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Investigating and Improving the Fermentation Conditions for Some *Aspergillus* and *Trichoderma* Species for the Production of Chitinase in Submerged Cultures

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ABSTRACT

The accumulation of chitinous waste from crustacean shells and fungal biomass poses significant environmental challenges globally, necessitating efficient biotechnological solutions. Chitinases produced by fungi are promising candidates for addressing this problem; however, the availability of potent fungal strains and optimized conditions for maximum enzyme yield remain limited. This study evaluated the chitinase production capability of 39 fungal species, comprising 207 isolates from the genera *Aspergillus* (98 isolates from 26 identified species and 3 unidentified species) and *Trichoderma* (109 isolates from 13 identified species and 23 unidentified species) at 30 ºC. Preliminary screening results revealed that 158 isolates exhibit positive activity (66 isolates of *Aspergillus* and 92 of *Trichoderma*), while 18 showed moderate capability. Among the 66 positive *Aspergillus* isolates, 53 shown strong chitinase activity, 12 exhibited moderate activity, and one displayed low activity. Among the 92 positive *Trichoderma* isolates, 85 exhibited high chitinase activity, 6 demonstrated moderate activity, and one was a low chitinase producer. In submerged fermentation (SmF), all fungi exhibiting elevated chitinase activity were examined. The findings indicated that *Aspergillus welwitschiae* AUMC 994 and *Trichoderma viride* AUMC 1782 were the most potent strains. The optimization of chitinase activity for both strains was achieved by modifying the pH, temperature, nitrogen source, and fermentation duration. At pH 8.0 and 30 ºC, *A. welwitschiae* AUMC 994 exhibited peak chitinase activity of 0.04 U/mL after

4 days utilizing yeast extract as the nitrogen source. *T. viride* AUMC 1782 achieved the maximum chitinase activity of 0.038 U/mL at pH 9.0 and 30 ºC after 3 days, utilizing yeast extract as a nitrogen source. This work presents fungal chitinases that may serve as interesting options for biocontrol agents against significant pests.

1.INTRODUCTION

Among the several secondary metabolites produced by microbes are enzymes, pigments, and antibiotics; these substances may have important practical uses for people [1]. Microbial enzymes have many benefits over those originating from plants or animals due to their diversified catalytic activity. Bacteria and fungi serve as sources for various industrial enzymes, including alginase, chitinases, lipase, proteases, and glutaminase [2, 3]. In nature, some bacteria and fungi produce chitinase. Because chitin, chitosan, and chitooligosaccharides are used in industry, there is a greater demand for chitinase with unique or preferred properties.

Chitin, the second most prevalent biodegradable polymer of N-acetylglucosamine. It can be found in the structures of arthropods, algae, fungi, plants, crustaceans, and insects [4]. The breakdown of chitin waste is greatly aided by chitinases. Globally, chitin waste presents serious environmental problems, and the production of chitinolytic enzymes is essential for a number of industrial uses [5, 6]. Numerous microorganisms, such as fungi and bacteria, exhibit the ability to generate chitinase such as *Aspergillus* sp., *Myrothecium anisopliae*, *Penicillium* sp., *Trichoderma harzianum*, *T. viride*, and *Verticillium lecanii*, [7].

Chitinases are glucosyl hydrolases that work on the β-1,4 linkages of chitin's N-acetylglucosamine monomers. Depending on how they cleave the chitin polymer, they are classified as endo-chitinases or exo-chitinases. N-acetylglucosamine and oligomers of β-1,4 N-acetylglucosamine are produced when the enzyme randomly breaks the β-1,4 link of chitin. Exochitinases fall into two groups: β(1,4)N-acetylglucosaminidase (EC 3.2.1.30), designed to attack oligomers produced by endochitinases, yielding N-acetylglucosamine, and chitobiases, which produce diacetylchitobiose from the non-reducing terminus of chitin [2, 3].

Chitinases play specialized roles in a wide variety of organisms from many kingdoms. Fungal chitinases are members of the GH 18 family and have a variety of physiological functions. In addition to using chitin for nutritional and energy needs, these include the stimulation of fungal growth, the facilitation of cell wall remodeling, the facilitation of mycoparasitism, the induction of autolysis, and applications in food, pharmaceuticals, medicine, agriculture, and environmental management [5, 9]. The main objectives are to: (1) identify the most efficient chitinase-producing strains within these genera; (2) optimize fermentation conditions (pH, temperature, nitrogen source, incubation duration) to maximize chitinase yield; and (3) evaluate the feasibility of employing these optimized strains as effective biocontrol agents in agricultural and environmental applications.

2. MATERIALS AND METHODS

2.1. Fungal strains

This study included 207 fungal strains that are related to *Aspergillus* (98) and *Trichoderma* (109). All fungi have been obtained from the culture collection of the Assiut University Mycological Centre (AUMMC).

2.2. Preliminary screening of chitinase activity on agar medium

The conversion of chitin into a soluble colloidal state was carried out following the method outlined by Gunalan et al. [11]. Sucrose-free Czapek's mineral medium [10] was used as a fermentation medium. The examined fungi were allowed to colonise for seven days before 50 µL of spore suspension was collected and inoculated into wells that were 5.0 mm in diameter created on the agar. After that, the plates were incubated at 30 °C for 72 h. A 0.25% iodine solution in water [10] was added to the plates after they had been incubated. The clear zone surrounding fungal colonies against the brown media colour indicated the chitinase activity.

2.3. Screening of chitinase activity in submerged fermentation (SmF)

A 250 mL Erlenmeyer flask with conical shape containing 50 mL of fermentation medium was used to cultivate each fungus strain demonstrating the greatest chitinase activity. Each flask was individually inoculated with a 5.0% spore solution derived from 7-day-old cultures., which contained 1.5×10^8 spores/mL. The flasks were subsequently agitated at 15o rpm for a duration of 7 days at a temperature of 30 °C.

2.4. Assay of chitinase and estimation of protein content

After fermentation, supernatant was separated via centrifugation (10,000 rpm for 10 minutes at 4 °C), to obtain chitinase. Chitinase activity was measured by mixing 0.5 mL of filtered crude enzyme with 0.5 mL of 1.0% colloidal chitin, made in a 50 mM Na-citrate buffer with a pH of 5.0. The reaction mixture was incubated at 40 ºC for 60. The solution was then subjected to a 10-minute boiling in a water bath after which 2 mL of 3, 5-dinitrosalicylic acid (DNS) were added [12]. After cooling, the absorbance was measured at 540 nm. Total protein was determined according to Lowry *et al*. [13]. As stated by AL-Kolaibe *et al*. [14], chitinase activity calculations were performed following Equation (1).

$$\textbf{Chitinase activity} = \left(\frac{\textbf{Absorbance x DF}}{\textbf{(X)(Y)(t)(slope)}}\right) \quad \text{U/mL/min} \quad (1)$$

Where: DF = the dilution factor for enzyme; x = the volume of enzyme used; y = the volume of hydrolysate used for assay; t = the reaction time; slope was determined by the standard curve of glucose.

2.5. Optimization of fermentation parameters

The potent strains' chitinase production was fine-tuned using the two factor at time (TFAT) protocol [14]. A total of 50 mL of fermentation medium supplemented with 1.0% chitin served as the only carbon source for The tests were conducted in 250 mL Erlenmeyer flasks.. An effective strain that was 7 days old was introduced to each flask independently using an injection of a spore suspension comprising 1.5×10^8 spores /mL.

Subsequently, the flasks were subjected to a range of fermentation conditions, including temperatures of 20, 25, 30, and 35 °C, and pH levels ranging from 4 to 10. $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, peptone, yeast extract, and beef extract, were the nitrogen sources that were varied over an incubation period of one to ten days, each at a concentration of 0.2%. Three separate experiments were carried out.

2.6. Morphological identification of the potent strains

Czapek's-Dox agar (Cz), malt extract agar (MEA), and Czapek's Autolysate yeast extract agar (CYA) were utilised for cultivating *A. welwitschiae* AUMC 994, while potato dextrose agar (PDA), corn meal agar (CMA), and oat agar (OA) were employed for the growth of *T. viride* AUMC 1782. Microscopic characteristics were analysed following seven days of incubation at 30 °C for *A. welwitschiae* AUMC 994, and after 3 days of incubation for *T. viride* AUMC 1782.

2.6. Genetic identification of the potent strains

For isolation of DNA, the procedure carried out by Moubasher *et al*. [15] was followed. PCR reaction was carried out by SolGent EF-Taq [16, 17], with ITS1 and ITS4 primers [18], using SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea). Sequences from species that are most similar were downloaded from the GenBank database. All sequences aligned by MAFFT [19], and optimized by BMGE [20]. Maximum-parsimony (MP) phylogenetic analysis was conducted by MEGA X (version 10.2.6) [21]. The most frugal trees' resilience was assessed by using 1000 bootstrap replications [22]. Modeltest 3.7's implementation of the AIC was employed to find the best optimum nucleotide substitution model [23]. The resulting tree was monitored by MEGA X, and saved as TIFF.

3. RESULTS

3.1. Preliminary screening of chitinase activity

Initial screening findings indicated that 158 strains had positive activity (66 strains of *Aspergillus* and 92 of *Trichoderma*), whereas 18 exhibited moderate ability. Of the 66 positive *Aspergillus* strains, 53 had strong chitinase activity, 12 showed moderate activity, and one revealed poor activity. Of the 92 positive *Trichoderma* strains, 85 displayed high chitinase activity, 6 showed moderate activity, and one was a low chitinase producer. In submerged fermentation (SmF), all fungi demonstrating heightened chitinase activity were investigated. The results demonstrated that *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782 were the most productive strains (Table 1).

Table 1. Screening of chitinase activity of 207 fungal strains belonging to *Aspergillus* (98 strains) and *Trichoderma* (109 strains) on sucrose-free Cz agar medium and in submerged cultures at 30 ºC.

Fungal species	No. of Isolates	Positive isolates	Preliminary screening			Secondary screening		
			H*	M	L	H	M	L
Aspergillus								
A. aculeatus	1	-	-	-	-	-	-	-
A. allahabadii	1	1	-	1	-	-	-	-
A. alutaceus	1	-	-	-				
A. aureoterreus	1	-	-	-	-	-	-	-
A. brasiliensis	1	-	-	-	-	-	-	-
A. carneus	2	2	-	1	1	-	-	-
A. clavatus	3	3	3	-	-	-	-	3
A. dentatus	1	-	-	-	-	-	-	-
A. flavus	23	21	21	-	-	-	10	11
A. flavipes	1	1	1	-	-	-	1	-
A. fumigatus	5	4	3	1	-	-	-	3
A. japonicus	2	1	-	1	-	-	-	-
A. melleus	1	-	-	-	-	-	-	-
A. nidulans	2	2	-	2	-	-	-	-
A. niger	27	23	18	5	-	1	7	10
A. niveus	1	-	-	-	-	-	-	-
A. ochraceus	2	-	-	-	-	-	-	-
A. parasiticus	4	4	3	1	-	-	2	1
A. quadrilineatus	2	-	-	-	-	-	-	-
A. rugulosa	1	-	-	-	-	-	-	-
A. sulphureus	2	1	1	-	-	-	-	1
A. sydowii	3	1	1	-	-	-	-	1
A. terreus	4	1	1	-	-	-	1	-
A. tupingensis	1	-	-	-	-	-	-	-
A. ustus	1	-	-	-	-	-	-	-
A. versicolor	1	-	-	-	-	-	-	-
A. welwitschiae	1	1	1	-	-	1	-	-
Aspergillus spp.	3	-	-	-	-	-	-	-
Trichoderma								
T. asperellum	2	2	2	-	-	-	1	1
T. atroviride	3	2	2	-	-	-	1	1
T. aureoviride	1	1	-	1	-	-	-	-
T. citrinoviride	4	2	2	-	-	-	-	2
T. hamatum	2	2	1	1	-	-	1	-
T. harzianum	25	21	18	2	1	-	10	8
T. inhamatum	3	3	2	1	-	-	1	1
T. koningii	23	20	20	-	-	-	9	11
T. longibrachiatum	14	13	13	-	-	1	5	7
T. polysporum	1	1	1	-	-	-	1	-
T. pseudokoningii	5	4	4	-	-	-	-	4
T. ressi	2	1	1	-	-	-	-	1
T. viride	1	1	1	-	-	1	-	-
Trichoderma spp.	23	19	18	1	-	-	15	3
Total	**207**	**158**	**138**	**18**	**2**	**3**	**65**	**70**

***** L= low producer (≤ 10 mm clear zone), M = moderate (11-19 mm), and High (≥20 mm)

3.2. Optimization of chitinase production by *A. welwitschiae* AUMC 994

The maximum chitinase activity of *A. welwitschiae* AUMC 994 has been determined to be 0.032±0.001 U/mL at pH 8.0 and 30 ºC (Figure 1). After four days, the activity rose to 0.04 U/mL when yeast extract served as the nitrogen supply (Figure 2).

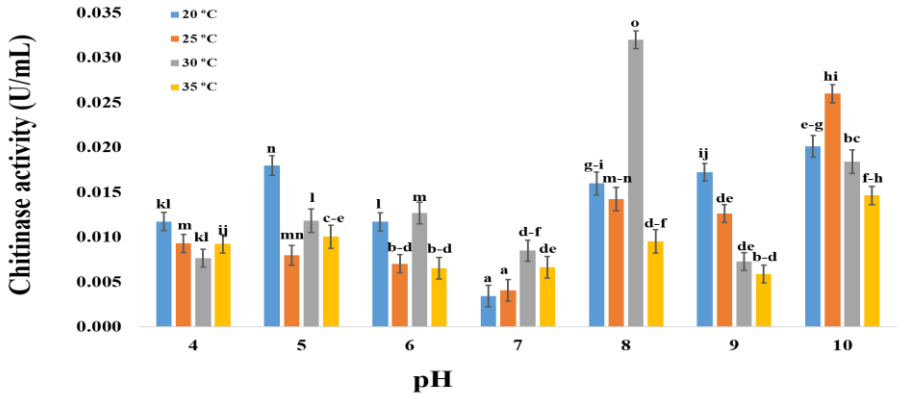

Figure 1. Effect of pH and temperature on chitinase production by *A. welwitschiae* AUMC 994.

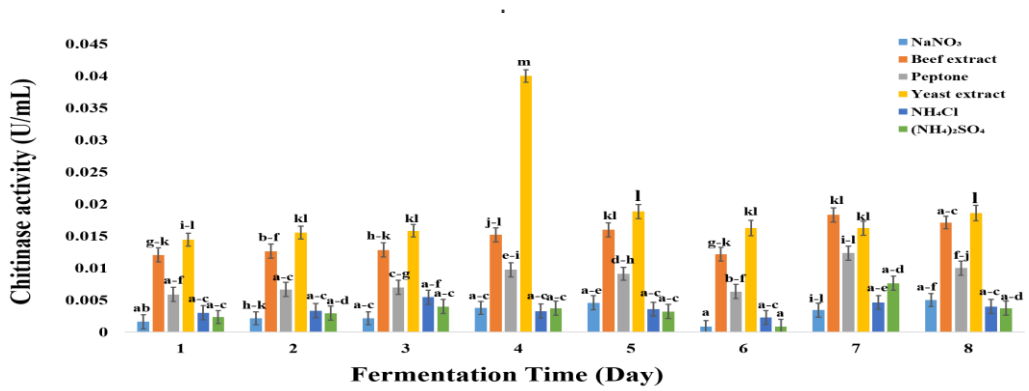

Figure 2. Effect of N_2-source and fermentation time on chitinase production by *A. welwitschiae* AUMC 994.

3.3. Optimization of chitinase production by *T. viride* AUMC 1782

At pH 9.0 and 30 ºC, *T. viride* AUMC 1782 had maximum chitinase activity of 0.031±0.001 U/mL (Figure 3), which increased to 0.038 U/mL after 3 days when yeast extract was employed as the nitrogen source (Figure 4).

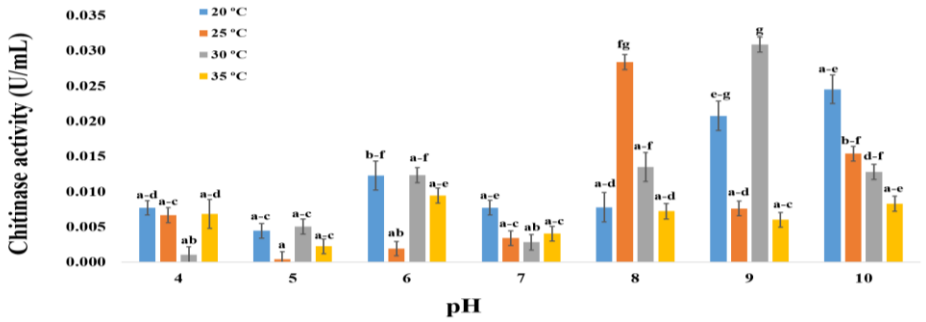

Figure 3. Effect of pH and temperature on chitinase production by *T. viride* AUMC 1782.

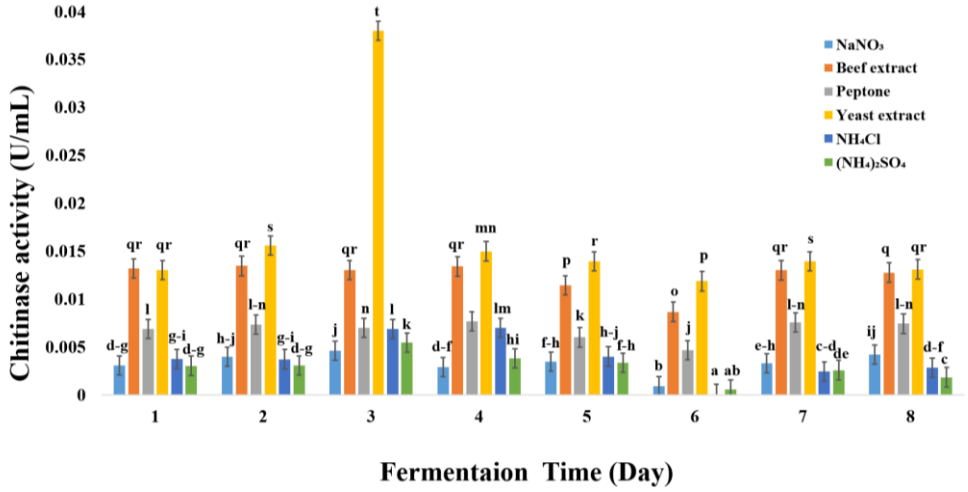

Figure 4. Effect of N_2.source and fermentation time on chitinase production by *T. viride* AUMC 1782.

3.4. Morphological and genetic identification of the potent strains

3.4.1. *Aspergillus welwitschiae* AUMC 994

The strain was identified morphologically as *A. welwitschiae* as it shared the identical morphological characteristics with the type species. It has long (3.0 mm × 15.0 µm), smooth-walled, hyaline conidiophores with large, globose, radiate conidial heads. Globose to subglobose vesicles (up to 80 µm) bearing hyaline to brown metulae (11-15 × 5-6 µm), and hyaline ampulliform phialides (5-11 × 3.0-3.5 µm) that produces brown, delicately to conspicuously roughed, globose to subglobose conidia (3.0-4.2 µm) (Figure 5). In the phylogenetic tree, the strain grouped on the same branch with *A. welwitschiae* strains CMV006G2 and GS-01-1 (Figure 6).

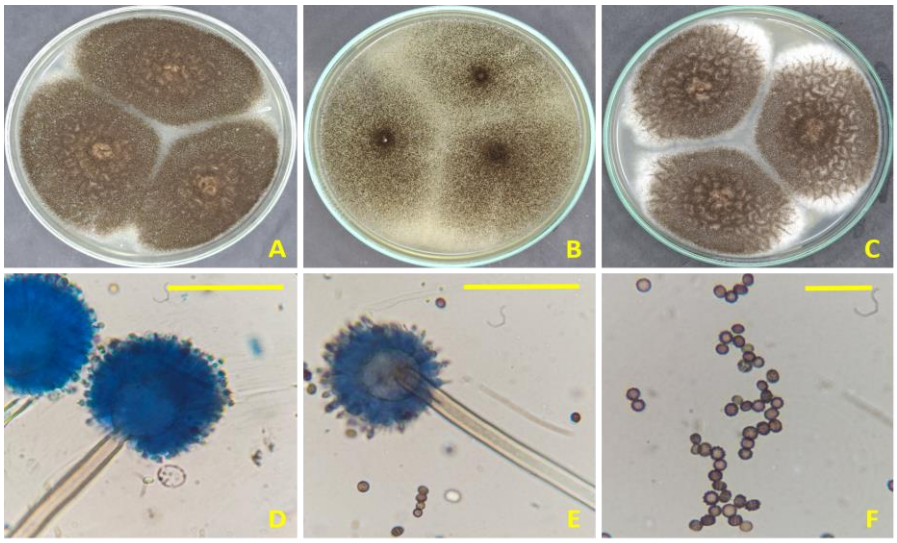

Figure 5. ***A. welwitschiae*** **AUMC 994:** (A–C) 7-day-old cultures on Cz, MEA, and CYA at 25 ºC. (D–E) Stipes with biserriate vesicles. (F) Conidia (Scale bars: D&E = 100 µm; F = 20 µm).

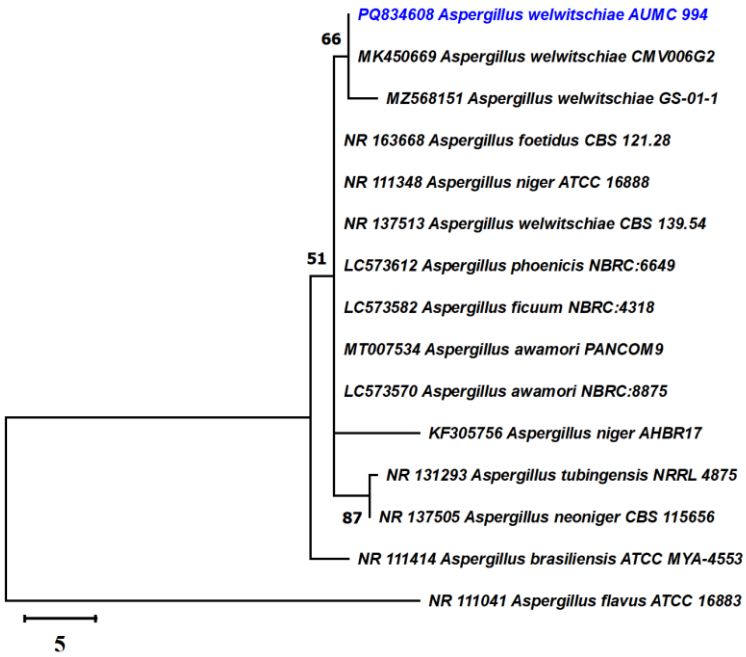

Figure 6. MP tree obtained by a heuristic search (1000 replications) utilising *A. welwitschiae* AUMC 994 ITS sequence compared to the closely hits of section Nigri. Bootstrap support percentages ≥50% are given adjacent to the respective nodes. *A. flavus* ATCC 16883 utilised as tree root.

3.4.2. *Trichoderma viride* AUMC 1782

Morphological identification of the *Trichoderma* strain revealed that it was *T. viride*. It has fast-growing colonies (50-90 mm) that forming dark bluish-green sporulation. Conidiophores not branched with loose arrangement. Lageniform 3, verticillate phaialides frequently paired (8-14 × 2.4-3.0 µm). Globose to ellipsoidal, bluish-green to dark green conidia (4.0-4.8 × 3.5-4.0 µm) (Figure 7). In the phylogenetic tree, the strain was grouped on the same branch with *T. viride* strains GT-8, NCFF 3 TV, and IARIT 13 (Figure 8).

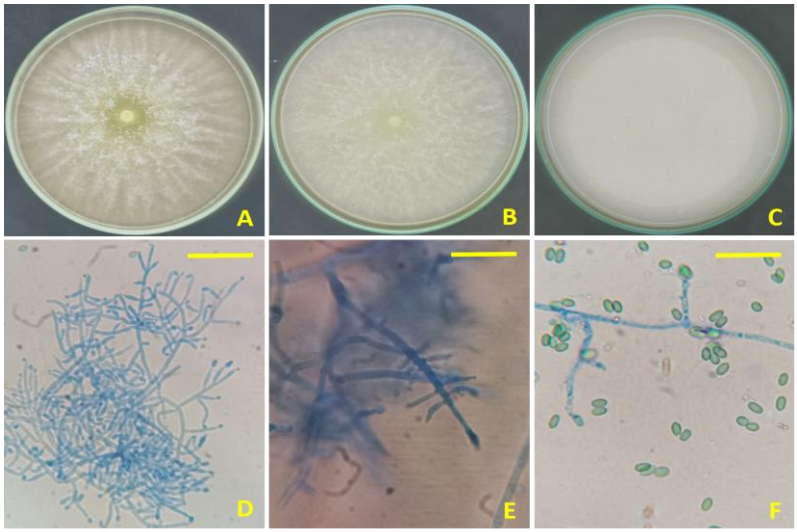

Figure 7. *T. viride* AUMC 1782: (A–C) Three-day-old colonies on PDA, CMA, and OA at 25 ºC. (D–E) Conidiophores and lageniform phialides. (F) Obovoid to ellipsoidal conidia (Scale bars = 20 µm).

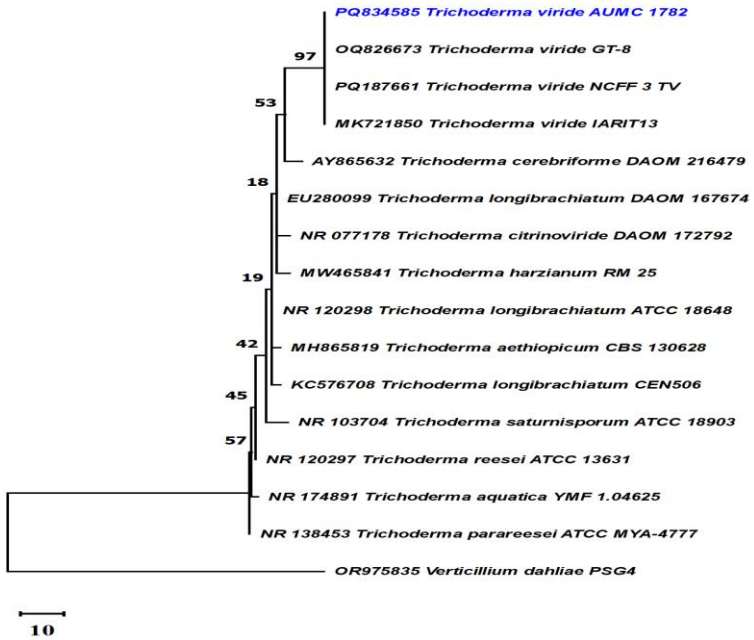

Figure 8. MP tree using a heuristic search (1000 replications) of the *T. viride* AUMC 1782' ITS sequence compared to the closely hits of *Trichoderma*. Bootstrap support values ≥50% are referred near the corresponding nodes. *Verticillium dahlia* PSG4 used as tree root.

4. DISCUSSION

This study evaluated the chitinolytic activity of different species of *Aspergillus* and *Trichoderma*. Over 76% of the fungi investigated in this study exhibited positive chitinase activity, with *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782 showing the highest chitinase output in submerged fermentation. The production of fungal chitinase mostly utilizes submerged fermentation due to its several benefits, including easier recovery of extracellular enzymes, enhanced mass transfer, and well-controlled process parameters. Filamentous fungi are one of numerous microbiological sources that can provide chitinases, enzymes with biotechnological uses. Among the many common uses for these enzymes are in biocontrol, medical applications, waste management, and the production of chito-oligosaccharides [5, 7]. The development of chitinases by several species of fungi has been documented through submerged (SmF) and solid-state fermentation (SSF) using substrates containing chitin [1, 4, 7, 24, 27].

Ongoing investigation of potential microbial sources of chitinase is necessary. This work sought to optimize chitinase synthesis by *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782, enhancing the comprehension of the independent variables affecting chitinase generation and determining optimal levels of activity and fermentation settings. Enzyme synthesis is affected by changes in molecular transport across cell membranes caused by changes in pH and temperature of the growth medium [28]. At 30 ºC, these results showed that the optimal pH levels for chitinase synthesis by *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782 were 8.0 and 9.0, respectively. *Thermothelomyces heterothalicus* PA2S4T, *T. viride* AUMC 13021, and *Acinetobacter terreus* all produced enzymes with optimal chitinolytic activity at pH 6.5, 4.5, and 5.6, respectively, according to these results [29,31].

Because it influences growth of microbes, by extension, enzyme synthesis for optimal output, the temperature of incubation is a crucial environmental component for enzyme synthesis by microbes. In this study, the effects of incubation temperatures ranging from 20 to 35 °C on chitinase synthesis were investigated. Both fungi showed their maximal chitinase activity at 30 °C, according to the results. Enzyme synthesis decreased as temperatures rose. The fungi's decreased metabolic activity, as mentioned before, could be the cause of this [32].

For optimal chitinase activity, each of the tested fungal strains used yeast extract as a nitrogen source. The synthesis of enzymes that play an essential role in numerous primary and secondary metabolic pathways relies on nitrogen supplies [33]. Yeast extract generated the maximum enzyme output when various nitrogen sources were added to the submerged fermentation medium of *T. viride*. Nampoothiri *et al*. [34] found that yeast extract was the most potent inducer of chitinase production by *T. harzianum*, and these results are in agreement with ours.

The current findings showed that the maximum chitinase production was demonstrated by *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782 after 4 and 3 days of incubation, respectively. In line with these findings, the amount of chitinase produced is dependent on the incubation time; as the incubation period advances, the enzyme output increases to a maximum and then decreases. Hypothesized causes of decreased output include enzyme degradation or nutrient depletion in the fermentation medium, which in turn could lead to inhibitory byproduct synthesis [33]. Previous research has shown that both *T. harzianum* and *T. virens* require an equal three-day incubation period to reach peak chitinase synthesis [35, 36].

5. CONCLUSION

This study investigated the chitinase production potential of various species within the genera *Aspergillus* and *Trichoderma* at 30 ºC. Preliminary screening results indicated that 76.0% of the total were positive for chitinase production. In SmF, the most productive fungi were *A. welwitschiae* AUMC 994 and *T. viride* AUMC 1782. Utilizing yeast extract as a nitrogen source, *A. welwitschiae* exhibited the most chitinase activity at pH 8.0 following 4 day, while *T. viride* attained maximum chitinase activity at pH 9.0 following three days of incubation. This study presents fungal chitinases as prospective biocontrol agents for significant uses in several agricultural domains. Future work should focus on scaling up the optimized fermentation processes and further exploring the industrial applicability of these fungal chitinases, particularly in biocontrol, waste management, pharmaceutical applications, and the production of value-added chito-oligosaccharides.

Ethics statement:

Not applicable.

Authors' contributions

Eman O. M. Othman: conceptualization, resources, formal analysis, writing—review and editing**, Ahmed M. Moharram:** conceptualization, visualization, supervision, writing—review, and editing. **Sedky H.A. Hassan:** conceptualization, visualization, supervision, writing—review, and editing **Osama A. M. Al-Bedak:** conceptualization,

investigation, supervision, resources, writing—review and editing, project management. All authors have read and approved the final manuscript and agree to its submission.

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Data Availability Statement:

All data generated or analyzed during this study are included in this manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Consent to Participate: The authors consented.

Consent for publication: The authors consented.

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