Abedin *et al. Annals of Microbiology* (2023) 73:27
https://doi.org/10.1186/s13213-023-01731-w

Annals of Microbiology

ORIGINAL ARTICLE

Open Access

Production, statistical evaluation and characterization of chitosanase from *Fusarium oxysporum* D18

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Abstract

Purpose The present research work focuses on the extraction of chitosanase enzyme from soil fungi. Chitosan hydrolysis by chitosanase is one of the most effective methods to produce chitosan oligosaccharides which are new biomaterials that have many biological activities such as antitumour, antioxidant, antidiabetic and antimicrobial.

Method A strain producing chitosanase was screened and identified as *Fusarium oxysporum* D18 with an accession number OL343607. Various physiological parameters (incubation type, carbon source, additive nitrogen source, statistical evaluation, solid state fermentation) were assessed to increase chitosanase production.

Results *Fusarium oxysporum* D18 produced a considerable value of chitosanase (1.220 U/ml). After 7 days of incubation, the best carbon source was lactose, and the best nitrogen source was ammonium chloride. Statistical evaluation was carried out by using Plackett–Burman and Box-Behnken designs. The highest chitosanase production (1.994 U/ml) was induced by the medium composition g/l: KH_2PO_4 (1.5), $MgSO_4$ (0.269), lactose (18), NH_4Cl (1.26), pH (6.68), using a 5-day-old inoculum and chitosanase activity was 1.63 folds that of the original medium. The production of chitosanase by *Fusarium oxysporum* D18 in solid state cultures using different solid substrates was studied and the best solid substrate for higher chitosanase activity (2.246 U/ml) was raw shrimp heads and shells and chitosanase activity was 1.13 folds that of the optimized liquid cultures. An extracellular chitosanase was isolated and partially purified by using 75% saturation of ammonium sulphate. The highest chitosanase activity (3.667 U/ml) with a specific activity of 0.390 U/mg protein was obtained at enzyme protein concentration of 9.391 mg/ml, substrate concentration of 1.2 % (w/v), V_{max} of the enzyme of approximately 0.430 U/mg protein, and K_M of 0.26 % (w/v), at pH 5.6 and reaction temperature of 50 °C. The activity of the purified and characterized chitosanase increased by 3 times than that the original isolate activity. The enzyme was thermostable and retained about 55% of its original activity after heating at 70 °C for 15 min. The enzyme preparations were activated by Ca^{2+} ions and inactivated by Zn^{+2}, Cu^{+2} ions, and EDTA.

Conclusion An antitumour activity of chitooligosaccharides produced by the chitosanase was applied to the MCF-7 (breast carcinoma cells) and they had a cytotoxicity inhibitory effect against them about IC_{50} = 448 μg/ml.

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Highlights

(1) We screened and identified a chitosanase producing strain from the soil as Fusarium oxysporum D18 with an accession number OL343607.

(2) The enzyme activity of chitosanase was greatly improved by optimizing the fermentation medium of the fungus.

(3) The activity of chitosanase was optimized by statistical evaluation that was carried out by using Plackett-Burman and Box-Behnken designs.

(4) The production of chitosanase by Fusarium oxysporum D18 in solid state cultures using different solid substrates was studied for increasing activity.

(5) Partial purification of the enzyme was performed by using 75 % saturation of ammonium sulphate.

(6) Physical and chemical properties of the enzyme were performed leading to a highly thermostable chitosanase.

(7) An antitumour chitosanase activity was applied to the MCF-7.

Keywords Chitosanase, Chitosan, Chitooligosaccharides (COS), Solid state fermentation (SSF), Anticancer activity

Background

Chitin is the second most abundant biopolymer on the planet after cellulose. Chitin, known as 2-acetamide-2-deoxy-β-d-glucan, has molecular weights ranging from hundreds of thousands to millions (Abo Elsoud and El Kady 2019). Its sources are very wide, common in insects and invertebrate exoskeletons and crustaceans. Chitosan, a D-glucosamine polymer, whose scientific name is 2-amino-2-deoxy-β-d-glucan, has a relative molecular weight ranging from hundreds of thousands to millions (Beier and Bertillsson 2013). It is present in the cell walls of a limited group of fungi in nature (Muzzarelli et al. 2012). It is usually prepared from chitin by the artificial deacetylation in the presence of alkali. Chitosan and its derivatives showed functional properties making them useful in many fields including food, cosmetics, medicine and pharmaceutical chitooligosaccharides (COS); are composed of 2 to 10 units of D-glucosamines; are easily absorbed in the intestine and quickly get into the blood flow (Chang et al. 2007; Aktuganov et al. 2019).

Conversion of chitosan into COS can be done either by acid or enzyme hydrolysis. Enzymatic hydrolysis has some advantages in producing COS (Cruz et al. 2004). It requires milder reaction conditions (relatively low temperatures and slightly acidic pH) and does not generate harmful by-products or wastes, which makes it safer and more environmentally friendly approach (Suresh 2019).

Chitosanase (EC 3.2.1.132) is an enzyme that catalyses the endo-type cleavage of β-1,4-linkages between D-glucosamine (GlcN) residues in chitosan, producing chitosan oligosaccharides and glucosamine (Weikert et al. 2017; Miguez et al. 2021). COS are new biomaterials that have been reported to have many biological activities such as antitumour, anti-HIV-1, antioxidant, antidiabetic and antimicrobial. They also had a number of potential applications in food, pharmaceutical and agricultural industries (Zhai et al. 2019). Chitosanases have many industrial and biotechnological applications since chitosanase-producing microorganisms have an application in the bioconversion of marine crustacean biomaterials to bioactive molecules such as antitumours and antioxidants (Wang et al. 2010). They can be also used to improve the resistance of plants against different phytopathogenic fungi (Gao et al. 2008). These various applications attract the research focus to improve the COS productivity to meet the industrial requirements.

Chitosanases have been found from many microorganisms, including fungi; although chitosanase has been studied for about 50 years (Choi et al. 2004), only certain microorganisms can produce it. The fungi producing chitosanase include *Aspergillus*, *Penicillium*, *Fusarium*, *Gongronella* and *Mucor*. Recent studies on chitosanases have been dominated by bacterial species belonging to the genera *Bacillus* (Durkin et al. 2009) and *Streptomyces*, and they have been purified and characterized. Few chitosanases have been reported to be produced by some plants (Thadathil and Velappan 2014).

The aim of the present work was the production of chitosanase from the most promising and potent fungus showing high chitosanase activity, identification by 18S-rRNA, statistical optimization, solid state fermentation, purification, characterization and application of chitosanase as an antitumour agent.

Materials and methods

Isolation of chitosanase-producing microorganisms

Seven soil samples were collected from different Egyptian sites for the isolation of chitosanase-producing microorganisms, 0.1 ml of soil suspension was transferred to the petri plates containing the chitosanase-detection agar medium (CDA) composed of (g/l): chitosan (10.0), glucose (10.0), yeast extract (2.0), $(NH_4)_2SO_4$ (2.0), KH_2PO_4

(2.0), $MgSO_4$ (0.24) and agar (15.0). The plates were incubated at 30°C and examined after 7 days of incubation for the appearance of a clear zone around colonies (Wangtueai et al. 2006; Myat et al. 2019).

Identification of the chitosanase-producing microorganism

The promising fungal isolate was selected for identification by 18S-rRNA sequence analysis amplified by polymerase chain reaction (PCR) (Hashem et al. 2018) at Sigma Scientific Services Company, Lebon building- La Cite mall - El Hossary - 6 of October, Cairo-Egypt.

Preparation of the fermentation liquid medium

Fermentation was performed in 250-ml Erlenmeyer flasks containing 50 ml medium (g/l): chitosan (10.00), yeast extract (2.0), KH_2PO_4 (2.0 g) and $MgSO_4$ (0.5). After sterilization, each flask was inoculated with 1 ml of fungal spore suspension and incubated for 7 days at 30°C in the static and shaker incubators. Afterwards, the contents of each flask were taken for analysis.

Assay for chitosanase activity

Chitosanase activity was assayed by measuring the reducing sugars liberated during the hydrolysis of chitosan with a DDA (degree of deacetylation) of 83%. The reaction mixture contained 1 ml of soluble chitosan (1 % (w/v)) and 1 ml of diluted enzyme solution at pH 5.6 using acetate buffer. Then the mixture was incubated at 37°C for 30 min (Chen et al. 2008; Abdel-Aziz et al. 2014).

One enzyme unit was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min. The resulting reducing sugar in the filtrate were measured using the modified dinitrosalicylic acid (DNS) method and were measured spectrophotometrically at 540 nm. The protein assay was determined by the method of Lowry et al. (1951).

Effect of different carbon sources on the production of chitosanase from Fusarium oxysporum D18

Chitosan was substituted by equal carbon amounts (10.0 g/l) of different carbon sources, one at a time (fructose, sucrose, dextrose, lactose, glucose and starch) and keeping other medium constituents at their basal level (Zhang and Zhang 2013; Liaqat et al. 2018).

Effect of different nitrogen sources on the production of chitosanase from Fusarium oxysporum D18

Yeast extract was substituted by potassium nitrate, ammonium sulphate, ammonium chloride and sodium nitrite as inorganic nitrogen sources in an equivalent nitrogen amount to that used in the fermentation medium (2.0 g/l), one at a time, and keeping other medium constituents at their basal level (Liaqat et al. 2018).

Statistical optimization

Statistical optimization with a two-level experimental design was carried out as follows, Plackett–Burman design followed by Box-Behnken design to optimize the variables of the highest effect.

Table 1 The Plackett–Burman experimental design for eight variables

Trial (no.)	Variable							
	A	B	C	D	E	F	G	H
	KH_2PO_4	$MgSO_4$	Lactose	NH_4Cl	pH	Inoculum age	Inoculum size	Medium volume
1	1	−1	1	−1	−1	−1	1	1
2	1	1	−1	1	−1	−1	−1	1
3	−1	1	1	−1	1	−1	−1	−1
4	1	−1	1	1	−1	1	−1	−1
5	1	1	−1	1	1	−1	1	−1
6	1	1	1	−1	1	1	−1	1
7	−1	1	1	1	−1	1	1	−1
8	−1	−1	1	1	1	−1	1	1
9	−1	−1	−1	1	1	1	−1	1
10	1	−1	−1	−1	1	1	1	−1
11	−1	1	−1	−1	−1	1	1	1
12	−1	−1	−1	−1	−1	−1	−1	−1
Control	0	0	0	0	0	0	0	0

Levels: high (+1), low (−1) and basal (0)

Plackett-Burman design

The variables that significantly influence chitosanase production were screened using a fractional factorial Plackett–Burman (PB) design (Plackett and Burman 1946). In this experiment, eight factors were screened in twelve combinations organized according to the Placket-Burman Design matrix as shown in Table 1. Using Microsoft Excel, statistical *t*-vales for equal unpaired samples were calculated for the determination of variable significance.

Box-Behnken design

It was used to obtain the optimum levels of the key factors determined by the Plackett-Burman design. Each factor was examined at three levels, high (+1), low (−1) and basal (0) (Box and Behnken 1960). This design was carried out according to the matrix represented in Table 2 (Liaqat et al. 2018). For predicting the optimum point, a 2nd-order polynomial model was fitted to correlate a relationship between the independent factors and the response (chitosanase production). For the three factors, the used equation is:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_1b_2x_1x_2 + b_1b_3x_1x_3 + b_2b_3x_2x_3 + b_{11}{x_1}^2 + b_{22}{x_2}^2 + b_{33}{x_3}^2$$

where y is the predicted response (chitosanase production); b_0 is the model constant; x_1, x_2, x_3 are the independent factors; b_1, b_2, b_3 are the linear coefficients; b_{12}, b_{13}, b_{23} are the cross-product coefficients; b_{11}, b_{22}, b_{33} are the quadratic coefficients.

The predicted optimum concentrations of the three significant factors were calculated using Microsoft Excel. The optimum value of chitosanase activity was determined by the solver function of Microsoft Excel. Three-dimensional surface plots were established by Statistica software.

Table 2 Box-Behnken matrix for three factors at three levels high (+ 1), low (− 1) and basal (0)

Trial	X1	X2	X3
1	− 1	− 1	0
2	− 1	1	0
3	1	− 1	0
4	1	1	0
5	0	− 1	− 1
6	0	− 1	1
7	0	1	− 1
8	0	1	1
9	− 1	0	− 1
10	1	0	− 1
11	− 1	0	1
12	1	0	1
13	0	0	0
14	0	0	0
15	0	0	0

Production of chitosanase by solid state fermentation (SSF)

Five grams of raw shrimp heads and shells, wheat bran and sugarcane bagasse was transferred to 250-ml Erlenmeyer flasks and moistened with 5 ml of media, then sterilized at 121°C for 15 min. After sterilization, each flask was inoculated with 1 ml of fungal spore suspension for enzyme production and then incubated at 25°C for 7 days.

At the end of the incubation period, the fermentation mass was extracted by the simple method of extraction using distilled water as extracting agent (Shehata and Abd El Aty 2015). The filtrate was used for the estimation of chitosanase activity and protein content. Different types and volumes of moistening agents and different incubation periods were studied.

Fractional precipitation of chitosanase by salting out with ammonium sulphate

The crude chitosanase solution was kept in an ice bath. This was followed by fractional precipitation of the enzyme produced from SSF by salting out with ammonium sulphate (Chasanah et al. 2011), the protein content and chitosanase activity of each fraction were measured.

Physical, chemical and kinetic characterization of chitosanase

Effect of enzyme concentration

Different enzyme concentrations from 3.13 to 12.52 mg/ml were used, then the chitosanase activity was measured (EL-Sayed et al. 2011). Each reaction mixture was incubated at 37°C for 30 min.

Effect of substrate concentration

Different substrate concentrations from 0.4 to 1.6 % (w/v) were used, and then the chitosanase activity was measured (Liang et al. 2016). K_M and $V_{\max}$ of chitosanase were estimated by linear regression technique utilizing Lineweaver–Burk plot according to the equation: (Gooch 2011)

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

where V is the enzyme velocity (activity), K_M is the Michaelis–Menten constant, $V_{\max}$ is the maximum enzyme activity, and S is the substrate concentration.

Effect of the incubation pH

The pH ranges from 5 to 6 of the chitosanase activity were determined by using two buffers (0.2 M of acetate buffer and of phosphate buffer) (Prakash and Gopal 2017).

Effect of the incubation temperature
Enzyme reactions were carried out at different incubation temperatures (25 to 60°C) for 30 min (Kassem et al. 2013).

Thermal stability
The enzyme solutions were pre-incubated for different time intervals (15, 30 and 60 min) at different temperatures (from 40 to 80°C) in the absence of substrate, and then the residual activity was measured (Kassem et al. 2013).

Effect of some activators and inhibitors on chitosanase activity
They were added to each reaction mixture at different concentrations (0.1, 0.01, 0.001 M), and the residual activity was measured and compared to the control. The tested substances were $ZnSO_4$, $CuSO_4$, $CaCl_2$ and EDTA (Prakash and Gopal 2017).

Evaluation of cytotoxic effect of chitosanase on mammalian cell lines: MCF-7 cells (human breast cancer cell line)
According to the procedure described by Mohan et al. (2021), the chitooligosaccharides produced by the partially purified chitosanase were used in this experiment which was performed at the Institute of Graduate Studies and Research, Alexandria, Egypt. Mammalian cell lines were obtained from VACSERA Tissue Culture Unit. The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 1% L-glutamine, HEPES buffer and 50 μg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub-cultured two times a week. Cytotoxicity was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay by seeding the cells in a 96-well plate at a cell concentration of 1×10^4 cells per well in 100 μl of growth medium. A fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial twofold dilutions of the tested chemical compound were added to confluent cell monolayers. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without the test sample and with or without DMSO. After incubation of the cells at 37 °C, various concentrations of the sample were added, and the incubation was continued for 24 h, and viable cell yield was determined by a colorimetric method. In brief, after the end of the incubation period, the media were aspirated and the crystal violet solution (1% (w/v)) was added to each well for at least 30 min. The stain was removed and the plates were rinsed using tap water until all the excess stain was removed. Then, glacial acetic acid (30% (v/v)) was added to all wells and mixed thoroughly, and the absorbance of the plates was measured after gently shaking on a microplate reader (TECAN, Inc.), using a test wavelength of 490 °nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated. The optical density was measured with the microplate reader (SunRise, TECAN, Inc. USA) to determine the number of viable cells and the percentage of viability was calculated as [1 − (ODt / ODc) × 100 % where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relationship between surviving cells and drug concentration is plotted to get the survival curve of each tumour cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each conc. using Graphpad Prism software (San Diego, CA. USA).

Results

Isolation and screening of chitosanase-producing fungi
Seven chitosanase-producing fungi were isolated. The most potent chitosan-degrading fungus which formed the largest clear zone on a CDA plate was selected and identified as *Fusarium oxysporum* D18 and the fungal strain nucleotide sequences were deposited in GenBank as *Fusarium oxysporum* D18 with accession number OL343607. Construction of the phylogenetic tree was done using the maximum likelihood method as shown in Fig. 1.

Studying the effect of some physiological factors on the chitosanase produced by Fusarium oxysporum D18
Under static incubation condition, the highest chitosanase activity (1.165U/ml) was obtained from *Fusarium oxysporum* D18 as compared to that obtained under shaken conditions after 7 days of incubation and the activity decreased gradually reaching the lowest activity after 11 days as shown in Table 3. Each treatment was carried out in triplicate, and the results obtained throughout the work were the arithmetic mean of at least 2 experiments.

As shown in Table 3, the enzyme activity of the culture filtrate was differentially affected by the nature of

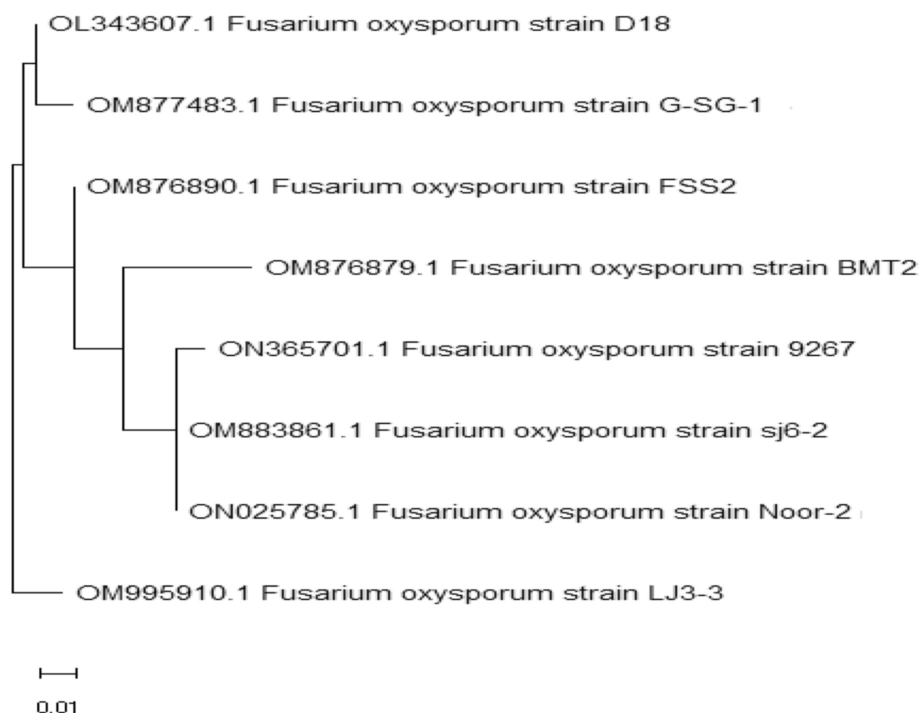

Fig. 1 Phylogenetic tree showing the relationship between *Fusarium oxysporum* D18 and the closely related strains

Table 3 Effect of different incubation periods, carbon and nitrogen sources on chitosanase production by *Fusarium oxysporum* D18

Variable	Dry weight (g)	Final pH	Protein content (mg /ml)	Chitosanase activity (U/ ml)
Incubation period (days)				
3	0.09	7	1.770	0.657
5	0.16	7	2.144	0.941
7	0.19	7	2.256	1.165
9	0.14	6	2.230	1.072
11	0.06	5.5	1.924	0.830
Carbon source				
Fructose	**0.07**	6.5	1.270	0.825
Sucrose	**0.10**	6	2.396	1.127
Dextrose	**0.06**	6	0.496	0.390
Lactose	**0.12**	6.5	2.428	1.191
Glucose	**0.09**	6	1.096	0.960
Starch	**0.06**	6	0.640	0.365
Chitosan	**0.10**	7	2.256	1.160
Nitrogen source				
Yeast extract	0.11	6	2.428	1.060
KNO_3	0.14	6	1.128	0.928
$(NH_4)_2SO_4$	0.25	6	2.23	1.133
NH_4Cl	0.07	4	2.574	1.220
$NaNO_2$	0.05	6	1.124	0.472

the carbon source. The most preferable carbon source for *Fusarium oxysporum* D18 was lactose, producing high chitosanase activity (1.191 U/ml) with maximum protein content (2.428 mg/ml), followed by chitosan.

Also, by testing of different nitrogen sources on the chitosanase production, it was found that ammonium chloride was the most preferable nitrogen source, yielding maximum chitosanase activity (1.220 U/ml) with protein content (2.574 mg/ml) followed by ammonium sulphate yielding chitosanase activity (1.133 U/ml) as shown also in Table 3.

Statistical optimization of the physicochemical parameters influencing chitosanase production by Fusarium oxysporum D18

Plackett-Burman design

All experiments were performed in duplicates and the average results of chitosanase activity were measured. The main effect of each variable on enzyme production was estimated and presented graphically in Fig. 2.

According to these suggestions, it can be predicted that the optimum medium for producing an extracellular chitosanase from the culture of *Fusarium oxysporum* D18 with a relatively high activity was (g/l): KH_2PO_4 (1.5), $MgSO_4$ (0.25), lactose (13), NH_4Cl (1.26), pH (7), using a 5-day-old inoculum, inoculum size (60 ml/l) and medium volume (35 ml).

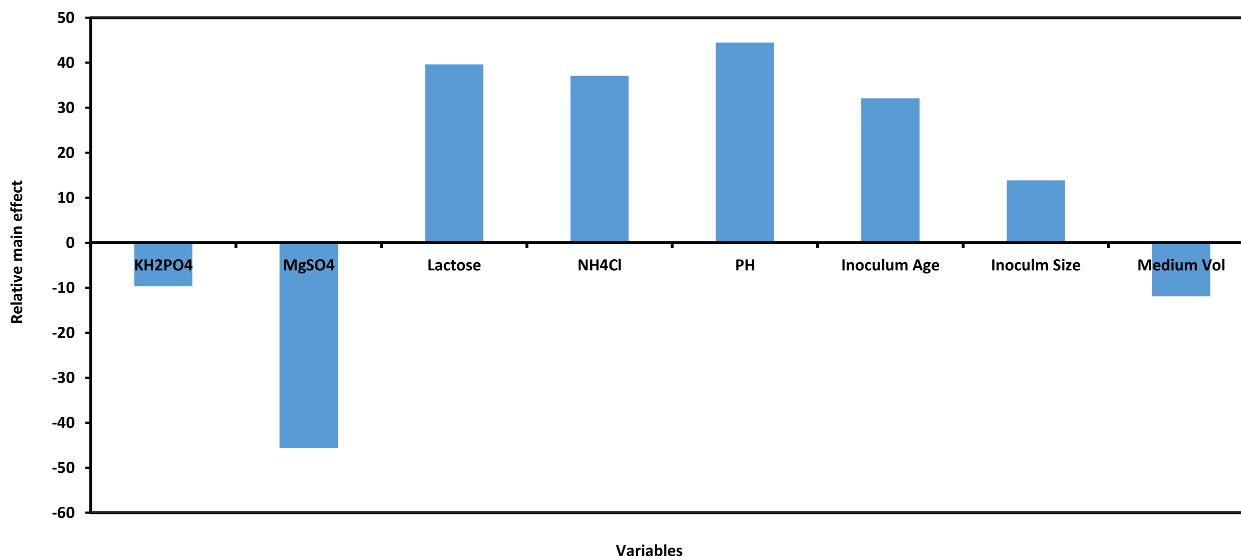

Fig. 2 The main effect of independent variables on the production of chitosanase by *Fusarium oxysporum* D18

On the basis of statistical analysis, the three variables evidencing the most significant effects on chitosanase production were $MgSO_4$ (t-value= 1.733), pH (t-value =1.678) and lactose (t-value =1.454).

Box-Behnken design

In this experiment, the three significant factors, $MgSO_4$, lactose and pH, were examined at three levels, which were high (+1), low (−1) and basal (0) included 15 trials. In all of these trials, non-significant factors were used at their near optimum levels, obtained from the Plackett-Burman design. The results were illustrated in surface plots in Fig. 3 to clarify the interaction between the three significant factors and chitosanase activity (response). The optimum levels of the three tested factors that were predicted to give the highest chitosanase activity were as follows (g/l): $MgSO_4$ (0.269), lactose (18) and pH (6.68).

Production of chitosanase by solid state fermentation (SSF)

The effect of different substrates on chitosanase production is shown in Fig. 4. The highest chitosanase activity (1.723 U/ml) was obtained when raw shrimp heads and shells were used, but wheat bran gave the lowest activity (0.283 U/ml).

By optimizing the (SSF) process for production of the enzyme, the highest chitosanase activity (2.246 U/ml) was obtained when raw shrimp heads and shells were moistened with 8 ml of the optimized medium after 5 days of incubation with (1.13 folds) that of the optimized liquid cultures as illustrated in Table 4.

Purification of chitosanase produced by Fusarium oxysporum D18 by salting out with ammonium sulphate

An extracellular chitosanase was isolated and purified from SSF medium of *Fusarium oxysporum* D18 by ammonium sulphate saturation. The results in Table 5 indicate that fractional precipitation with ammonium sulphate brought about 15.13% protein recoveries.

The recovered protein showed its peak value at the fraction precipitated with 75% ammonium sulphate saturation. This fraction has the highest protein content (7.83 mg/ml) represented about 28.57 % of the total recovered protein. The chitosanase activity of the fraction precipitated with different ammonium sulphate saturations increased gradually up to the 75% fraction, where the maximum activity (3.44 U/ml) was obtained. The activity of this fraction was 26.72 % of the total recovered activity. Higher or lower ammonium sulphate saturation showed lower chitosanase activity. The total recovered activity obtained by ammonium sulphate fractions was 9.69 % of the activity present in the crude enzyme solution.

Physical, chemical and kinetic characterization of chitosanase

Influence of enzyme protein concentration on chitosanase was assayed toward a parallel relationship that occurred between the enzyme concentration and chitosanase; the maximum specific activity of the chitosanase (0.374 U/mg protein) was obtained at an enzyme protein concentration of 9.391 mg/ml. Lower protein concentrations showed a decrease in enzyme activity.

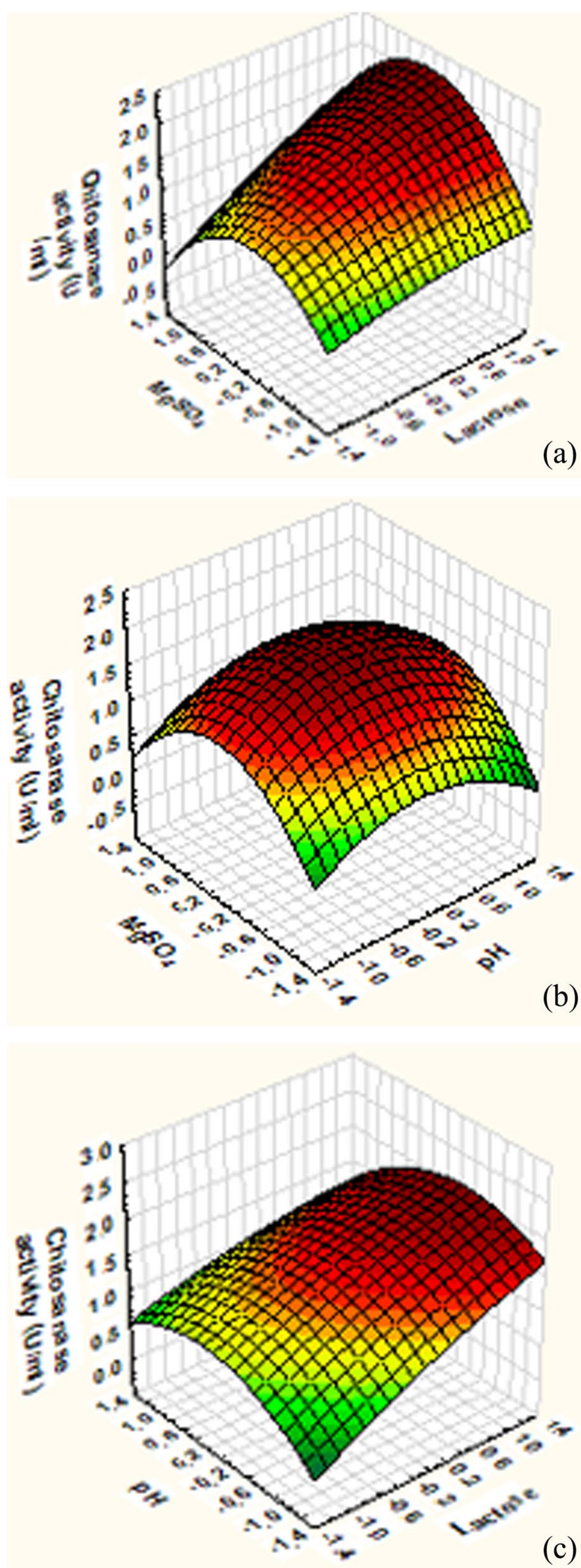

Fig. 3 Response surface plots elucidating interaction between critical factors influencing chitosanase production by *Fusarium oxysporum* D18

Substrate (chitosan) concentration dependence of the enzyme activity was also characterized with a parallel relationship as shown in Fig. 5 which represents the Michaelis-Menten non-linear regression curve. The maximum activity was obtained at a substrate concentration of 1.2 % (w/v) and the chitosanase activity at that point reached 3.498 U/ml with a specific activity of 0.372 U/mg protein. Further increases in substrate concentration yielded a slightly lower enzyme activity. The maximum value of enzymatic activity (V_{max}) was approximately 0.430 U/mg protein as extrapolated by the Lineweaver–Burk plot. The value of K_M is the substrate concentration required to attain half of the maximum enzyme velocity. The value of K_M thus obtained was 0.26 % (w/v). Smaller K_M value is a representative of powerful affinity towards substrate. If an enzyme has a small value of K_M, it achieves its maximum catalytic efficiency at low substrate concentrations. Hence, the smaller the value of K_M, the more efficient is the catalyst.

Influence of pH on the chitosanase stability was assayed toward soluble chitosan under limitedly narrow range of values (pH 5–6). As expected, the optimum pH value of the reaction was 5.6 at which the maximal enzyme activity was recorded (0.366 U/mg protein) with relative chitosanase activity (100%). Above or below this pH value, the activity of the partially purified enzyme was gradually decreased.

Temperature dependence of the enzyme activity was characterized with pronounced optimum activity (0.390 U/mg protein) at 50°C after salting out with ammonium sulphate. A further increase in temperature resulted in a gradual decrease in activity.

The thermostability of the partially purified chitosanase was studied; the results indicated that the chitosanase was stable over a wide range of temperatures. The stability of it in the absence of chitosan depends on the temperature of heating and the time of exposure. The enzyme became more activated when exposed to 50°C for 15 and 30 min, and this activity increased by 39.27 and 20.49%, respectively, more than that of the control. The enzyme was nearly not affected by increasing the temperature of heat treatment to 60°C for 15 min, but it retained about 27% of its original activity after 60 min. The enzyme was thermostable and retained about 55 and 30% of its original activity after heating at 70°C for 15 and 30 min of exposure, respectively.

The partially purified chitosanase was assayed in presence of some metal ions (activators and inhibitors). The results indicated that the enzyme was activated in the presence of Ca^{+2}. While the results showed that Zn^{+2}, Cu^{+2} ions and EDTA inhibited chitosanase activity. 0.001 M of $CaCl_2$ slightly increased the activity by about 8%,

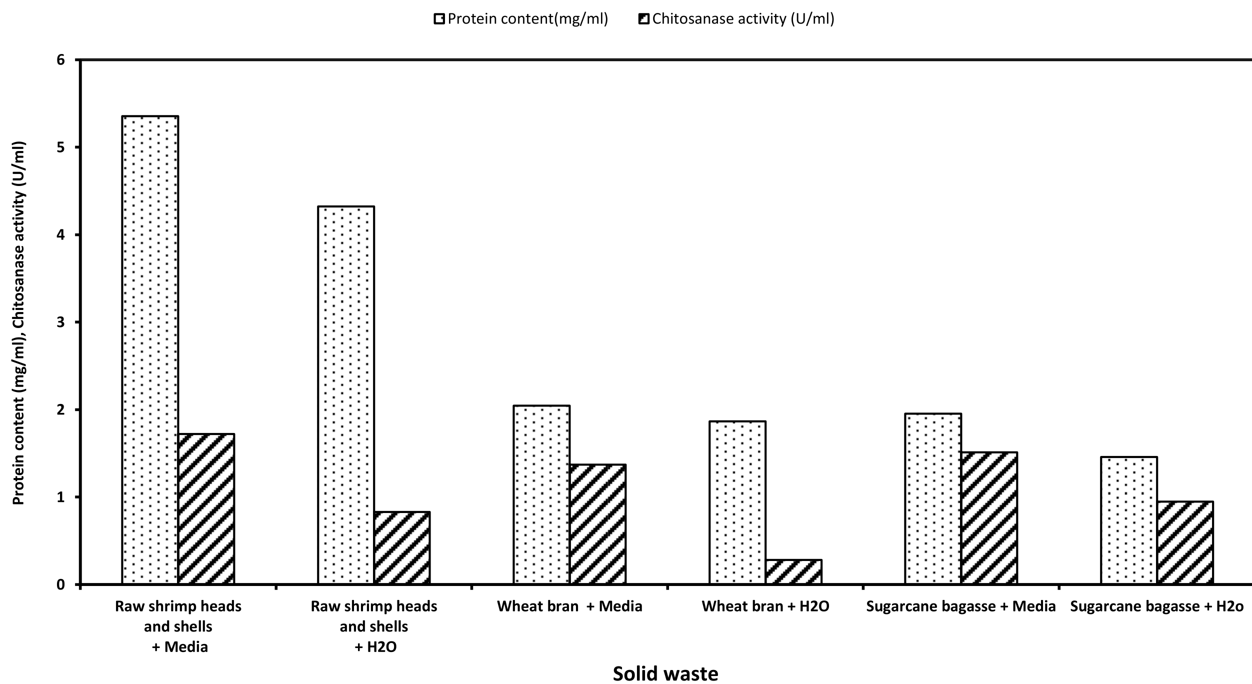

Fig. 4 Experimental results of the (SSF) for chitosanase activity produced by *Fusarium oxysporum* D18

Table 4 Effect of moisture content on chitosanase produced by *Fusarium oxysporum* D18 using raw shrimp head and shell solid waste

Moistening agent	Volume of moistening agent (ml)	Final pH	Protein content (mg /ml)	Chitosanase activity (U/ml)
Optimized medium	2	7	3.088	0.948
	5	8	5.354	1.723
	8	9	5.732	1.985
	11	9	4.968	1.814
Distilled water	2	7	2.410	0.404
	5	8	4.322	0.829
	8	8	3.446	0.768
	11	9	3.096	0.506

Table 5 Fractional precipitation of chitosanase using different saturations of ammonium sulphate

Precipitating agent	Concentration (%)	Final pH	Protein content (mg / ml)	Total protein content (mg/ fraction)	Recovered protein (%)	Chitosanase activity (U / ml)	Specific activity (U/ mg protein)	Total activity (U/ fraction)	Recovered activity (%)
Control	-	6	2.715	543	100	1.990	0.733	398	100
Ammonium sulphate	25	6	1.926	5.778	1.064	0.627	0.326	1.881	0.473
	35	6	2.436	7.308	1.346	1.139	0.468	3.417	0.859
	50	6	3.586	10.758	1.981	1.910	0.533	5.73	1.440
	65	6	6.100	18.300	3.370	2.766	0.453	8.298	2.085
	75	6	7.826	23.478	4.324	3.436	0.439	10.308	2.590
	85	6	5.518	16.554	3.049	2.982	0.540	8.946	2.248
Total				82.176	15.134			38.850	9.693

and by increasing the concentration to 0.01 and 0.1 M, it activated the enzyme by 20.50 and 28.70%, respectively. 0.001 M of $CuSO_4$ brought about 5% increase in the enzyme activity, and increasing the concentration to 0.01 and 0.1 M resulted in a decrease in the activity, reaching 5.74 and 17.06%, respectively.

On the other hand, 0.01 M of $ZnSO_4$ reduced the relative activity by about 35.85%, and 0.01 M of EDTA brought about 80% inhibition of the enzyme activity, while 0.1 M of EDTA totally inhibited the enzyme activity.

Evaluation of the cytotoxic effects of the partially purified chitosanase on mammalian cell lines: MCF-7 cells (human breast cancer cell line)

In this experiment, the chitooligosaccharides, which are produced by the partially purified chitosanase, were used to estimate an antitumour activity against mammalian cell lines and MCF-7 cells (human breast cancer cell line) and they had the highest cytotoxicity inhibitory effect against them at concentration of about IC_{50} = 448 μg/ml. Where IC_{50} is the concentration required to cause toxic effects in 50% of intact mammalian cells, it was estimated from graphic plots of the dose-response curve for each conc. At this concentration, the cells had the lowest viability (30.21%).

Discussion

On studying the effect of some physiological factors on the chitosanase produced by *Fusarium oxysporum* D18, the highest activity of chitosanase produced by *Fusarium oxysporum* D18 (1.165U/ml) was obtained after 7 days of static incubation. This result was in agreement with Shehata and Abd El Aty (2015), who reported that the optimum incubation period for the chitosanase production by *Chaetomium globosum* KM651986, *Aspergillus fumigatus* and *Aspergillus terreus* were 7 days at 28°C.

In contrast, it was demonstrated by Singh and Vidyasaga (2017) that colloidal chitosan was the best inducer for chitosanase production by *Aspergillus fumigatus*. However, Abdel-Aziz et al. (2014) showed that molasses is the best C-source for chitosanase activity by *Mucor rouxii* and biomass yield followed by chitosan.

The majority of microorganisms produced chitosanase by induction (Pagnoncelli et al. 2010; Hosny et al. 2016), but some microorganisms constitutively produced chitosanase without chitosan. Our strain used in this work also produced the enzyme in the absence of chitosan as an inducer and this characteristic has advantages at the fermentation stage because chitosan increases the viscosity of the culture medium and makes production costs high (Choi et al. 2004).

These results were different from those obtained by Singh and Vidyasaga (2017) who reported that yeast extract was the optimal nitrogen source for *Aspergillus fumigatus*.

Statistical evaluation of the physicochemical parameters influencing chitosanase production by *Fusarium oxysporum* D18 was carried out by using Plackett-Burman and Box-Behnken designs.

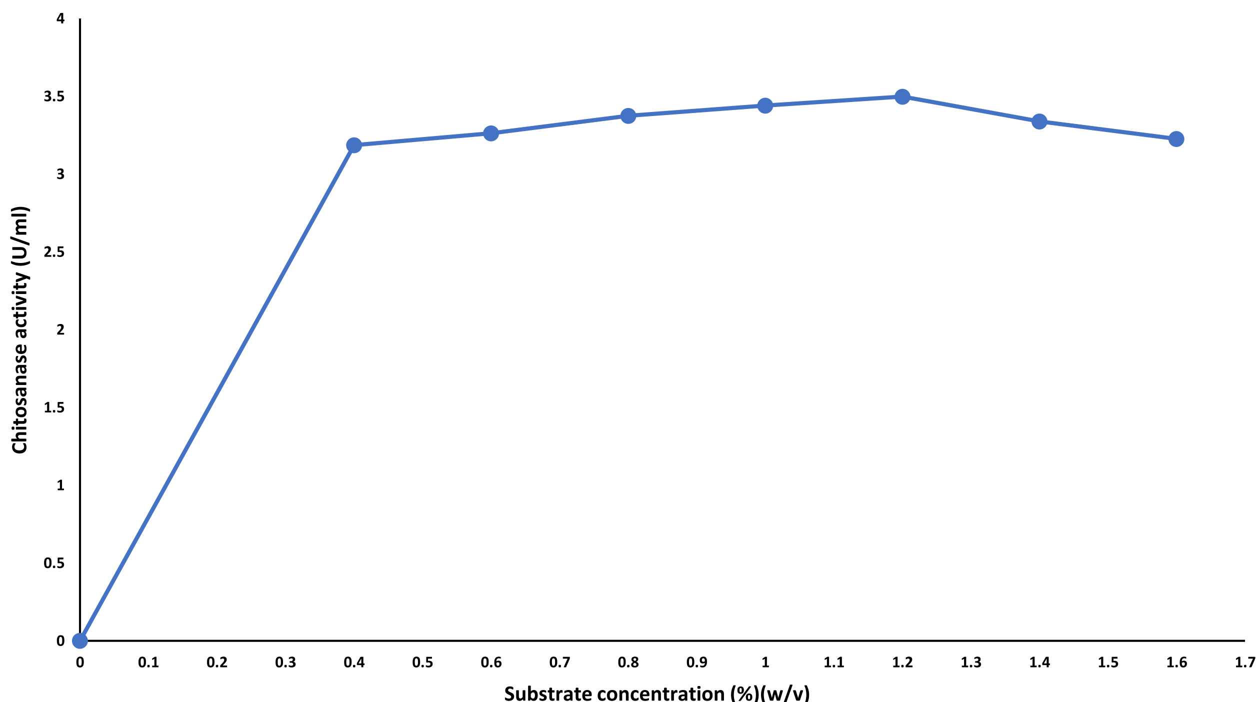

Fig. 5 Michaelis–Menten non-linear regression curve for chitosanase produced by Fusarium oxysporum D18

By applying Plackett-Burman design, the most significant factors affecting chitosanase production were $MgSO_4$, pH and lactose. It was conducted that the highest chitosanase production (1.994 U/ml) was induced by the medium composition g/L: KH_2PO_4 (1.5), $MgSO_4$ (0.269), lactose (18), NH_4Cl (1.26), pH (6.68), using a 5-day-old inoculum. These results were in accordance with those obtained by Zhang and Zhang (2013), who found that chitosanase production by *Aspergillus fumigatus* YT-1 was significantly affected by $MgSO_4$. Zhang et al. (2012) reported that pH factor had a significant effect on chitosanase production by *Aspergillus* sp. QD-2.

On production of chitosanase by (SSF), the results obtained in this manuscript were in agreement with those results previously noted by Nidheesh et al. (2015), who reported that *Purouroeocillium lilacinum* CFRNT12 produced chitosanase with an activity of 2.34 U/g IDS (initial dry substrate) under SSF by the use of solid shrimp by-product as a substrate. On the other hand, wheat bran was chosen by Xu et al. (2021) as a supplementary carbon source for high chitosanase production by *Streptomyces*.

Purification of chitosanase produced by *Fusarium oxysporum* D18 was performed by salting out with ammonium sulphate; the crude extract of chitosanase produced from SSF medium of *Fusarium oxysporum* D18 was precipitated with finely powdered 75% ammonium sulphate saturation.

El-Sayed et al. (2012) reported that using acetone for precipitation of chitosanase revealed unsuitability as precipitating agent due to the poor yield obtained relative to the crude enzyme. Hence, the crude enzyme was precipitated by using ammonium sulphate with different saturations. Also, chitosanase produced by *Gongronella butleri* was partially purified by ammonium sulphate precipitation (Seki et al. 2019). Pagnoncelli et al. (2010) found that the crude chitosanases hydrolyzed soluble chitosan into biofunctional oligomers (COS) and the use of crude enzymes from *Paenibacillus ehimensis* B-23118 instead purified ones were of industrial interest because enzyme purification steps were expensive.

Upon studying of some physical, chemical and kinetic characterization of chitosanase, it was found that the highest activity of chitosanase produced by *Fusarium oxysporum* D18 was observed at a substrate concentration of (1.2 % (w/v)). The same result was obtained by Sarni and Dali (2016), who reported that the optimum concentration of the substrate required for maximum chitosanase activity produced by *Klebsiella* sp. was 1.0 % (w/v). Cheng and Li (2000); Aktuganov et al. (2003); Chen et al. (2005) reported that the optimum concentration of the substrate required for maximum chitosanase activity produced by *Aspergillus* sp., *Bacillus* sp. 739 and *Aspergillus* sp. CJ22-326, respectively, was from 0.5 to 1.0 % (v/v). K_M (0.26% (w/v)) and $V_{\max}$ (4.04 U/ml) of chitosanase were estimated by linear regression technique utilizing Lineweaver–Burk method (Gooch 2011). Cao et al. (2022a, b) found that K_M and $V_{\max}$ of the recombinant chitosanase (*Po*CSN75A) were 0.27 mg/ml and 4.36 U/ml respectively. The K_M and $V_{\max}$ of the chitosanase (Csn-SH) were 0.50 mg/mL and 140.05 μmol mg^{-1} min^{-1}, respectively (Cui et al. 2021).

In this manuscript, chitosanase showed the highest activity at pH 5.6. This result showed an agreement with Jiang et al. (2021) who reported that the recombinant chitosanase (PbCsn8) was most active at pH 5.5. Also, Hosny et al. (2016) reported that the optimum pH of the chitosanase from *Dothideomycetes* sp. css035 and *Aspergillus fumigatus* KB-1 using acetate buffer was pH 5.5. Also, Cao et al. (2022a, b) found that pH 5.5 was optimum for chitosanase produced by *Penicillium oxalicum* M2. In contrast, Guo et al. (2022) reported that the optimum pH that produced the highest activity of the recombinant chitosanase (SaCsn46A) was 6.2.

The influence of temperature on chitosanase was also examined and the result obtained in this study was in agreement with the study carried out by El-Sherbiny (2011) who reported that the activity of the partially purified chitosanase from *Chaetomium globosum* KM651986 and *Streptomyces cyaneogriseus* was found optimal at 50°C and at lower or higher temperatures, the activities were reduced. Chiang et al. (2003) found that the optimum activity for the purified chitosanase produced from *Bacillus* sp. was at 45 °C. *Kitasatospora setae* KM-6054 chitosanase which was cloned in *E. coli* had the highest activity at 60 °C (Xu et al. 2023). Also, recombinant chitosanase (CsnS) showed its highest activity at 60°C (Zheng et al. 2021).

Thermostability of chitosanase produced by *Fusarium oxysporum* D18 was examined. The enzyme was thermostable and retained about 55% of its original activity after heating at 70°C for 15 min.

These results indicated that chitosanase had a thermal stability and agree with the results obtained by El-Sherbiny (2011) who reported that the purified chitosanase produced by *Streptomyces cyaneogriseus* was stable at 40 and 50°C when it was incubated without substrate for 30–90 min, and gradually inactivated at 60 and 70°C and significantly lost activity above 70°C. Han et al. (2018) reported that the engineered chitosanase (CsnA) from *Renibacterium* sp. QD1 retained about 40% activity after being kept at 60°C for 60 min. The recombinant chitosanase (CsnQ) could retain 77.72 and 71.79% of the original activity after 60 min of incubation at 20 and 30 °C, respectively. The residual activity of purified CsnQ

reduced dramatically after 60 min of incubation at temperatures above 30 °C (Ma et al. 2020).

The activity of the partially purified chitosanase was tested in presence of some metal ions. The enzyme preparations were activated by Ca^{2+} ions and inactivated by Zn^{+2}, Cu^{+2} ions and EDTA.

The results of this study showed an agreement with those obtained by Chasanah et al. (2011) who reported that the chitosanase produced by *Aeromonas media* KLU 11.16 was activated by the presence of Ca^{+2} and was inhibited by $Zn^{2\,+}$, Cu^{2+} and EDTA (concentration of 1mM of EDTA decreased significantly (100%) the chitosanase activity). In contrast, Aktuganov et al. (2022) found that chitosanase produced by *Bacillus Thuringiensis* B-387 was slightly suppressed by Ca^{+2}. Cheng and Li (2000) reported that the purified chitosanase of *Aspergillus* sp. was greatly enhanced by Cu^{+} ions at low concentrations.

This result differed from that obtained by Liang et al. (2016) who reported that the activity of chitosanase produced by *Bacillus mycoides* was nearly unaffected by Ca^{2+}, but agreed with them when they reported that the enzyme was inhibited by Cu^{+}.

An antitumour activity of chitooligosaccharides produced by chitosanase was applied to the MCF-7 cells (human breast cancer cell line), and they had a cytotoxicity inhibitory effect against them at concentration about $IC_{50} = 448$ μg/ml, where IC_{50} is the concentration required to cause toxic effects in 50% of intact mammalian cells. At this concentration, the cells had the lowest viability (30.21%).

Other results were obtained by Hashem et al. (2018) who reported that the chitooligosaccharides produced by chitosan hydrolysis using chitosanase obtained from *Dothideomycetes* sp. NRC-SSW had a stronger cytotoxic activity against human liver cancer cells (Hep-G2) in comparison to breast cancer cell line (MCF7) as the IC_{50} of Hep-G2 was 12 μg/ml while the IC_{50} of MCF-7 was 85.5 μg/ml. Also, Chokradjaroen et al. (2018) reported that chitooligosaccharides have a cytotoxic effect against human uterine cervix cancer cell line (HeLa cells), human breast adenocarcinoma cell line (MCF-7 cells) and human lung cancer cell line (H460 cells). The IC_{50} of chitooligosaccharides against cancer cells HeLa, MCF-7 and H460 were approximately 2.3, 2.0 and 4.1 mg/ml, respectively. Chitooligosaccharides can easily permeate the cancer cells. Chitooligosaccharides derivatives greatly limit the formation of new blood vessels in tumours, thereby blocking tumour progression and metastasis. It has the ability to decrease free radicals in normal cells, which are implicated in cellular damage (Pavan et al. 2022).

Conclusion

In this study, a chitosanase-producing fungus, *Fusarium oxysporum* D18, was isolated from soil, and it showed high chitosanase activity after optimization by statistical evaluation using Plackett-Burman and Box-Behnken designs of different physical and chemical factors. Physical and chemical properties of the partially purified enzyme were performed leading to a highly thermostable antitumour chitosanase with high cytotoxicity against mammalian cell lines: MCF-7 cells.

Acknowledgements
The financial support received from the Botany and Microbiology Department, Faculty of Science, Alexandria University is gratefully acknowledged.

Human and animal rights
No human subjects or livestock were included in this research.

Informed consent
The scientists certify that this study adhered to ethical and professional standards.

Authors' contributions
Prof. Dr. RM.A.A: participate in the development of the research plan, statistical experiments, statistical tables, figures, preparation, writing and revision of the manuscript. Dr. AE.AE-S: supervising the student in preparing and conducting laboratory and statistical experiments, statistical tables, figures, preparation and revision of the manuscript. DR.M.AE: conducting laboratory and statistical experiments, writing and revision of the thesis.

Funding
Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB).

Availability of data and materials
Available from the Botany and Microbiology Department.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 8 May 2023 Accepted: 17 July 2023
Published online: 19 August 2023

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