungi capable of invading the ocular surface

* Corresponding author at: Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Al Majmaah, Saudi Arabia.

E-mail address: m.palanisamy@mu.edu.sa (P. Manikandan).

¹ Equally contributed to this work.

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(Mravičić et al., 2012). *Fusarium*, *Aspergillus*, *Curvularia*, *Bipolaris* and yeast fungi such as *Candida* (Thomas, 2003) are the most common causative agents of keratomycosis. In the Southern part of India, the major etiologic agents of fungal keratitis are *Fusarium* and *Aspergillus* (Gopinathan et al., 2002; Manikandan et al., 2013; Srinivasan, 2004). Interestingly, *Aspergillus* spp. are the second leading etiological agents of mycotic keratitis, invasive aspergillosis and superficial infections (Hedayati et al., 2007). Fungi secrete several extracellular hydrolytic enzymes like keratinases, collagenases, gelatinases, phospholipases, lipases and acid proteinases in culture media (Khan et al., 2010). These enzymes not only have a major role in the metabolism but also serve as virulence factor by causing potential harm to the host cells to satisfy the nutritional needs of the pathogen. *Aspergillus* spp. produce a range of extracel-

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lular enzymes that are used to break down complex polysaccharides into simple sugars to be assimilated and used for growth and reproduction, also for survival on host organism.

Research on extracellular enzymes production as a virulence factors for *Aspergillus* isolated from ocular infection remains unexplored (Bouchara et al., 1995; Latgé, 1999; Tomee and Kauffman, 2000). Fungi secrete several extracellular hydrolytic enzymes such as keratinases, collagenases, gelatinases, phospholipases, lipases, and acid proteinases in culture media (Khan et al., 2010). Extracellular proteinases help in the adherence and survival of the pathogen on mucosal surfaces (Borg and Röchel, 1988), invasion of host tissues (Odds, 1985; Röchel, 1986) and digestion of immunoglobulins (Röchel, 1986; Yuan and Cole, 1987) and corneal matrix degradation (Gopinathan et al., 2001). Park et al. (2013) (Park et al., 2013) reported that lipolytic enzymes also have been implicated in fungal virulence and has been extensively studied in *Candida* species. Khan et al. (2010); (Alp and Arikian, 2008; Khan et al., 2010) stated that lipase of *Aspergillus* species has a role in tissue damage. Elastase cleaves the peptide bonds in elastin, aiding in the digestibility of this elastic protein. The keratomycosis aided by the extracellular enzymes of *Aspergillus* thus will add to the severity of the infection. Against this background, the present *in vitro* analysis was undertaken with the objective of examining the role of the extracellular enzyme activities as putative virulence factors in *Aspergillus* keratitis.

2. Materials and methods

2.1. Isolation and identification of *Aspergillus* spp.

Corneal scrapings were collected by an ophthalmologist from the patients with suspected keratomycosis at Aravind Eye Hospital and Postgraduate Institute of Ophthalmology (Coimbatore, Tamilnadu, India) during 2013–2015. The collected material was inoculated directly onto 5% sheep blood agar, Chocolate agar, brain heart infusion broth and potato dextrose agar (PDA) (HiMedia, Mumbai, India) and also spread on a glass slide for direct microscopy after 10% KOH wet mount. The Culture plates were incubated at 37 °C (for bacteria) and 27 °C (for fungi), examined daily, and discarded after 1 week if no growth were present. The fungi that were initially identified based on colony morphology on SDA were further characterized microscopically after lactophenol cotton blue staining (Harris, 2000). Suspected *A. flavus* isolates were further screened on *Aspergillus* differentiation agar (ADA) to differentiate other similar morphological species of *Aspergillus* genera (Rodrigues et al., 2007). All the isolates were stored in screw capped tubes containing 0.85% saline at 4 °C.

2.2. Fungal inoculum preparation

The test isolates *Aspergillus* were grown on potato dextrose agar slants and incubated at 28 °C for seven days. Sterile saline (0.9% NaCl, 2 mL) was added to the culture slant, and the conidia were harvested after gentle vortexing and the mycelial remnants from the conidial suspension were separated by filtration through sterile cotton-wool. The conidial suspension was used as inoculum.

2.3. Extracellular enzyme assays

DNase test agar, rose Bengal elastin agar, tributyrin agar and skim milk agar were aseptically prepared and autoclaved for assays of DNase, elastase, lipase and protease, respectively. All the chemicals were purchased from HiMedia, Mumbai. For keratinase assay, basal medium (BM) overlaid with keratin azure (Sigma-Aldrich, USA) was used (Scott and Untereiner, 2004). The assay media were

inoculated with 30 µl of spore suspension and incubated at 28° C in darkness. After incubation, DNase assay plates were flooded with 1 N hydrochloric acid for clarity in the zone of hydrolysis (Sánchez and Colom, 2010). Elastase (Kothary et al., 1984), lipase (Griebeler et al., 2011) and protease assay plates (J Sharma et al., 2005) were observed for the zone of clearance after fungal mats were removed with the help of a sterile cotton swab (Mythili et al., 2014). Keratinase activity was evaluated visually from the release of azure dye into the colourless lower layer of BM after third day and within three weeks of inoculation (Scott and Untereiner, 2004).

For DNase, elastase, lipase and protease enzymes, enzyme activity index (EAI) was calculated by the following formula (Blanco et al., 2002);

$$\text{Enzyme activity index (EAI)} = \frac{\text{Zone diameter (ZD)}}{\text{Growth diameter (GD)}}$$

The mean EAI was calculated from three observations and the calculated values were grouped under four classes as high EAI (2.0 to 1.75), medium EAI (1.75 to 1.25), low EAI (1.25 to 1.10) and negligible or no EAI (1.10 to 1.00).

3. Results

A total of 1628 ocular specimens were collected from suspected cases of keratitis, from which 85 isolates of *Aspergillus* species were obtained. These isolates were further analysed for microscopic and macroscopic morphology so as to identify at the species level. Microscopic characteristics such as stipes colour, surface appearance, vesicle serration, shape of vesicle and conidia surface were observed after staining with LCB and compared with the standard morphological characteristics (Diba et al., 2007) and they were identified as *A. flavus* (n = 53), *A. fumigatus* (n = 14), *A. terreus* (n = 9), *A. tamarii* (n = 6) and *A. niger* (n = 3).

The extracellular enzyme assay revealed that all the *A. flavus* isolates had one or the other enzyme activities and each isolate were found to have different enzyme activity indices (EAIs) ranging from 1 to 1.97 (Table 1). Among the 47 isolates tested for DNase activity, medium EAI was observed for 16 isolates, low EAI value for 30 isolates, negligible or no EAI value for one isolate and none of the isolate showed high EAI value (Fig. 1). The highest (1.59) and lowest (1.08) was exhibited by the isolate AF29 and AF18 respectively. For elastase enzyme 26, 9, 4 and 8 isolates exhibited high, medium, low and negligible or no EAI, respectively. The elastase EAI ranged from 1.0 to 1.97, the highest value of 1.97 (AF05) and the minimum value of 1.0 (AF10, AF11, AF12, AF13, AF17 and AF18). While analyzing lipase activity indices, it was found that 12, 29 and 6 isolates had medium, low and negligible or no EAI respectively. Of 47 isolates tested for protease activity a total of 12, 30 and 5 isolates exhibited medium, low and negligible or no EAI respectively. The highest value for protease activity was found to be 1.55 and the least value of 1.00. A total of five isolates (AF04, AF08, AF14, AF21 and AF27) were positive for keratinase production.

Majority of the *Aspergillus* isolates produced protease (n = 75; 88% of 85) followed by lipase (n = 59; 69% of 85), DNase (n = 35; 41% of 85), elastase (n = 26; 31% of 85) and keratinase (n = 13; 15% of 85) (Figs. 1 and 2). The EAI for the enzymes DNase, elastase, protease and lipase ranged between 1.01 and 1.98. DNase, protease and lipase showed a maximum EAI of 1.98 and lowest EAI value of 1.01, whereas elastase EAI was found to be varying between 1.26 and 1.92. Of 53 isolates of *A. flavus* 88.68% (n = 47), 71.7% (n = 38), 37.74% (n = 20), 35.85% (n = 19) and 13.21% (n = 7) had protease, lipase, DNase, elastase and keratinase activities, respectively. All the isolates of *A. tamarii* (n = 6) and *A. niger*

Table 1
Frequency distribution of EAls of *Aspergillus* isolates from keratitis.

Enzymes	Isolates	Enzyme activity indices (EAls)									
		1.0–1.10	1.11–1.20	1.21–1.30	1.31–1.40	1.41–1.50	1.51–1.60	1.61–1.70	1.71–1.80	1.81–1.90	1.91–2.0
DNase	<i>A. flavus</i> (n = 53)	1	10	7	1	1	-	-	-	-	-
	<i>A. fumigatus</i> (n = 14)	-	2	-	-	1	-	-	-	-	1
	<i>A. terreus</i> (n = 9)	-	1	1	2	-	-	-	-	-	-
	<i>A. tamaraii</i> (n = 6)	1	2	1	1	-	-	1	-	-	-
	<i>A. niger</i> (n = 3)	-	-	1	-	-	-	-	-	-	-
Elastase	<i>A. flavus</i> (n = 53)	-	-	1	1	-	2	2	3	6	4
	<i>A. fumigatus</i> (n = 14)	-	-	-	-	-	2	-	-	-	1
	<i>A. terreus</i> (n = 9)	-	-	-	-	-	-	1	-	1	-
	<i>A. tamaraii</i> (n = 6)	-	-	-	-	1	-	-	-	-	-
	<i>A. niger</i> (n = 3)	-	-	-	1	-	-	-	-	-	-
Protease	<i>A. flavus</i> (n = 53)	7	17	17	2	3	1	-	-	-	-
	<i>A. fumigatus</i> (n = 14)	3	1	2	3	-	-	2	-	-	-
	<i>A. terreus</i> (n = 9)	2	3	1	1	-	-	1	-	-	-
	<i>A. tamaraii</i> (n = 6)	-	3	1	-	-	-	1	-	-	1
	<i>A. niger</i> (n = 3)	-	2	1	-	-	-	-	-	-	-
Lipase	<i>A. flavus</i> (n = 53)	6	18	3	8	3	-	-	-	-	-
	<i>A. fumigatus</i> (n = 14)	1	2	-	2	-	-	-	1	-	-
	<i>A. terreus</i> (n = 9)	2	-	2	1	-	1	-	-	-	1
	<i>A. tamaraii</i> (n = 6)	-	-	1	2	-	-	1	1	-	-
	<i>A. niger</i> (n = 3)	1	-	2	-	-	-	-	-	-	-

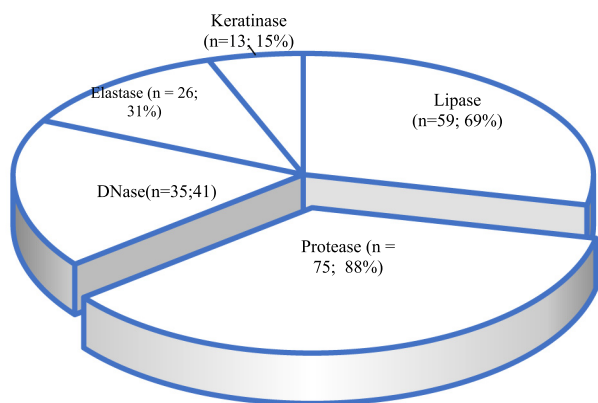


Fig. 1. Total number (%) of *Aspergillus* isolates (n = 85) with different extracellular enzyme activity.

(n = 3) exhibited protease activity. A total of 7, 5 and one isolates of *A. flavus* (13.21% of 53), *A. fumigatus* (35.71% of 14) and *A. terreus* (11.11% of 9), respectively showed keratinase activity. The extracellular enzyme activity indices of *Aspergillus* isolates were calculated and tabulated as frequency table (Table 1). On DNase test agar, isolates of *Aspergillus* exhibited varying magnitude of enzyme activity. One of the isolates of *A. flavus* showed very low/negligible EAI (1.0 to 1.10). Relatively low EAI (1.11 to 1.30) was exhibited by 17 isolates and medium EAI (1.31 to 1.60) was shown by 2 isolates and none of the isolates of *A. flavus* showed high EAI (1.61 to 2.0). High DNase EAI (1.61 to 2.0) was exhibited by one isolate of each *A. fumigatus* and *A. tamaraii*. *A. terreus* had low to medium EAI (1.11 to 1.40) and *A. niger* exhibited low EAI (1.21 to 1.30).

4. Discussion

Certain extracellular enzymes such as DNase, elastase, protease, lipase, keratinase, etc., are known to play an important role in the process of infection by pathogenic including fungi (U Gajjar, 2019).

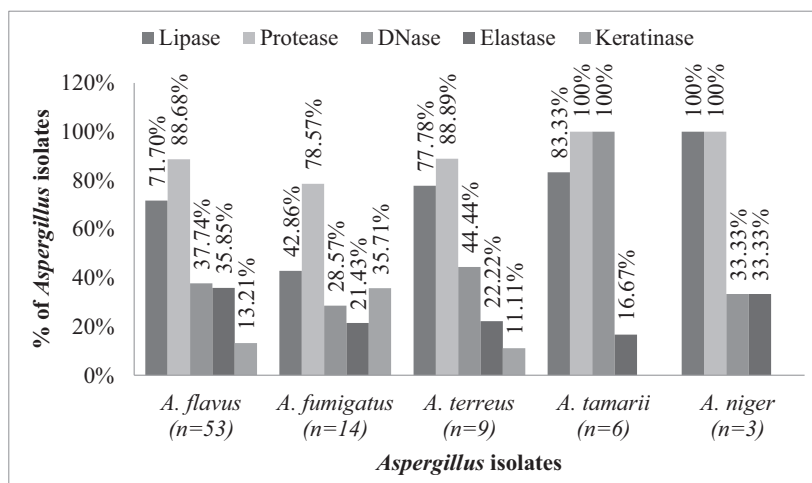


Fig. 2. Percentage distribution of species of *Aspergillus* showing various extracellular enzyme activities.

In the present study five clinically important enzymes viz., DNase, elastase, protease, lipase and keratinase were screened for their activity in order to evaluate the overall spectrum of extracellular enzyme activities of *Aspergillus* (n = 85) strains isolated from fungal keratitis.

Most of the isolates (n = 25; 96.15% of 26) of *Aspergillus* showed medium to high elastase activity. Fifteen isolates of *A. flavus* showed high elastase activity indices (1.61 to 2.0) and one isolate each of *A. tamarii* and *A. niger* showed medium elastase activity indices (1.31 to 1.60). For protease, exactly, 34 (64.15% of 53) isolates of *A. flavus* showed relatively low EAls (1.11 to 1.30) and high EAls were exhibited by *A. fumigatus* (n = 2), *A. tamarii* (n = 2) and *A. terreus* (n = 1). A total of 21 and 11 isolates of *A. flavus* showed relatively low and medium lipase activity, respectively. Two isolates of *A. tamarii* and one isolate each of *A. fumigatus* and *A. terreus* had high lipase activity.

In this study, the possible role of the extracellular enzymes as a putative virulence factor in *Aspergillus* keratitis was explored. It was observed that the isolates of *Aspergillus* were able to produce an array of extracellular enzymes. *Aspergillus* isolates had lesser activities of DNase (n = 35) and keratinase (n = 15) when compared to protease, elastase and lipase. The DNase enzyme activity was analysed by observing the halo zone around the colony, similar to that of Sanchez and Colom (Sánchez and Colom, 2010) where 85 *Cryptococcus* isolates were reported to be positive for DNase activity. In the present study, 69% (n = 59) and 88% (n = 75) of *Aspergillus* isolates produced lipase and protease, respectively. Higher lipase activity in *Fusarium* isolates was observed by St. Leger et al. (St. Leger et al., 1986). Similar to the present study, Gajjar et al. (U Gajjar, 2019) and Selvam et al. (Selvam et al., 2014) reported 83% of protease activity in *Aspergillus* isolates. Alp and Arikan (Alp and Arikan, 2008) investigated the production of extracellular elastase, acid proteinase, and phospholipase from the clinical isolates of *Aspergillus* and found that 84.9, 27.4, and 65.8% of *Aspergillus* isolates were able to produce elastase, acid proteinase and phospholipase, respectively and also reported that none of the *A. niger* isolate produced elastase. Whereas, in the present study, 31% of *Aspergillus* isolates produced elastase and one isolate among 3 isolates of *A. niger* also produced elastase activity. The production of elastin lytic enzymes by *Aspergillus* suggests that elastase play a role in pathogenesis (Blanco et al., 2002; Kolattukudy et al., 1993; Kothary et al., 1984; Rhodes et al., 1988). Kothary et al. (Kothary et al., 1984) stated that non-elastase producing environmental *Aspergillus* isolates were relatively less virulent compared with high elastase producers. Bazan (Bazan, 2005) stated that lipid and lipid mediated compounds play an important role in the recovery of corneal inflammations in a complex manner. These probably answer the critical role of extracellular lipase produced by the test isolates of *Aspergillus* from keratomycosis patients.

Although the present study detected keratinase activity only among 15% *Aspergillus*, Friedrich et al. (Friedrich et al., 1999) and Sharma et al. (Sharma et al., 2011) reported keratinase activity in many of these species. Oyeleke et al. (Oyeleke and Auta, 2010) stated that *Aspergillus* yielded high amount of protease enzymes. In the present study, protease activity was profoundly seen among *Aspergillus* isolates (88% of 85). Protease is proven to be associated with cornea that showed progressive ulcer along with the presence of dense inflammatory cells (Gopinathan et al., 2001). Burda and Fisher (Burda and Fisher, 1960) and Dudley and Chick (DUDLEY and CHICK, 1964) have demonstrated extracellular protease activity in corneal matrix degradation in mycotic keratitis using rabbit model. The pathogenic role of extracellular protease in keratitis has been reported by various authors (Barletta et al., 1996; Mahmoud et al., 2007; Zhu et al., n.d.).

5. Conclusion

The present study clearly indicates the potential role of extracellular enzymes of *Aspergillus* spp. in the onset and progression of keratitis. However, the number of isolates *Aspergillus* keratitis cases is low and the problem remains to be further clarified by concordant examination of other potential virulence factors. Further studies on the multifactorial impact on virulence of the isolates causing fungal keratitis are required.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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