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PRODUCTION, OPTIMIZATION AND APPLICATION OF CHITOSANASE ENZYME FROM MARINE ACTINOMYCETES - *STREPTOMYCES MASSASPOREUS*

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Abstract

Chitooligosaccharides has wide range of applications in medical and pharmacological fields due to water solubility and non toxicity properties. The present study was production of the chitosanase enzyme from marine *Streptomyces massasporeus*. The chitosanase producing actinomycetes isolates are isolated from marine wastes dumped soil, Out of 146 isolates,4 isolates were prominent of forming clear zone on the media.One among the isolate S15 produced highest chitosanase activity was identified as *Streptomyces massasporeus* KR873429 based on the molecular characterization. The chitosanase enzyme was optimized through RSM and the production of enzyme was increased to 1.913 U/ml.The crude enzymes were prepared and concentrated through (80% saturation) ammonium sulphate precipitatin followed by dialysis. The chitoligosaccarides hydrolysate was prepared with partially purified S15 chitosanase enzyme.The prepared COS mixture shown significant biocompatibility by MTT assay.

Key words chitosan ,chitosanase,bioactivity ,chitooligosaccharides, COS hydrolysates

Introduction

Chitosan is the second important natural biopolymer next to cellulose. It is an exoskeleton component of crabs, shellfish, insects and crustaceans (Berger *et al*., 2004). The properties of biocompatibility, biodegradation, non toxicity makes chitosan to be used in biomedical and pharmaceutical formulations. The antimicrobial activity of chitosan depends on molecular weight (MW), viscosity (Liu *et al*., 2006) and solubility (Yang, Chou and Li, 2005). The solubility of chitosan decreases with increasing molecular weight and increasing pH(Jia and Xu, 2001).These drawbacks can be overcome by using low molecular weight chitosan and chitooligosaccharides for several applications (Koping *et al*., 2003).

Chitooligosaccharides (COS) possesses short glucosamine units and free amino groups which help in water solubility (at neutral pH).The water solubility, low viscosity properties of enhances the wide potential applications of COS. It can be prepared chemically, physically or enzymatically from chitosan. Enzymatic hydrolysis with glycoside hydrolases like chitinases or chitosanases, is a promising approach since, the chemical composition (FA), sequence (PA) and DP of the resulting COS mixture depends on the substrate and the enzyme specificity.

Chitosanase enzymes hydrolysis glycosidic bonds of chitosan (Park *et al*., 2009).It is found widely in bacteria, fungi, plants and actinomycetes. Among bacteria *Bacillus* sp is dominant, *Streptomyces* sp is dominant among actinomycetes and fungi are less reported (Cheng and Li, 2000). The pure COS fractions applications are limited due to high costs of enzymatic hydrolyses. Generally purification procedures are time consuming and challenging task. The proposed current work was focused on enzymatic hydrolysate (mixture of COS after the enzymatic hydrolysis) formulation for novel bioactivities in order to reduce the cost of application.

Screening of the chitosanase producing organism

The marine muddy soil samples, fish market soil samples (waste dumped) were collected aseptically from various places of Tamilnadu, Kerala and Pondicherry. Soil suspension was prepared by adding one gram of collected sample to five ml of sterile water. Then the prepared suspension plated on CDA (Chitosanase Detection Agar) plates (Cheng and Li, 2000) (comprising basal medium M9 with (0.5% and 1%) chitosan) incubated for 3-4 days at room temperature. The zone formed strains on the CDA plates were chosen and stored in slants for further studies.

Assays of chitosanase activity (DNS- Miller, 1959)

Briefly, the crude enzyme solution (0.5 ml) added to chitosan substrate (0.5ml) at 55^{o}C. After 30 min incubation, 3ml of DNS (3, 5-Dinitrosalicylic acid) was mixed then in boiling water for 3 min. The absorbance of the mixture was measured at 540 nm. The protein content (Lowery *et al*., 1951) of the crude extract was compared with bovine serum albumin as the calibration standard.

PCR amplification

The genomic DNA of the isolates prepared (Vijay Kumar *et al*.,2010),PCR amplication was performed with primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1942R (5′-AAGGAGGTGATCCAACC-3′). Applied Bio-systems Big Dye Version 3.1 kit on an ABI-PRISM 3730 DNA Sequencer was used for amplification using forward and reverse primers. Chromas (Version 2.01) used for correcting ambiguous sequences and assembled with Bio-Edit (Version 7.0.9.0). The species identification was checked with homologous sequences of isolate using the BLASTn program (NCBI) and submitted to GenBank with accession number.

Phylogenetic analysis of isolate

UPGMA method was used for 16S rDNA sequences of the strains evolutionary relationship for construction of the phylogenetic tree (Sneath and Sokal, 1973) in MEGA5. The maximum composite likelihood method (Tamura, Nei and Kumar, 2004) was used for computing. (Tamura *et al*., 2007).

Culture conditions optimization for the effective production of chitosanase enzyme

The production media CDA, was subjected for the optimization production of chitosanase enzyme. The preliminary parameters such as pH, temperature, incubation time, medium, substrate concentration and nutritional parameters were primarily screened by one at a time strategy test.

The strains were grown for 9 days for checking optimum incubation time. The optimum temperature was determined by incubating at 22°C, 27°C, 30°C, 37°C, 45°C and 55°C for an optimized period of time. The pH value on the chitosanase production was determined by varying pH of the culture medium. The experiments were carried out in triplates.

Different concentrations of chitosan (0.05%, 0.2%, 0.25%, 0.5%, 1%, 1.5%, 2% and 2.5%) was checked to determine the substrate concentration. The effects of various carbon (starch,glucose,fructose,xylose,D-glucosamine) and nitrogen sources (ammonium chloride,yeast extract, peptone, glutamic acid ammonium sulfate,potassium nitrate) of 0.5%, 1%, 2% were used as nutritional supplement in media. The experiments were carried out in triplates.

Central composite design for the production of chitosanase on M9 medium

Based on the basic preliminary optimization described above, central composite design (CCD) with five factor analysis was carried. Each factor is varied over 5 levels (alpha = 1.682), 2 axial points (+ and − alpha), 2 factorial points (+ and −1), and 1 centre point resulting in a total of 53 experimental trials involving the replications. The dependent variables chosen was Chitosanase activity (U/mg). The independent variables chosen were Chitosan concentration (%) (0.5-2.5) A;Carbon sources (%) (10-30) B;Nitrogen sources (%) (5-15) C; pH (5-8) D and Temperature (^{o}C) (25-45) E.

The results obtained from the above were subjected for Analysis of variance (ANOVA). The effect of variables in the response surface regression procedure (1) shown below

$$Y = \beta_0 + \beta_1 * A + \beta_2 * B + \beta_3 * C + \beta_4 * D + \beta_5 * E + \beta_{12} * AB + \beta_{13} * AC + \beta_{14} * AD + \beta_{15} * AE + \beta_{23} * BC + \beta_{24} * BD + \beta_{25} * BE + \beta_{34} * CD + \beta_{35} * CE + \beta_{45} * DE + \beta_{11} * A^2 + \beta_{22} * B^2 + \beta_{33} * C^2 + \beta_{44} * D^2 + \beta_{55} * E^2 \quad (1)$$

where Y is the measured response, β0 is the intercept term, β_1, β_2, β_3, β_4 and β_5 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} and β_{55} are quadratic coefficient, β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and $\beta_{45}$are interaction coefficient and A, B, C, D and E are coded independent variables.

The quality of fit was expressed by R^2, and its significance by F-test. The response surface equation was optimized for variables using Design Expert software version 8.0.7.1. The optimum concentration of each parameter was identified on the contour and hump in 3D plots.

Purification of enzyme -Ammonium sulfate fractionation

Ammonium sulphate was added to 80% saturation in increments with gentle stirring to dissolve and equilibrate the additions. The proteins then precipitated were centrifuged for 15min at 4°C. The resultant pellets were added to phosphate buffer (5ml) at pH 7.0. The remaining was applied again with ammonium sulphate to achieve 100% (w/v) saturation and both were dialysed.

Chitooligosaccharides Preparation

Chitooligosaccharides are prepared by adding 1ml of the enzyme to 1ml of 1% chitosan for 30 min at 45°C. The solution was then heated for 5 min at 100°C and allowed for cooling. The chitooligosaccharides present in the supernatant was separated and precipitated with 0.25M of sodium hydroxide (Zhang *et al*., 1999). The precipitate was centrifuged for 2 min at 1,000 rpm and then aliquots were lyophilized. Bioactive COS prepared by varying the incubation time from minutes to hours of 1% chitosan with partially purified enzyme .

MTT assay

The in vitro proliferation assay of embryonic 3T3 normal fibroblasts (0.1 ml) cells added to in 96 well plates left for 12 hrs for the cells to adhere. The chitooligosaccharide containing medium of various concentration was then added. The cell growth after 72 hrs of incubation the cytotoxic effect of the chitooligomers was determined by MTT method**.**The cells are washed with PBS at 37°C and supplemented with 100µl of serum free medium with 10 µl of MTT solution and incubated for 4 hrs. Each well was added with 100 µl of solubilization solution at 37°C to dissolve formazan crystals and then agitated for 10 min .The absorbance was measured at 570 nm and cell viability percentage was calculated.

Result & Discussion

Screening of the chitosanase producing organism

The clear zone producing organisms on the CDA plates was indicated chitosanase enzyme as shown in Figure 1.The growth and clear zone was observed in the CDA of both 0.5%

and 1%. It is evidenced from the present study that the higher chitosanase producers were predominantly isolated fish wastes dumped soil than seawater and muddy soil samples (Table 2). Most of higher zone producers of actinomycetes isolates were isolated from the fish wastes dumped soil sample. The actinomycetes isolates were named as S1,S2, …& A1,A2 ….

Chitosanase enzyme activity

The maximum chitosanase activity was observed on the fourth day of the incubation. The maximum chitosanalytic activity of the isolates were (S15 was 1.464 U/ml, S15.1 was 1.221 U/ml, S18 was 1.274U/ml, A22 was 1.227 U/ml, and A4 was 1.169 U/ml) chosen were further studies(Table 4.2)..

DNA Sequencing and species identification - Phylogenetic analysis of isolate

The phylogenetic tree was constructed based on evolutionary relationship of the isolates with closely related species using 16S rRNA (Figure). The sequences were submitted to GenBank and their accession number was provided. The isolate S15 are the organisms identified (Table 1) with their accession number: *Streptomyces massasporeus* - KR873429 (S15) https://www.ncbi.nlm.nih.gov/nuccore/KR873429

Culture conditions optimization for the chitosanase enzyme production

In our present study strain S15, A4 produced higher activity on the 4th day (Kim and Ji, 2001) . A22,S15.2,S18 showed higher activity on the third day S18 actinomycetes isolates showed maximum production on 3rd and 4th day (Figure 3).

The strains S15.1 showed higher activity at pH 6 as the report of Cheng *et al*. (2012) on *Aspergillus fumigatus*. Few of the isolates S 15,A22 A4,S18 shown the maximal production in the pH 6.5 exactly matched the results of Yoon *et al*. (1998); Kobayashi *et al*., (2011). Hence the optimum pH for the production of the chitosanase of the present study was fixed as 6.5 which got altered from the initial pH for the isolation of the organism. The temperature optimization for the maximum chitosanase production in our study was 28 º C for matched the results of Abdel, Kahil and Keera, (2014).

S15.1, S18 showed maximum activity at 1% and all other the isolates S15,A22,A4 showed maximal production at 1.5% substrate concentration (Figure).Hence 1 and 1.5% was finally selected for the optimal growth of the isolates and for the production of chitosanase enzyme(Kim and Ji, 2001). 1% xylose as carbon source increased the production chitosanse enzyme in all the isolates of actinomycetes. Compared with all the nitrogen sources ammonium chloride, peptone has influenced the production in actinomycetes.

Central composite design of S15 chitosanase production media

The model for the chitosanase production was done by analysis of variance (ANOVA) and the results are shown in Table 4.12. *F* value of S15 is 48.651 for chitosanase production implies the model is significant. The coefficient of determination R^2 was 0.969 where 96.9 %

indicating that the mathematical model of the experimental value is reliable for chitosanase activity. The regression equation coefficients were calculated:

Specific activity of S15 (U/mg) = +1.85 + 0.015* A +0.12* B-0.051* C - 0.029 * D -0.095 * E-0.039 * A * B+0.031* A * C + 0.021 * A * D + 0.020 * A * E - 8.719E-003* B * C-7.187E-004 * B * D-0.011* B * E+0.03 * C * D - 6.969E-003* C * E-9.094E-003* D * E-0.31* A^2-0.29 * B^2 - 0.30* C^2 - 0.32 * D^2 - 0.25* E^2

3D surface contour graphs Figure 4.7a showed the increase of chitosanase production when chitosan concentration and carbon source increases as well as by increasing the chitosan concentration (Figure 4.7b) and nitrogen source .All the plots (Figures 4.7 c,d., e, f, g, h, i, j) showed the optimum level responses for the variable.

From our study maximum S15 chitosanse production was 1.913 U/ml when the substrate concentration was 1.5%, carbon (xylose) concentration was 20%, and nitrogen concentration (peptone) was 10%, pH 6.5 and temperature 27.5 º C.

Molecular weight determination by SDS –PAGE

The crude enzyme S15 showed to be approximately 29kDA (Figure 4.12a-c). Chen *et al.* (2005) stated that the endochitosanase enzyme belong to the range from 20 to 50 kDa, while exo-chitosanases ranged from 97 to 135 kDa. From this it is obvious that the present study enzymes fall into the endo chitosanase class.

MTT assay

The *in vitro* biocompatibility efficacy of chitoligosaccharides in 3T3 embryonic fibroblast cells using MTT assay for assessing cell proliferation and cell toxicity. The cell viability of S15 chitoligosaccharides were above 75% after 72 hours of incubation was considered to be non cytotoxic .The antiproliferative activity of prepared COS (Figure 4.21b) compared with control proved to be 100% biocompatibility as cell death or inhibition of growth were noticed in treated wells.

In MTT assay our results were similar to De Assis *et al.* (2012) and were similar to Jeon and Kim, (2000). S15 hydrolysates did not significantly inhibit the cell proliferation. The test COS consists of the mixture of oligomers even in the higher concentration the antiproliferative activity is very lower. Hence the chitooligosaccharides prepared from chitosanase could be considered as biocompatible.

Figure 1 Chitosanase producing actinomycetes S15 on CDA plates

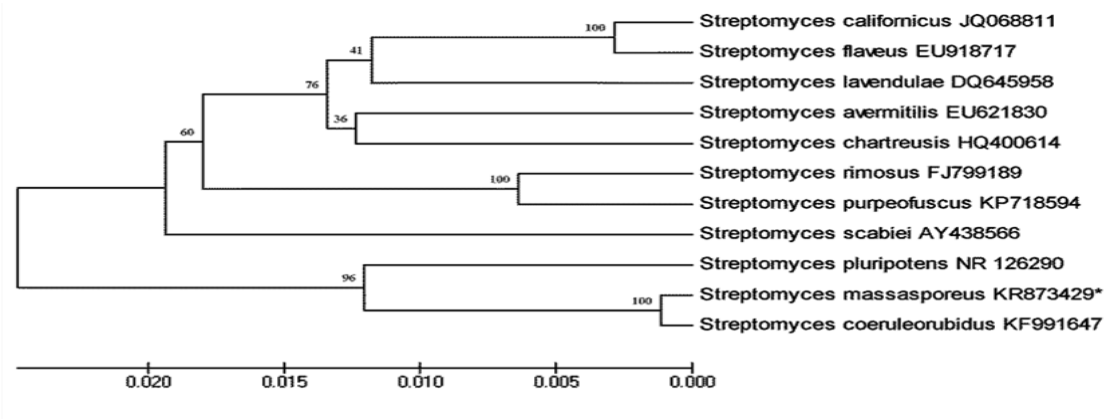
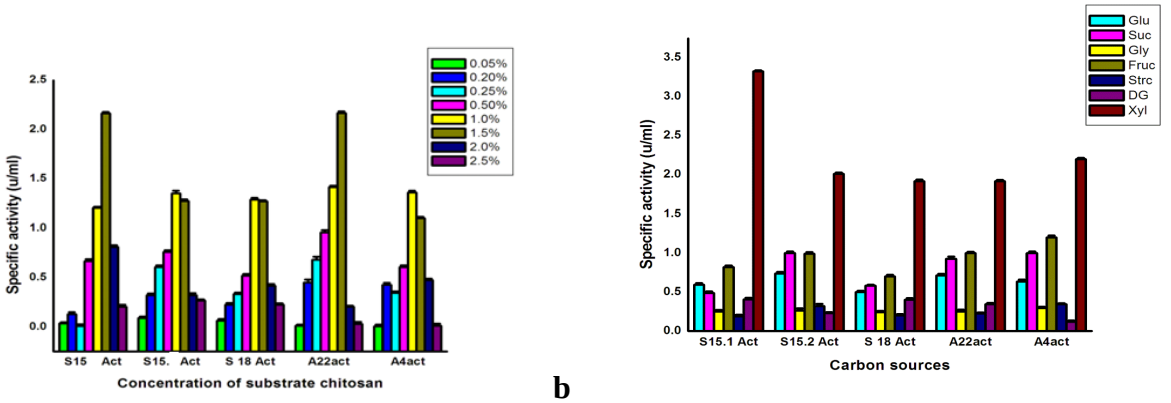
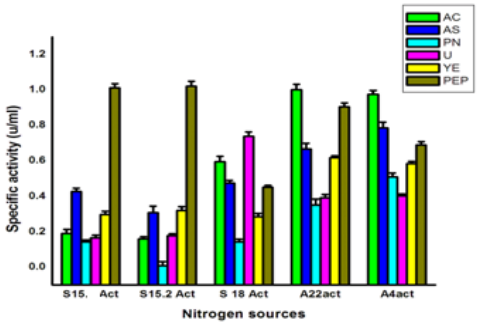


Figure 2 Dendrogram showing the relatedness of 16S rDNA between isolate S15 and selected related references derived from Genebak database



b



c

Figure 3 Optimization of a)Substrate concentration ,b)Carbon source ,c)Nitrogen source of actinomycete isolates for chitosanase enzyme prodution

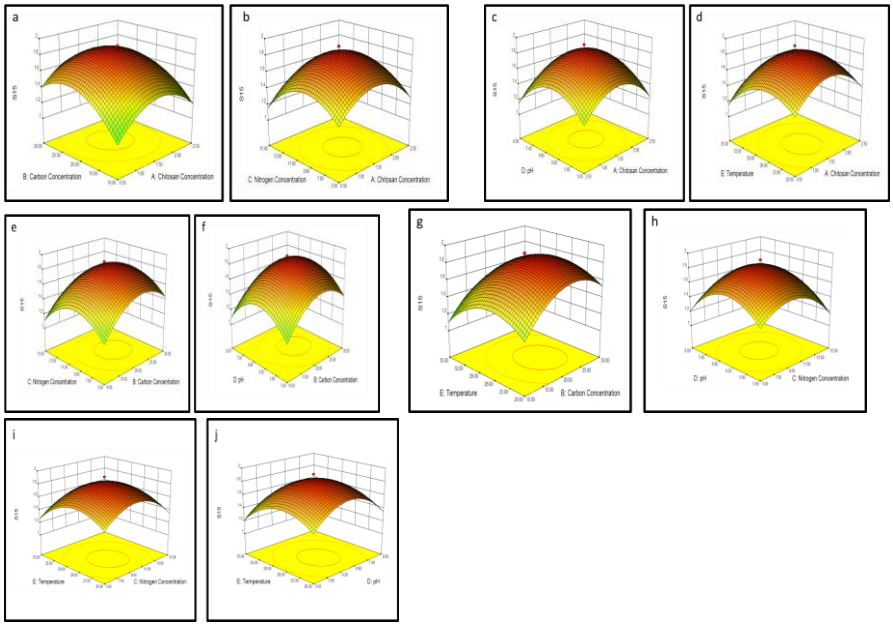


Figure 4 a-j 3D surface plot of S15 on chitosanase activity

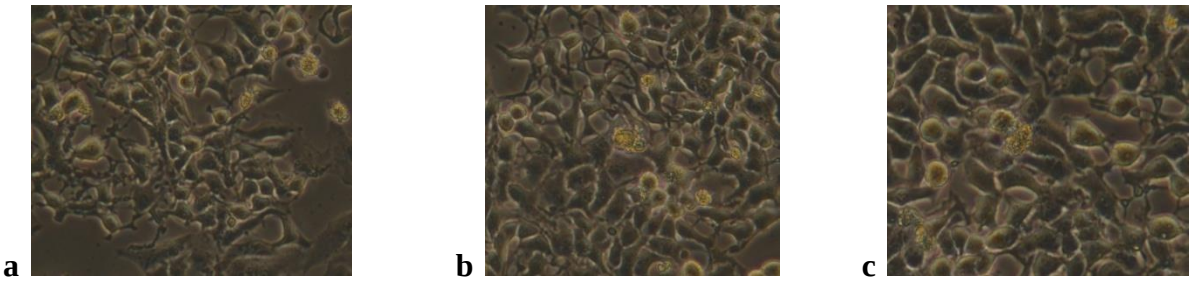


Figure 5 Cytotoxicity assay of S15chitooligosaccharide on normal 3T3 fibroblast cells at various concentrations a) 50 µg/ml b)100 µg/ml c) 500 µg/ml

Table 1 Chitosanse activity of the isolates

Isolates	Biomass (g/L)	Specific activity (U/ml)
A22	2.67 ± 0.36	1.227
S15	2.53 ± 0.44	1.464
S15.1	2.32 ± 0.32	1.221
S18	2.45 ± 0.23	1.274
A4	2.38± 0.22	1.169

Table 2 ***Optimization of the pH in M9 media for the chitosanase production***

	A22act	A4act	S15.1 Act	S15 Act	S 18 Act
4.5	0.481±0.018^{a}	0.474±0.008^{a}	0.155±0.000^{a}	0.010±0.000^{a}	0.309±0.001^{a}
5.0	0.902±0.003^{b}	0.957±0.014^{b}	0.666±0.000^{b}	0.229±0.003^{b}	0.380±0.001^{b}
5.5	0.926±0.005^{b}	0.963±0.002^{b}	0.847±0.003^{c}	0.871±0.003^{c}	0.858±0.002^{c}
6.0	1.123±0.004^{c}	1.127±0.000^{c}	1.136±0.032^{d}	1.227±0.002^{d}	1.203±0.004^{d}
6.5	1.125±0.005^{d}	1.256±0.006^{d}	1.221±0.001^{d}	1.428±0.004^{e}	1.307±0.003^{e}
7.0	1.016±0.170^{e}	1.022±0.000^{e}	0.307±0.005^{e}	0.389±0.005^{f}	0.380 ±0.000^{b}
7.5	0.703±0.003^{f}	0.766±0.006^{f}	0.300±0.001^{e}	0.362±0.002^{g}	0.306 ±0.005^{a}
8.0	0.600±0.006^{g}	0.711±0.002^{g}	0.140±0.002^{a}	0.503±0.005^{h}	0.260±0.002^{f}
8.5	0.293±0.003^{h}	0.224±0.002^{h}	0.125±0.005^{a}	0.528±0.016^{i}	0.009±0.001^{g}
9.0	0.122±0.002^{i}	0.107±0.000^{i}	0.010±0.000^{f}	0.008±0.000^{j}	0.250±0.001^{h}

Values were given as mean ± S.D; Each column with different superscript letters Indicates are significantly different determined by one-Way ANOVA Post-hoc Tukey's test ($p \leq 0.05$).

Table 3 Experimental designs used in RSM studies by using five independent variables showing observed values of specific activity for S15 chitosanase production

Standard Order	A - Chitosan Concentration (%)	B - Carbon sources (%)	C - Nitrogen sources (%)	D – pH	E – Temperature (°C)	Actual Chitosanase activity (U/ml)
1	0.50	10.00	5.00	5.00	20.00	0.431
2	2.50	10.00	5.00	5.00	20.00	0.198
3	0.50	30.00	5.00	5.00	20.00	0.913
4	2.50	30.00	5.00	5.00	20.00	0.501
5	0.50	10.00	15.00	5.00	20.00	0.237
6	2.50	10.00	15.00	5.00	20.00	0.312
7	0.50	30.00	15.00	5.00	20.00	0.531
8	2.50	30.00	15.00	5.00	20.00	0.420
9	0.50	10.00	5.00	8.00	20.00	0.210
10	2.50	10.00	5.00	8.00	20.00	0.430
11	0.50	30.00	5.00	8.00	20.00	0.421
12	2.50	30.00	5.00	8.00	20.00	0.512
13	0.50	10.00	15.00	8.00	20.00	0.032
14	2.50	10.00	15.00	8.00	20.00	0.432
15	0.50	30.00	15.00	8.00	20.00	0.632
16	2.50	30.00	15.00	8.00	20.00	0.512
17	0.50	10.00	5.00	5.00	35.00	0.321
18	2.50	10.00	5.00	5.00	35.00	0.232
19	0.50	30.00	5.00	5.00	35.00	0.510
20	2.50	30.00	5.00	5.00	35.00	0.578
21	0.50	10.00	15.00	5.00	35.00	0.011
22	2.50	10.00	15.00	5.00	35.00	0.321
23	0.50	30.00	15.00	5.00	35.00	0.164
24	2.50	30.00	15.00	5.00	35.00	0.437
25	0.50	10.00	5.00	8.00	35.00	0.032

Standard Order	A - Chitosan Concentration (%)	B - Carbon sources (%)	C - Nitrogen sources (%)	D – pH	E – Temperature (°C)	Actual Chitosanase activity (U/ml)
26	2.50	10.00	5.00	8.00	35.00	0.201
27	0.50	30.00	5.00	8.00	35.00	0.412
28	2.50	30.00	5.00	8.00	35.00	0.327
29	0.50	10.00	15.00	8.00	35.00	0.120
30	2.50	10.00	15.00	8.00	35.00	0.110
31	0.50	30.00	15.00	8.00	35.00	0.410
32	2.50	30.00	15.00	8.00	35.00	0.309
33	1.50	20.00	10.00	6.50	27.50	1.913
34	1.50	20.00	10.00	6.50	27.50	1.400
35	1.50	20.00	10.00	6.50	27.50	1.913
36	1.50	20.00	10.00	6.50	27.50	1.913
37	1.50	20.00	10.00	6.50	27.50	1.913
38	1.50	20.00	10.00	6.50	27.50	1.913
39	1.50	20.00	10.00	6.50	27.50	1.913
40	1.50	20.00	10.00	6.50	27.50	1.913
41	0.50	20.00	10.00	6.50	27.50	0.111
42	1.50	20.00	10.00	6.50	27.50	0.203
43	1.50	10.00	10.00	6.50	27.50	0.001
44	1.50	30.00	10.00	6.50	27.50	0.521
45	1.50	20.00	10.00	6.50	27.50	0.410
46	1.50	20.00	10.00	5.00	27.50	0.112
47	1.50	20.00	10.00	5.00	27.50	0.015
48	1.50	20.00	10.00	6.50	27.50	0.843
49	1.50	20.00	10.00	6.50	35.00	0.057
50	1.50	20.00	10.00	6.50	27.50	1.913
51	1.50	20.00	10.00	6.50	27.50	1.623
52	1.50	20.00	10.00	6.50	27.50	1.913

Table 4.12 ANOVA for response surface quadratic model for S15

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Block	0.030528	1	0.030528		
Model	21.6719	20	1.083595	48.65848	< 0.0001
A-Chitosan Concentration	0.010173	1	0.010173	0.456835	0.5041
B-Carbon Concentration	0.623269	1	0.623269	27.98768	< 0.0001
C-Nitrogen Concentration	0.111755	1	0.111755	5.018313	0.0324
D-pH	0.035827	1	0.035827	1.608783	0.2141
E-Temperature	0.387802	1	0.387802	17.41414	0.0002
AB	0.047973	1	0.047973	2.154191	0.1523
AC	0.030443	1	0.030443	1.367023	0.2512
AD	0.014578	1	0.014578	0.65461	0.4246
AE	0.012207	1	0.012207	0.548153	0.4646
BC	0.002433	1	0.002433	0.109232	0.7432
BD	1.65E-05	1	1.65E-05	0.000742	0.9784
BE	0.004073	1	0.004073	0.182876	0.6719
CD	0.049849	1	0.049849	2.238454	0.1447
CE	0.001554	1	0.001554	0.069783	0.7934
DE	0.002646	1	0.002646	0.11883	0.7326
A^2	5.590041	1	5.590041	251.019	< 0.0001
B^2	4.938995	1	4.938995	221.784	< 0.0001
C^2	5.26543	1	5.26543	236.4424	< 0.0001
D^2	6.209754	1	6.209754	278.847	< 0.0001
E^2	3.858998	1	3.858998	173.287	< 0.0001
Residual	0.690351	31	0.022269		

Lack of Fit	0.404012	22	0.018364	0.577208	0.8592
Pure Error	0.28634	9	0.031816		
Cor Total	22.39278	52			

R-Squared	0.969
Adj R-Squared	0.949
Pred R-Squared	0.913

Conclusion

The present study was focused on to produce mixture of compound or hydrolysate. Hence the chitooligosaccharide hydrolysate was prepared with partially purified chitosanse enzymes of the present study. The antimicrobial efficiency of COS gel were checked against pathogens and The COS hydrolysate activity was higher. MTT assay with normal 3T3 embryonic fibroblast cells lines has proved the biocompatibility COS hydrolysate. COS hydrolysate will an alternative for the available technologies to the mark for effective management of wound dressing.

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