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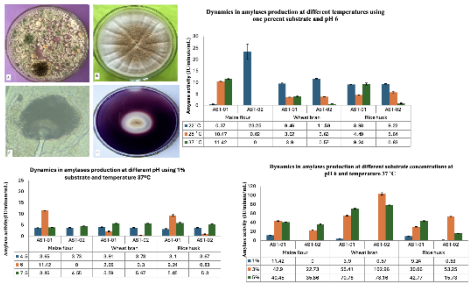
Enzymatic Potential and Antifungal Susceptibility Profile of Aspergillus terreus Against Essential Oils

Saba Sana[1]*, Nida Sana[2], Areeba Nadeem[1], Layyaba Nazir[1], Rukhma Sattar[1], Sikander Ali[1], Najeeb Ullah[3], Rania Ali El Hadi Mohamed[4], Nawal Al-Hoshani[4], Maher S. Alwethaynani[5], Fakhria A. Al-Joufi[6], and Tariq Aziz[7]*

[1] *Dr. Ikram-ul-Haq Institute of Industrial Biotechnology, Government College University, Lahore, PAKISTAN*
[2] *Institute of Social and Cultural Studies, The University of Punjab, Lahore, PAKISTAN*
[3] *Food and Biotechnology Research Centre, PCSIR Labs Complex, Lahore, PAKISTAN*
[4] *Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, SAUDI ARABIA*
[5] *Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Alquwayiyah, Riyadh, SAUDI ARABIA*
[6] *Department of Pharmacology, College of Pharmacy, Jouf University, 72341 Aljouf, SAUDI ARABIA*
[7] *Laboratory of Animal Health, Hygiene and Food Quality University of Ioannina, Arta, GREECE*

Abstract: ***Aspergillus terreus*** **is a pathogen as well as an industrial organism. Current study deals with in vitro evaluation of** ***A. terreus*** **as a potential candidate for starch-based industries and susceptibility of toxigenic isolates to plant essential oils. Conventionally identified** ***A. terreus*** **soil isolates (n=25) were screened for mycotoxin production by TLC. Non- toxigenic isolates were selected for starch hydrolysis using starch agar. Toxigenic** ***A. terreus*** **were selected for antifungal activity of plant-derived essential oils (*Nigella sativa, Elettaria cardamomum, Eucalyptus globulus, Cinnamomum verum Cuminum cyminum,* and *Syzygium aromaticum*) by agar well diffusion and micro-broth dilution method. The lowest minimum inhibitory concentration (MIC ≥ 0.65±0.22µL/mL) was observed for *Cuminum cyminum.* So, it was used for inhibitory effect on growth and toxin production in broken grains. *Cuminum cyminum* EO inhibited the growth of *A. terreus* (0.00±0.00 cfu/g) in inoculated group and OTA production was also close to uninoculated group. Starch hydrolysis to colony diameter ratio helped for selection of isolates for further experiments. In starch hydrolysis, two isolates AST-01 and AST-02 produced highest ratios, which were 2.25±.08 and 1.76±.04 respectively. Influence of maize flour, wheat bran and rice husk with varying concentration, incubation temperature and pH were evaluated on starch hydrolysis potential. The hydrolytic potential quantified by dinitorosalisylic acid (DNS) method. *A. terreus* AST-02 had the highest hydrolytic potential (102.96±2.61IU) under incubation of 3% wheat bran at 37 °C and pH 6.0. It is concluded that *A. terreus* is a potential candidate for starch-based industries and further crop contamination by toxigenic species could be curtailed using essential oil as a feed preservative.**



Keywords: stored grains, fungal contamination, antifungal potential, amylase activity (IU), essential oils, OTA

1 Introduction

Amylases are hydrolytic enzymes which produce reducing sugars by breaking up O linked glycosidic bonds present in starch. The three major amylases alpha (α) amylase (EC 3.2.1.1), Beta (ß) amylase (EC 3.2.1.2), and glucoamylase (EC

*Corresponding author: Saba Sana, Dr. Ikram-ul-Haq Institute of Industrial Biotechnology, Government College University, Lahore, PAKISTAN. Tariq Aziz, Laboratory of Animal Health, Hygiene and Food Quality University of Ioannina, Arta, GREECE
E-mail: saba.sana@gcu.edu.pk (SS), iwockd@gmail.com (TA)
ORCID ID: https://orcid.org/0000-0003-0905-8076 (TA)
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3.2.1.) are produced by plants, humans, animals, parasites (*Ascaris suum*) and microorganisms[1]. Starch based industries are potential users of amylases. Amylases are being utilized in fuel production, paper, textile, detergent, brewing, pharmaceutical, chocolate and food industry[1, 2]. Microbial sources of amylases are gaining importance in industrial processes due to high yield and easy manipulation of microorganisms. Bacteria and fungi are frequently used to produce amylases. However fungal amylases are preferred over bacterial amylases because of stability, easy extraction from mycelia of mold species, acquisition of GRAS (Generally Regarded as Safe) status, ability to tolerate low water activity and hyperosmotic conditions. It is estimated that α amylase accounts for approximately 25% of the global enzyme market, with fungal derived enzymes contributing to about by α amylase and fungal enzyme have 40% share in it[2].

Among fungi, *Aspergillus* species are the highest producers of amylases. Amylase biosynthesis is influenced by physico-chemical parameters including temperature, initial pH of medium, spore inoculum, starch source, metal ions, surface acting agents, agitation, and incubation time[3]. Several *Aspergillus* species have been studied for amylase production and optimization including *A. niger, A. flavus, A. tamarii* and *A. oryzae*[4-6]. *A. terreus* is a saprophytic fungus present in soil. It is well known for production of valuable secondary metabolites (lovastatin) and hydrolytic enzymes[7, 8]. One of the secondary metabolites produced by fungi is the mycotoxin. It is estimated that 25% of world's crops are contaminated with mycotoxins[9]. Mycotoxins are thermostable[10], low molecular weight metabolites that escape from detection by immune system and produce toxifies to human, animal, plants and bacteria. There are more than 400 mycotoxins. However, major classes are aflatoxins[11], ochratoxins, zeralenone, fumonisins and patulin. Among ochratoxin there are 20 subtypes and Ochratoxin A (OTA) is the most toxic mycotoxin. OTA is produced by several fungi including *A. terreus*[12]. Activities during crop cultivation, temperature, humidity, insect infestation cause contamination of grains by toxigenic fungal species[13].

Mycotoxin production is typically associated with toxigenic strains that possess active biosynthetic gene clusters responsible for mycotoxin biosynthesis, whereas non-producer strains either lack these genes or harbor these in a silenced or mutated form[14]. Notably, the metabolic pathways involved in mycotoxin biosynthesis differ significantly between the two groups. The resulting enzyme profiles are different in toxin producers compared to non-producers. For instance, in the case of terrein biosynthesis, the gene cluster is comprised of 11 genes (*terA*-*terJ* and *terR*), with *terA* encoding a non-reducing polyketide synthase that acts as the main enzyme. Whereas the expression of these cluster genes strictly depends on *terR*. Overexpression of regulatory genes (*terR*) leads to activation of the cluster and subsequent toxin production, whereas reduced expression results in silencing of the pathway and no toxin production. This expression rate is highly influenced by environmental conditions. Terrein is a significant example of production of secondary metabolite as a result of environmental stress[15]. The environment serves as a major source of fungal contamination, carrying spores that readily settle on exposed food commodities. The hot and humid environment, insect infestation, and inadequate ventilation promote fungal colonization and facilitate mycotoxin production[12, 13]. These environmental and post-harvest handling factors significantly increase the risk of fungal contamination and toxin accumulation in stored food and feed products[9]. Therefore, optimal storage conditions can prevent toxins production by the toxigenic strains[16].

Several physical, chemical and biological methods are under investigation to curtail contamination of toxigenic fungi in crops. Each and every method has pros and cons[12]. One approach is to use a preservative to prevent the fungal growth during storage conditions. Essential oils of plants are recognized as a source of compounds. These compounds have different biological properties; antibacterial, antifungal, antiparasitic, antioxidant, and anti-inflammatory activities[17, 18]. Antifungal potential of essential oils has also been reported against *A. terreus*[19]. The inhibitory effect is associated with cell membrane disruption, inhibition of ergosterol (an important component of fungal cell membrane) synthesis and interference with fungal enzyme activity. Due to lipophilic nature of essential oils, these can easily penetrate fungal cell and cause cell death by bringing desirable changes in membrane permeability, component synthesis and affecting the enzymatic activity[20]. Use of essential oils as antifungal is a natural, environment friendly and sustainable approach to reduce fungal and mycotoxin contamination in feed. As these are easily biodegradable and GRAS (Generally Regarded as Safe) status [21].

In the present study indigenous isolates of *A. terreus* were explored and optimized to determine standard requirements of temperature, pH, substrate, and concentration for the highest quantity of amylases production. The susceptibility of toxigenic *A. terreus* to different essential oils; *Nigella sativa Elettaria cardamomum, Eucalyptus globulus, Cinnamomum verum, Cuminum cyminum* and *Syzygium aromaticum* was also evaluated.

The study explores the potential of *A. terreus* as an industrial candidate for starch-based industries, while simultaneously addressing its role as a toxigenic organism that poses a threat to stored grains and animal feed. This dual approach was undertaken due to the dual nature of *A. terreus* by serving as both a valuable bioresource and a potential contaminant. By screening indigenous soil isolates, non-toxigenic strains were identified and optimized for

enhanced amylase production under varying physicochemical conditions, supporting their application in industrial processes. Conversely, toxigenic strains were assessed for their susceptibility to plant-derived essential oils, offering a natural and eco-friendly solution to reduce fungal growth and ochratoxin contamination in grains. Integrating these two perspectives not only supports the safe industrial utilization of *A. terreus* but also contributes to the development of sustainable strategies for post-harvest grain protection which ultimately enhance food and feed safety.

2 Materials and Methods

Indigenous amylase producing *Aspergillus terreus* isolates were used for enhanced production of amylases in an optimization study. Physico-chemical parameters of pH, temperature and substrates with three different concentrations were used to evaluate their influence on amylase production. Further, the susceptibility of Ochratoxin A (OTA) producing *A. terreus* to different plant essential oils were also determined.

2.1 Sample collection

Soil samples (n=145) were collected from livestock farms of District Lahore, Pakistan. Soil (10g) was taken from a depth of 10cm with help of sterile spatula. Soil was transferred to sterilized polyethylene bags and transported at low temperature to Mycology Laboratory, Department of Microbiology, University of Veterinary and Animal Sciences Lahore [22].

2.2 Isolation of *Aspergillus terreus*

A. terreus was isolated from soil following protocol used by Waksman[23]. One mL of soil suspension (10% in phosphate buffer saline) was inoculated on sterile Sabouraud's dextrose agar (SDA; g/L: Peptone 10g, Dextrose 40g, Agar agar 15g) under sterile conditions followed by incubation at 25 ± 3 ˚C for 72 hours under humid conditions. Discrete colonies were purified by subculturing on SDA plates.

2.3 Identification of *Aspergillus terreus*

Purified colonies were identified as *A. terreus* based on colony and microscopic characters[24]. The Recto and verso morphological features studied were color, texture and pigment production on SDA. Microscopic examination was carried out using slide culture method[25]. Briefly, one drop of molten SDA was placed on a glass slide. A scanty of spores was shifted from purified culture to the SDA drop on glass side. A cover slip was placed over it and incubated at 25 ± 3 ˚C. Slides were observed at 100x and 400x magnification under a compound microscope (Meiji Techno) to view hyphae, foot cell, spores and additional structures after staining with lacto phenol cotton blue.

2.4 Toxigenic Potential of *Aspergillus terreus*

A. terreus isolates were screened for toxin production by thin layer chromatography. Inoculum was standardized by spore counting through Neubauer chamber. Five mL of inoculum containing 10^6 spores of *A. terreus* per mL of PBS) was inoculated into 100mL of Sabouraud's dextrose broth (SDB) in conical flasks and incubated at 25 ± 3 ˚C for a period of 45 days in the dark in a shaking incubator for mycotoxin production. Mycotoxins were extracted by adding sodium chloride (2.5g), distilled water (5mL), methanol (5mL) and chloroform (45mL) into 12.5g autoclaved homogenized fungal culture. This mixture was placed at 37 ˚C for 30 minutes at 150 rotations per minute (rpm) followed by filtration by Whatman filter paper. The filtrate was concentrated and spotted on silica gel coated thin layer chromatographic (TLC) plates at a distance of 1.5 cm from bottom of plate. Plates were placed in TLC tank containing mobile phase (Chloroform 95mL; Methanol 5 mL) till mobile phase front reaches 15 cm from bottom. Plates were dried and observed under ultraviolet light of 365nm in wood's lamp. Toxigenic isolates were used in Phyto inhibition experiment and non-toxigenic isolated were used for amylase production and optimization studies[26].

2.5 Antifungal activity of EOs

Antifungal activity of plant essential oil (EO) of *Nigella sativa, Elettaria cardamomum, Eucalyptus globulus, Cinnamomum verum, Cuminum cyminum* and *Syzygium aromaticum* was determined by agar well diffusion assay following the microbroth dilution method. *A. terreus* spore suspension was prepared and standardized to 10^6 spores/mL by counting chamber. One mL of culture was mixed with molten SDA and poured into plate. After solidification, wells were made and sealed with molten agar. Each EO (100µL) was poured into wells followed by incubation at 25 ± 3 ˚C for 3-5 days. Zone of Inhibition was measured in millimeter. Essential oils which produced ZOIs were processed for minimum inhibitory concentration determination by the microbroth dilution method. Two-fold serial dilution of EOs was prepared in a 96 wells microtitration plate using SDB upto the 10^{th} well. Standard fungal culture (100µL) was poured

into 1st to 11th well (Growth control). The 12th well served as broth control. Plates were incubated at 25°C ± 3 for 3-5 days [27]. Presence or absence of growth was observed in control and test wells.

2.6 Inhibitory effect of EO on fungal growth and toxin production

Inhibitory effect of essential oil of *Cuminum cyminum* was evaluated against toxigenic *A. terreus.* The Experiment was conducted in nine groups, which these three were treatment groups having different substrate; maize wheat and rice grains. In each group, broken grains (100g) were mixed with essential oil (specific quantity) inoculated with one mL of standard inoculum of *A. terreus* followed by incubation for 45 days at room temperature. Moisture level of 60% was maintained during the whole experiment. Three groups were positive control for maize, wheat and rice. In these groups essential oil was not mixed with grains. Negative control groups were kept without inoculation of fungi and essential oil and termed as uninoculated group. The rest of the condition were constant for whole experiment**.** Fungal load (cfu/g) was determined by plate count and aflatoxin was quantified by HPLC post incubation. Phyto inhibition activity against fungi and toxin was observed by comparing the treatment groups data with that of control group[27].

1. Inoculated Treated: Broken grains (wheat/maize/rice)100 g with 60% moisture content + EO+ *A.terresus* spores
2. Inoculated: Broken grains (wheat/maize/rice)100 g with 60% moisture content + *A. terresus* spores
3. Uninoculated: Broken grains (wheat/maize/rice)100 g with 60% moisture content

2.7 Amylase production

Non-toxigenic *A. terreus* isolates were assessed for amylase production by iodine test. *A. terreus* from pure culture was inoculated on starch agar plates (g/L: meat extract 3g; peptic digest of animal tissue 5g; soluble starch 2g; agar agar 3%; pH 7) by spot culture technique followed by incubation at 25 ± 3 °C for 72 hours. Lugol's iodine (potassium iodide 10g in distilled water 20mL; iodine 5g; distilled water up to 100mL) was poured on starch agar plates having fungal growth. Zone of hydrolysis in millimeter (mm) were observed to determine production of amylases[28].

2.8 Optimization of physicho-chemical parameters

Physicho-chemical parameters were optimized for enhanced production of amylases by the method described by Singh et al. with modifications[29]. A standardized inoculum (10^6 spores per mL of PBS) was prepared using Neubauer chamber for spore counting. Inorganic broth (g/L: sodium nitrate 1g; dipotassium hydrogen phosphate 1g; magnesium sulphate 0.5g; ferrous sulphate 0.01g) was prepared. Inorganic broth was used for preparation of 1percent concentrations of maize flour with initial pH value 6 in 250 mL Erlenmeyer flask and after inoculation of standardized inoculum it was incubated at 22°C for a period of seven days. This process was carried out for 3 and 5% concentrations of maize flour with initial pH value of 4.5 and 7.5 and incubation temperature 28°C and 37 °C. Similar optimization experiment was conducted using wheat bran and rice husk on above mentioned physical parameters.

2.9 Qualitative detection of amylases

After seven days of incubation, crude amylases were obtained by filtering fungal broth culture through Whatman no. 1 filter paper. Amylases production was detected by iodine test. One mL from filtrate was transferred to a glass test tube. Lugol's iodine (20µL) was added to it and color development was recorded[30].

2.10 Quantification of amylases

Quantity of amylases produced under different physico-chemical parameters was determined by dinitrosalicylic acid (DNS) method[31]. One mL from filtrate was mixed in two mL phosphate buffer (pH 6) and two mL starch solution (1%) followed by incubation in water bath at 37 °C for 30 minutes. DNS reagent (3, 5-dinitrosalicylic acid 1g; distilled water 50mL; sodium potassium tartrate tetrahydrate 30g; 2N sodium hydroxide 20mL; distilled water 100mL) was added and placed at boiling temperature till change in color appeared. Experiment was conducted with a control containing three mL distilled water and seven mL DNS reagent. Optical density (OD) values were recorded at 540 nm using spectrophotometer (BMS UV-2800). Amylases quantity was expressed in terms of unit activity (IU/mL/minute). It is the amount of amylase that produce one µmol of reducing sugar released at one minute under assay conditions.

2.11 Statistical analysis

Data from preliminary screening and optimization experiments were analyzed using statistical package for social sciences (SPSS Version 10) by analysis of variance (ANOVA)[32] followed by Duncan's multiple range tests (post-hoc).

3 Results

3.1 Isolation and Identification

Out of 145 soil samples 25 isolates were identified as *A. terreus* based on morphological and microscopic examinations. Isolates of *A. terreus* were initially white on SDA with cottony texture. Upon maturation, these turned to cinnamon brown with a white periphery on obverse side of plate. While pale color was observed on reverse side of plate. Microscopic examination using slide culture method revealed presence of hyaline septate hyphae, smooth hyaline conidiophores, vesicle, metulae and phialides. Primary culture plate morphology and microscopic characteristics of *A. terreus* are presented in **Fig. 1**. Identified *A. terreus* were screened for mycotoxin production by thin layer chromatography. Out of 25 isolates 10 isolates were toxigenic and 15 were non-toxigenic.

3.2 Antifungal activity of EO

OTA producing *A. terreus*[10] were used in phyto-inhibition experiment. According to agar well diffusion assay *C. verum*, *S.aromaticum* and *C. cyminum* produced the highest mean ZOI (50.67 ± 0.57mm). The highest activity was of *C.verum* (mean ZOI 54.67 ± 0.57mm) followed by *Cuminum cyminum* (22.67 ± 0.57mm) and *Syzygium aromaticum* (20.67 ± 0.57mm). *Zingiber officinale* and *Eucalyptus globulus* did not give antifungal activity against toxigenic *A. terreus.* All EO produced ZOIs were used for determination of MIC. The lowest MIC was observed for *Cuminum cyminum* which was MIC ≥ 0.65 ± 0.22μL/mL (**Table 1**).

3.3 Inhibitory effect of EO on fungal growth and toxin production

The *Cuminum cyminum* EO produced the lowest MIC value was used for in the next experiment on broken grains of wheat, maize and rice. The results of the fungal counts and aflatoxins are presented in **Table 2**. Antifungal potential of EO against *Aspergillus terreus* was evaluated in an experiment. Toxigenic *A. terreus* was inoculated into grains of three categories; wheat, maize and rice in broken form. Each category of grains was provided with 60% moisture content.

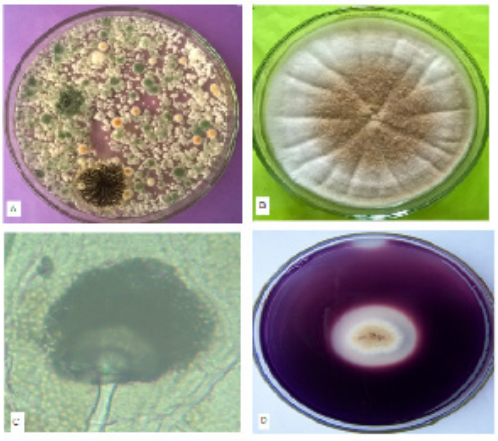

Fig. 1 *Aspergillus terreus.* **A:** Primary culture; **B**: Mscroscopic view of *Aspergillus terreus;* **C:** Microscopic view of *Aspergillus terreus* at 40X; **D:** Zone of hydrolysis on starch agar by *Aspergillus terreus.*

Table 1 Antifungal activity of Essential oils (ZOI) against toxigenic *Aspergillus terreus* (n=10).

Sr. No.	Essential Oils	ZOI in mm (Mean ± SD)	MIC μL/ML (Mean ± SD)
1.	*Zingiber officinale*	00.00 ± 0.00[a]	-
2.	*Eucalyptus globulus*	00.00 ± 0.00[a]	-
3.	*Syzygium aromaticum*	20.67 ± 0.57[b]	1.30 ± 0.45[a]
4.	*Cuminum cyminum*	22.67 ± 0.57[c]	0.65 ± 0.22[a]
5.	*Cinnamomum verum*	50.67 ± 0.57[d]	2.60 ± 0.9[b]

*Means values having same superscripts differ non-significantly and with different superscripts differ significantly column-wise.

After 45 days *A. terreus* count was performed. A group in each category was left untreated. This untreated group was also inoculated with *A. terreus.* It was named as inoculated group. Counts (CFU/g) were converted into log and statistical analysis was performed. In inoculated group, 45 days count of *A. terreus* was highest (8.95 ± 0.01) in wheat. EO proved to be antifungal as it inhibited the growth of *A. terreus* and no fungal counts were detected after 45 days of incubation in inoculated treated group similar to uninoculated group. So, log cfu/g were determined as 0.00 ± 0.00 in these groups. As far as anti-toxigenic potential is concerned in stored grains of wheat, inhibitory effect was observed on OTA production by EO as the toxin quantities were close to uninoculated group. The OTA was quantified as 0.05 ± 0.00 and 0.04 ± 0.00 in inoculated treated and uninoculated stored grains. Inoculated group have 13.82 ± 0.36 ppb of OTA. In case of maize similar pattern was observed, inhibitory effect was detected on cfu/g of stored maize in inoculated treated group. No fungal counts were observed in inoculated treated and uninoculated groups in contrast to inoculated group, which have 8.93 ± 0.01 cfu/g. The quantity of OTA was approximately similar in uninoculated (0.043 ± 0.00) and inoculated treated (0.05 ± 0.01) groups of stores maize. However, in inoculated maize 13.82 ± 0.30 ppb of OTA was detected. In contrary to wheat and maize rice have the lowest fungal counts (8.60 ± 0.01cfu/g) and OTA (9.47 ± 0.71ppb)

Table 2 Inhibitory Effect of *Cuminum cyminum* Essential oil against OTA producing *Aspergillus terreus* (n=10).

S. No.	Substrates	Groups	Fungal Load in log cfu/g (Mean ± S.D)	OTA ppb (Mean ± S.D)
		Uninoculated	0.00 ± 0.00[a]	0.04 ± 0.00[a]
1.	Wheat	Inoculated	8.95 ± 0.01[c]	13.82 ± 0.36[c]
		Inoculated Treated	0.00 ± 0.00[a]	0.05 ± 0.00[a]
		Uninoculated	0.00 ± 0.00[a]	0.043 ± 0.00[a]
2.	Maize	Inoculated	8.93 ± 0.01[c]	13.82 ± 0.30[c]
		Inoculated Treated	0.00 ± 0.00[a]	0.05 ± 0.01[a]
		Uninoculated	0.00 ± 0.00[a]	0.05 ± 0.00[a]
3.	Rice	Inoculated	8.60 ± 0.01[b]	9.47 ± 0.71[b]
		Inoculated Treated	0.00 ± 0.00[a]	0.04 ± 0.00[a]

*Means values having same superscripts differ non-significantly and with different superscripts differ significantly column-wise.

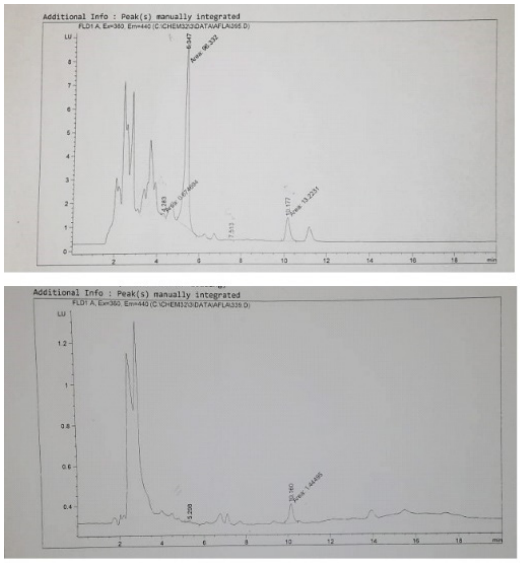

Fig. 2 Representative chromatogram of OTA produced by *Aspergillus terreus.* Retention time (10 minutes) is the identification of OTA and peak areas for first chromatogram (above) is 13.22 and 2nd chromatogram (lower) is 1.44.

in inoculated groups. However, a reduction in fungal counts and OTA production was observed in treated groups as fungal counts (0.00 ± 0.00 cfu/g) and OTA (0.04 ± 0.00 ppb) in inoculated treated groups were close to uninoculated groups. The representative chromatograms for OTA are shown in **Fig. 2**.

3.4 Amylase production potential

Non-toxigenic *A. terreus*[15] were assessed for amylase production on starch agar. Five isolates among 15 showed hydrolysis of starch on starch agar after pouring Lugol's iodine. However, only two isolates showed complete hydrolysis (no color around the fungal colony) of starch, while remaining three showed partial hydrolysis (yellow and brown zones around the fungal colony). Mean zone of hydrolysis, colony diameter and zone to colony diameter ratio are given in **Table 3**. The highest zone of hydrolysis to colony diameter ratio was recorded for AST-01 (2.25 ± 0.08) followed by AST-02 (1.76 ± 0.04), AST-03 (1.12 ± 0.01), and AST-05 (1.03 ± 0.00). The lowest ratio was of AST-04 (1.02 ± 0.00). The best producer (AST-01) has the lowest colony diameter (16 ± 1.00) and the zone of hydrolysis is 36 ± 1.00.

3.5 Optimization of fermentative parameters

In optimization experiment for different temperature at one percent substrate (wheat, maize, and rice) pH 6, *A. terreus* (AST-01) gave the highest amylase activity of 11.59 IU/ minute/ mL at 22˚C with one percent wheat bran. On the other hand, for AST-02 the highest amylase activity (23.25 IU/ minute/ mL) was measured at 22 with 1% maize flour at pH 6. Results for optimization of temperature for amylase production are mentioned in **Fig. 3**. **Figure 4** describes the dynamics in amylase production of selected *A. terreus* isolates under different pH. Optimum amylase production of AST-01 was obtained at pH 6 while AST-02 showed negligible amylase activity at pH 6 using 1% maize flour as a substrate and temperature 37˚C. However, AST-02 isolate of *A. terreus* produced 5.67 IU/minute/mL at 7.5 pH value. Which was highest for this isolate in this experiment of pH optimization. In optimizing the substrate, wheat bran at 3% proved the best substrate for *A. terreus* amylase production. AST-01, and AST-02 both revealed the increase in amylase activity with 3% substrate concentration of wheat bran at pH 6 and temperature 37˚C (**Fig. 5**).

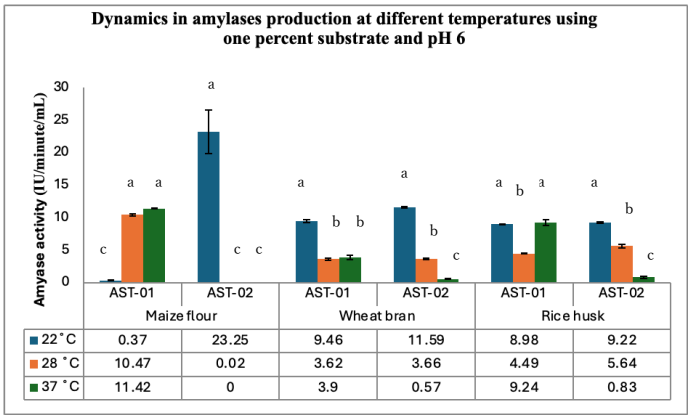

Fig. 3 Dynamics in amylase production at different temperatures.

Table 3 Zone of hydrolysis of starch, colony diameter and zone of hydrolysis to colony diameter ratio of non-toxigenic *A. terreus* isolates (n=5).

Sr. No.	Isolate	Mean ±SD Zone of Hydrolysis (mm)	Mean ±SD Colony diameter (mm)	Mean ±SD (ZOH/CD ratio)
1	AST-01	36 ± 1.00^{b}	16 ± 1.00^{a}	2.25 ± 0.08^{d}
2	AST-02	37 ± 1.00^{b}	21 ± 1.00^{b}	1.76 ± 0.04^{c}
3	AST-03	45 ± 1.00^{c}	40 ± 1.00^{d}	1.12 ± 0.01^{b}
4	AST-04	48 ± 0.00^{d}	47 ± 0.00^{e}	1.02 ± 0.00^{a}
5	AST-05	28 ± 1.00^{a}	27 ± 1.00^{c}	1.03 ± 0.00^{a}

*Means values having same superscripts differ non-significantly and with different superscripts differ significantly column-wise.

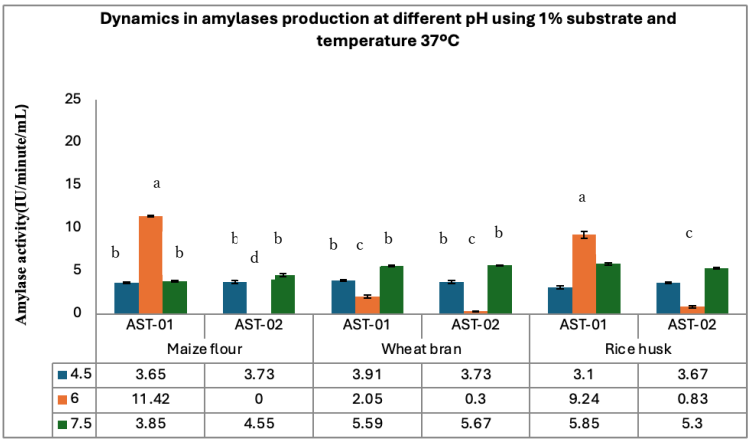

Fig. 4 Dynamics in amylase production at different pH.

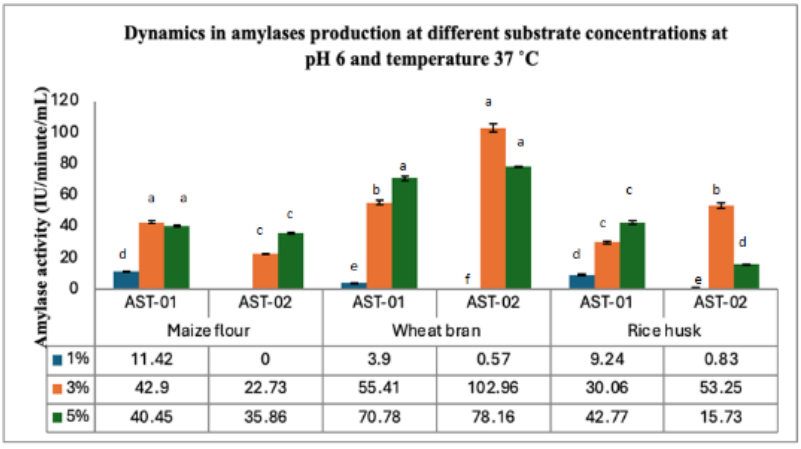

Fig. 5 Dynamics in amylase production at different substrate concentrations.

4 Discussion

A. terreus is a soil borne fungus and can be frequently isolated from soil. It is an important specie of genus *Aspergillus* for production of significant products including drugs (lovastatin) and hydrolytic enzymes[33]. In present study indigenous *A. terreus* soil isolates were evaluated for mycotoxin production by TLC, and non-toxigenic isolates were assessed for amylases production. Five isolates were found starch hydrolyzing in preliminary screening on starch agar. In previous study; Vassileva et al. reported presence of starch hydrolyzing *A. terreus* along with other fungal species in soil [33]. *A. terreus* produced a zone of hydrolysis of 10 mm on starch agar in contrast to present study. In present study the highest zone of hydrolysis was 48 mm (AST-04) and lowest was 28mm (AST-05). Ogbonna et al. screened soil *A. terreus* with 43 mm diameter on starch agar and colony diameter of 5 cm (55 mm) on seventh day incubation[34]. Results of this study are closely related to AST-03, and AST-04 which produced zones of 45 mm, and 48 mm respectively and opposite to AST-01 (36 mm), AST-02 (37 mm), and AST-05(28 mm). The colony diameter of AST-04 (4.7 cm or 47 mm) after 72 hours of incubation is found close to Ogbonna's *A. terreus* isolates[34]. These comparisons showed the importance of indigenous isolates in enzyme production and justify their further exploration for industrial applications.

Production potential of enzymes by fungal species is influenced by several physico-chemical factors including temperature, pH, nitrogen source, and carbon source[35, 36]. In the present study optimal conditions for AST-01, and AST-02 were 37˚C, and 22 ˚C temperature, pH 6 and maize flour (3%) and wheat bran (3%) respectively for amylases production. The results are in contrast to *A. terreus* optimal conditions (temperature 45˚C, pH 4, and rice husk 2%) for amylases (glucoamylase)[6]. The optimal parameters of present study are opposite to results of [3]. In his study, pearl millet was found the best substrate at temperature 27-36 ˚C and pH 7 for amylases production by *A. terreus*. However, the

optimal pH (5.03) recorded by Ogbonna et al. for *A. terreus* was in agreement with the present study's findings of optimal pH 6 [34].

A. niger has been reported as amylase producers in several studies[5, 7, 29, 40]. Ogbonna et al. results of *A. niger* for zone of hydrolysis (55 mm), and colony diameter are different from present study[34]. In this study *A. terreus* (0.375 mmol / L) gave highest amylases production as compared to *A. niger* (0.281 mmol/ L) opposite to zone of hydrolysis on starch agar (*A. niger* produced wider zone). In contrast to Ogbonna's findings, in the present study the highest amylases quantified by DNS method were found (102.96 ± 2.61 IU) for AST-02 [31]. Sundar et al. evaluated effect of temperature, pH, and nitrogen concentration on amylases production by *A. niger* and found 28 ˚C, and pH 7 as the best fermentation condition for amylases production in contrast to both isolates in the present study[37]. Varalakshmi et al. found highest amylase in term of activity at pH 9.5 at room temperature[38]. An acidic optimal pH reported by Hernandes et al. for *A. niger* differ from the present study[39]. Suganthi et al. used agro-industrial wastes as substrates for *A. niger* at different physico-chemical parameters, and found oil cake as the best substrate. The optimum temperature was 37°C, comparable to AST-01 [40].

Sani et al. studied *A. niger,* and *A. flavus* fermentation parameters and found optimal conditions using cassava peel as amylase inducer [4]. The conditions found optimum were 28˚C and pH 7 for both types of fungi, which are different from the present study. Moreira et al. studied on *A. tammari*[41] and Sivaramakrishnan et al. on *A. oryzae* for amylases production[5]. The results of these studies (optimal temperature 30ºC) and (pH 6.9 for *A. tammari* and pH 5 for *A. oryzae*) are contrary to the present study. Wheat bran was found as better inducer for amylase production by *A. oryzae* in solid state fermentation, which is in agreement with the results of AST-02. The differences between present study and previously reported work are likely due to variations in isolates and physico-chemical parameters selected for optimization. Indigenous *A. terreus* can be used on industrial scale for amylases production under optimized conditions.

The above comparisons clearly demonstrate that enzyme production potential is singnificantly affected by the both fungal specie variations, as well as the fermentation parameters for enzyme production. The current findings emphasize the potential of indigenous *A. terreus* isolates, particularly AST-02, for industrial amylase production under optimized conditions; low-temperature and slightly acidic conditions using cost-effective substrates like wheat bran.

The study's findings demonstrate that certain essential oils show significant antifungal activity against toxigenic *Aspergillus terreus.* According to the above-mentioned study results, *Cinnamomum verum*, *Cuminum cyminum*, and *Syzyium aromaticum* produced the highest mean zone of inhibition (ZOI) of 54.67 ± 0.57 mm, 22.67 ± 0.57 mm, and 20.67 ± 0.57mm respectively while, *Zingiber officinale* and *Eucalyptus globulus* did not reveal any antifungal activity. The *Cuminum cyminum* had the lowest MIC value that was 0.65 ± 0.22 µL/mL. The results are in contrast to the findings of Takesh et al., who reported 2% *cumin* as the inhibitory concentration against *A. terreus*[42]. The study of Tan et al. showed that *Zingiber officinale* had a MIC of 625 µg/mL with *A. niger* [43], whereas clove (28.14 mm ZOI) showed results comparable to the present study findings, highlighting its antifungal potential across different aspergilli. The present study is in contrast with the study of Elgat et al. which showed that *Eucalptus* had a MIC of 12 µg/mL with *A. terreus* [44]. The present study is opposite to the study of Angelini et al., which indicated the cumin EO had the lowest MIC against all fungi including *A. terreus*[45]. This study confirms the inhibitory effect of essential oils on *A. terreus*, which produces OTA on different substrates like wheat, maize, and rice. This study suggests that inoculation with *A. terreus* resulted in the maximum fungal growth and OTA production on all the above-mentioned substrates. These essential oils treatment significantly reduces the fungal load and OTA production on all the substrates, inhibiting both of these near zero. The variations in different studies highlight the significance of geographical origin, oil composition, and target fungal isolates in determination of antifungal activity.

The study by Naz et al. investigated the antifungal activity of EOs against OTA produces by *A. parasiticus* using grains as a substrate [27]. The lowest OTA was observed at 10% was 8.16 ± 0.05 ng/g. It increased with the increase in the moisture content of inoculated grains, while no increase in OTA was noticed in un-inoculated and in the grains inoculated with the *C. vernum* essential oil. Another study also reported the anti-toxigenic effect of EOs in grains. The selected EOs presented antifungal activity against two toxigenic fungi; *P. verrucosum,* and *A. ochraceus.* The *C. zeylanicum,* and *C. martini* EOs showed better antifungal activity as compared to other EOs. The *C. zeylanicum, and C. martini* EOs completely inhibited the growth and OTA production of *P. verrucosum* and *A. ochraceous* at 1500, and 2500 mg/g in maize grains, respectively. These results support the findings of current data [14]. This study and earlier data suggest that EOs can be used for minimizing the fungal growth and OTA production in food sources, validating the current study's conclusion that EOs, particularly *C. verum*, *C. cyminum*, and *S. aromaticum*, presents promising, eco-friendly alternatives to chemical fungicides for managing *A. terreus* and OTA contamination in food commodities.

5 Conclusion

It is concluded that *A. terreus* has potential for use in biofuel production or starch-based industries and its starch hydrolytic potential can be enhanced by optimizing fermentation parameters. Notably, the plant essential oils exhibited significant antifungal and anti-ochratoxin production potential in stored grains of wheat, maize and rice, highlighting their potential as natural food preservatives for safe food. These findings suggest the use of EOs of plants is an eco-friendly and sustainable approach for controlling fungal contaminants and OTA production contributing to a safer food supply chain.

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Statement of Novelty

This is the first study to evaluate *Aspergillus terreus* from two perspectives; as an industrial candidate for starch-based industries and as a hidden menace of the livestock sector. This research not only provides a sustainable approach for industrial practices but also highlights the eco-friendly and cost-effective mitigation strategies to control fungal feed contaminants.

Ethical Approval

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

All the data generated in this research work has been included in the manuscript.

Authors' Contributions

Conceptualization, Saba Sana; methodology, Nida Sana; software, Areeba Nadeem; validation, Maher S. Alwethaynani; formal analysis, Layyaba Nazir, investigation, Rukhma Sattar; resources, Rukhma Sattar and Sikander Ali; data curation, Fakhria A. Al-Joufi.; writing—original draft preparation, Najeeb Ullah.; writing—review and editing, Rania Ali El Hadi Mohamed and Nawal Al-Hoshani; visualization, Rania Ali El Hadi Mohamed and Nawal Al-Hoshani; Supervision, Tariq Aziz and Saba Sana; project administration, Tariq Aziz.

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