Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2021 18(2): 1313-1324. **OPEN ACCESS**

Optimization of chitinase production from *Aspergillus terreus* and its activity against root-knot nematode *Meloidogyne incognita*

Sultanh A. Alzahrani[1], Nuha M. Alhazmi[1] and Tarek A. A. Moussa[1, 2*]

[1]University of Jeddah, College of Science, Department of Biology, Jeddah, **Saudi Arabia**
[2]Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, **Egypt**

***Correspondence:** tarekmoussa@yahoo.com **Received 26-02-2021, Revised: 19-04-2021, Accepted: 20-04-2021 e-Published: 21-04-2021**

Exploration of microbial communities for the synthesis of chitinolytic enzymes is an important characteristic in biodiversity. Chitinolytic microbial species can be used for degradation of chitin instead of chemical treatment, which has lesser environmental impact because of its ecofriendly. The filtrate of twenty fungal isolates was tested for nematode mortality using root-knot nematode *Meloidogyne incognita* larvae. The isolate HA4 gave the highest mortality percentage (97% mortality). HA4 was selected and identified on molecular basis using ITS rDNA genes as *Aspergillus terreus* TNS05 (LC57100). The maximum dry biomass was gained on medium 6 (10.7 g l^{-1}) and the final pH reach its maximum value on medium 2 (7.8) and the lowest was attained in medium 2 (5.1). The highest value of economic coefficient (Ec) (73.9%) was detected in medium 7. The highest chitinase activity was observed in medium 7 (9.4 U ml^{-1}) and the least activity (0.2 U ml^{-1}) was obtained in medium 14. By calculating each factor individually, the highest chitinase activity was attained at chitin (1.0 g%), urea (0.75 g%), yeast extract (2.2 g%), ammonium sulfate (2.5 g%), potassium dihydrogen phosphate (4.5 g%) at agitation rate (250 rpm) and pH value (6.0). By comparing to the different media composition confirmed that medium 7 is the best medium for chitinase activity. The highest biomass protein content was attained in medium 16 (932.4 mg g^{-1}), in case of filtrate protein, the highest content was attained in medium 16 (2127.4 mg ml^{-1}). The best medium for total reducing sugars (TRS) in *A. terreus* biomass was 9 (33.6 mg g^{-1}), the highest direct reducing sugars (DRS) detected in medium 18 (7.3 mg g^{-1}) and non-reducing sugar (NRS) reached the highest in medium 9 (29.6 mg g^{-1}). In culture filtrates, the medium 9 was the best for TRS (513.2 mg ml^{-1}), medium 7 (363.0 mg ml^{-1}) for DRS and medium 12 (266.6 mg ml^{-1}) for NRS.

Keywords: chitinase, *Aspergillus terreus*, *Meloidogyne incognita,* optimization, root-knot

INTRODUCTION

Plant nematodes are widely distributed in agricultural soil and are responsible for mild to heavy loss in variety of crops (Kushwaha & Sarma, 2015). The annual global loss of crops is more than US$ 100 billion (Degenkolb & Vilcinskas, 2016a,b).

The chemical nematicides efficiency is a disagreement point because their non-specificity in mechanisms, non-selective, expensive, and toxic to both invertebrate and vertebrate (Degenkolb & Vilcinskas, 2016a,b; Li *et al.*, 2020). In addition, another disadvantage is that the development of the eggs did not affect because the chitin is the main component of nematodes eggshell in many layers, that gives resistance to chemical nematicides. Thus, the biological agents are costly effective and eco-friendly to control

nematodes.

More than 50,000 compounds bio-actively have been identified from the microorganisms extracts with a diverse structure chemically and showed agrochemical activity, antitumor, and antimicrobial (Kasim, 2016). The top producers of bioactive compounds are bacteria (17%), actinomycetes (45%), and fungi (38%) (Devi *et al.*, 2012). In the development of secondary metabolites, fungi are the most important microorganisms that produce compounds in the culture media that are not required for their growth and generate extracellular capacity (Suay et al. 2000; Zhang et al. 2009).

Many researchers have indicated that fungi are promising biological control agents because of their high reproductive abilities, target specific activity, short generation time, and resting stage. Some nematophagous fungi have shown promising results as a biocontrol agent against the harmful plant parasitic nematodes (Jatala, 1986; Stirling, 2011). Natural antagonists of nematode parasites are nematophagous fungi, which provide an ecophysiological source of new biocontrol strategies (Degenkolb & Vilcinskas, 2016a,b). The fungal conidia must sprout on the body of their host to be considered nematophagous, and the hyphae must enter the nematode and make it sick (Sexton & Howlett, 2006). They comprise more than 200 taxonomically diverse species.

Secondary metabolites are compounds like enzymes, enzyme inhibitors, mycotoxins, pesticides, hormones, and antibiotics that stimulate plants and animals the growth (Demain & Fang, 2000). Fungi produce active compounds that act as nematicides (Chitwood, 2003), and *Aspergillus* spp. are promising candidates for nematicide studies. In this study screening of number of fungal isolates for the control on root-knot nematode *Meloidogyne incognita* and the ability to produce chitinase enzymes and its optimization using statistical method was also studied.

MATERIALS AND METHODS

Collection of soil samples

The soil samples were collected from seven different sites in Jeddah-Makkah road and from different elevations, then preserved in sterile zipper plastic bags with collection place and sample number labelling (Table 1). Then taken the soil samples to the laboratory until to be use.

Fungal isolation from soil samples

Fungi were isolated from subsurface layer of soil (ca. 15-30 cm) by using two methods dilution plate and soil plate methods (Johnson & Curl, 1972) in which six plates/sample were used. Potato Dextrose Agar (PDA) mediums (Pronadisa, Madrid, Spain) were used in isolation techniques, supplemented with chloramphenicol (50 ppm) and Rose Bengal (1/15000) in the 1st isolation.

Dilution Plate Method (Waksman, 1922)

Soil dilutions were carried out by suspending 1.0 g of soil in sterile distilled water (10 ml). 1.0 ml of 10^{-4} and 10^{-5} dilutions were added to PDA plates for fungal isolation and avoiding the congestion of colonies. Rose Bengal was used to prevent the bacterial growth. The plates were incubated for 4-7 days at 28±2ºC and checked daily. The colonies were isolated in pure cultures.

Soil Plate Method (Warcup, 1950)

About 0.005 g of soil was spread in the sterile Petri dish bottom and then cooled (40-45ºC) PDA medium was added. The plates were incubated at 28±2ºC for 4-5 days.

Three plates from each soil samples, and each morphologically unique fungal colony was sub-cultured and purified. The purified fungal isolates were maintained on PDA slant and stored at 4ºC.

Preservation of fungal isolate

The stock cultures were maintained in the refrigerator and subcultures were carried out at monthly intervals. Fresh cultures were prepared at four-month intervals by streaking onto PDA plates from stock slants to check for purity and then sub-culturing on fresh PDA slants. Both plates and slants were incubated at 28±2ºC.

Effect of different fungal isolates on nematode mortality

The culture filtrate was tested for nematode mortality. One ml culture filter with 1 ml of nematode solution was incubated for 24, 48 and 72 h. Microscopic examination: After 24h, it was examined and counted the destroyed wall of nematode, which expressed as mortality percentage.

Molecular identification of the most potent isolate

Internal Transcript Spacer (ITS) was used to identify the most potent fungal isolate according to the manufacturer's instructions using the QIAamp DNA Mini Kit (Qiagen, France). The fragments

containing ITS1 and ITS2 were amplified with primers ITS1, ITS2, ITS3, and ITS4 (ITS1: 5´-TCCGTAGGTGAACCTGCGG-3´; ITS2: 5´-GCTGCGTTCTTCATCGATGC-3´; ITS3: 5´-GCATCGATGAAGAACGCAGC-3´; ITS4, 5´-TCCTCCGCTTAT TGATATGC-3´). The products of PCR were sequenced (Macrogen, South Korea). The sequence was aligned using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted for accession number.

Dry biomass determination

According to Taguchi experiment (Taguchi & Rafanelli, 1994), different media were used to cultivate *A. terreus* using chitin as substrate. In 250-ml flasks, fungal discs (10 mm) were inoculated and incubated at 28°C for 7 days, then the cultures were filtered through Whatman No. 1 and the fungal biomasses were dried at 80°C till constant weight obtained.

Chitinase production

According to Taguchi method (Taguchi & Rafanelli, 1994) large number of experimental conditions were established to enhance their efficiency, reduce experimental errors and reproducible of the experiments in laboratory. Eight factors (pH, chitin, urea, inoculums size, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4 and agitation) which showed significant effects on the enzyme production (Zeilinger *et al.*, 1999) were considered on the present experimental situation (Table 2).

Each factor has three levels and the number of experiments was assigned by symbolic arrays L18 (means 18 different media). All factors have been represented with three levels except pH value (2^1) with a layout of L18 ($2^1 \times 3^7$) (Table 3). Eighteen media were generated from this statistical method according to Taguchi method (Monreal & Reese, 1969) where 18 probabilities were resulted from the variation of the eight factors.

Submerged fermentation experiment were done in 100 ml medium containing (%g.l^{-1}): chitin (1.0, 1.5 and 2.0); yeast extract (1.0, 1.5 and 2.0); $(NH_4)_2SO_4$ (2.0, 2.5 and 3.0); urea (0.5, 0.75 and 1.0); KH_2PO_4 (4, 4.5 and 5); 1 ml of salt solution containing ($CaCl_2.2H_2O$, 0.6; $MgSO_4$, 0.2; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.H_2O$, 0.0028; $CoCl_2.6H_2O$, 0.0032), and pH adjusted to 6.0 or 6.5. Five-millimeter diameter fungal discs from the growing margins of 5-day-old *A. terreus* culture were added with concentrations 5.0, 7.0 and 10.0 discs. The flasks were incubated at 28±2°C for 5 days in shaking incubator (New Brunswick Scientific Co., Inc. Edison, N.J. USA) at 150, 200 and 250 rpm. The fungal cultures were filtered through Whatman No. 3 filter paper followed by filtration through 0.2 mm bacterial filter, then filtrate used for enzyme assay and biochemical tests.

Chitinase activity

The chitinase activity (U mg^{-1}) was analyzed in culture filtrates as described by (Bradford, 1976). The dinitrosalicylic acid (DNS) method was used to measure the chitin-released reducing saccharides. The mixture contained 200 µl of 0.5% colloidal chitin (citrate buffer 0.05 M, pH 6.6) and 200 µl of culture filtrate incubated at 37°C for 1 h. To determine the released reducing sugars, by adding 1 ml of DNS was added to the reaction mixture, then boiled for 5 min in water bath, cooled to room temperature, and centrifuged for 5 min at 6,000 rpm. The absorbance was measured with a spectrophotometer (Evolution 100, Thermo Scientific) at 540 nm. Protein concentration (mg ml^{-1}) was determined (Bradford, 1976), using bovine serum albumin (Sigma-Aldrich, USA) as the standard.

Biochemical analysis

Carbohydrate content

Extraction of carbohydrate from fungal biomass was done as described by (Nemec *et al.*, 1989). Total Reducing Sugars (TRS) and Direct Reducing Sugars (DRS) were measured spectrophotometrically using modified Nelson's solution (Naguib, 1964). Results are expressed as µg reducing sugars g^{-1} dry biomass.

Protein content

Protein from fungal biomass was extracted as described by Osherov and May (Osherov & May, 1998) and measured as described by Bradford (Bradford, 1976).

Statistical analysis

Each experiment was replicated 3 times in a completely randomized design. The data was subjected to analysis of variance (ANOVA) by software SPSS, version 26.0 (IBM Inc., NC, USA).

RESULTS

Screening for nematode mortality

All filtrates of the fungal isolates were tested for nematode mortality using root-knot nematode *Meloidogyne incognita* larvae. The fungal isolate

showed diverse effect on the larvae as shown in Table 4. The highest mortality percentage was obtained by the fungal isolate HA4 (97% mortality) followed by HA1 and HA2 (96 and 94% mortality, respectively). Based on this experiment, the fungal isolate HA4 was selected for further experiments.

Molecular identification of the isolate HA4

As a result of the ITS sequence alignment of the fungal isolate HA4, it is identified as *Aspergillus terreus* TNS05 as shown in Figure 2. The sequence was submitted to the GenBank and had accession number (LC57100).

Dry biomass and final pH

The weight of *A. terreus* TNS05 dry biomass varied according to the composition of medium (Table 5). When the fungus was grown on the different media, the maximum growth was gained on medium 6 (10.7 g l^{-1}), followed by media 2 and 13 (9.4 and 9.2 g l^{-l}, respectively).

The final pH changed moderately to higher values than the initial to reach its maximum value on medium 2 (7.8) and the lowest value was attained in media 2 and 17 (5.1 and 5.6, respectively). The economic coefficient (Ec) indicated higher values usage of chitin. The highest value (73.9%) was detected in medium 7 (Table 5).

Chitinase activity

The results showed that the activity of chitinase was influenced by the different compositions of the medium (Table 6). The highest chitinase activity was observed in medium 7 (9.4 U ml^{-1}), the least chitinase activity (0.2 U ml^{-1}) was obtained when using medium 14. When the chitinase activity was referred to each factor individually the highest chitinase activity was attained at chitin (1.0 g), urea (0.75 g), $(NH_4)_2SO_4$ (2.5 g), yeast extract (2.2 g), KH_2PO_4 (4.5 g) at agitation rate (250 rpm) and pH value (6.0) (Figure 1). By comparing these concentrations to the different media composition confirmed that medium 7 is the best medium for chitinase activity.

Protein content

The highest biomass protein content in dry biomass was attained in medium 16 (932.4 mg g^{-1}), whereas the lowest biomass protein was attained in medium 2 (226.0 mg g^{-1}). In case of filtrate protein, the highest content was attained in medium 16 (2127.4 mg ml^{-1}) and in medium 12 gave the lowest protein content (978.4 mg ml^{-1}) (Table 6).

Carbohydrate content

It was found that from Table 7, the best medium for TRS in *A. terreus* biomass was 9 (33.6 mg g^{-1}), while media 7 and 12 was accompanied by the least mycelial TRS (3.4 and 3.2 mg g^{-1}, respectively). In case of DRS, the highest value was detected in medium 18 (7.3 mg g^{-1}), while it was declined significantly in media 12, 15 and 11, where 1.9, 1.8 and 1.3 mg g^{-1} were recorded, respectively. The least DRS in biomass was detected in medium 1 (0.6 mg g^{-1}). The highest biomass NRS was attained in medium 9 (29.6 mg g^{-1}), significant decline was detected in media 10, 11, 18, 1 and 17. Media 12 and 7 recorded the least amount of NRS (1.3 and 0.5 mg g^{-1}, respectively).

Table 1: Soil samples collected from different places in Jeddah

Soil Sample Number	Location	The Location Coordinates With GPS
1	MAKKAH MC	21 37 15"N 39 17 26"E, 93 E, 90 m Elevation
2	JEDDAH HA=Al Harazat	21 35 37"N 39 12 2" E, 135 SE, 30 m Elevation
3	JEDDAH SA= Al safa	21 28 41"N 39 20 10"E, 21 N, 110 m Elevation
4	JEDDAH FA=Al Faisaliyah	21 34 50"N 39 10 52"E, 153 SE, 20 m Elevation
5	JEDDAH FA1= Al Faisaliyah	21 34 50"N 39 10 52"E, 69 E, 20 m Elevation
6	JEDDAH FA2= Al Faisaliyah	21 34 50"N 39 10 52"E, 284 W, 20 m Elevation
7	JEDDAH FA3= Al Faisaliyah	21 34 50"N 39 10 52"E, 330 NW, 20 m Elevation

Table 2: Medium components and experimental variables at different levels, studied in Taguchi DOE

Serial no.	Factor	Level 1	Level 2	Level 3
1	pH	6.0	6.5	-
2	Agitation (rpm)	150	200	250
3	Chitin (w/v)	1.0	1.5	2.0
4	Urea (w/v)	0.50	0.75	1.0
5	Inoculum (disk)	5	7	10
6	Yeast extract (w/v)	1.0	1.50	2.0
7	$(NH_4)_2SO_4$ (w/v)	2. 0	2.5	3.0
8	KH_2PO_4 (w/v)	4.0	4.5	5.0

Table 3: Taguchi designed of experiment (DOE methodology)

Media no.	Factors levels							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	1	1	1	1	1	1	1	1
2	1	1	2	2	2	2	2	2
3	1	1	3	3	3	3	3	3
4	1	2	1	1	2	2	3	3
5	1	2	2	2	3	3	1	1
6	1	2	3	3	1	1	2	2
7	1	3	1	2	1	3	2	3
8	1	3	2	3	2	1	3	1
9	1	3	3	1	3	2	1	2
10	2	1	3	3	3	2	2	1
11	2	1	1	1	1	3	3	2
12	2	1	2	2	2	1	1	3
13	2	2	1	2	3	1	3	2
14	2	2	2	3	1	2	1	3
15	2	2	3	1	2	3	2	1
16	2	3	1	3	2	3	1	2
17	2	3	2	1	3	1	2	3
18	2	3	3	2	1	2	3	1

Table 4: Screening the efficiency of fungal isolates on root-knot nematode *Meloidogyne incognita* larvae

Fungal Isolate	Mortality (%)
AS1	41
AS2	40
FA1	75
FA1(2)	0
FA2	40
FA3	0
HA1	94
HA2	96
HA3	76
HA4	97
HA5	62
HA6	38
HA7	0
HA8	0
HA9	0
HA10	0
HA11	0
MC1	39
MC2	22
MC3	0

Table 5: Effect of media composition on dry biomass (g.l^{-1}) and final pH of *A. terreus* after 8 days incubation period at 28°C

Media no.	Dry biomass (g l^{-1})	Initial pH	Final pH	Ec (%)
1	4.6±0.14	6.0	6.7	37.2
2	9.4±0.11	6.0	7.8	39.4
3	4.4±0.12	6.0	5.1	42.8
4	4.4±0.21	6.0	6.0	41.3
5	7.8±0.14	6.0	6.5	38.9
6	10.7±1.45	6.0	6.7	39.5
7	5.4±0.16	6.0	6.4	73.9
8	8.6±0.24	6.0	7.6	38.5
9	3.6±0.21	6.0	6.8	40.0
10	2.6±0.28	6.5	7.3	37.7
11	1.6±0.16	6.5	6.6	46.8
12	4.0±0.21	6.5	7.0	43.1
13	9.2±0.28	6.5	6.6	38.8
14	4.3±0.18	6.5	7.3	41.4
15	6.4±0.28	6.5	6.5	40.0
16	2.8±0.17	6.5	7.2	36.7
17	8.6±0.17	6.5	5.6	37.9
18	3.4±0.21	6.5	6.5	42.4

Ec= Economic coefficient= utilized sugar/dry biomassX100.

Table 6: Effect of media composition on chitinase activity (U ml^{-1}) and protein content in biomass (mg g^{-1}) and culture filtrates (mg ml^{-1}) of *A. terreus* after 8 days incubation at 28°C

Media no.	Chitinase activity (U ml^{-1})	Total protein	
		biomass (mg g^{-1})	Filtrate (mg ml^{-1})
1	1.0±0.04	243.6±3.5	981.4±2.5
2	2.5±0.14	226.0±0.4	1297.0±6.7
3	0.7±0.03	619.0±7.5	1194.4±6.0
4	0.7±0.08	586.0±0.4	1609.2±1.4
5	1.3±0.04	782.2±1.8	1576.0±1.8
6	0.6±0.04	314.2±1.1	1143.0±0.7
7	9.4±0.04	793.6±5.3	1504.0±3.2
8	2.5±0.06	693.3±0.7	1290.4±1.1
9	1.7±0.03	412.1±1.2	1235.8±3.9
10	0.8±0.03	717.0±3.5	1787.4±0.7
11	4.1±0.02	571.0±3.9	1423.2±7.1
12	1.9±0.08	423.1±0.2	978.4±2.5
13	1.3±0.05	345.7±1.2	1712.8±4.6
14	0.2±0.01	568.3±1.9	1675.8±3.5
15	1.1±0.04	352.6±0.4	1777.0±5.3
16	1.7±0.03	932.4±2.8	2127.4±3.9
17	0.6±0.04	701.4±0.7	1144.0±2.5
18	1.8±0.01	725.8±6.7	1364.8±3.9

Table 7: Effect of different media composition on the total reducing sugars (TRS), direct reducing sugars (DRS) and non-reducing sugars (NRS) in mycelial mats and culture filtrates of *A. terreus* after 8 days incubation at 28°C

Media no.	Carbohydrate contents					
	Mycelia (mg g^{-1})			Filtrate (mg ml^{-1})		
	TRS	DRS	NRS	TRS	DRS	NRS
1	5.9±0.05	0.6±0.07	5.3±0.06	399.0±1.63	135.0±1.49	264.0±1.56
2	6.5±0.06	2.3±0.03	4.2±.05	324.6±1.27	205.8±1.49	118.8±1.38
3	7.8±0.01	3.1±0.04	4.7±0.03	392.1±1.63	303.0±1.69	89.1±1.66
4	4.7±0.09	2.5±0.02	2.2±0.06	217.7±1.27	137.4±1.56	80.3±1.42
5	16.0±0.21	4.1±0.09	11.9±0.15	400.4±1.49	210.8±1.69	189.6±1.59
6	20.9±0.19	2.2±0.01	18.7±0.10	399.2±1.41	142.2±1.63	257.1±1.52
7	3.4±0.02	2.9±0.01	0.5±0.02	417.4±1.56	363.0±1.49	54.4±1.53
8	18.4±0.04	2.8±0.06	15.6±0.05	396.7±1.34	297.4±1.63	99.4±1.49
9	33.6±0.05	4.0±0.51	29.6±0.23	513.2±1.13	269.5±1.41	243.7±1.27
10	13.1±0.53	3.0±0.07	10.1±0.30	445.0±1.56	191.2±1.13	253.8±1.35
11	8.3±0.04	1.3±0.12	7.0±0.08	202.6±1.27	121.8±1.49	80.8±1.38
12	3.2±0.04	1.9±0.05	1.3±0.05	393.2±1.27	126.6±1.56	266.6±1.42
13	6.7±0.04	2.3±0.04	4.4±0.03	442.0±1.56	211.9±1.56	230.1±1.40
14	5.5±0.08	3.7±0.05	1.8±0.62	360.8±1.49	293.3±1.56	67.6±1.15
15	27.2±0.11	1.8±0.06	25.4±0.85	354.8±1.27	233.3±1.56	121.6±1.42
16	6.4±0.02	2.3±0.04	4.1±0.03	318.4±2.26	234.6±1.56	83.8±1.91
17	9.1±0.04	4.0±0.05	5.2±0.04	366.2±3.61	121.4±1.49	244.8±2.55
18	10.0±0.06	4.0±0.03	6.0±0.04	201.4±1.34	142.6±1.56	58.8±1.45

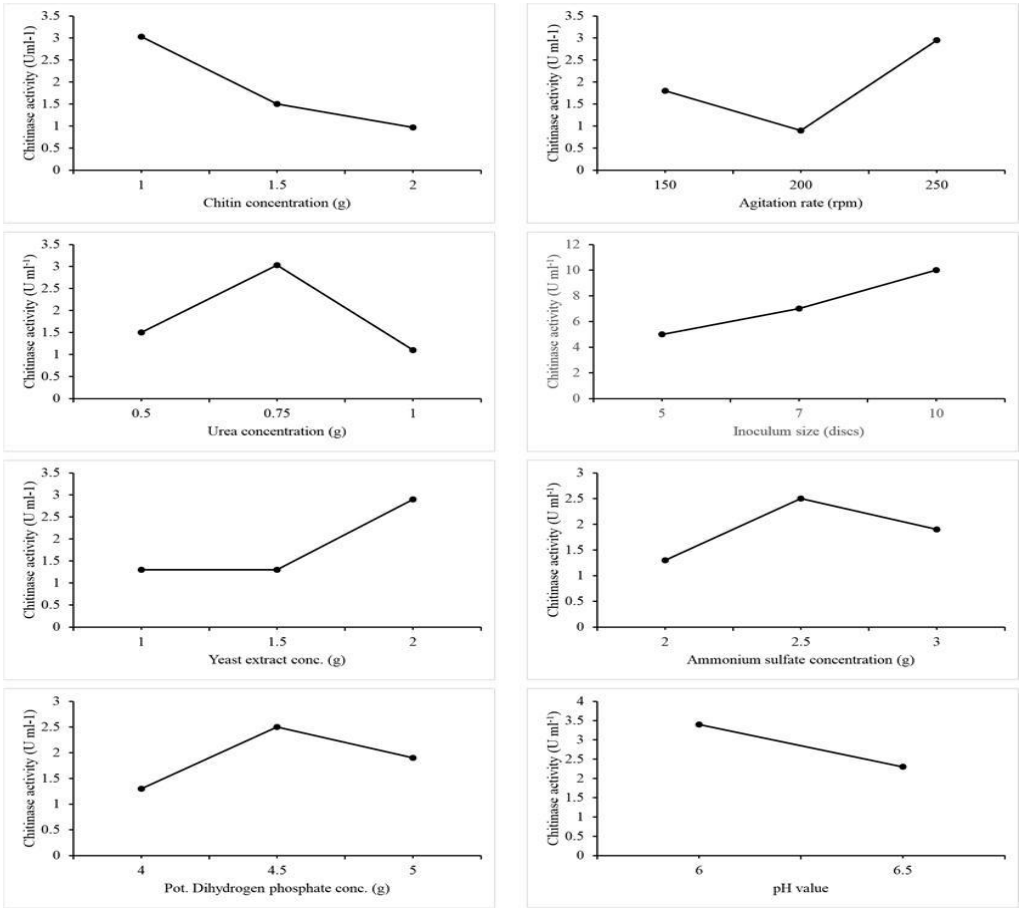

Figure 1: Assessment the composition of medium used individually on the chitinase activity.

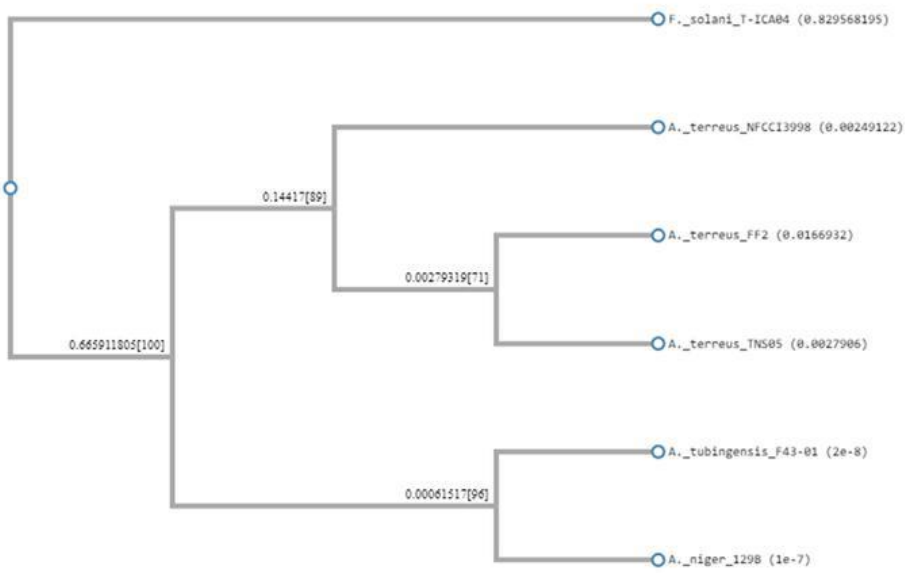

Figure 2: Phylogentic tree of the fungal isolate HA4 (*A. terreus* TNS01) and some related fungal isolates from the database (*A. terreus* NFCCI3998 (KX792117), *A. terreus* FF2 (MH065614), *A. niger* 129B (KU847851), *A. tubingensis* F43-01 (KX664400) and *F. solani* T-ICA04 (KJ620371)

In culture filtrates of *A. terreus* (Table 7), The data indicated that the medium 9 was the best for TRS (513.2 mg ml^{-1}), the TRS values showed sharp decrease in media 2, 16, 4 and 11, which recorded 324.6, 318.4, 217.7 and 202.6 mg ml^{-1}, respectively. However, the least TRS content was attained on medium 18 (201.4 mg ml^{-1}). The highest DRS was in medium 7 (363.0 mg ml^{-1}), the lowest value was detected in medium 17 (121.4 mg ml^{-1}). The NRS were highest when medium 12 was utilized (266.6 mg ml^{-1}), the least NRS value was recorded on medium 7 (54.4 mg ml^{-1}) in the culture filtrate of *A. terreus*.

DISCUSSION

Chitinases (E.C. 3.2.1.14.) are hydrolyzing enzymes that hydrolyze chitin to its components. The chitinase had different properties according to origins, which play a crucial role in the parasitism and nutrition of fungi and bacteria (Sahai & Manocha, 1993; Gooday, 1997).

The fungal isolate showed diverse effect on the larvae where the highest mortality percentage was obtained by the fungal isolate HA4 (97% mortality) followed by HA1 and HA2 (96 and 94% mortality, respectively).

The antagonism between plant parasite nematode and fungi are the most studied models due to the high economic concern. Fungi that attack nematodes are called nematophagous fungi. (Gortari & Hours, 2008). The *in vitro* activity of some fungi (*Arthrobotrys*, *Acremonium*, *Cylindrocarpon*, *Aspergillus*, *Dactylella*, *Monacrosporium*, *Lecanicillium*, *Fusarium*, *Paecilomyces*, *Pochonia*, *Pyrenochaeta*, *Penicillium*, *Verticillium* and *Trichoderma*) has been measured using the severity of egg infection and egg-parasitic index (EPI). The effects of the crude and/or purified substances on the egg hatching, mortality and the 2nd juveniles stage (J2) mobility was also measured (Chen & Chen, 2002; Olivares-Bernabeu & López-Llorca, 2002; Mukhtar & Pervaz, 2003; Khan et al. 2004; Park et al. 2004). In several cases, the percentages of hatching reductions, morphological alterations, and the viability of larvae have been examined. The compounds in filtrates with harmful effects on J2 nematodes and on the eggs have been considered as nematicides (Meyer et al. 2000; SILVA et al. 2002; Mukhtar & Pervaz, 2003; Juba et al. 2004; Adekunle & Akinsanmi, 2005).

Based on the molecular identification using the ITS sequence of the fungal isolate HA4, it is identified as Aspergillus terreus TNS05 (LC57100).

Arthrobotrys, *Acremonium*, *Cylindrocarpon*, *Aspergillus*, *Dactylella*, *Monacrosporium*,

Lecanicillium, *Fusarium*, *Paecilomyces*, *Pochonia*, *Pyrenochaeta*, *Penicillium*, *Verticillium* and *Trichoderma* are the most common genera related to nematodes parasitism (Chen & Chen, 2002; Olivares-Bernabeu & López-Llorca, 2002; Verdejo-Lucas et al., 2002; Sun et al., 2006).

A well-established practice is statistical methods for developing the best medium formulation for optimizing metabolite production (Baltz et al. 2010)

The chitinase activity was varied according to the medium used. The highest chitinase activity was observed in medium 7 (9.4 U ml^{-1}), where chitin (1.0 g), urea (0.75 g), yeast extract (2.2 g), KH_2PO_4 (4.5 g), and $(NH_4)_2SO_4$ (2.5 g) at agitation rate (250 rpm), pH value (6.0) and incubation period 8 days.

The highest chitinase production was attained when shrimp shell and were used as substrates. The production of *Aspergillus niger* LOCK 62 chitinase was maximum after 6 days incubation (Brzezinska & Jankiewicz, 2012). On sucrose-peptone-yeast extract medium, a marine soil isolate of *Aspergillus terreus* with chitinase activity was cultivated. In the production medium, colloidal chitin substituted sucrose (Narayanan et al. 2013). Maximum production of *Aspergillus terreus* chitinase was obtained when 2% of shrimp-shell powder was used as a sole carbon source at 30°C and pH 5 for five days under shaking conditions (Aida et al., 2014).

There chitinase activity was greater for *Paecilomyces* sp. in culture medium C than in the basal medium (Homthong et al. 2016). From *Aeromonas* sp. high levels of extracellular chitinase may be produced in chitin-containing media as carbon source. The effects of medium composition and physical parameters were studied on the production of chitinase by Aeromonas sp. It was found that the optimized medium contains 0.75% (w/v) colloidal chitin (Al-Ahmadi et al. 2008).

Trichoderma lixii IG127
and *Clonostachys rosea* IG119, were tested in media containing 1% colloidal chitin, 1% yeast nitrogen base, for chitinase production in shaken cultures (Pasqualetti et al. 2019). In the Trichoderma asperellum chitinase production medium, culture filtrate supplemented with 0.1% colloidal chitin was used. Six days of growth at 30°C and pH 6.0 are the optimal conditions for maximal chitinase production (Gueye et al., 2020).

The highest biomass protein content was attained in medium 16 (932.4 mg g^{-1}), while the highest filtrate protein was attained in medium 16 (2127.4 mg g^{-1}). The best medium for TRS in biomass was 9 (33.6 mg g^{-1}), while DRS was detected in medium 18 (7.3 mg g^{-1}), and the highest NRS was attained in medium 9 (29.6 mg g^{-1}). In culture filtrates the medium 9 was the best for TRS (513.2 mg ml^{-1}), the medium 7 (363.0 mg ml^{-1}) for DRS and medium 12 (266.6 mg ml^{-1}) for NRS.

Biomasses grown on starches from potatoes and cereals were characterized by large amounts of chitin and polysaccharides in *Cunninghamella elegans*, and glucose gave rise to a biomass rich in acidic polysaccharides and lipids. Biomasses grown on steep corn liquor, on the other hand, were low in acidic polysaccharides and rich in protein when N sources and micronutrients were added (Tigini et al. 2012).

Since it is commonly understood that fungi respond to the growth substratum and other environmental physicochemical properties, their cell wall composition, their physicochemical properties, and the morphological aspect of the colony are significantly modified. (Tekerlekopoulou et al. 2010).

CONCLUSION

The maximum dry biomass was gained on medium 6, while the highest chitinase activity and economic coefficient (Ec) were observed in medium 7 and the least activity was obtained in medium 14. By calculating each factor individually, the highest chitinase activity was attained at chitin (1.0 g%), urea (0.75 g%), yeast extract (2.2 g%), ammonium sulfate (2.5 g%), potassium dihydrogen phosphate (4.5 g%) at agitation rate (250 rpm) and pH value (6.0). By comparing to the different media composition confirmed that medium 7 is the best medium for chitinase activity.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

No acknowledgement

AUTHOR CONTRIBUTIONS

TAAM and NMA supervised and designed the experiments and also wrote the manuscript. SAA, collected soil samples and performed the experiments. TAAM, NMA and SAA carried out data analysis. TAAM reviewed the manuscript. All authors read and approved the final version.

REFERENCES

Adekunle OK, Akinsanmi OA. 2005. Bioactivity of Fusarium oxysporum f. sp. glycines and Sclerotium rolfsii filtrates on egg hatching, survival and infectivity of juveniles of Meloidogyne incognita race 2. *Australian Journal of Experimental Agriculture* 45: 99–102.

Aida FM, Al-Nusarie S, Taghreed S. 2014. Production, optimization, characterization and antifungal activity of chitinase produced by Aspergillus terrus. *African Journal of Biotechnology* 13.

Al-Ahmadi KJ, Yazdi MT, Najafi MF, Shahverdi AR, Faramarzi MA, Zarrini G, Behravan J. 2008. Optimization of medium and cultivation conditions for chitinase production by the newly isolated: Aeromonas sp. *Biotechnology* 7: 266–272.

Baltz RH, Demain AL, Davies JE. 2010. *Manual of industrial microbiology and biotechnology*. American Society for Microbiology Press.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72: 248–254.

Brzezinska MS, Jankiewicz U. 2012. Production of antifungal chitinase by Aspergillus niger LOCK 62 and its potential role in the biological control. *Current microbiology* 65: 666–672.

Chen F, Chen S. 2002. Mycofloras in cysts, females, and eggs of the soybean cyst nematode in Minnesota. *Applied Soil Ecology* 19: 35–50.

Chitwood DJ. 2003. Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture–Agricultural Research Service. *Pest Management Science: Formerly Pesticide Science* 59: 748–753.

Degenkolb T, Vilcinskas A. 2016a. Metabolites from nematophagous fungi and nematicidal natural products from fungi as alternatives for biological control. Part II: metabolites from nematophagous basidiomycetes and non-nematophagous fungi. *Applied microbiology and biotechnology* 100: 3813–3824.

Degenkolb T, Vilcinskas A. 2016b. Metabolites from nematophagous fungi and nematicidal natural products from fungi as an alternative for biological control. Part I: metabolites from nematophagous ascomycetes. *Applied microbiology and biotechnology* 100: 3799–3812.

Demain AL, Fang A. 2000. The natural functions of secondary metabolites. *History of modern biotechnology I*: 1–39.

Devi LS, Khaund P, Nongkhlaw FMW, Joshi SR. 2012. Diversity of culturable soil micro-fungi along altitudinal gradients of Eastern Himalayas. *Mycobiology* 40: 151–158.

Gooday GW. 1997. The many uses of chitinases in nature. キチン・キトサン研究 3: 233–243.

Gortari MC, Hours RA. 2008. Fungal chitinases and their biological role in the antagonism onto nematode eggs. A review. *Mycological Progress* 7: 221–238.

Gueye N, Kumar GK, Ndiaye M, Sall SYD, Ndiaye MAF, Diop TA, Ram MR. 2020. Factors affecting the chitinase activity of Trichoderma asperellum isolated from agriculture field soils. *Journal of Applied Biology & Biotechnology Vol* 8: 41–44.

Homthong M, Kubera A, Srihuttagum M, Hongtrakul V. 2016. Isolation and characterization of chitinase from soil fungi, Paecilomyces sp. *Agriculture and Natural Resources* 50: 232–242.

Jatala P. 1986. Biological control of plant-parasitic nematodes. *Annual review of phytopathology* 24: 453–489.

Johnson LF, Curl EA. 1972. *Methods for research on the ecology of soil-borne plant pathogens*. Burgess Minneapolis.

Juba J, Huettel R, Liu XZ, Nitao J, Humber R, Meyer S. 2004. Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility. *Nematology* 6: 23–32.

Kasim AA. 2016. Influence of bioactive metabolites extracted from three species of nematode-trapping fungi on the growth of Escherichia coli and Staphylococcus aureus. *World Journal of Biology and Biological Sciences* 4: 9–14.

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of Paecilomyces lilacinus protease and chitinase on the eggshell structures and hatching of Meloidogyne javanica juveniles. *Biological control* 31: 346–352.

Kushwaha RK, Sarma BK. 2015. Studies on some nematophagous fungi for management of root knot diseases of tomato. *Journal of Biotechnology and Crop Science* 4: 56–60.

Li J, Meng Z, Li N, Dong B, Ji X, Zhang S, Qiao K. 2020. Evaluating a new non-fumigant nematicide fluopimomide for management of southern root-knot nematodes in tomato. *Crop Protection* 129: 105040.

Meyer SLF, Massoud SI, Chitwood DJ, Roberts DP. 2000. Evaluation of Trichoderma virens and Burkholderia cepacia for antagonistic activity against root-knot nematode, Meloidogyne incognita. *Nematology* 2: 871–879.

Monreal J, Reese ET. 1969. The chitinase of Serratia marcescens. *Canadian Journal of Microbiology* 15: 689–696.

Mukhtar T, Pervaz I. 2003. In vitro evaluation of ovicidal and larvicidal effects of culture filtrate of Verticillium chlamydosporium against Meloidogyne javanica. *Int J Agric Biol* 4: 576–579.

Naguib MI. 1964. EFFECT OF SEVIN (N-METHYL-I-NAPHTHYL CARBAMATE) ON CARBOHYDRATE+ NITROGEN METABOLISM DURING GERMINATION OF COTTON SEEDS. *Indian Journal of Experimental Biology* 2: 149.

Narayanan K, Chopade N, Raj PV, Subrahmanyam VM, Rao JV. 2013. Fungal chitinase production and its application in biowaste management.

Nemec S, Phelps D, Baker R. 1989. Effects of dihydrofusarubin and isomarticin from Fusarium solani on carbohydrate status and metabolism of rough lemon seedlings. *Phytopathology* 79: 700–705.

Olivares-Bernabeu CM, López-Llorca LV. 2002. Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. *Revista iberoamericana de micología* 19: 104–110.

Osherov N, May GS. 1998. Optimization of protein extraction from Aspergillus nidulans for gel electrophoresis. *Fungal Genetics Reports* 45: 38–40.

Park J, Hargreaves JR, McConville EJ, Stirling GR, Ghisalberti EL, Sivasithamparam K. 2004. Production of leucinostatins and nematicidal activity of Australian isolates of Paecilomyces lilacinus (Thom) Samson. *Letters in Applied Microbiology* 38: 271–276.

Pasqualetti M, Barghini P, Giovannini V, Fenice M. 2019. High production of chitinolytic activity in halophilic conditions by a new marine strain of Clonostachys rosea. *Molecules* 24: 1880.

Sahai AS, Manocha MS. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host—parasite interaction. *FEMS Microbiology Reviews* 11: 317–338.

Sexton AC, Howlett BJ. 2006. Parallels in fungal pathogenesis on plant and animal hosts. *Eukaryotic Cell* 5: 1941–1949.

SILVA GH, OLIVEIRA DF, CAMPOS VP. 2002. Purificação de metabólitos fúngicos com efeitos tóxicos sobre Meloidogyne incognita. *Fitopatologia brasileira* 27: 594–598.

Stirling GR. 2011. Biological control of plant-parasitic nematodes: an ecological perspective, a review of progress and opportunities for further research. *Biological Control of Plant-Parasitic Nematodes*:: 1–38.

Suay I, Arenal F, Asensio FJ, Basilio A, Cabello MA, Díez MT, García JB, del Val AG, Gorrochategui J, Hernández P. 2000. Screening of basidiomycetes for antimicrobial activities. *Antonie van Leeuwenhoek* 78: 129–140.

Sun M-H, Gao L, Shi Y-X, Li B-J, Liu X-Z. 2006. Fungi and actinomycetes associated with Meloidogyne spp. eggs and females in China and their biocontrol potential. *Journal of invertebrate pathology* 93: 22–28.

Taguchi G, Rafanelli AJ. 1994. Taguchi on robust technology development: bringing quality engineering upstream.

Tekerlekopoulou AG, Tsiamis G, Dermou E, Siozios S, Bourtzis K, Vayenas D v. 2010. The effect of carbon source on microbial community structure and Cr (VI) reduction rate. *Biotechnology and bioengineering* 107: 478–487.

Tigini V, Prigione V, Donelli I, Freddi G, Varese GC. 2012. Influence of culture medium on fungal biomass composition and biosorption effectiveness. *Current microbiology* 64: 50–59.

Verdejo-Lucas S, Ornat C, Sorribas FJ, Stchiegel A. 2002. Species of root-knot nematodes and fungal egg parasites recovered from vegetables in Almería and Barcelona, Spain. *Journal of Nematology* 34: 405.

Waksman SA. 1922. A method for counting the

number of fungi in the soil. *Journal of bacteriology* 7: 339.

Warcup JH. 1950. The soil-plate method for isolation of fungi from soil. *Nature* 166: 117–118.

Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP. 1999. Chitinase Gene Expression during Mycoparasitic Interaction ofTrichoderma harzianumwith Its Host. *Fungal Genetics and Biology* 26: 131–140.

Zhang Y, Mu J, Feng Y, Kang Y, Zhang J, Gu P-J, Wang Y, Ma L-F, Zhu Y-H. 2009. Broad-spectrum antimicrobial epiphytic and endophytic fungi from marine organisms: isolation, bioassay and taxonomy. *Marine drugs* 7: 97–112.