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Improved production and partial characterization of chitosanase from a newly isolated *Chaetomium globosum* KM651986 and its application for chitosan oligosaccharides

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ABSTRACT

Chaetomium globosum KM651986 was selected as the best chitosanase producing fungus. It was identified on the basis of morphological and molecular taxonomy and got the Genbank accession number KM651986. The fungus showed the highest chitosanase activity of 540.51±0.47U/gds in the wheat bran solid state fermentation medium supplemented with 1% chitosan. The important factors affecting chitosanase production were statistically optimized using Response Surface Methodology. The optimal fermentation conditions increased the chitosanase activity up to 1758.75 U/gds, an approximate 3.25-fold improvement as compared to the previous yield with un-optimized medium. Partial purification of chitosanase by using ammonium sulphate (30-70 %) precipitation showed high yield 72.73% and purification fold 4.2. The partially purified enzyme was optimally active at pH 5.0 acetate buffer and temperature at 50°C. The enzyme presented moderate thermal stability. The K_m and V_{max} of chitosanase were calculated to be 0.24 mg/ml and 40µmol/min, respectively. The activity of chitosanase was markedly enhanced by Na^{+2}, Mn^{2+}, mercaptoethanol and EDTA and slightly inhibited by Fe^{3+}, K^{+}, Ag^{+} and Ba^{2+}. The crude enzyme was used to hydrolyze the soluble chitosan and the TLC result showed that the chitosan oligosaccharides obtained from the hydrolysis mainly dimers to hexamers. The obtained chitosan oligosaccharides showed valuable biological applications as antioxidant and antimicrobial activities. This is the first report on the statistical optimization and characterization of Chaetomium globosum KM651986 chitosanase enzyme and its applications for chitosan oligosaccharides.

Key words: Chitosanases, *Chaetomium globosum* KM651986, optimization**,** Chitosan oligosaccharides, Antioxidant, Antimicrobial.

INTRODUCTION

Chitosanases (EC 3.2.1.132) are produced by many micro-organisms, including bacteria, actinomycetes and fungi**.** They are generally endo-splitting enzyme and could hydrolyze the β-(1→4) glycosidic bonds of chitosan into chitosan oligosaccharides and glucosamine **[1]**. Many efforts are being made to found a new source for production of this important enzyme.

Chitosan is a polysaccharide of β-1,4-linked glucosamine (GlcN) residues, partially substituted with *N*-acetyl groups. Applications of chitosan have been developed in industries, such as food production and pharmaceutical industry **[2].** Studies on chitosan have attracted interest for oligosaccharide conversion because oligosaccharides are not only water-soluble but also possess versatile functional properties such as inhibiting the growth of fungi and bacteria, activating immune responses and exerting antitumour activity **[3-6]**. It has been reported that oligomers of lower molecular weight exhibit better biological activities than that of chitosan **[7].**

Enzymatic processes to produce chitosan oligosaccharides is popular by right of low production cost, low environmental impact and high reproducibility **[8,9]**, and its ideal alternative to traditional chemical degradation which needs acid hydrolysis and results in low yields of oligosaccharides.

Chitosanases are still unavailable in bulk quantities for commercial applications because of low enzymes productivity by chitosanolytic strains **[10].** Therefore, this problem could be resolved by isolating new chitosanolytic strains with high chitosanase productivity, optimizing fermentation conditions for chitosanase production and reducing chitosanase production cost. Consequently, using cheaper natural substrates such as, wheat bran for chitosanase production is popular, as this approach not only improve chitosanase productivity, but also reduce chitosanase production cost. The use of agricultural residues in microbial fermentations is being encouraged due to their ease of availability and inexpensiveness **[11].**

The objective of this study was to optimize the production of chitosanase from a newly isolated *Chaetomium globosum* KM651986 by using of response surface methodology (RSM). Furthermore characterization of partially purified chitosanase, the products of enzymatic hydrolysis of chitosan were identified and studied. Evaluation of antioxidant and antimicrobial activity of chitosan oligosaccharides was also achieved.

EXPERIMENTAL SECTION

2.1. Chemicals

Chitosan (75-85% deacetylated, low molecular weight) was purchased from Sigma-Aldrich (MO, USA). Chitosan oligomers (G1-G6) were purchased from Wako chemical (Oska, Japan). Other reagents were of analytical grade.

2.2. Isolation and identification of fungi

Different shrimp shell wastes were collected in sterile clean plastic bags. They were washed using sterile distilled water and blotted between two folds of sterilized filter paper and transferred to Czapek's Dox agar plates supplemented with antibiotic to prevent the bacterial growth. The plates were incubated at 28°C for up to 10 days, during which the developing fungi were isolated. All fungal isolates were maintained at 4 °C on potato dextrose agar (PDA) slants **[12]** in microbiology laboratory, chemistry of natural and microbial products department, NRC.

The pure fungal isolate was identified in the National Research Centre, Chemistry of Natural and Microbial Products Dept., Microbial Culture Collection Unit (MCCU) based on the morphological characterization according to its colonial and microscopic properties comparing with fungal species described by **[13-16].**

Molecular identification of the selected fungal isolate based on their internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Mycelia were collected by centrifugation and DNA was extracted by using protocol of Gene Jet Plant genomic DNA purification Kit (Thermo # K0791). PCR made by using Maxima Hot Start PCR Master Mix (Thermo# K0221), and PCR clean up to the PCR product made by using GeneJET™ PCR Purification Kit (Thermo# K0701) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). Finally, sequencing to the PCR product performed in GATC Company, German by use ABI 3730xl DNA sequencer, using forward and reverse primers, and by combining the traditional Sanger technology with the new 454 technology.

Purified DNA was subjected to PCR amplification using a pair of ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers for ITS-rDNA amplification **[17].** Sequence data was analyzed in the Gene Bank database by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared to all of the sequences in the database to assess the DNA similarities **[18].**

The nucleotide sequence of the fungal isolate determined in this study has been deposited in the GenBank under the accession number (KM651986).

2.3. Inoculum preparation

Inoculum was prepared by incubating the culture on PDA slants at 28 ◦C for about 7 days, until sufficient sporulation was observed. The spores were harvested using 10 ml sterile distilled water. One ml of the spore suspension containing about 1×10^7 spores/ml was used as inoculum.

2.4. Fermentation media for chitosanase production

Two different fermentation media were screened for the best chitosanase production.

2.4.1. Medium I (SMF)

Modified Czapek's-Dox medium (MD) containing (1%) soluble chitosan as carbon source, with some modifications composed of (g/L): 2.0 $NaNO_3$, 5.0 yeast extract, 1.0 KH_2PO_4 , 0.5 $MgSO_4.7\ H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$, initial pH 5.5 **[19].** This medium used as synthetic submerged fermentation medium (SMF).

The fermentation medium was dispensed in 250 ml conical flasks, each containing 50 ml. The submerged flasks were inoculated with 1ml spore suspension of each tested fungal species and incubated for 7 days at 28°C under static and shaking conditions (using shaker incubator, 200 rpm).

2.4.2. Medium II (SSF)

Solid state fermentation of wheat bran alone and another supplemented with (1%) soluble chitosan were carried out in Erlenmeyer flasks (250 ml) with 2g of the solid substrate, moistened with 10 ml distilled water. Each flask was covered with hydrophobic cotton and autoclaved at 121 ◦C for 20 min. After cooling, each flask was inoculated with 1ml spore suspension and incubated at 28 °C for 7 days in a static mode.

When fermentation was completed, 50ml of distilled water were added to the fermented matter, and the mixture was mixed thoroughly on a rotary shaker (200 rpm) at room temperature (28 ± 2 ◦C) for 30 min. The mixtures were filtered through muslin cloth. After centrifugation of the filtrate at 4 ◦C for 10 min, the supernatant was collected as the crude enzyme solution **[20].**

2.5. Statistical optimization of chitosanase enzyme

Response surface methodology of central composite design (CCD) used for statistical optimization of *Chaetomium globosum* KM651986 chitosanase enzyme.

Table (1): Central composite design (CCD) consisting of 20 experiments for three experimental factors in coded and actual values for the production of *Chaetomium globosum* KM651986 chitosanase enzyme

Trial number	Factor levels							
	Chitosan conc. (A, %)		Incubation temp. (B, °C)		Incubation period (C, day)		Chitosanase activity (U/gds)	
	Coded	Actual	Coded	Actual	Coded	Actual	Observed	predicted
1[c]	0	1.50	0	31.50	0	9.00	1758.75	1756.43
2	+1	2.00	+1	35.00	-1	7.00	745.82	602.68
3	+1	2.00	+1	35.00	+1	11.00	498.25	469.47
4	0	1.50	- 1.682	25.61	0	9.00	516.46	325.14
5[c]	0	1.50	0	31.50	0	9.00	1755.85	1756.43
6	-1	1.00	+1	35.00	-1	7.00	568.05	481.11
7	- 1.682	0.66	0	31.50	0	9.00	805.40	836.74
8	0	1.50	0	31.50	- 1.682	5.64	643.02	704.99
9[c]	0	1.50	0	31.50	0	9.00	1756.55	1756.43
10	-1	1.00	-1	28.00	0	9.00	272.38	398.44
11[c]	0	1.50	0	31.50	0	9.00	1755.34	1756.43
12	+1	2.00	-1	28.00	+1	11.00	332.17	402.02
13	0	1.50	0	31.50	+1.682	12.36	506.55	468.74
14	+1.682	2.34	+1	35.00	+1	11.00	689.25	571.87
15	0	1.50	+1.682	37.39	0	9.00	111.67	327.14
16[c]	0	1.50	0	31.50	0	9.00	1757.66	1756.43
17[c]	0	1.50	0	31.50	0	9.00	1758.55	1756.43
18	+1	2.00	-1	28.00	-1	7.00	673.44	773.74
19	-1	1.00	-1	28.00	-1	7.00	534.49	546.19
20	-1	1.00	0	31.50	+1	11.00	949.17	941.99

[C] *Central points.*

The 3–factor–5–level central composite design (CCD) with twenty experiments were carried out to determine the optimal values of chitosan concentration (A), incubation temperature (B) and incubation period (C) and to develop a mathematical correlation between the three important variables and chitosanase activity (Y). All three variables were investigated at low level (-1), zero level (0) and high level (+1), respectively, with α = 1.682. Codes and actual values of variables and matrix of CCD along with chitosanase activity of each trial are shown in **Table (1)**. Statistical analysis of CCD is shown in **Table (2).** The behavior of the system was explained by the following quadratic model equation.

$$Y_{(activity)} = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

where $Y_{(activity)}$ was the predicted production of chitosanase (U/gds), $\beta 0$ intercept, $\beta 1$, $\beta 2$ and $\beta 3$ linear coefficients, $\beta 11$, $\beta 22$ and $\beta 33$ quadratic coefficients and $\beta 12$, $\beta 13$ and $\beta 23$ interactive coefficients. A, B and C were the

independent variables corresponding to the concentration of Chitosan, incubation temperature and incubation period respectively. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) and the quadratic models were represented as contour plots (3D) using Design-Expert® 8 software from Stat-Ease, Inc. **[21].**

2.6. Preparation of soluble chitosan

The 1% soluble chitosan was prepared by dissolving one gram of chitosan in 40 ml of deionized water and 9 ml of 1.0 M acetic acid. The solution was stirred for 2 h and the pH was adjusted to 6.0 with 1.0 M sodium acetate. This solution was finally made up to 100 ml by adding 0.05 M acetate buffer pH 6.0 **[22].**

Table (2): Analysis of variance (ANOVA) for response surface quadratic model

Term	Coefficient Estimate	Standard Error	F-value	P-value Prob>F
Intercept	1756.43	52.07	44.17	Model < 0.0001significant
A-Chitosan conc.	31.29	34.54	0.82	0.3864
B- Incubation temp.	0.59	34.54	2.956	0.9866
C- Incubation period	-70.24	34.54	4.13	0.0694
AB	-26.50	45.13	0.34	0.5702
AC	-55.99	45.13	1.54	0.2431
BC	59.63	45.13	1.75	0.2159
A^2	-306.55	33.63	83.10	0.0001
B^2	-505.68	33.63	226.13	0.0001
C^2	-413.50	33.63	151.20	0.0001

R-Squared = 0.9755, Adj R-Squared = 0.9534, Pred R-Squared =0.8111, Adeq Precision=15.856, Std. Dev.= 127.66, Mean= 919.44, C.V. %= 13.88.

2.7. Assay of chitosanase activity

Low molecular weight chitosan was used as the substrate in the chitoanase assay. The enzyme activity was determined by adding 0.5 ml of 1% soluble chitosan and 0.5 ml of enzyme .The incubation was carried out at 50 °C for 60 min. Then the sample was boiled for 10 min to stop the reaction and analyzed its reducing sugar content. The amount of reducing sugar was measured using the modified dinitrosalicyclic acid (DNS) method **[23].** One enzyme unit was defined as the amount of enzyme required to produce 1μmol of reducing sugar with glucosamine as the standard per min.

2.8. Protein determination

Protein content was determined by the method of Bradford **[24]** using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard.

2.9. Partial purification of chitosanase enzyme

Solid ammonium sulphate was added to the culture filtrate at different concentration 0-30, 30-70 and 70-100 % saturation. The precipitate was collected by centrifugation and dissolved in 0.1 M sodium acetate buffer pH 5. The enzyme solution was dialyzed against distilled water.

2.10. Physicochemical properties of partially purified chitosanase

2.10.1. Effect of temperature on chitosanase activity

The maximum activity of chitosanase was determined at different temperature (30 – 70 ^oC).

2.10.2. Effect of pH on chitosanase activity

Partially purified enzyme was assayed with acetate buffer (0.1 M, pH 3.2-5.8).

2.10.3. Thermal stability

The maximum activity of enzyme was determined after heated at different temperatures for varying time intervals. Temperatures, of 30, 40, 50 and 60 ^oC were applied, each for time intervals of 15, 30, 45 and 60 min. The remaining enzyme activity was then assayed.

2.10.4. Substrate specificity

Substrate specificity of the enzyme was determined by using different substrates, each substrate was dissolved in the suitable buffer.

2.10.5. Effect of activators and inhibitors on enzyme activity

This effect was done by preincubation of different metals with chitosanase at concentration of 1.0 mM for 30 min at 9^oC before determination of enzyme activity. EDTA and mercaptoethanol were also tested.

2.10.6. Determination of Michaelis' constant (K_m)
The K_m value of the partially purified enzyme was determined according to the method of Lineweaver and Burk **[25].**

2.11. Hydrolytic analysis by thin layer chromatography (TLC)
Products analysis using low molecular weight chitosan with chitosnase enzyme at various time intervals were separated by TLC on silica gel plates (Whatmann, GF-254) using n-propanol : water : concentrated ammonia 7:2:1 (v: v : v) as solvent. Spots were visualized by charring with 10% H_2SO_4 in ethanol and each spot was identified by comparison with chitosan oligosaccharides (G1-G6).

2.12. 1,1–diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity assay
DPPH assay was carried out as described by Amarowicz et al. **[26].** A total of 0.5 mg of chitosan oligosaccharides was adjusted to a volume of 4.0 ml, and then 0.5 ml of 1mM methanolic solution of DPPH was added to the sample solution. The contents were stirred vigorously for 15 seconds and then left to stand at room temperature for 30 min. Decrease in colorization was measured spectrophotometrically at 517 nm.

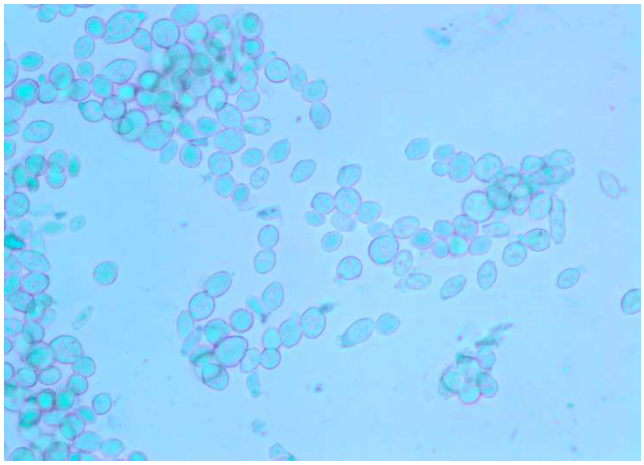
The antioxidant activity was calculated using the equation shown below: Antioxidant activity = [(absorbance of control – absorbance of test sample)/absorbance of control] x 100.

2.13. *In-vitro* antimicrobial activity of chitosan oligosaccharide
The chitosan oligosaccharides were tested against a panel of gram positive and gram negative bacterial pathogens, and fungi. Antimicrobial tests were carried out by the agar well diffusion method **[27]** using 100 mL of suspension containing 1 $x10^8$ CFU/mL of pathological tested bacteria and 1 x 10^6 CFU/mL of fungi spread on nutrient agar and Sabourand dextrose agar respectively. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 200 µL of chitosan oligosaccharides. The inoculated plates were then incubated for 24 h at 37 ºC for bacteria and 48 h at 28 ºC for fungi. After incubation time, antimicrobial activity was evaluated by measuring the zone of inhibition of the tested microorganisms.

RESULTS AND DISCUSSION

3.1. Identification of fungal isolates.
Five different fungal isolates were isolated from shrimp shell wastes collected in Egypt, and identified morphologically as *Chaetomium globosum*, *Aspergillus fumigatus*, *Aspergillus parasiticus*, *Aspergillus niger* and *Aspergillus terreus.* The best fungus for chitosanase production was further studied and characterized taxonomically by molecular and conventional methods. Data derived from morphology, and phylogenetic analyses identified this fungus as *Chaetomium globosum* (GenBank Accession number KM651986) with 93% similarity **Fig. (1).**

A



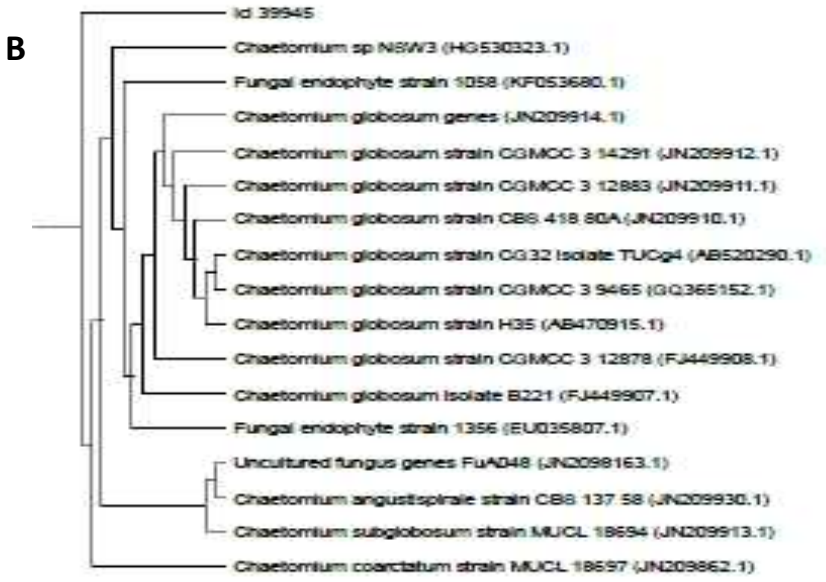


Fig. (1): A, Photo of *Chaetomiumglobosum* KM651986ascosporesunder microscope.B, Phylogenetic tree based on 18S rRNA gene sequences showing closest relatives. GenBank sequence accession numbers are indicated in parentheses after the strain names

3.2. Potentiality of production of chitosanase among tested fungi

The first experiment was performed to evaluate the best conditions for production of chitosanase enzyme and the best producing strain. Five fungal isolates belonging to two genera were examined to investigate their capacity to produce chitosanase in two different fermentation media under different conditions **Table (3).** The results showed that, the chitosanase activity among the fungal species of the genus *Aspergillus* is quite different and less than that of *C. globosum* KM651986 in submerged fermentation medium (SMF) and solid state fermentation medium (SSF). Several species particularly *Aspergilli* were listed by other investigators as chitosanase producers such as *A. oryzae*, *A.* sp J22-326, *A. fumigatus*, *A.* sp Y2K and *A. flavus* and others **[12, 28, 29, 30, 31, 4]**. But the novel in this study was that the fungal isolate *C. globosum* KM651986 showed the highest chitosanase activity than the extensively studied fungal isolates of the genus *Aspergillus sp.*

C. globosum KM651986 showed chitosanolytic activity of (435.12±0.52 U/gds) in solid state fermentation medium of wheat bran alone, and when this medium supplemented with 1% chitosan the activity increased to (540.51±0.47 U/gds), This suggested that Chitosan is favorable inducer for the enzyme synthesis and that agree with study of Chen et al. **[32]** on chitosanase production by a mutant *Aspergillus sp.* CJ 22-326-14. However, the activity was low when the submerged fermentation medium of Czapek's-Dox used, **Table (3).**

Table (3): Chitosanolytic activity of different fungal isolates grown on two different media with different conditions

Fungal isolates	Medium I (SMF)		Medium II (SSF)	
	MD Static (U/ml)	MD Shaking (U/ml)	WB (U/gds)	WB+Chitosan (1%) (U/gds)
Chaetomium globosum	7.33±0.06	4.21±0.12	435.12±0.52	540.51±0.47
Aspergillus fumigatus	5.51±0.12	3.23±0.42	420.32±0.14	442.07±0.06
Aspergillus parasiticus	2.64±0.14	1.73±0.17	84.56±0.62	183.86±0.86
Aspergillus niger	1.32±0.32	1.17±0.15	156.40±0.05	277.51±0.04
Aspergillus terreus	6.11±0.03	4.68±0.28	193.27±0.01	313.75±0.31

**(MD) Modified Czapek's-Dox medium used as synthetic submerged fermentation medium (SMF).*
** * (WB) Wheat bran medium used as natural solid state fermentation medium (SSF).*
All the experiments were carried out in duplicate and the average values are reported as mean ± SD calculated using MS Excel.

Chitosanase activity of all screened isolates was higher in the wheat bran SSF medium than SMF medium, which indicated that the components in wheat bran could satisfy the nutrition requirement of these strains but with different degree **[33, 34].** Furthermore, using a certain amount of wheat bran in the medium could reduce chitosanase production cost. Wheat bran was also added in the medium for chitosanase production by *Aspergillus fumigatus* YT-1 **[35]** and *Trichoderma koningii* **[36]**.Therefore, the natural wheat bran medium under SSF conditions used as a basal medium for *C. globosum* KM651986 chitosanase production.

Considering the abilities of influencing the chitosanase induction, the concentration of the complex carbon source chitosan, the incubation temperature and the incubation period were chosen to investigate their influences on the production of chitosanase enzyme, under SSF conditions from the most favorable fungal isolate *C. globosum* KM651986, by response surface methodology of central composite design (CCD).

3.3. Central composite design (CCD)

To examine the combined effect of these three important factors, a central composite design (CCD) was employed within a range of –α (-1.68) and +α (+1.68) in relation to the production of chitosanase.

The prediction and observation responses along with design matrix are presented in **Table (1)** and the results showed that, the minimum response of 111.67U/gds chitosanase activity was obtained with 37.39 ºC incubation temperature. The maximum response of 1758.75 U/gds chitosanase activity was obtained with 31.5 ºC. These results indicated that, the further increment in temperature up to 37.39 ºC reduced the activity to 15- fold, compared to that obtained at suitable temperature of 31.5 ºC.

The second-order regression equation provided the levels of chitosanase activity as a function of chitosan concentration, incubation temperature and incubation period can be presented in terms of coded factors as in the following equation:

chitosanase activity (U/gds)=+1756.43 +31.29*A +0.59*B -70.24*C -26.50*A*B -55.99*A*C +59.63*B*C -306.55*A^2 -505.68*B^2 -413.50* C^2.

The statistical significance of the equation was checked by F-test and the analysis of variance for response surface quadratic model is shown in **Table (2).** ANOVA of regression model demonstrates that the model is significant as it is evident from the Fischer test with very low probability value. The value of lack of fit, Model F and model P>F were found to be 16135.22, 44.17 and 0.0001 respectively, indicating that model was significant. The goodness of fit of the model was checked by the determination coefficient (R^2). The coefficient of regression (R^2) was calculated to be 0.9755. The value of the adjusted regression coefficient (Adj R^2 = 0.9534) was also high, which advocates for high significance of the model. At the same time relatively low coefficient variation (CV=13.88%) confirm the precision and reliability of the experiment was performed.

From **Table (2)** it can be seen that values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A^2, B^2, C^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.

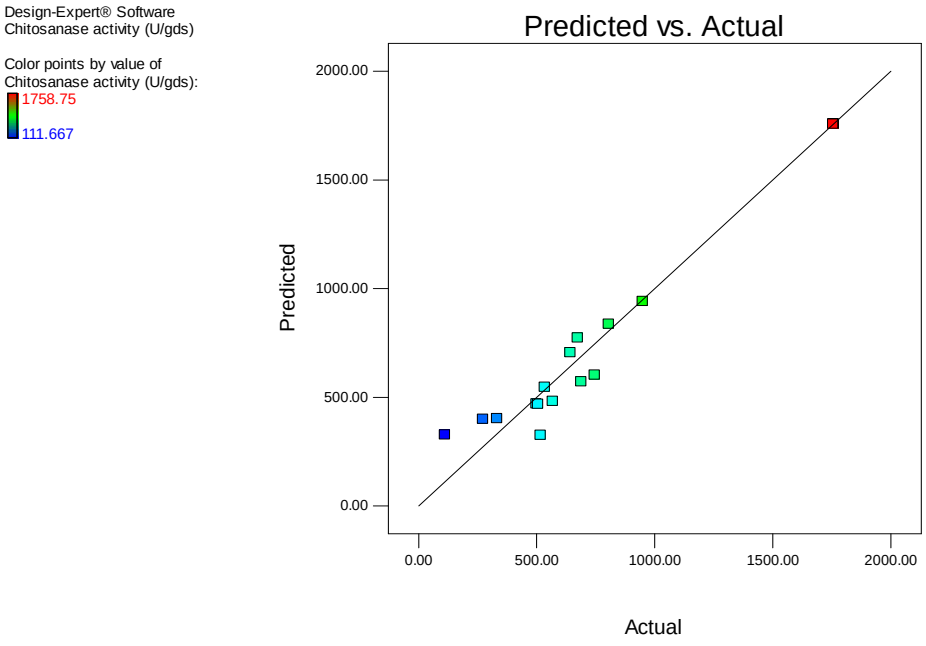


Fig. (2): Predicted v/s actual chitosanase production by*Chaetomium globosum* KM651986

The relationship between the actual and predicted chitosanase activity (response) is shown in **Fig. (2).**The cluster of measurements near the diagonal line in the parity plot indicates a good fit of the model and demonstrates a satisfactory correlation between the actual and predicted values.

Three dimensional (3D) response surface plots of chitosanase production based on the final model are depicted in **Fig. (3)** which were generated in pair-wise combination of the three factors while keeping the other one at its optimum level**.** The response at the central point corresponds to a maximum degree of achievable chitosanase activity for that factor.

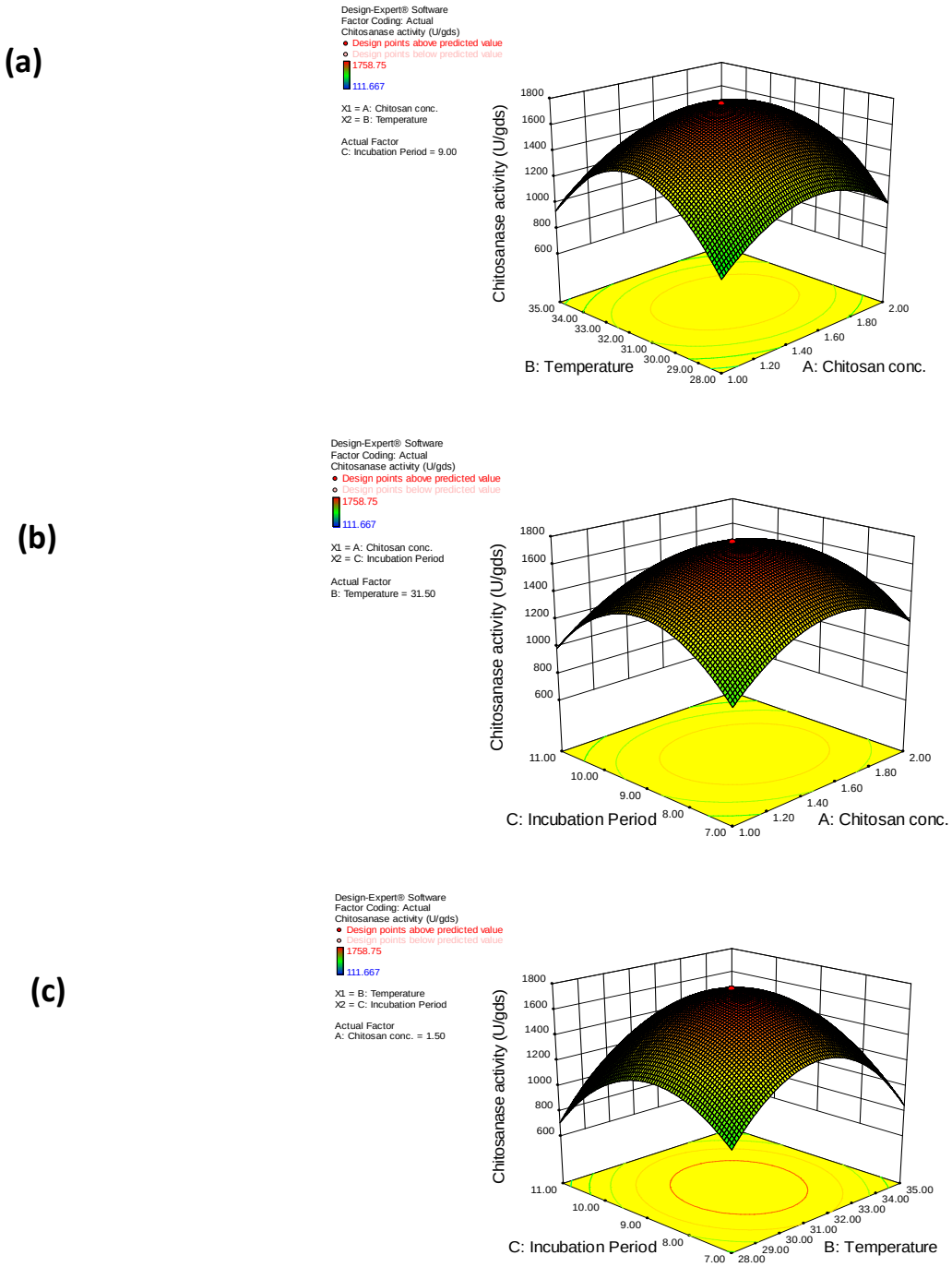


Fig. (3): Three dimensional response surface plot for the effect of (a) chitosan conc. and temperature (b) chitosan conc. and incubation period (c) temperature and incubation period

The validation was carried out under the following optimum conditions of the media. 2g/flask wheat bran moistened with 10 ml distilled water, as solid state fermentation medium supplemented with 1.5% chitosan and incubated at 31.5 ºC for 9 days incubation period in 250ml Erlenmeyer flasks. The experimental chitosanase production of 1758.75U/gds was obtained which is closer to the predicted chitosanase production of 1756.43U/gds; this result indicated the validity and the effectiveness of the proposed model.

The optimization of CCD resulted in 3.25-fold increase in chitosanase production. This reflects the necessity and value of optimization process. This result is nearly similar to the fold enhancement in chitosanase activity of *Aspergillus fumigatus* YT-1 after optimization **[35].**

3.4. Purification of chitosanase

Chitosanase was partially purified from the culture filtrate by ammonium sulphate precipitation (30-70 %). Data showed good yield (72.73 %) and purification fold (4.2). **Table (4)**

Table (4): Partially purification of chitosanase

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	660	46	14.35	100	1
30-70 % ($NH_4)_2SO_4$	480	8	60	72.73	4.2

3.5. Physicochemical characterization of chitosanase

3.5.1. Effect of temperature

The activity of partially purified chitosanase from *Chaetomium globosum* KM651986 was found optimal at 50°C **Fig. (4A).**At lower or higher temperatures, the activities were reduced at 30 and 70°C, respectively. This optimal temperature was slightly lower than that of *Aspergillus fumigatus* KB-1(60°C) **[30]**, but higher than those of *Bacillus subtilis* IMR-NK1 (45°C) **[37].**

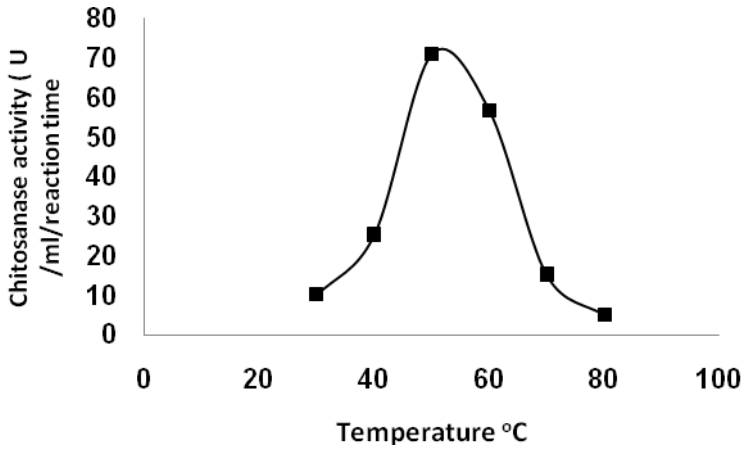
3.5.2. Effect of pH

The optimal pH of crude chitosanases was at pH 5.0, **Fig. (4B).** the optimal pH's for various chitosanases were in a broad range of 4.0-8.0 **[38]** depending on the microbial strains. Eron and Lee **[30]** reported that the optimum pH of the chitosanase from *Aspergillus fumigatus* KB-1 using acetate buffer was pH 5.5.Where chitosanase from *Penicillium janthinellum* exhibited optimum activity at pH 7–9**[39].**

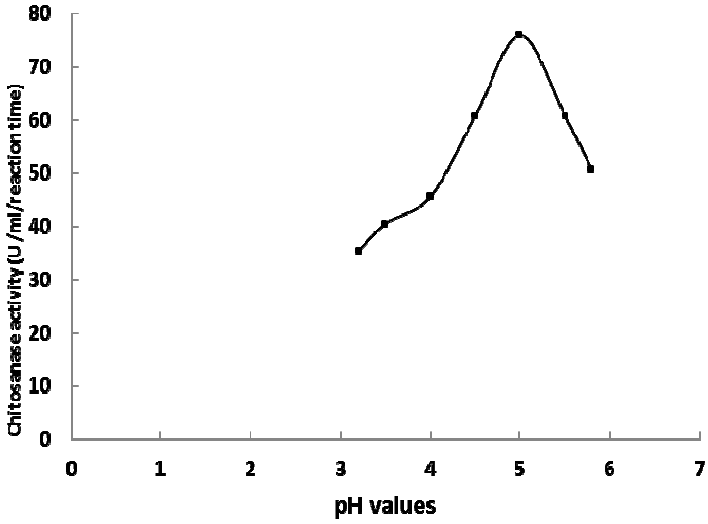
3.5.3. Thermal stability

In the absence of substrate, chitosanase is stable at temperature below 40^{o}C when heated for 60min, and it loses 70% of its activity when heated at 60 ^{o}C for 30 min., this result indicated that chitosanase has a moderate thermal stability **Fig. (4C),** these results to some extend better than chitosanase from *Penicillium janthinellum* which was stable at 25–50 °C. **[39].**

(A)



(B)



(C)

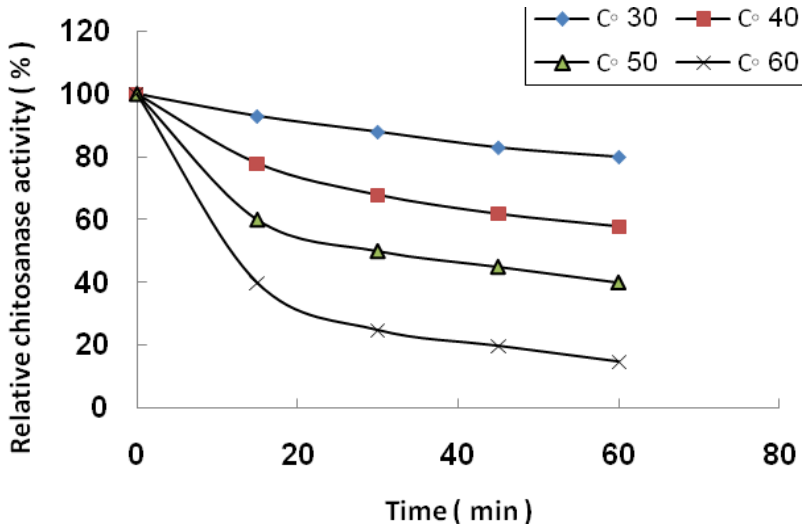


Fig. (4): A, Effect of different temperatures onchitosanase activity. B, Effect of different pH values of acetate buffer on chitosanase activity.C, Effect of different temperature on chitosanase stability at different time

3.5.4. Kinetic determination

K_m and V_{max} were calculated as 0.24 mg/ml and 40µmol/min **Fig. (5).** the lower K_m indicated that the isolated enzyme had a relatively high affinity for chitosan.

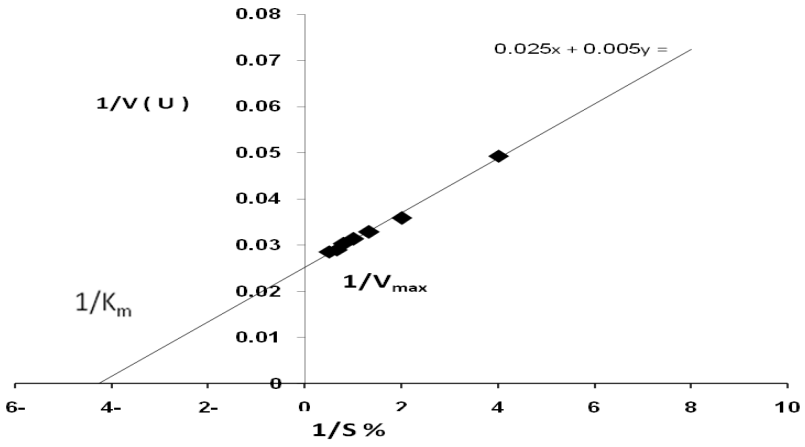


Fig. (5): Lineweaver-Burk plot for hydrolysis of chitosan by chitosanase

3.5.5. Effect of substrate specificity

Substrate specificity of chitosanase was summarized in **Table (5).** The enzyme efficiently hydrolyzed soluble chitosan but exhibited little activity on colloidal chitin. Most fungal chitosanases hydrolyze chitosan efficiently and chitin to a lesser extent.

3.5.6. Effect of activators and inhibitors

As shown in **Table (6)** The chitosanase enzyme activated by the presence of Na^{+}, Mn^{+2} , Mercaptoethanol and EDTA at concentration 1.0mM .

The chitosanase was almost completely inactivated by Cu^{+2}, Hg^{+2}, Mg^{+2} and Ca^{+2} at 1.0mM concentration and significantly inactivated by Ag^{+}, Fe^{+2} and Ba^{+2}. However, the other ions have little effect on chitosanase activity.

3.6. Analysis of the reaction products

The hydrolytic products of chitosan (DDA 75-85 %) by the enzyme were analyzed by thin layer chromatography (TLC) **Fig. (6).**

A TLC analysis revealed that the chitosanase had liberated chitosan oligomers (G6-G2) from chitosan. The length of the chain depends on the incubation time, when the incubation time increased the length of oligomers decreased. The data verify that the best yield of chitosan were obtained within 5 and 10 min hydrolysis. After overnight incubation only glucosamine in the hydrolysate suggesting an exo-type cleavage manner to release a single G1 (glucosamine) residue from chitosan.

Table (5): Substrate specificity of *Chaetomium globosum* KM651986 chitosanase enzyme

Substrate	Relative activity (%)
Chitosan	100
Lichenin	40
Salicin	30
Xylan	20
Pectin	15
Colloidal chitin	10
Pullulan	0
Laminarin	0
Cellulose	0
Galactomnnan	0

Table (6): Effect of some activators and inhibitors on chitosanase activity under standardized conditions

Activator and inhibitors (1.0 mM)	Relative activity (%)
$NaNO_3$	110
$MnSO_4$	100
$CoCl_2$	80
K_2SO_4	60
$AgNO_3$	50
$FeSO_4$	45
$BaCl_2$	30
$CuSO_4$	0.0
$Hg(NO_3)_2$	0.0
$MgCl_2$	0.0
$CaCl_2$	0.0
Mercaptoethanol	105
EDTA	95

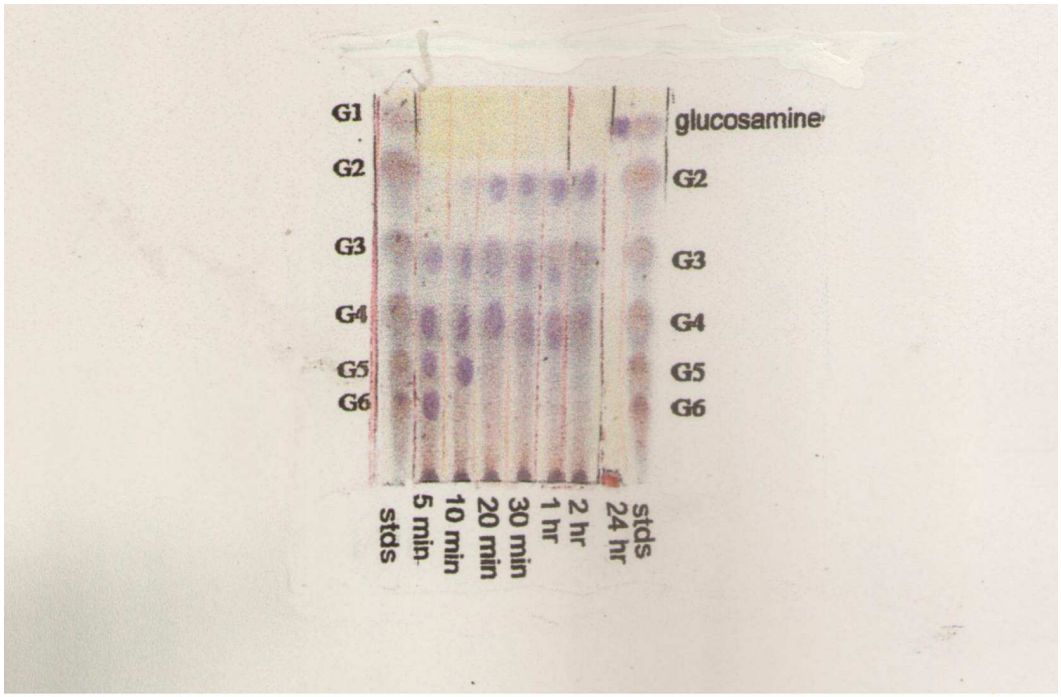


Fig. (6): Thin layer chromatography pattern of chitosan hydrolysis, the figure showed different chitosan oligosaccharidesstandards (G1-G6) and the product of enzymatic hydrolysis after 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 24 h respectively

3.7. Antioxidant activity

Fig. (7) Represent the antioxidant activities of the end products of hydrolysis of chitosan at 5, 10, 20 and 30min and 1h, 2h and 24h. From the figure it showed that when the incubation time increased the antioxidant activity decreased. These results revealed that when the number of chain oligomers increased the antioxidant activity increase. Tokoro *et al*. **[40]** reported that the high degree polymerized (DP) oligomers such as chitopentoes and chitohexose are more biologically active than the low DP ones (chitotriose and chitobiose).

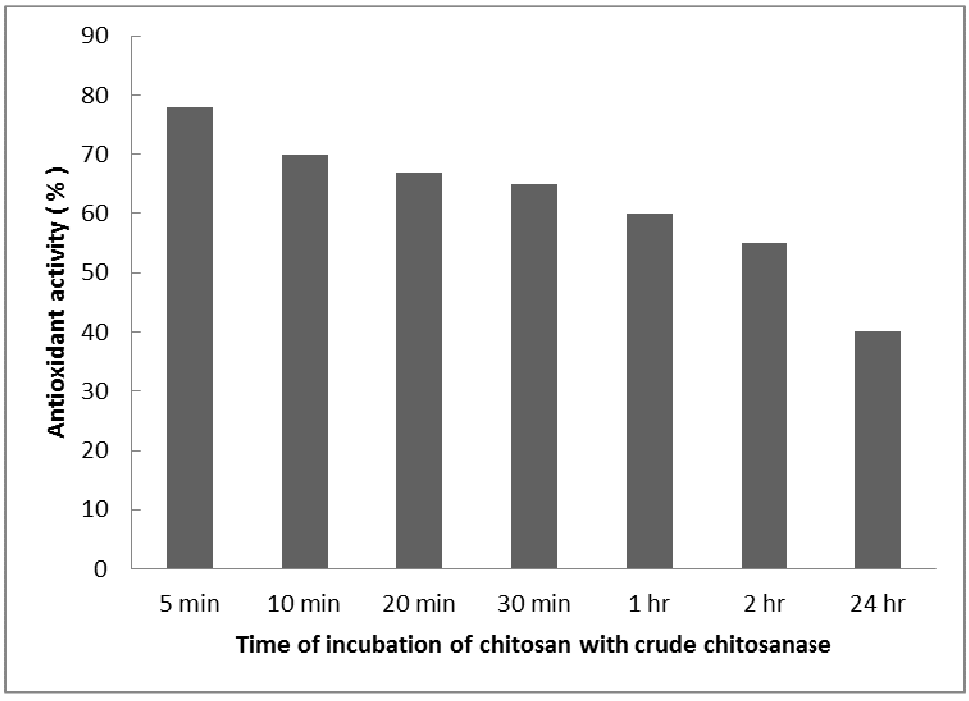


Fig. (7): Antioxidant activities of the end product hydrolysate of chitosan at different time intervals

3.8. *In- vitro* antimicrobial activity of chitosan oligosaccharides

Antimicrobial activity of the chitosan oligosaccharide was tested using agar well diffusion method **[27]**. The activity of chitosan oligosaccharides were studied against the *Staphylococcus aureus* (ATCC29213), *Bacillus subtilis*

(ATCC6633), *Pseudomonas aeruginosa* (ATCC27953), *Escherichia coli* (ATCC25922) and *Candida albicans* (NRRL Y-477).

The results in **Table (7)** revealed that, the inhibitory activity against Gram negative bacteria was higher than Gram positive bacteria and the yeast , chitosan oligosaccharides display good activity against *E. coli, P. aeruginosa* and *B. subtilis* with zones of inhibition 22, 20 and 18 mm, respectively. In addition, it showed activity against *C. albicans* with zone of inhibition 17mm. it is obvious that, the less zone of inhibition of 15mm appeared with *S. aureus*. Kumar *et al*. **[41]** reported that the antimicrobial effects of chitosan oligosaccharieds may be due to staking of chitosan oligosaccharieds molecules on the microbial cell surface creating a polymer membrane, which blocks the transport of nutrients towards the cell membrane of the microorganisms, impairing the physiological activities of the microorganisms, and killed them.

Table (7): Antimicrobial activity of the chitooligosaccharide (COS)

Diameter of inhibition zones in millimeters (mm)*				
Gram positive bacteria		Gram negative bacteria		Fungi
Staphylococcus aureus (ATCC29213)	*Bacillus subtilis* (ATCC6633)	*Escherichia coli* (ATCC25922)	*Pseudomonas aeroginosa* (ATCC27953)	*Candida albicans* (NRRL Y-477)
15	18	22	20	17

**Antimicrobial activity expressed as inhibition diameter zones in millimeters (mm) of (COS) against the pathological strains based on well diffusion assay.*

CONCLUSION

Chaetomium globosum KM651986, showed the highest chitosanase activity under SSF medium of wheat bran. The goal was to optimize the production of chitosanase by RSM. Production of chitosan oligomers from chitosan hydrolysis has considerable attention for their application, mainly in medicine which showed antioxidant and antimicrobial activities.

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