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Chitinase of Marine *Penicillium chrysogenum* MH745129: Isolation, Identification, Production and Characterization as Controller for Citrus Fruits Postharvest Pathogens

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Abstract

Marine waste is one of the most environmental problems. Chitinase is essential in decomposition of chitin, resulting in the utilization of chitin as a renewable resource. Marine fungi *Penicillium chrysogenum* MH745129 was isolated from red sea water and identified by 18S rRNA. It was selected for chitinase production from medium containing different chitin waste sources as shrimp shell powder, fish shell powder, chitin, sawdust and alginate. Addition of dextrose to fermentation media containing shrimp shell powder increases the enzyme activity to 78.2U/mL. The optimum reaction mixture conditions of partially purified chitinase activity was obtained using 60% acetone at 40 °C, pH 6.0 at 40 min, and it was stable at 50°C for 60 min. The kinetic constants K_m and V_{max} determined for chitinase with colloidal chitin as substrate was 6.26 mg/ml and 68.5 U/ml, respectively. The chitinase molecular weight was found to be 42 kDa. In vitro partially purified chitinase was significant in reduction, reduced the linear mycelial growth of both *P. digitatum* and *P. italicum*. Also in vivo all treatments reduced significantly postharvest disease incidence of Valencia orange and Lime fruits compared to the control. After 20 days storage period, 100% of control fruit developed green and blue mold in Valencia orange and lime fruits.

Keywords: Marine fungi isolation and identification, Marine wastes, Chitinase, Partial purification, Biocontrol.

1. Introduction

Chitin, α-1, 4-connected a polymer of N-acetyl-D-Glucosamine, is the second most bounteous poly-saccharide in nature alongside cellulose, and to a great extent it exists in squanders from handling of marine nourishment items (crab, shrimp and krill shells just as fish scales) (Pointing and Hyde, 2001).

Marine squanders are viewed as the extraordinary wellsprings of chitin. Rinaudo (2006) referenced that more than 80,000 tons of chitin were created from marine squanders each year, which should be all the more viably used to keep away from the destructive effect on nature. The waste created from the overall generation and preparing of shellfish and fish scales is a difficult issue of developing greatness. This rich waste may present ecological danger because of the simple disintegration (Mejia-Saules *et al*., 2006; Darwesh *et al.,* 2018a).

Chitinases (EC 3.2.1.14) having a place with the group of glycosyl hydrolases, catalyze the hydrolysis of chitin, (GlcNAc) residues (Taib, *et al.*, 2005). Chitinases are recorded as Glycosyl Hydrolases (GH) family-18 and GH family-19. Family 19 is commonly profoundly preserved and contains primarily plant chitinases. Family 18 incorporates countless developed chitinases from plants, animals, bacteria and fungi (Zees *et al*., 2005).

Interest for industrial enzymes, especially of microbial inception, is consistently expanding inferable from their applications in a wide assortment of procedures. Among the microorganisms, filamentous fungi are especially fascinating because of their simple cultivation and generation of extracellular enzymes of industrial potential, i.e. textile, animal feed, baking, pulp and paper industries, leather, chemical and biomedical industry, agriculture, food technology, pharmaceuticals, medicine (Falch, 1999; Hasanin *et al.*, 2018; Hasanin *et al.*, 2019), estimation of fungal biomass (Miller *et al*., 1998), mosquito control (Mendonsa *et al.*, 1996) and waste-management industry (Usai, *et al.*, 1987; Darwesh *et al.*, 2019).

Chitinases utilized likewise, in the biocontrol of irritations that assault diverse plantations, causing financial misfortune around the world, diminishing or killing the utilization of pesticides, and limiting the negative effect on nature. What is more, these enzymes can be utilized for the segregation of protoplasts from fungus and yeasts, for the arrangement of single-cell protein, and for the treatment of chitinous waste from the fishery business (Rathore and Gupta 2015). Chitinases produced N-acetyl-D-Gluco-samine as imperative remedial operator in the treatment of osteoarthritis and bioactive chit-oligosaccharides as vital antitumor mediators (Lodhi *et al*., 2014).

Antifungal chitinases have additionally been investigated by Berini *et al.* (2017). Chitinases serve to

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assault fungal pathogens that comprehend chitinous ingredients for self-defense in plants (Singh *et al*., 2007). Accordingly, chitinases are increasing much consideration around the world. Marine fungi turned out to be a rich wellspring of new biologically active ordinary products (Ghanem *et al*., 2010, Gomes *et al*., 2001). The vast majority of these microorganisms cultivate in an interesting and extraordinary environment and in this way they have the ability to deliver exceptional and unordinary secondary metabolites (Fang *et al*., 2005). Shrimp shell waste is tried basically for the generation of bioactive saccharides (Das, Neeraja *et al*., 2012), for example, N-acetylglucosamine which is utilized for the creation of beautifiers and nutritious enhancements (Chen *et al*., 2010).

In Egypt, the citrus business relies upon manufactured fungicides as ordinary repetition for the control of post-gather citrus organic product rots. Loss of viability of few fungicides, expanding good and steady evaluating of buildup limits, is difficulties for the industry. There is a developing need to create elective methodologies for controlling post-harvest rot pathogens. Post-collect rot of citrus organic product brought about by *Penicillium (p.) digitatum* (green fungus), *Penicillium italicum* Whemer (Blue shape) and *Geotrichum candidum* (Bitter rot) have been accounted for everywhere throughout the world and denotes to real losses in production throughout harvest, packing and exportation (Joseph and Korsten, 2003).

This study describes the isolation, identification of fungal strain as well as the production and characterization of an extracellular chitinase under submerged fermentation using shrimp shell wastes as an inducer. Moreover, the antimicrobial potential and in vivo experiment of the chitinase were also evaluated, the efficiency of partially pure enzymes on the mycelial growth of some plant pathogens and their efficacy in the control of citrus molds under storage conditions.

2. Materials and Methods

2.1. Microorganisms

2.1.1. Producer strain

The marine fungal strain *Penicillium chrysogenum* MH 745129 isolated from red sea water and identified by18S rRNA.

2.1.2. Test strains

Penicilium digitatum and *Penicilium italicum* were isolated from rotting citrus fruit and morphology identified according to Simmsons (2007) at Plant Pathology Department, National Research Centre, Giza, Egypt. These isolates were kept on potato dextrose medium and stored at 4°C until used.

2.2. Fungal strain isolation and medium used

Isolation and purification of fungi were carried out using single spore and hyphal tips technique and individually transferred to Glucose peptone medium (GPM) agar slants (Atalla and Nour El-Din, 1993). The medium was composed of (800 mL sea water and 200 mL distilled water): Glucose 1.0 g/L, peptone 0.5 g/L, yeast extract 0.1 g/L, agar 15 g/L, (Jenkins *et al*., 1998**).** The strain was kept and stored at 4°C.

2.3. Molecular identification of fungal isolate

2.3.1. DNA isolation

DNA extraction was done by using the protocol of Gene Jet genomic DNA purification Kit (Thermo# K0791) as follows manufacture of the kit.

2.3.2. PCR amplification and Sequencing

The PCR amplification of 18S rDNA region was carried out following the manufacture of Maxima Hot Start PCR Master Mix (Mix (Thermo) #K0221). The 18srDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify a 1500 bp fragment of the 18SrDNA region. The ITS1–5.8S–ITS2 genomic region was amplified from genomic DNA using the forward primer ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and the reverse primer ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Elshahawy *et al.*, 2018).

The PCR reaction was performed with 10µl of genomic DNA as the template, 1µL of 18SrRNA Forward primer, 1µL of 18SrRNA reverse primer 13 µL Water, nuclease-free and 25 µL Maxima® Hot Start PCR Master Mix (2X) in a 50µL reaction mixture as follows: activation of Taq polymerase at 95 °C for 10 minutes, 35 cycles of initial den. 95°C for 10 min, dent. 95°C for 30 sec, annealing 55°C for 1 min, extension 72°C for 1min, final extension 72°C for 15min. After completion, the PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 mg/mL), to ensure that a fragment of the correct size had been amplified.

The amplification products were purified with K0701 Gene JET™ PCR Purification Kit (Thermo**).** Afterward, the samples became ready for sequencing in ABI Prism 3730XL DNA sequencer and analysis on GATC Company.

2.3.3. Phylogenetic analysis and tree construction

Phylogenetic data were obtained by aligning the nucleotides of different 18S rRNA retrieved from a BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. Phylogenetic and molecular evolutionary analyses were conducted using Mega 6 program (Barakat *et al.,* 2017). All analyses were performed on a bootstrapped data set containing 100 replicates (generated by the program).

2.4. Preparation of sea wastes substrates

Fish and shrimp shells were collected, washed with tap water and dried in oven at 70 °C. The dried shells were ground into fine powder added and mixed by shaking. Shrimp-shell powder and fish shell powder were added separately to the fermentation medium in comparison with chitin powder, sawdust and alginate as substrate.

2.5. Fermentation condition

Fermentation was carried out in 250 mL Erlenmeyer flask each containing 50 ml of fermentation medium consist of (g/L): Different chitin sources, 20.0; Sodium nitrate 2.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01 and autoclaved at 121°C for 15 min. One ml of 10^6 spore suspension of *Penicillium chrysogenum* MH745129 was inoculated in each flask and incubated at 28-30 °C for 7 days at 150 rpm. The enzyme activity was

determined under the standard conditions mentioned in chitinase assay.

2.6. Chitinase assay

Colloidal chitin was prepared from chitin powder (Sigma-Aldrich Corp. St. Louis, MO USA) according to the method described by Reid and Ogryd-Ziak (1981). Determination of enzyme activity was carried out according to the method of Reid and Ogryd-Ziak (1981). Take 1 mL of 1% colloidal chitin in citrate phosphate buffer (pH 6.6) in test tubes, one ml of culture filtrate was added and mixed by shaking. Tubes were incubated in a water bath at 37°C for 60 minutes, then cooled and centrifuged before assaying. Reducing sugars were determined in 1ml of the supernatant by 3, 5-dinitrosalysilic acid (DNS). Optical density was measured at 540 nm.

2.7. Optimization of medium composition on chitinase production

2.7.1. Effect of different chitin sources

The chitinase production was carried out by using different polysaccharides from marine origin: shrimp-shell powder and fish shell powder in comparison with chitin powder, sawdust and alginate. One ml of 10^6 spore suspension of *Penicillium chrysogenum* MH745129 was inoculated in each flask and incubated at 28-30 °C for 7 days at 150 rpm. The enzyme activity was determined under the standard conditions mentioned in chitinase assay.

2.7.2. Effect of different concentrations of shrimp shell

Fifty mL of sterile fermentation media prepared with shrimp shell powder at different concentration ranged from 10 to 50 g/L. The enzyme activity was determined under the standard conditions mentioned in chitinase assay.

2.7.3. Effect of different carbon sources

The effect of additional different carbon sources (glucose, lactose, fructose, xylose, dextrose and sucrose), supplemented in medium at a concentration of 1% was tested for their ability to enhancement of chitinase activity. The inoculated flasks were incubated at 28-30 °C for 7 days at 150 rpm. Chitinase assay was measured as mentioned above.

2.7.4. Effect of nitrogen sources

The influence of using various nitrogen sources on the chitinase production was investigated. Different nitrogen sources such as sodium nitrate, potassium nitrate, ammonium nitrate and yeast were used in comparing with medium free of nitrogen source. The inoculated flasks were incubated at 28±2 °C for 7 days at 150 rpm, and then chitinase assay was detected.

2.7.5. Effect of different incubation period

The production medium was inoculated by the selected strain and incubated at different incubation periods (3, 5 and 7 days). Chitinase activity and mycelium dry weight were determined by filtering the mycelial mat on pre-weighted filter paper (e.g. Whatman No.1) that has been set to dry at 105°C until constant weight and weight it again.

2.8. Characterization of partially purified chitinase enzyme

2.8.1. Partial purification of chitinase

The crude enzyme obtained from *Penicillium chrysogenum* MH745129 culture was precipitated by adding 4:1 cooled acetone to stirred supernatant as described by Darwesh *et al.* (2019). The mixture was centrifuged at 10,000 rpm for 15 min at 4°C and the precipitated proteins were resuspended in the suitable amount of buffer.

2.8.2. The protein content measurement

The protein content was estimated by using the Lowry *et al*. [26] with bovine serum albumin (BSA), (1mg/mL) as standard.

2.8.3. Effect of different pH values, temperatures and reaction time on chitinase activity

The activity of the partially purified chitinase enzyme was measured at different pH values (3.6-7.0). The enzyme activity was determined at different temperatures of the reaction mixture (10-60 °C) and different reaction times (10-60 min).

2.8.4. Determination of thermal temperature stability of chitinase activity

Thermal chitinase stability was determined by incubating the enzyme at different temperature levels ranging 10-70 °C for 10-60 min; residual enzyme activity was measured at slandered conditions.

2.8.5. Determination of kinetic parameters (Km and Vmax) using Lineweaver-Burk plots

The kinetic parameters (*Km* and *Vmax*) were determined for the partially purified chitinase and calculated from Lineweaver –Burk plots (1934).

2.8.6. Polyacrylamide gel electrophoresis

The molecular weight of purified chitinase was estimated using a sodium dodecylsulphate polyacrylamide gel electrophoresis (12%SDS–PAGE) according to Laemmli (1970). After running at 120 V and 40 mA for 45 min, the gel was stained using Coomassie blue-silver. The Precision Plus Protein™ (Bio-Rad®) (10–250 kDa) was used as molecular mass marker (Darwesh *et al*., 2018b).

2.9. In vitro: Antifungal activity of partially purified chitinase

Antifungal activity of the partially purified enzyme obtained from *Penicillium chrysogenum* MH745129 on shrimp shell waste was studied by determining their inhibitory effect on linear growth of *Penicilium (P.) digitatum* and *P. italicum* on PDA medium using the well-plate diffusion method as described by Marrez *et al.* (2019). Pathogenic fungi over laid on PDA plate and after 30 min three wells (5mm. in diameter) were made in each plate and inoculated with 20 µL of partially purified enzyme in contrast with the same volume of water as a control. The plates were kept for 2 h at 4°C then incubated at 28°C for 5 days. Diameter of linear growth and reduction was measured.

2.10. Management of green and blue mold disease of orange and lime fruits during storage

Commercially harvested navel oranges (*Citrus sinensis* L. Osbeck) and Lime (*Citrus aurantifolia* F. Muell), with a healthy appearance from citrus orchards, were used in this experiment. Highly aggressive isolates of *Penicillium digitatum* and *Penicillium italicum* originally isolated from the rotted citrus fruit were used as pathogenic fungi. Isolates were grown on potato dextrose agar (PDA) at 25°C for 7 days. Spore suspension (10^6 spores /mL) was obtained by flooding 7th day old cultures of the pathogen with sterile distilled water containing 0.01% (v/ v) Tween 80 (Zhang, 2013).

The citrus fruits were coated with either partially pure chitinase enzyme. The efficacy of coated citrus and/or lime fruits against mold incidence under stress of artificial infestation was evaluated during storage conditions. Fruits were surface sterilized by dipping them into 1% (v: v) sodium hypochlorite for 3 min. Then, fruits were rinsed 3 times with sterile distilled water and blotted dry on sterile filter paper.

The fruits were wounded by a sterilized needle at one marked point and dipped individually into partially purified enzyme; then the treated fruits were artificially inoculated by spraying with tested fungi (having a mean of 10^6 spores/mL). Thereafter, all treated fruits were air dried, placed into carton boxes (46x23x30 cm) with a capacity of 15 fruits/box, covered with plastic sheets to maintain a relative humidity–RH (90–95%), and stored in a fruit store at 20±2°C for 4 weeks. Five boxes as replicates were used for each treatment as well as the control. Decayed fruits were counted periodically every week. The percentage of total disease incidence was calculated at the end of the storage period.

2.11. Statistical analyses

Statistical analyses were performed with descriptive statistics (mean) and inferential tests (ANOVA followed by Turkey test) to determine statistically significant differences ($P < 0.05$) between treatments with Sigma Stat Software (Neler, *et al*., 1985).

3. Results and Discussion

3.1. Fungal strains isolation and selection the promising isolate

A total of 25 marine fungal isolates were tested for their ability of chitinase production. Out of them, RSW_SEP2 isolate was the most promising and selected for fully identification, production, characterization and biocontrol applications.

3.2. Molecular identification of the isolate

3.2.1. DNA isolation and PCR amplification

The genomic DNA of RSW_SEP2 isolate was applied to PCR using general primers to amplify the ITS1 and ITS4 region between the tiny and great nuclear rDNA, counting the 18S rDNA. These primers amplified a DNA fragment of about 579 bp. The outcome was in accordance with (Freeman, *et al.*, (2000); Rasu, *et al.*, 2012) who denoted that these primers are accurate for fungi and amplified a DNA fragment of about 560 bp using some fungi.

The nucleotide sequence (579 bp) of strain *Penicillium chrysogenum* RSW_SEP2 was blasted with the available Gen Bank resources using NCBI-BLAST search (www.ncbi.nlm.nih.gov/BLAST) to contest the RSW_ SEP2 isolate with those of *Penicillium chrysogenum* strains. The results displayed the great sequence similarity species (99%) with *P. chrysogenum* strains.

3.2.2. Alignment, phylogenetic tree construction and GC%

The phylogenetic tree (Figure 1) showed that strain RSW_SEP2 is greatest intimately correlated to *Penicillium chrysogenum*. Consequently, it was suggested a name of *P. chrysogenum* RSW_SEP2. The percent of Guanidine + Cytosine is individual of various universal features used to identify organism genomes. The G+C content of the genomic DNA was 58% mol% for RSW_SEP2 strain was achieved from the phylogenetic examination. The results were in agreement with those by Storck (1966) and Nakase (1971) who indicated the GC content of fungi ranges from 31.5 to 63% based on each class. The compositional variety also decreases from classes and subclasses to genera and species.

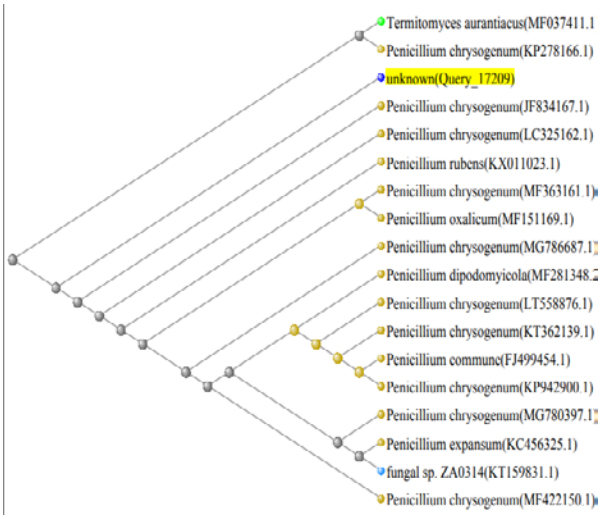

Figure 1. Phylogentic relationships of *Penicillium chrysogenum* isolate and closely related species from the GenBank database, according to 18S rDNA sequence similarity.

3.2.3. Nucleotide sequence ID

The nucleotide sequences of 18S rRNA gene of *Penicillium chrysogenum* RSW_SEP2 has been deposited in GenBank under accession number**:** MH745129.

3.3. Optimization of medium composition on chitinase production

3.3.1. Effect of different sources of chitin on chitinase activity

Determination of chitinase activity was measured using shrimp-shell powder, fish shell powder, chitin powder, sawdust and alginate as different sources of chitin. The results in Figure 2 showed maximum chitinase activity of 71.4 U/mL using shrimp shell powder followed by chitin powder of 60.7 U/mL. These results were coincided with Farag *et al*. (2014) who found that maximum chitinase activity obtained from *A. terreus* in fermentation medium containing shrimp shell powder as a chitin source. In addition, Rattanakit *et al*. (2007) and Krishnaveni and Ragunathan (2014) obtained the maximal chitinase production from fish-scales by *A. terreus* and shell fish

wastes by *Bionectria* CBNR BKRR, respectively. On the other hand, these results were constricted with Maria *et al*. (2009) and Sharmistha *et al*. (2012) who found that chitinolytic fungi produce maximum chitinase activity from colloidal chitin.

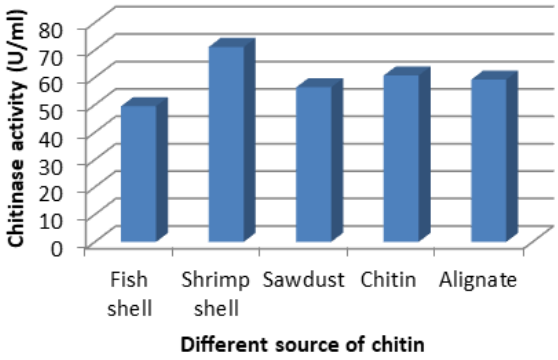

Figure 2. Effect of different source of chitin on chitinase production by *Penicillium chrysogenum* MH 745129.

3.3.2. Effect of different concentrations of shrimp shell powder

Optimization of shrimp shell powder concentration was carried out using different concentration of chitin ranged from (10-50) g/L. The results in Figure 3 showed the highest activity of 76.8 U/mL was achieved at 20 g/L of shrimp shell followed 75.4 U/ml using 30 g/L then the activity gradually decreased. These results were not in harmony with Jesus *et al*. (2006) who found that the 60 g/L of shrimp shell powder gave the maximum chitinase activity from *Serratia marcescens*.

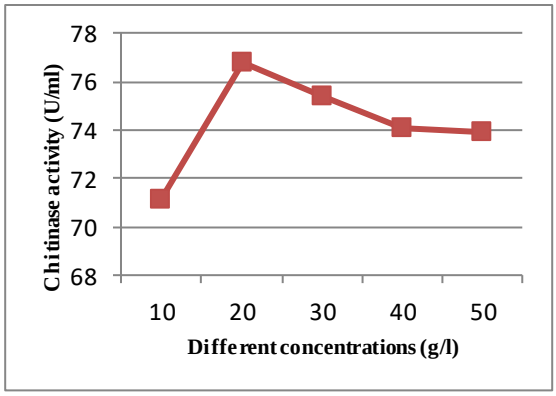

Figure 3. Effect of different concentration of shrimp shell on chitinase activity by *Penicillium chrysogenum* MH 745129.

3.3.3. Effect of different carbon source

The results in Figure 4 indicated that the maximal chitinase activity was 78.2 U/mL in presence of dextrose followed by xylose and fructose. These results were in agreement with several authors such as Sandhya *et al.* (2005); Nawani *et al.* (2002) and Farag *et al*. (2014) they reported that the addition of different sugars to the production medium increased the chitinase activity by different strains as *Trichoderma harzianum* and *A. terreus*. In contrast, Sharmistha *et al*. (2012) found that the addition of maltose, sucrose, xylose, lactose, glucose and fructose decreased chitinase production from *Serratia marcescens*.

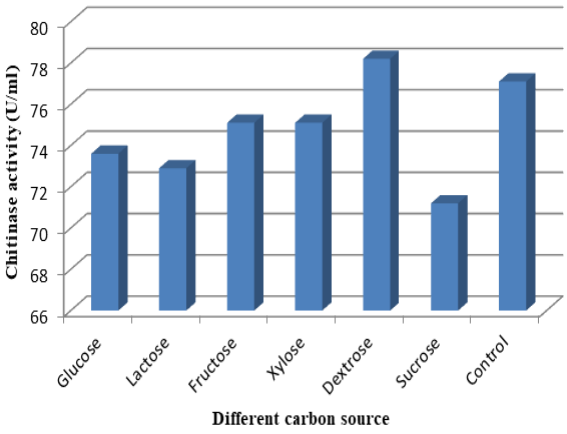

Figure 4. Effect of different carbon source on chitinase by *Penicillium chrysogenum* MH 745129.

3.3.4. Effect of different nitrogen source

The results in Figure 5 showed that the maximal chitinase activity of 92.4 U/mL was obtained from medium containing potassium nitrate using shrimp shell as substrate followed by sodium nitrate and ammonium nitrate.

These results counteracted with several authors who mentioned that 1% of ammonium sulphate were most suitable for chitinase production from *A. terreus* and (Farag *et al*., 2014). Also, the addition of 1% yeast extract increased the chitinase activity by *S. marcescens*, *Alcaligenes xylosoxydans* and *Paenibacillus Sabina* JD2 strains (Ulhoa and Peberdy, 1993 and Sharmistha *et al*., 2012).

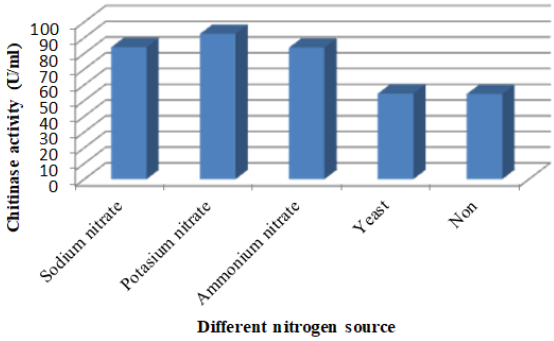

Figure 5. Effect of different nitrogen source on chitinase activity by *Penicillium chrysogenum* MH 745129.

3.3.5. Effect of different incubation period

Effect of different incubation time and mycelium dry weight was evaluated after (3, 5, 7 and 10 days). The results in Table 1 showed that chitinase activity increased gradually till reached its maximum activity of 78.20 U/mL on the fifth day in fermentation medium containing dextran then the enzyme activity gradually decreased by increasing the incubation period. The highest mycelium dry weight was 1.91 g/L at the same time. The outcome of these results was in agreement with Krishnaveni and Ragunathan (2014) who indicated that the maximum chitinase activity from *F. solani* CBNR BKRR was 5 days. On the contrary, the maximum chitinase activity was found 3 and 4 days from *Oidium caricae* and *Tichoderma harziaum*, respectively (Synowiecki and Al- Khateeb, 2003 and Ghanem *et al*., 2011).

Table 1. Effect of different incubation period on chitinase activity by *Penicillium chrysogenum* MH 745129.

Different incubation period (days)	Chitinase activity (U/mL)	Mycelium dry weight (g)
3	40.02	0.88
3D	42.50	0.90
5	76.40	1.49
5D	78.20	1.91
7	52.51	1.51
7D	46.09	1.88
10	42.17	1.23
10D	36.48	1.42

D = with dextran

3.4. Characterization of partially purified chitinase

The crude enzyme obtained from the harvested culture was precipitated by adding to 60% saturation of acetone. The enzyme activity was 50.33 U/mL, the total protein 19.78 U/mg and the specific activity 2.54U/mg protein.

3.4.1. Different reaction temperature

The partially purified chitinase was measured in different temperature (10-60^{o}C) of reaction mixture to select the optimum reaction temperature. The results in Figure 6 showed that the 40 ^{o}C was optimized for chitinase activity. These results were close to that obtained from Ekundayo *et al*. (2016) who found that 50 ^{o}C had the optimal chitinase activity from *T. viride*; while Badiaa *et al*. (2010) found that the optimum reaction temperature for the chitinase produced from *P. rifitoensis* strain M2-26 was at 70^{o}C.

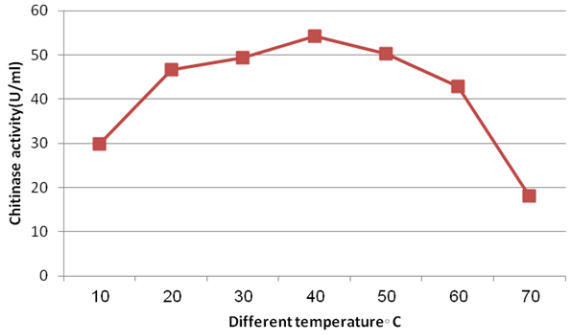

Figure 6. Effect of different temperatures on chitinase activity by *Penicillium chrysogenum* MH 745129.

3.4.2. Different pH values

The influence of different pH values ranging from (3.6-7.0) on chitinase activity was carried out. The results in Figure 7 showed that the enzyme activity increased till reaching its maximum activity of 4.97 U/mL at pH 6.0 then the enzyme activity decreased with the increasing of pH value. These results were almost near to the results that obtained by Hammami *et al*. (2013) who mentioned that the optimum pH value of the partially purified chitinase from *B. Cereus* was pH 6.5. On the contrary, pH value ranged from 4.5-5.0 was the most suitable for chitinase activity from different strains as *T. lanuginosus* SY2 and *T. viride* (Guo *et al*., 2008; Mendana *et al*., 2011 and Ekundayo *et al*., 2016).

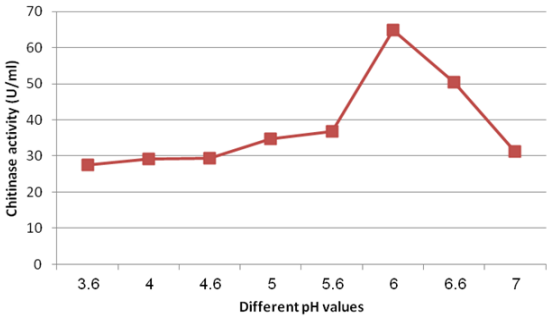

Figure 7. Effect of different pH values on chitinase activity by *Penicillium chrysogenum* MH 745129

3.4.3. Different reaction times

Different reaction mixture time (10 - 60 min) was investigated to select the best reaction time. The maximal chitinase activity of 65.11 U/mL was obtained at 40 min of reaction time as shown in Figure 8. On the other hand, the enzyme activity above and below 40 min activity decreased. These results were closer to those obtained by Farag *et al*. (2014) and Masilamani *et al*. (2012) who found the maximum chitinase activity of *A. terreus* and *Metapenaeus dobsonii* at 30 min.

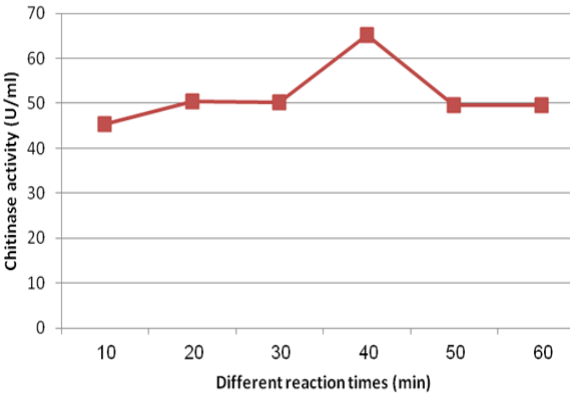

Figure 8. Effect of different reaction times (min) on chitinase activity by *Penicillium chrysogenum* MH 745129.

3.4.4. Thermal stability

The results in Table 2 showed that the enzyme activity was stable at 50 °C temperature for 60 min, and then the chitinase activity decreased with the increasing of the temperature. The obtained results were in agreement with Ekundayo *et al*. (2016) who found that the chitinase activity of *Trichoderma viride* culture was stable at temperature ranged from 40-50 °C. On the other hand, these results were contracted with Nampoothiri *et al*. (2004) and Jenifer *et al*. (2014) who observed that the optimal chitinase activity was 40 °C was optimal the relative chitinase activity.

Table 2. Thermal stability of chitinase from *Penicillium chrysogenum* MH 745129.

Temp °C	Chitinase activity (U/mL)					
min	20	30	40	50	60	70
10	47.87	46.41	46.45	47.45	49.59	44.52
20	46.96	47.09	42.00	49.19	49.66	45.04
30	46.17	47.61	43.60	48.71	49.70	47.66
40	46.10	46.81	46.87	48.56	49.91	42.04
50	46.06	45.89	44.92	48.41	50.53	37.60
60	45.24	44.84	44.21	47.99	49.66	37.27

3.4.5. Determination of kinetic parameters (Km and V max) by Lineweaver –Burk plots

The kinetic characters of the partially purified enzyme using chitin as substrate were determined and calculated from Lineweaver–Burk plots (1934). The estimated K_m and V_{max} values of the chitinase acting on chitin as substrate were 6.26 mg/ml and 68.5 U/mL (Figure 9). The K_m value (6.26 mg/mL) was greater than that obtained for the chitinase from *G. catenulatum* (Ma *et al*., 2012) but lower than those obtained for the chitinases from *T. harzianum* CECT 2413 (3.6 mg/mL) and *Serratia marcescens* B4A (8.3 mg/ml) (De La Cruz *et al*., 1992 ; Zarei *et al*., 2011).

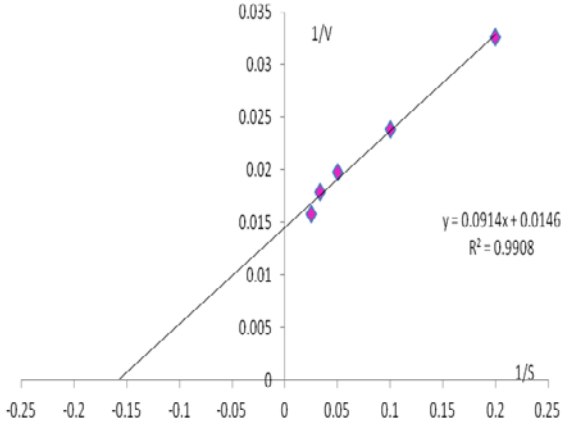

Figure 9. Lineweaver-Burk plot relating *Penicillium chrysogenum* MH745129 chitinase reaction velocity to chitinase concentration.

3.4.6. Determination of chitinase molecular weight

The molecular weight of chitinase was found to be 42 kDa estimated by 12% SDS-PAGE (Figure 10). This value was near to the value estimated for the chitinases produced from *A. niger* LOCK 62 and *A. fumigatus* YJ-407, with a molecular weight of 43 kDa and 46 kDa (Swiontek Brzezinska and Jankiweicz, 2012; Xia *et al.,* 2001). Rattanakit *et al*. (2007) purified 3 chitinases from *Aspergillus* sp. with molecular weights of 45, 51, and 73 kDa. In addition, the diverse chitinases produced by NRRL 2129 strain and *A. terreus* showed molecular weights of 82.7, 44.6, 28.2, 26 kDa and 60 kDa respectively (Farag *et al.*, 2016; Binod *et al*., 2005). Furthermore, this value lay in the range in which diverse molecular weight (27-190 kDa) has been stated for other fungal chitinases (Nagpure and Gupta, 2012; Saraswathi and Ravuri, 2013; Li, 2006).

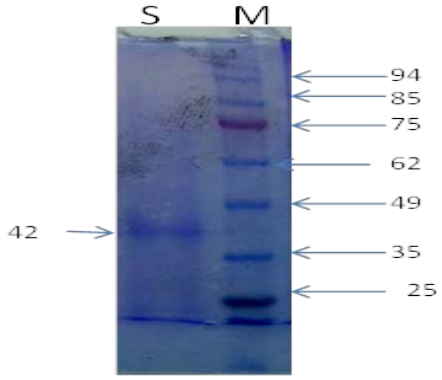

Figure 10. Molecular weight of partially purified chitinase from *Penicillium chrysogenum* MH 745129 by SDS page

3.5. In vitro: antifungal activity of partially purified enzyme on linear growth of P. digitatum and P.italicum

The *in vitro* suppressive effect of partially purified enzyme from marine fungal strain *Penicillium chrysogenum* MH745129 against the linear mycelial growth of *P. digitatum* and *P. italicum* are shown in Table (3). The results indicated that the partially pure enzyme significantly reduced the linear mycelial growth of both *P. digitatum* and *P. italicum* compared with control.

Table 3. Reduction in fungal growth in response to partially pure enzyme on linear growth (mm) and reduction of *P. digitatum* and *P. italicum* in vitro conditions.

	Penicilium digitatum		*Penicilium italicum*	
Treatment	Growth	%Reduction	Growth	%Reduction
Partially pure enzyme	27b	70	25b	72.2
Control	90a	-----	90a	-----

Data in each column with the same letter is not significantly different (*P*=0.05) according to Tukey test (Neter *et al.,* 1985).

3.6. In vivo: protective effect of partially purified enzyme on postharvest diseases of Valencia orange and Lime fruits

The results in Table 4 showed that in vivo the protective effect of partially purified enzyme against postharvest diseases of Valencia Orange and Lime fruits caused by *P. digitatum* and *P. italicum* as disease incidence (%) after 20 days of treatment and inoculation, when the fruit was stored at 20±2 ^{o}C. Results indicated that all treatments significantly reduced postharvest disease incidence (%) of Valencia Orange and Lime fruits compared to the control. After 20 days storage period, 100% of control fruit developed green and blue molds in Valencia orange and Lime fruits.

The deterioration of chitin, an essential piece of the worldwide carbon and nitrogen cycles, depends basically on microbiological cultivations. Chitin can be utilized by microbial populaces as the sole wellspring of these 2 components thought about basic waste, shrimp squander is prepared for animal feed and is likewise utilized in farming as a shoddy normal nitrogen compost.

Table 4. Protective effect of partially pure enzyme against postharvest diseases of Valencia orange and lime fruits.

Treatments	Valencia orange fruits		Lime fruits	
	Green mold	Blue mold	Green mold	Blue mold
Partially pure enzyme	7.0b	10.0b	9.0b	12.0b
Control	100a	100a	100a	100a

Data in each column with the same letter is not significantly different (P=0.05) according to Turkey test (Neter *et al*., 1985).

Moreover, Rattanakit *et al*. (2007) illustrated that *Aspergillus* sp. cultivated on medium containing shrimp waste, blends the equivalent or higher measures of chitinolytic enzymes than when cultivated on medium enhanced with colloidal chitin.

The most vital plant pathogens include: Fusarium, Penicillium, Alternaria, Botrytis, Ramularia, Monilinia, Cladosporium and Aspergillus. These molds assault numerous agricultural plants, including vegetables, fruit, and pretty flowers. So as to ensure the harvests individuals apply distinctive kinds of product assurance synthetic fungicides prepared by chemical combination. There are likewise numerous biotic mediators' fighting fungal pathogens, comprising different bioactive compounds of microbiological source, e.g. chitinases (CHIs). The capacities of microorganisms to deliver antifungal CHIs have been investigated. The enzymes created by the family of storing fungi named *Trichoderma* are of significant biotechnological importance (Benitez *et al*., 2004).

Utilization of arrangements containing this fungus in biological control of fungi improvement is conceivable because of the generation of such enzymes as CHIs or glucanases. The Exo-α-1.3glucanase could tie to cell walls of different phytopathogenic organisms, for example *Aspergillus niger* and *Rhizoctonia solani.* The CHI viably restrains the development of *R. solani, Marchophominia phaseolina, Fusarium* sp (Monteiro *et al*., 2010).

Various investigations showed the likelihood of utilizing them in chitinolytic enzymes production with fungal action against some phytopathogenic fungi. In biological control of fungal phytopathogens, utilization of agents containing different metabolites of microorganisms, including chitinases, has all the earmarks of being the more efficient, since they show more grounded fungicidal action than purified chitinases. The use of agents creating a consortium of chitinolytic producers seems to obtain better results in the fight against phytopathogenic fungi.

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