Full Paper

Anticancer and antifungal efficiencies of purified chitinase produced from Trichoderma viride under submerged fermentation

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Introduction

Chitin, a poly-β-1,4-N-acetylglucosamine is the most abundant polysaccharide found in nature after cellulose (Deshpande, 1986). Chitin is the fundamental structural constituent of the cell wall of most fungi, but is susceptible to innumerable bacterial and fungal species acting as antagonists, due to their synthesizing of chitinases (Sahai and Manocha, 1993). About 75% of the whole weight of shellfish, such as crabs and shrimp are considered as waste, and chitin forms 20–58% of its dry weight (Wang and Chang, 1997). The use of chitinous residues often results in corrosive chemicals resulting in high cost, low product yields and ecological toxicity. So chitinivorous micro-organisms may present a most frugal and eco-friendly approach to treat these chitinous wastes (Singh et al., 2009).

Chitinases are produced by various filamentous fungi involving Trichoderma, Neurospora, Agaricus, Beauveria, Penicillium, Aspergillus, Conidiobolus, Metharhizium, Stachybotrys and Mucor (Duo-Chuan, 2006).

Chitinases can be used in the production of functional chitin-oligosaccharides; in making of single cell protein and in the preparation of fungal protoplasts (Patil et al., 2000). Chitinases are predominately helpful in agriculture as biocontrol agents against fungal phytopathogens be-

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cause they cause lysis of the fungal cell wall and they offer a nontoxic alternative to chemical fungicides (Maisuria et al., 2008).

As normal mammalian cells do not contain chitin, theoretically chitinase treatment will not hurt them. Interestingly, when chitinase was introduced to a culture medium of cultured cancer cells, significant cell surface damage was observed, ultimately leading to cell death (Pan et al., 2005).

The present work reports on the production, purification, and characterization of chitinase from Trichoderma viride, the antitumor efficiency of chitinase against different types of cancer cell line, and its antifungal activity against phytopathogenic fungi.

Materials and Methods

Isolation and screening of chitinase producing fungi. Fungi used in this study were isolated from Mangrove soil of Ras Mohammed protected area at Sharm El-Sheikh, Egypt. The dilution plate technique was employed for the isolation of fungal strains (Palaniswamy et al., 2008). The fungal strains were identified by Assiut University Mycological Centre (AUMC), Egypt. All cultures were recorded in AUMC with their accession numbers. These fungi were cultured on Czapek’s Dox medium for 7 days every four weeks at 30°C. The fungi were subjected to rapid assay of chitinase production by chitin agar plates containing colloidal chitin 10 g/l, (NH₄)₂SO₄ 2 g/l, KH₂PO₄ 0.7 g/l, HgSO₄·7H₂O 0.5 g/l, FeSO₄·7H₂O 0.01 g/l, agar 15 g/l, pH 7.0, and incubated at 30°C for 7 days. Then, the fungus with the highest clear zone was selected for further investigation.

Production of chitinase. Production of chitinase was achieved in 250 ml Erlenmeyer flasks each containing 20 ml of screening medium. Each flask was inoculated with 1 ml of a spore suspension (10⁶ spore/ml) prepared from 5-day old slants of Trichoderma viride AUMC 13021. The flasks were incubated in a shaking incubator (150 rpm) for 4 days at 30°C.

Production of colloidal chitin. Colloidal chitin was obtained from the chitin by the modified method of Hsu and Lockwood (1975), with the following composition: chitin powder (40 g) was slowly added to 600 ml of concentrated HCl and maintained at 30°C for 60 min with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to two liters of water at 4–10°C. The suspension was obtained by filtration with suction on a coarse filter paper and washed by suspending it in about 5 L of distilled water three times.

Crab and shrimp shell powder. Shells of crab and shrimp were gathered, washed several times in warm tap water, then distilled water and air dried in an oven for 24 h at 60°C. After drying, the shells were ground to fine particles.

Optimization of fermentation parameters for maximum production of chitinase by T. viride under submerged fermentation (SMF). The chitinase production parameters were optimized under SmF. These parameters were determined using different sources of chitin; namely, colloidal chitin, shrimp-shell powder, crab-shell powder, chitin powder, chitosan and demineralized chitin, effect of different concentrations of colloidal chitin (0.4–1.8%), incubation period (2–7 days), incubation temperature (20–50°C), initial pH of the medium (5.5–9; pH was adjusted using 1N NaOH or 1N HCl), The effect of shaking velocity (100, 125, 150, 175 and 200 rpm) and the effect of different carbon and nitrogen sources were tested as additives to salt basal medium with 1% (w/v). Starch, glucose, fructose, maltose, lactose and sucrose were used as carbon sources, and beef extract, peptone, yeast extract, urea, malt extract, casein and gelatin were used as nitrogen sources.

Chitinase assay. The activity of chitinase was measured by calculating the amount of liberated reducing sugar (N-acetylglucosamine (NAGA) from the disintegration of colloidal chitin (Thamthiankul et al., 2001). The mixture of reaction containing 1.0 ml 1% (w/v) colloidal chitin (in 0.02 M phosphate buffer, pH 5.5) and 1.0 ml of diluted enzyme solution was incubated for 30 min at 40°C, then 0.5 ml of 3,5-dinitrosalicylic acid (DNS) reagent was added and boiled for 10–15 min, the resulted color was calculated at 530 nm. One unit of chitinase activity is defined as the amount of enzyme that liberates 1 μmol of N-acetylglucosamine under standard assay conditions.

Protein determination. The protein content of the enzyme preparations was measured by the method of Lowry et al. (1951).

Enzyme purification. Enzyme purification was started with precipitation of 400 ml of crude enzyme preparation (CEP) by the gradual addition of (NH₄)₂SO₄ using the range of saturation from 50 to 90%. The precipitated protein was gathered by centrifugation, dissolved in a minimal of 0.02 M phosphate buffer, pH 5.5, loaded on a Sephadex G-100 gel filtration column (1.5 × 45 cm) and eluted with the previous buffer. The fractions recording the highest chitinase activity were gathered, pooled, concentrated and introduced to an ion-exchange chromatography column (1.5 × 45 cm) containing diethyl amino ethyl cellulose (DEAE-cellulose). The proteins were eluted in a stepwise gradient on NaCl (0–1.0 M) at a flow average of 24 ml/h. The fractions reporting the highest activity of purified chitinase were desalted by dialysis, lyophilized and stored at 0°C for further studies.

Molecular weight determination. SDS-PAGE on a 12% w/v acrylamide gel was performed for the determination of the approximate molecular weight in accordance with the procedure of Gooday (1990). The gel was stained with 0.25% Coomassie Brilliant Blue R-250 and destained by washing overnight with a mixture of acetic acid-methyl alcohol-water (5:5:1 v/v).

Determination of kinetic parameters. The optimum pH of the chitinase activity was evaluated by measurements at 30°C in different buffers covering the pH range of 5.0–9.0. These buffer solutions were 0.1 M phosphate, pH 5.0–7.0, 0.1 Tris-HCl, pH 7.0–8.0 and 0.2 M Glycine-NaOH, pH 8.5–9.0. The pH stability of the enzyme was calculated by measuring its residual activity after incubation for different time periods in the buffers with different pH.
values. The optimum temperature of the chitinase activity was determined by incubating the enzyme at various temperatures ranging from 20–60°C in 0.1 M phosphate buffer, pH 6.5. Thermostability of the purified chitinase was calculated after incubating the purified enzyme at different temperatures for different time periods in the same buffer. The enzyme was incubated with gradual concentrations of colloidal chitin, and different kinetic parameters were calculated. Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) of enzyme activity were calculated by linear regression from a Lineweaver-Burk plot (Lineweaver and Burk, 1934). The effect of various cations (i.e. Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Hg$^{2+}$ and Mn$^{2+}$), sodium dodecyl sulphate (SDS), ethylenediamine-tetraacetate (EDTA), phenylmethylsulfonyl fluoride (PMSF) and iodoacetate, on the enzyme activity was tested. The enzyme was preincubated with these components (metal ions were added as chlorides) for 15 min at 1 or 10 mM then the relative enzyme activity was estimated.

**Determination LD$_{50}$ of chitinase enzyme by intraperitoneal injection in Sprague Dawley Rats.** Sprague Dawley rats weighing between (120–150 grams) purchased from the Egyptian organization for biological products and vaccines. The rats were housed in airy plastic cages under a controlled environmental condition cycle (12 h dark, 12 h light at 25°C) in groups of 5 animals per cage, with free access to food and water. The procedure was performed according to Bass et al. (1982). For the determination of the acute lethal dose (LD$_{100}$) and the median lethal dose (LD$_{50}$) of purified chitinase, doses from 10 to 40 mg/kg body weight with an increasing factor of 1.2 were used. Mortality was recorded after 24 hours, and LD$_{50}$ was calculated as follows:

$$\log LD_{50} = \log LD_{next below 50\%} + (\log increasing\ factor \times proportionate\ distance)$$

Proportionate distance $= (50\% – mortality\ below\ 50\%/% mortality\ above\ 50\% – mortality\ next\ below\ 50\%)$.

**Antitumor assay.** The anticancer efficiency of the purified chitinase as an antitumor was measured against: human hepatocellular carcinoma cell line (HEPG2); human breast adenocarcinoma cell line (MCF-7), human colorectal carcinoma cells (HCT-116) and human cervical carcinoma cell line (Hela). Carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, USA). The antiproliferative effect was measured using MTT assay (Renugadevi and Venus, 2012). Dulbecco’s modified Eagle’s medium (DMEM) amended with 1% l-glutamine, HEPES buffer and 50 µg/ml gentamycin was used for cultivation of the cells for 48 h at 37°C in 5% CO$_2$. The formed monolayer of cells was exposed to various concentrations of purified chitinase (2:60 µg/ml) for MCF7 and HCT-116 cell lines and (60:140 µg/ml) for Hela and Hep G2 cell lines, the incubation was resumed for 48 h and the yield of viable cells was measured by a colorimetric method.

Cell viability (%) = (OD of treated cells/OD of control cells × 100).

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Table 1. Effect of various parameters on chitinase production by *Trichoderma viride* under submerged fermentation.

| China source | Collodial chain | Shrimp-shell powder | Chitin powder | Digestion powder | Glucosel | Cellulase | Cargo | Lipase | Xylanase | Cellulase | Cargo | Xylanase |
|--------------|----------------|---------------------|--------------|-----------------|---------|----------|-------|-------|----------|----------|-------|---------|
| (1%) (w/v)   | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) | Specific Enzyme activity (U/mg) |
|--------------|----------------|---------------------|--------------|-----------------|---------|----------|-------|-------|----------|----------|-------|---------|
| 10.14 ± 0.03 | 8.13 ± 0.10    | 6.82 ± 0.01         | 5.32 ± 0.15  | 4.05 ± 0.06     | 3.93 ± 0.17 | 3.42 ± 0.14 | 2.11 ± 0.06 | 1.83 ± 0.04 | 1.53 ± 0.12 | 1.21 ± 0.06 | 0.93 ± 0.08 | 0.72 ± 0.04 |
| 2.13 ± 0.09  | 1.80 ± 0.05    | 1.53 ± 0.15         | 1.22 ± 0.04  | 1.05 ± 0.14     | 0.93 ± 0.08 | 0.83 ± 0.04 | 0.62 ± 0.03 | 0.45 ± 0.02 | 0.39 ± 0.01 | 0.30 ± 0.00 | 0.25 ± 0.00 | 0.20 ± 0.00 |
| 2.82 ± 0.02  | 2.52 ± 0.01    | 2.22 ± 0.00         | 1.92 ± 0.00  | 1.62 ± 0.00     | 1.32 ± 0.00 | 1.02 ± 0.00 | 0.72 ± 0.00 | 0.52 ± 0.00 | 0.40 ± 0.00 | 0.30 ± 0.00 | 0.20 ± 0.00 | 0.15 ± 0.00 |
| 2.82 ± 0.02  | 2.52 ± 0.01    | 2.22 ± 0.00         | 1.92 ± 0.00  | 1.62 ± 0.00     | 1.32 ± 0.00 | 1.02 ± 0.00 | 0.72 ± 0.00 | 0.52 ± 0.00 | 0.40 ± 0.00 | 0.30 ± 0.00 | 0.20 ± 0.00 | 0.15 ± 0.00 |
Anticancer and antifungal efficiencies of *Trichoderma viride* chitinase

| Purification step       | Total activity (U) | Total protein (mg) | Specific activity (U/mg protein) | Yield (%) | Purification fold |
|-------------------------|-------------------|-------------------|----------------------------------|-----------|------------------|
| CEP                     | 18396             | 480               | 38.33                            | 100.0     | 1.00             |
| Ammonium sulphate (65%) | 16654             | 340               | 48.98                            | 90.5      | 1.28             |
| Sephadex G-100          | 14988             | 200               | 74.94                            | 81.5      | 1.96             |
| DEAE-Cellulose          | 13450             | 64                | 210.16                           | 73.1      | 5.48             |

**Fig. 1.** SDS-PAGE of purified chitinase from *T. viride* Lane 1, chitinase sample, Lane M, molecular weight standards (Marker).

**Table 2.** Purification of chitinase from *Trichoderma viride*.

Antifungal efficacy of purified chitinase against phytopathogenic *Fusarium oxysporum f. sp. lycopersici race 3* (the causal agent of tomato wilt). The fungal growth inhibition capacity of the purified chitinase was determined by dual plate assay (Huang et al., 2005). *Fusarium oxysporum f. sp. lycopersici race 3* obtained from Agricultural Research Service Culture Collection through USDA was placed at the center of a PDA plate. Two holes were punched into two opposite corners of plate, pipette with 200 μl purified enzyme (2.13 mg/ml proteins). Plates were incubated at 28°C for 5 days. Following incubation, growth diameter of the fungal pathogen was measured. In the control plate, the two holes were pitted by sterile distilled water. Result was expressed as the mean of the percentages of inhibition of *Fusarium oxysporum f. sp. lycopersici race 3* growth in the existence of the purified chitinase.

Percentage of inhibition = [1 – (fungal growth/control)] × 100.

**Statistical analysis.** The recorded data were analyzed with SPSS version 20, in which the equations of the hypothesis tests, including the mean, standard deviation, and T-statistics value were used. The obtained data were statistically compared with the highest value which is marked as (*). Results were considered highly significant, significant, or non-significant, where *p* ≤ 0.01, *p* ≤ 0.05 and *p* > 0.05, and represented by HS, S and NS, respectively.

**Results**

**Screening of fungal isolates for chitinase producers**

According to colloidal chitin degradation and zone of clearance on chitin agar plates, twenty two fungal isolates were evaluated for their ability to produce chitinase belonged to nine genera: namely, *Aspergillus*, *Cunninghamella*, *Emericella*, *Fusarium*, *Humicola*, *Paecilomyces*, *Penicillium*, *Trichoderma* and *Rhizopus* (data not shown). Based on the maximum chitinase production, *Trichoderma viride* AUMC 13021 was recorded as having the most potential of the investigated fungi, with a maximum zone diameter of (2.8 ± 0.13 cm) and diameter colony of (1.6 ± 0.09 cm). Hence, this isolate was selected for the subsequent experimentation under submerged fermentation (SmF).

**Optimization of chitinase production**

Microbial enzyme biosynthesis is influenced by the prevailing cultural and environmental conditions. In this connection, a series of experiments were carried out to study the effect of those conditions on the enzyme productivity of *Trichoderma viride* AUMC 13021 to determine the maximum production of chitinase under SmF. The productivity of chitinase under various culture conditions and additives were used is shown in Table 1. The maximum enzyme yield was obtained at 1.4% of colloidal chitin, 96 h of incubation, 35°C, pH 6.5 and 125 rpm and using 1% maltose and 1% yeast extract as supplementation of salt basal medium.

**Chitinase purification**

The results of the purification procedure for the chitinase from the crude enzyme preparation (CEP) of *T. viride* are summarized in Table 2. The results showed that partial purification of the crude chitinase was achieved by fractional precipitation with ammonium sulphate using the range of saturation from 50–90%. Among all the fractions, the 65% ammonium sulphate fraction showed the highest protein recovery and highest chitinase recovered activity (90.5%), about 1.28-fold purification. The semipurified chitinase was then injected into a column of Sephadex G-100. The total protein and chitinase activity recovered from the Sephadex G-100 reached about 81.5% with purification of 1.96-fold of the applied sample. The most active fractions (number 14 to 20) from the gel filtration on Sephadex G-100 column were applied to an ion-exchange chromatography column containing diethyl amino ethyl cellulose (DEAE-cellulose). The fractions (18–23) showed the highest specific activity of 210.16 U/mg protein, purification of 5.48-fold and recovery of 73.1%.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The results (Fig. 1) showed the SDS-PAGE electrophoresis of the purified chitinase, indicating the presence of a single protein band. The purified chitinase showed a molecular mass of 62 kDa in gel electrophoresis.

Impact of pH on the enzyme activity and stability

A high chitinase activity was recorded at pH values between 5.0 and 7.5 with a maximum activity at pH 6.5 (Fig. 2A). It retained full activity after one hour of incubation at pH 6.5 and about 66% of the original activity after 30 min was restored at pH 8.0 (Fig. 2B).

Impact of temperature on enzyme activity and stability

Maximum activity of purified *T. viride* chitinase was recorded at 40°C (Fig. 2C). At higher temperatures, a gradual decrease in enzyme activity was monitored. The results showed that the purified enzyme retained about 83% of its activity at 45°C for 60 min and retained about 70% of its original activity at 50°C for 30 min (Fig. 2D).

**\( V_{\text{max}} \) and **\( K_m \)** calculation

\( V_{\text{max}} \) of purified enzyme was calculated to be 90.8 U/ml. The \( K_m \) value was calculated from the Lineweaver-Burk plot of reciprocals of initial velocities and substrate concentrations to be 6.66 mg/ml.

Effect of metal ions and inhibitors on chitinase activity

Metal ions may serve as activators or inhibitors in numerous enzymatically catalyzed reactions. Therefore, the effect of some metal ions on chitinase activity was investigated (Table 3). The results showed that Ca\(^{2+}\) and Mn\(^{2+}\) act as potent activators, where the enzyme activity was significantly increased to 132% and 127%, respectively, of the original activity at 10 mM. On the other hand, the enzyme activity was inhibited in the presence of Hg\(^{2+}\),

| Components                              | Conc. (mM) | Relative enzyme activity (%) |
|-----------------------------------------|------------|------------------------------|
| Control                                 | 0.0        | 100                          |
| Metal ions*                             |            |                              |
| Ca\(^{2+}\)                              | 1          | 100                          |
| Ca\(^{2+}\)                              | 10         | 132                         |
| Co\(^{2+}\)                              | 1          | 88                           |
| Co\(^{2+}\)                              | 10         | 45                           |
| Cu\(^{2+}\)                              | 1          | 82                           |
| Cu\(^{2+}\)                              | 10         | 44                           |
| Mg\(^{2+}\)                              | 1          | 100                          |
| Mg\(^{2+}\)                              | 10         | 119                          |
| Zn\(^{2+}\)                              | 1          | 45                           |
| Zn\(^{2+}\)                              | 10         | 30                           |
| Hg\(^{2+}\)                              | 1          | 70                           |
| Hg\(^{2+}\)                              | 10         | 28                           |
| Mn\(^{2+}\)                              | 1          | 100                          |
| Mn\(^{2+}\)                              | 10         | 127                          |
| Enzyme inhibitors                       |            |                              |
| Sodium dodecyl sulphate (SDS)           | 1          | 86                           |
| Sodium dodecyl sulphate (SDS)           | 10         | 70                           |
| EDTA                                    | 1          | 80                           |
| EDTA                                    | 10         | 65                           |
| PMSF                                    | 1          | 99                           |
| PMSF                                    | 10         | 97                           |
| Iodoacetate                             | 1          | 99                           |
| Iodoacetate                             | 10         | 94                           |

*Metal ions were added as chlorides.
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The impact of various enzyme inhibitors on the activity of *T. viride* chitinase was also evaluated. Purified chitinase was significantly inhibited by sodium dodecyl sulphate and EDTA retaining only about 70% and 65% of its initial activity, respectively. While the activity of enzyme was apparently not influenced by PMSF and iodoacetate.

The injection of doses started from the concentration 10 to 40 mg/kg body weight. The results represented in Table 4 revealed that the median lethal dose (LD50) was approximately 18.43 mg/kg body weight or 3873.16 units/kg body weight for purified chitinase.

**Cytotoxic activity of purified chitinase**

MTT assay showed that the purified chitinase has a high toxic effect to MCF7 with an IC50 value of 20 μg/ml (Fig. 3A) and a moderate cytotoxic effect to HCT-116 cell lines with an IC50 value of 44 μg/ml (Fig. 3B). Moreover, our results showed that there is no inhibitory effect of purified chitinase on the growth of Hela and Hep G2 cells lines (Figs. 3C and D, respectively).

**Medial lethal dose (LD50) of chitinase**

The dual plate method showed that 45% inhibition of *Fusarium oxysporum f. sp. lycopersici* race 3 (the causal agent of tomato wilt)

Microscopic examination of mycelia near the zone of inhibition showed vacuolated and fragmented hyphae (Fig. 4D) compared to mycelia observed on the control plate (Fig. 4C).

**Discussion**

The production of microbial chitinases engaged great awareness in the last few decades, and microorganisms which produce a complex of mycolytic enzymes are con-

| Dose (mg/kg b.wt.) | Number of animals | Survivals | Deaths | % Mortality |
|-------------------|------------------|-----------|--------|-------------|
| 35.83             | 10               | 0         | 10     | 100%        |
| 29.85             | 10               | 2         | 8      | 80%         |
| 24.88             | 10               | 4         | 6      | 60%         |
| 20.73             | 10               | 5         | 5      | 50%         |
| 17.28             | 10               | 6         | 4      | 40%         |
| 14.4              | 10               | 7         | 3      | 30%         |
| 12                | 10               | 9         | 1      | 10%         |
| 10                | 10               | 10        | 0      | 0%          |

*Increasing factor (approx.) = 1.2 * Proportionate distance = 0.45.

![Fig. 3. Cytotoxic activity of purified chitinase of *T. viride* against different lines.](image)
Also the optimum temperature for chitinase production (Sandhya et al., 2004; Wasli et al., 2009, respectively).

The shaking velocity is an important agent impacting enzyme productivity. This may be caused by the mechanical forces that can lead to vacuolation of older hyphal compartments, which may lead to weakened hyphae and/or enhancing hyphal fragmentation (Paul et al., 1994). A similar finding was obtained by Bhattacharya et al. (2016) who recorded that maximum production of chitinase from Bacillus pumilus was at 120 rpm.

Among the various carbon sources which were introduced as an extra complement in the media for maximum chitinase production, maltose exerted the highest increase in the enzyme production in comparison with other carbon sources. Maltose was also found to be the best carbon source for chitinase production by T. harzianum (Sandhya et al., 2004).

Nitrogen sources play a crucial role in the synthesis of enzymes involved in various primary and secondary metabolic pathways (Karthik et al., 2014). Supplementation of the SmF medium of T. viride with various nitrogen sources showed that yeast extract recorded the highest enzyme production. These findings are in line with the work of Nampoorthiri et al. (2004) who recorded yeast extract as the best inducer for the production of chitinase by T. harzianum.

The purified chitinase from T. viride was successfully purified by ammonium sulfate fractionation, gel-filtration, and ion-exchange chromatography with a purification of 5.48-fold and a recovery of 73.1%. A similar result was obtained by Fleuri et al. (2009) who recorded a purification of 6.65-fold for purified chitinase from Cellulosimicrobium cellulans.

The appearance of chitinase as a single band with a molecular mass of 62 kDa ensures the homogeneity and purity of the enzyme. These results are in agreement with previous findings on other chitinase from Cellulosimicrobium cellulans and Aspergillus terreus (Farag et al., 2016; Fleuri et al., 2009, respectively).

The purified chitinase of Trichoderma viride AUMC 13021 showed maximum chitinase production compared with other fungal strains. Similar results were obtained by Duo-Chuan et al. (2004) and Sudhakar and Nagarajan (2011) who recorded that T. roseum and T. harzianum, respectively, were the potent fungi for chitinase production.

Submerged fermentation has been employed predominantly for fungal chitinase production because it has many advantages, such as well-controlled process parameters, increased mass transfer, achievement of an oxygen delivery mechanism, and easy recovery of extracellular enzymes (Stoykov et al., 2015). The production of chitinase under submerged conditions has been recorded by Sandhya et al. (2004) and Fleuri et al. (2009).

Similar results were concluded that the colloidal chitin was the best carbon source for the maximum production of chitinase from T. harzianum (Sandhya et al., 2004). The incubation time has an effect on the production of chitinase, the enzyme productivity increases to a maximum level over a period of time after which it decreases with increasing time. The reason for this decrease in production may be attributed to the consumption of nutrients in the fermentation medium or may be due to the synthesizing of inhibitory products in the medium resulting in the suppression of enzyme secretion or deterioration of the enzyme itself (Karthik et al., 2014). The same incubation period (4 days) was previously reported for the maximum production of chitinase by T. harzianum and T. virens (Sandhya et al., 2004; Wasli et al., 2009, respectively). Also the optimum temperature for chitinase production from T. harzianum was 35°C (Sudhakar and Nagarajan, 2011).

The initial pH of the culture medium is an important agent affecting the enzyme production, since it can affect the fungal growth by influencing the accessibility of medium nutrients. The maximal productivity was recorded at pH 6.5 and this finding was in coincidence with that recorded by many researchers and confirms that production of chitinase occurs in an acidic environment (Wasli et al., 2009; Zhang et al., 2004).

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The purified chitinase of Trichoderma viride was found to have a good activity in a slightly acidic value (pH 6.5) and this optimum pH is equal to that reported for purified chitinase from Thermomyces lanuginosus (Prasad and Palanivelu, 2012).

The purified chitinase from T. viride exhibited a maximum activity at 40°C, and a decreasing activity above this temperature may be explained by the fact that the temperature increases the reaction speed and also affects the rate of enzyme destruction, producing a gradual falling in the concentration of active enzyme. Our results are in complete accordance with the optimum temperature for the most fungal chitinase which range from 25 to 50°C (Karthik et al., 2014; Lee et al., 2009). The Michaelis constant ($K_m$) value of the purified enzyme was calculated to be 6.66 mg/ml and the same value was recorded for chitinase from Bacillus sp. (Cheba et al., 2017).

The purified chitinase was enhanced by Ca$^{2+}$ and Mn$^{2+}$. These results agree with fungal chitinase reported for...
Hydrate parts of these glycoproteins, or glycolipids, are not involved in the binding of substrate to the purified enzyme. Also the slightly inhibitory effect of a reducing agent such as iodoacetate provides evidence for the absence of -SH group in the active sites of enzyme.

The median lethal dose of *T. viride* chitinase was explored by intraperitoneal injection in Sprague Dawley rats. It was about 18.43 mg/kg body weight, so chitinase enzyme is a promising antitumor agent due to mild (LD$_{50}$) compared with median lethal dose of the known antitumor drug paclitaxel which recorded 19.5 mg/kg in rats (Hureaux et al., 2010).

The antitumor studies on the cell line showed that chitinase has an efficiency against MCF7 and HCT-116 cell lines, and no cytotoxic effect against Hela and HepG2 cells. Our results are in agreement with the previous findings of Pan et al. (2005) who reported two different chitinase samples purified from *Streptomyces griseus* and *Serratia marcescens* which caused structural damage to MCF-7 breast cancer cells *in vitro* and selectively lysed tumor cells in mice. This effect may be due to the presence of new polycarbohydrates on the surface of many cancer cells which react with chitinase. After the carbohydrate parts of these glycoproteins, or glycolipids, are cross digested by chitinase, their original function is damaged, and the tumor cells die. As normal mammalian cells do not contain chitin, in theory chitinase treatment will not hurt them (Pan et al., 2005). Moreover, not only breast cancer is affected by chitinase, but also human lung cancer, colon cancer, bladder cancer, melanoma and sarcoma cancer xenografts are all sensitive to chitinase treatment. Only the human prostate cancer line we developed, which grew slowly in SCID mice (Pan, 2012).

Similar results are reported by Viana et al. (2017) who reported that cytotoxicity of chitinase isoforms from *Calotropis procera* latex against HCT-116, OVCAR-8 and SF-295 (glioblastoma) tumor cell lines with IC50 ranging from 1.2–2.9 µg/ml. The real mechanism of the antitumor effect of chitinase is not clear yet, thus future studies are necessary.

This antifungal activity of purified *T. viride* chitinase is largely attributed to the action of purified chitinase in the lysis of the fungal cell wall providing evidence of its potential as a biocontrol agent against phytopathogenic *Fusarium oxysporum* f. sp. *lycopersici* race 3 the causative factor of tomato wilt. The starting interest in examining race 3 is due to the plurality of commercial tomato cultivars which are still sensitive to race 3 (Reis and Bioteux, 2007). Several studies have reported that chitinases produced from different microorganisms can cause deformation and inhibition of viable fungal hyphae (Fadhil et al., 2014). Similar reports observed that the purified endochitinase of *Trichoderma* exhibited antifungal efficiency against *Sclerotium rolfsii*, *Aspergillus flavus* and *Fusarium moniliforme* (El-Katatny et al., 2005). Moreover, the chitinase purified from *A. terreus* inhibited the growth of *A. oryzae*, *Penicillium oxysporum* and *Fusarium solani* (Farag et al., 2016). Other purified chitinases were reported to show an antifungal effect like inhibition of germination of spores and germ tube elongation (Lin et al., 2009). Our results are in good agreement with *Bacillus pumilus* chitinase against *Fusarium oxysporum* and *Rhizoctonia solani* (Agarwal et al., 2017).

**Conclusion**

A locally isolated fungal strain of *T. viride* was used for the production of chitinase in a submerged culture condition. The highest yield of chitinase was obtained (38.33 U/mg) under optimized conditions. The chitinase (62 kDa) was purified to homogeneity with an overall yield of 73.1% and 5.48 purification fold from a cell-free extract by simple procedures of precipitation, gel filtration and ion exchange chromatography. The purified chitinase has a good activity at a slightly acidic value and over a wide range of temperature. Also, the purified chitinase selectively lysed MCF7 and HCT-116 cell lines *in vitro*. Therefore, we can state that chitinase is a promising candidate for cancer therapy. The results of the dual plate assay emphasized that the purified chitinase inhibited *Fusarium oxysporum* through lytic mechanism, so it may be used as a biocontrol agent, as an alternative to harmful chemical pesticides. To the best of our knowledge, this is the first report to determine the median lethal dose (LD$_{50}$) of chitinase.

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