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Production, Optimization, Characterization and Application Of Chitosanase enzyme from *Bacillus tequilensis*

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Abstract

Chitosanase enzymes have the ability to break down chitosan to hitosan oligosaccharidewhich exhibits a significant antimicrobial, antitumor activities. In this study, chitosanase enzyme was prepared from *Bacillus tequilensis* (S9) which was isolated from poultry soil sample containing decomposed chicken feathers. The production medium was optimized and the higher enzyme activity was observed on the 3rd day. Themaximum chitosanase production of S9 was 4.112 U/ml on shrimp shell media after optimization. The molecular weight of the S9 chitosanase was 40 kDa (crude enzyme), 29 kDa (partially purified). Furthermore, the prepared Chitooligosaccharides hydrolysategel had stronger antibacterial activity against diabetic foot ulcer pathogens and has potential biocompatibility.

Key words: *Bacillus sp*, Chitosanase, Chitooligosaccharides, antibacterial activity, HETCAM

Introduction

Chitosan, second largest polymer next to cellulose, a functional polysaccharidethat has emerged as biomaterial in various industries such as food, pharmaceutical, wastewater treatments, textile and other industries (Choi *et al*. 2004). However, due to its higher molecular weight and viscosity, chitosan possess a challenge for absorption in thehuman intestine (Xiao *et al*. 2010). On the other hand, chito oligosaccharide, with its low viscosity and solubility at neutral pH, exhibits greater bioactivity compared to chitosan (Kim and Rajapakse 2005). Chitooligomers are primarily obtained through chemical hydrolysis, physical methods and enzyme hydrolysis (Li *et al*. 1992). Among these

methods, enzymatic hydrolysis is preferred due to its controllable nature, mild hydrolysis conditions and minimal environmental pollution (Zheng and Zhu 2002). The biodegradable nature and enzymatic hydrolysis of chitosan make it a promising material for drug delivery systems, applications in the biomedical and biotechnological fields.

Chitooligosaccharides are synthesised by specific enzymes (chitinase, chitosanase, gluconase, and protease) and non-specific enzymes (Kim and Rajapakse 2005). Chitosanase stands out among all the specific enzymes as it is particulary effective in converting chitosan into chitooligosaccharides unlike the other enzymes which have varying substrates activities; chitosanase exclusively hydrolyse chitosan (Shadia *et al*. 2008) into glucosamine oligomers (Yoon *et al*. 1998). Numerous chitosanases have been produced from microorganisms like actinomycetes, fungi and bacteria (Kim *et al*. 2004). These microbial chitosanases exhibit different hydrolytic activity patterns, either endo-type cleavage or exo-type cleavage (Xiong *et al*. 2009). Exotype chitosanase activity releases a single glucosamine residue and glucosmine oligomers, while endotype chitosanase produces COS with greater degree of polymerization which are functionally active (Sinha *et al*. 2012).

Even though microbial chitosanses produces chitooligosccharides, the costly purification process makes it expensive to be utilised in a large-scale production. Therefore, exploration of industrial applications for these enzymes requires the screening of promising microbial isolates with high chitosanase activity, as well as to develop simple efficient methodology for extracting and purifying chitosanolytic enzymes (Chen *et al*. 2005; Shimosaka *et al*. 1995). This focus led to the isolation of a promising chitosanase producing microbial isolate for biologically functional chitooligosaccharides. Our isolate *Bacillus S9* exhibits promising chitosanase enzyme activity for degrading chitosan. The optimal production condition of chitosanase was analysed and characterized. In addition, characteristics and antibacterial activity of chitooligosaccharides was also reported and discussed in detail.

Materials and Methods

Screening of chitosanase producing organisms

The chitin rich sources of poultries chicken feather dumped pit soil samples from various places of Tamil Nadu were collected. The two sets of samples were collected one from the pit that had been filled for 1 month and another from a pit that had been filled for 6 months taken from the depth of 10cm. The soil samples were diluted serially,

and then the suspension (0.1ml) plated and incubated on to the CDA plates (Cheng and Li 2000) at 30° C for 3-5 days. The zone of clearance on the CDA plates indicated the existence of chitosanase producing organism

Quantification of Chitosanase activity

Enzyme activity was quantitatively estimated by measuring the reducing sugar liberated while break down of chitosan with DNS (Miller 1995). The crude extract's protein content was checked by Lowery *et al*. (1951).

Optimization of Chitosanase production

Four medias such as nutrient broth with 1% chitosan, CDA broth, Minimal Salt Chitosan, Enrichment medium/Shrimp shell media (Shrimp shell powder-15g, Ammonium phosphate -1g/l, Magnesium sulphate heptahydrate 0.3g/l, Peptone -1.5g/l,Yeast extract-1g/l,Potassiun dihydrogen phosphate-,0.3g/l,Nacl -1.5g/l) were used to determine chitosanase production. After incubating the cultures (24 hrs at 37° C)were centrifuged for 15 minutes (10,000 rpm) and supernatant was used for quantitativeassay.

Screening of chitosanase production using shrimp powder by Plackett-Burman design (PBD)

In this study, the aim was to optimize chitosanase production using RSM. The independent factors: shrimp shell powder, $(NH_4)2SO_4$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, Yeast extract, Nacl, Peptone, cultivation temperature and initial pH were screened for significant chitosanase production. The factors were analysed at low level (–1) and high level (+1) individually. The trials conducted in triplicate and activity was measured (Table 2).

Central composite design (CCD) for media using shrimp shell powder

The correlation between the variables such as $(NH_4)2SO_4$ (X1), shrimp shell powder (X2) and $MgSO_4.7H_2O$ (X3) was determined using central composite design (CCD). The variables were examined at three different levels: low level (-1), zerolevel (0) and high level (+1). The codes, variable levels, CCD matrix and chitosanase activity for each trial are presented.

The data obtained from RSM regarding the chitosanase production underwent analysis of variance (ANOVA). The RSM data obtained were analysed using response

surface regression method (1) provided to elucidate the variables impact of quadratic, cross and linear product terms

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

In the above, Y represents the measured response, β0 denotes 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} indicates quadratic coefficients, β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and β_{45} represents interaction coefficient. The coded independent variables are A, B, C, D and E. The optimal concentrations of variables were determined by analyzing contour plots. The model evaluation in ANOVA, with the coefficient of determination R^2 indicating the quality of fit and F-test determines the statistical significance. The optimization of the maximum yield was conducted basedon the equation by Design Expert software version 8.0.7.1. Through 3D plots, contour, and hump,the optimal concentration of each parameter was identified.

Characterization and Identification of the Isolates

The genomic DNA of microbial isolates producing chitosanase were extracted using STE buffer and chloroform extraction. The quality and quantity were assessed through agarose gel electrophoresis, with Ethidium Bromide (EtBr) staining at a concentration of 0.5µg/ml. 16S rDNA was subseuently amplified using the isolated genomic DNA with forward primer 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and reverse primer 1942R (5´-GGTTACCTTGTTACGACTT-3´).

The Qiaquick PCR purification kit PCR manufactured by QIAGEN; USA was used to purify 16SrDNA. The forward and reverse primers utilized for amplifying 16S rDNA region in BigDye Version 3.1 kit. The sequencing reaction was carried out on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems). Ambiguous sequences were corrected with Chromas (Version 2.01) and assembled with BioEdit (Version 7.0.9.0). All the processed sequences were submitted to NCBI databank using Bankit tool and are assigned with accession number (Figure 3)

Purification of Enzyme-Ammonium sulphate fractionation

During the enzyme purification process, ammonium sulphate was slowly mixed to reach level of 80% saturation, with gently stirring to ensure complete dissolution and

equilibrium. The proteins then precipitated were subjected to centrifugation for 15min at 4°C. The resultant was then mixed with 5ml of phosphate buffer (pH 7.0). The remaining supernatant was further saturated with ammonium sulphate for 100% (w/v) saturation and both fractions were dialysed.

The partially purified enzyme molecular weight was determined with SDS-PAGE as described by Lammeli, 1970. The bands obtained for the enzyme samples were compared with protein marker.

Partially purified enzyme stability

The enzyme was assayed for pH stability at pH (3-9) and the enzyme was incubated in buffer with above pH ranges without chitosan substrate for 90 min assayed for enzyme activity. Similarly, the enzyme solution was analysed for optimum temperature 30-90 ◦C and the thermal stability for 90 min at optimal pH (El-Sherbiny 2011).

Preparation of Chitooligosaccharides

Chitooligosaccharides were prepared through hydrolysis of chitosan using partially purified chitosanase enzyme. The enzyme (1ml) was combined with 1% chitosan (1ml) for 30 minutes at 55°C. The reaction was then terminated by boiling the mixture for 5 minutes at 100°C. After cooling the solution was mixed with 0.25M of sodium hydroxide and subsequently centrifuged for 2 minutes at 1,000 X g. The chitooligosaccharides present in the supernatant was separated and stored for further studies (Zhang *et al*. 1999).

Preparation of chitooligosaccharide gel

The chitooligosaccharide solution of about 0.5ml was taken and 1.5ml of 10%ormaldehyde solution were added and kept undisturbingly for the formation of the hydro gel. The hydrogels were synthesised through crosslinking of the chitooligosaccharides with formaldehyde following the method of Lin and Metters (2006). Inorder to determine the time and the amount of formaldehyde solution required for the hydrogel formation, five samples of chitooligosaccharides (0.1 ml) are added with different volumes of formaldehyde solution. Water was then added until resultingreach 12.1 g. The gel formation crosslinking time was fixed between 5 and 120 min tillit reaches transparent.

Characteristics of chitooligosaccharides hydrogel -Swelling test of chitooligosaccharide gel Samples

A weighted amount of chitooligosaccharide gel samples was immersed in phosphate buffer of pH (7.4, 6.8 and1.2) at 35^{o} C until they swell at the equilibrium. The excess water on surface was removed and were weighted. The swelling ratio could be calculated as following

$$\text{Swelling ratio \%} = [(W_s - W_d) / W_d] \text{ X } 100$$

where**,**

W_s - Weight in wollen state, W_d -Weight in dry state.

Antimicrobial activity of chitiooligosaccharides

The antimicrobial properties of prepared COS hydrolysate were tested against multi drug resistant diabetic foot ulcer pathogens *Staphylococcus aureus, Escherichia coli* was procured from KMCH Laboratories, Coimbatore and Vellore CMC, Tamilnadu. The chitooligosaccharide hydrolysates at different concentrations starting from 10 to 100µlt were taken in well and left to incubate for 24 hours at 37°C.

Hen's egg test on chorioallantoic membrane (HET – CAM)

The CAM assay was carried out as outlined Budai and Varnagy (2000) to investigate the allergic reaction or biocompartibility of COS mixture which was applied on the surface of chorio-allantonic membrane (CAM) and evaluated the irritant endpoints (vascular lysis, hemorrhage and coagulation) developments and test substances qualitative assessments of irritational potential.

All the samples were administered on the chick eggs g Chorio-allantoic membrane surface which was then observed using stereo-zoom microscope. The Irritation Score (IS) calculation was carried out done as per IS [B] analysis method (NIH, 2006). An IS score could be calculated as below,

$$\left(\left(\frac{(301 - \textit{Hemorrhage time})}{300}\right) \times 5\right) + \left(\left(\frac{(301 - \textit{Lysis time})}{300}\right) \times 7\right) + \left(\left(\frac{(301 - \textit{Coagulation time})}{300}\right) \times 9\right)$$

where, Hemorrhage time = time (in seconds) of the first appearance of blood hemorrhages, Lysis time = time (in seconds) of the first appearance of vessel lysis, Coagulation time = time (in seconds) of first appearance of protein coagulation.

Figure. 1. Isolates producing clear zone of halos in CDA plates

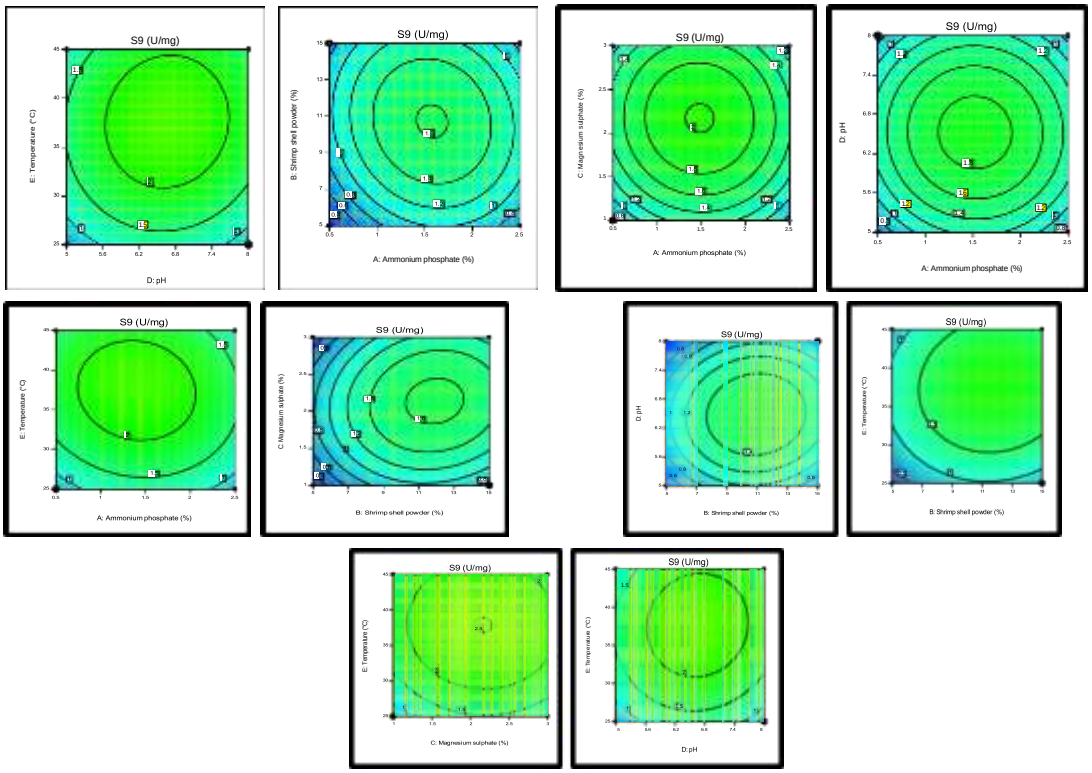

Figure. 2.a-j Contour plot of S9 on shrimp media on chitosanase activity

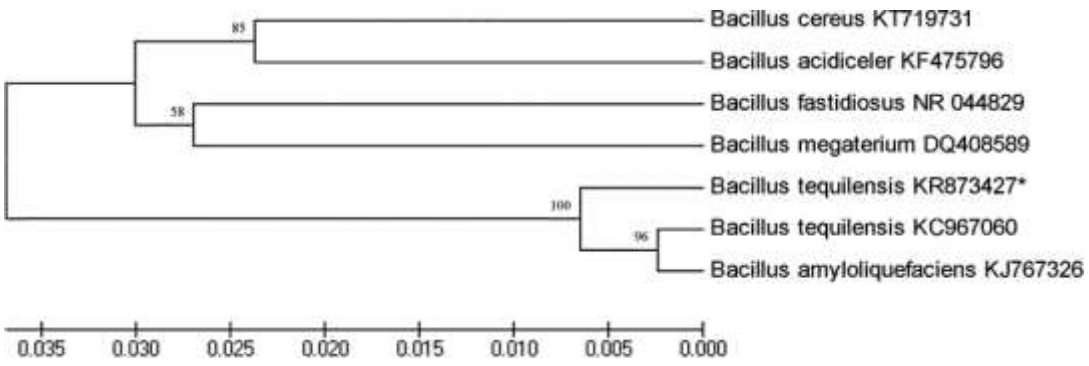

Figure. 3. Phylogentic relatedness of 16S rDNA between isolate S9 *Bacillus tequilensis*

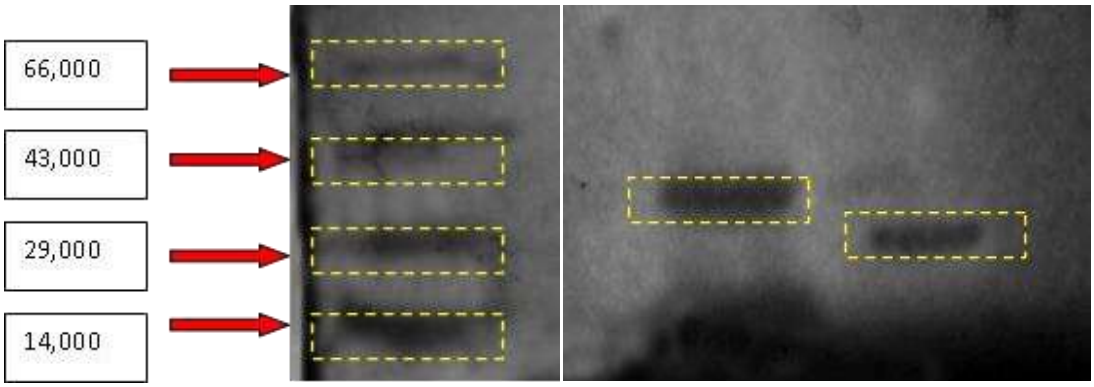

Figure. 4. Molecular weight determination of the chitosanase by SDS PAGE of crude enzyme of S9 and S9 partially purified purified enzyme

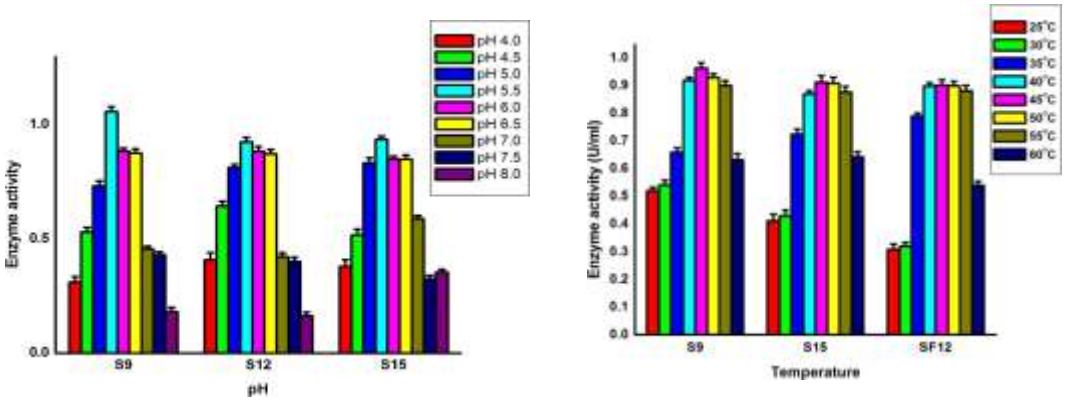

Figure. 5. Effect of pH, Temperature on the partially purified enzyme activity of the isolate S9

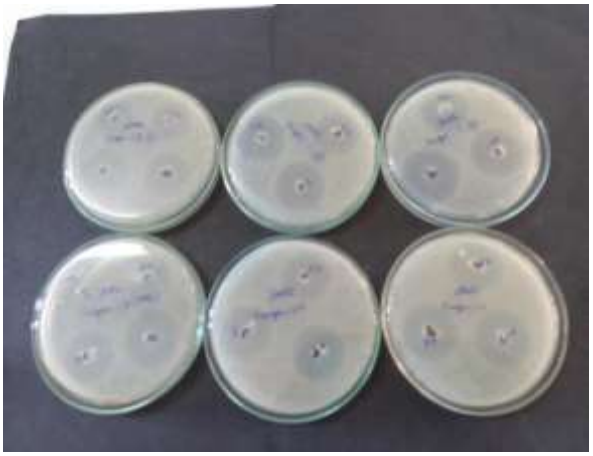

Figure. 6. Antimicrobial activity of COS by well diffusion method

Figure. 7. COS hydrogel

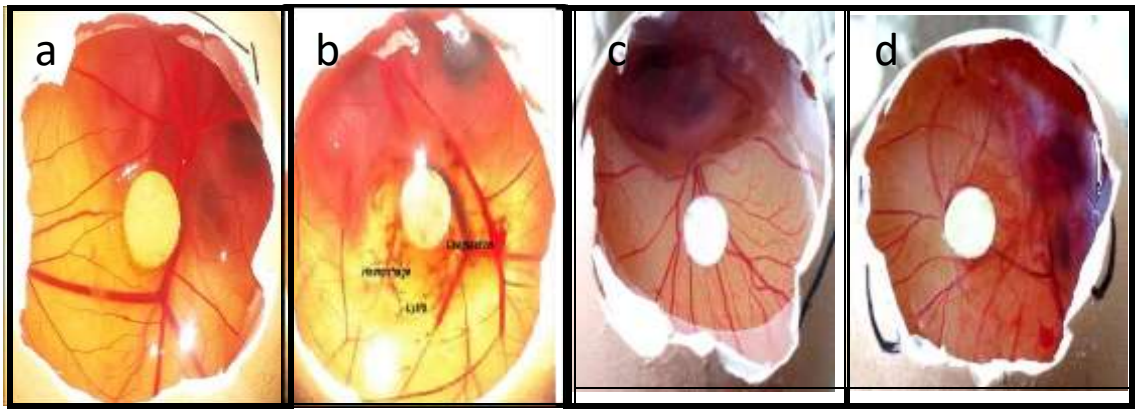

Figure. 8. Effect of chitoligosaccharide hydrolysate on CAM assay (a) negative control, (b) positivecontrol, (c) Chitosan control, (d) S9

Table 1: Microorganisms isolated from different soil samples

SOIL SAMPLES	No. of samples	Bacteria	
		ZP	NZP
Chicken feather dumped soil-Fresh (Uthukuli poultry)	6	1	22
Chicken feather dumped soil - 1 month soil (Uthukuli poultry)	7	7	18
Chicken feather dumped soil – 6-months soil (Erode poultry)	6	-	37
Chicken feather dumped soil – 1 month soil (Namakkal poultry)	6	**5**	12
Chicken feather dumped soil – 1month soil (Erode poultry)	4	8	5
Chicken wastes dumped soil – 6 months (Namakkal poultry)	7	-	-
TOTAL	**36**	**21**	**94**

Table 2: Culture Media Optimization for Chitosanase production

Microbial strains	Nutrient broth with 1%chitosan	Minimal salt Medium	M9/CDA broth	Shrimp shell with M9
S9	**0.743±0.632 [a]**	**0.312±0.021 [a]**	**1.260 ± 0.001 [a]**	**1.048±0.041 [a]**
S3	0.452±0.01[a]	0.071±0.000 [b]	1.222 ± 0.000 [b]	0.943±0.297 [b]
S7	0.032±0.21 [a]	0.548±0.232 [c]	1.181 ± 0.165 [c]	0.432±0.231 [c]
S15	0.13±0.0201 [a]	0.632±0.117 [d]	1.164 ± 0.002 [d]	0.765±0.154 [d]
S1	0.137±0.064 [a]	0.712±0.021 [c]	1.121 ±0.324 [e]	0.811±0.064 [e]
S 8	0.217±0.311 [d]	0.432±0.121 [a]	1.174 ± 0.612 [f]	0.789±0.332 [f]
S2	0.118±0.091 [e]	0.554±0.123 [e]	1.141 ±0.011 [c]	0.927±0.311 [g]
S4	0.201±0.132 [f]	0.642±0.112 [a]	1.147 ± 0.065 [g]	0.753±0.000 [h]
S5	0.211±0.378	0.571±0.062 [a]	1.161 ± 0.012 [h]	0.819±0.438 [h]
S6	0.203±0.121 [c]	0.661±0.143 [f]	1.183 ± 0.145 [i]	0.747±0.601 [i]
S10	0.275±0.331 [a]	0.665±0.112 [g]	1.227 ± 0.721 [j]	0.832±0.321 [k]
S11	0.105±0.112 [a]	0.567±0.022 [h]	1.169 ±0.412 [k]	0.642±0.022 [m]
S12	0.654±0.129 [a]	0.554±0.132[j]	1.124 ± 0.234 [l]	0.714±.0171 [l]
S14	0.432±0.211 [a]	0.443±0.543 [i]	1.141 ± 0.126 [m]	0.732±0.123 [n]

Table 3: Codes and levels of variables and statistical analysis of Plackett-Burman design

Terms	Code Levels		T-value	P-value
	–1	1		
$(NH4)_2SO_4$	1.0	2.0	42.30	0.015
Yeast extract	1.5	3	–1.15	-0.305
KH_2PO_4	2.0	4.0	-2.255	0.225
Peptone	1.0	2.0	–9.45	0.067
Shrimp waste powder	10.0	15.0	18.00	0.035
NaCl	1.5	3.0	–10.00	0.063
$MgSO_4 \cdot 7H_2O$	1.5	2.5	11.15	0.046
pH	4.5	5.5	–1.50	0.374
Temperature	25	35	–1.70	0.339

Outline criterion: 0.05, a significant at 5 % level

Table 4: Experimental designs used in RSM studies by using five independent variables showing observed values of specific activity for S9 chitosanase activity in shrimp media

Run	Ammonium phosphate (g)	Shrimp shell powder (g)	Magnesium sulphate (g)	pH	Temperature (°C)	Actual S9 chitosanase activity (U/ml)
1	0.5	15	3.0	8	25	0.873
2	0.5	15	1.0	8	25	0.654
3	2.5	5	3.0	5	45	0.632
4	2.5	15	3.0	5	25	1.027
5	0.5	15	1.0	5	25	1.002
6	1.5	10	2.0	6.5	35	2.156
7	2.5	5	3.0	5	25	0.597
8	0.5	15	3.0	5	45	1.476
9	0.5	15	3.0	5	25	1.481
10	2.5	15	3.0	8	25	1.076
11	2.5	15	1.0	5	25	0.441
12	0.5	5	3.0	5	45	0.61
13	2.5	15	1.0	8	45	0.961
14	2.5	5	1.0	8	45	0.931
15	0.5	5	1.0	8	25	0.3318
16	2.5	15	1.0	5	45	1.09
17	2.5	15	3.0	8	45	1.076
18	0.5	15	1.0	8	45	1.876
19	0.5	15	1.0	5	45	1.038
20	0.5	5	3.0	8	45	0.54
21	1.5	10	2.0	6.5	35	4.112
22	0.5	5	1.0	5	25	0.431
23	2.5	5	1.0	5	45	0.532
24	2.5	15	1.0	8	25	1.211
25	0.5	5	1.0	8	45	0.756
26	1.5	10	2.0	6.5	35	4.112
27	0.5	5	3.0	8	25	0.37
28	1.5	10	2.0	6.5	35	4.112
29	0.5	5	3.0	5	25	0.539
30	1.5	10	2.0	6.5	35	4.112
31	2.5	5	1.0	5	25	0.831
32	0.5	15	3.0	8	45	2.517
33	1.5	10	2.0	6.5	35	4.112
34	2.5	5	1.0	8	25	0.21
35	2.5	15	3.0	5	45	1.42

Run	Ammonium phosphate (g)	Shrimp shell powder (g)	Magnesium sulphate (g)	pH	Temperature (°C)	Actual S9 chitosanase activity (U/ml)
36	2.5	5	3.0	8	25	0.721
37	1.5	10	2.0	6.5	35	4.112
38	1.5	10	2.0	6.5	35	4.112
39	0.5	5	1.0	5	45	0.799
40	2.5	5	3.0	8	45	0.32
41	1.5	10	3.0	6.5	35	0.983
42	1.5	10	2.0	6.5	35	1.31
43	1.5	10	2.0	6.5	35	3.461
44	1.5	10	2.0	6.5	35	0.921
45	1.5	10	2.0	5	35	0.311
46	1.5	10	2.0	6.5	25	0.111
47	1.5	10	2.0	6.5	35	0.328
48	1.5	10	2.0	6.5	35	3.461
49	1.5	10	2.0	6.5	35	0.008
50	1.5	10	2.0	6.5	58	0.172
51	1.5	10	2.0	6.5	35	2.86
52	0.5	10	2.0	6.5	35	0.04

Table 5: ANOVA for response surface quadratic model for S9

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Block	1.17	1	1.17		
Model	80.33	20	4.02	17.73	< 0.0001
A-Ammonium phosphate	3.46	1	3.460	1.528	0.9691
B-Shrimp shell powder	2.91	1	2.91	12.86	0.0011
C-Magnesium sulphate	0.32	1	0.32	1.42	0.2417
D-pH	1.362	1	1.362	6.012	0.9387
E-Temperature	0.56	1	0.56	2.47	0.1261
AB	0.28	1	0.28	1.25	0.2718
AC	0.023	1	0.023	0.10	0.7526
AD	0.011	1	0.011	0.051	0.8234
AE	0.30	1	0.30	1.31	0.2610
BC	0.31	1	0.31	1.38	0.2486

BD	0.13	1	0.13	0.59	0.4499
BE	0.21	1	0.21	0.93	0.3417
CD	0.035	1	0.035	0.15	0.6976
CE	0.029	1	0.029	0.13	0.7227
DE	0.16	1	0.16	0.72	0.4031
A^2	19.06	1	19.06	84.18	< 0.0001
B^2	12.73	1	12.73	56.21	< 0.0001
C^2	17.04	1	17.04	75.22	< 0.0001
D^2	23.08	1	23.08	101.92	< 0.0001
E^2	23.32	1	23.32	102.96	< 0.0001
Residual	7.02	31	0.23		
Lack of Fit	3.43	22	0.16	0.39	0.9649
Pure Error	3.59	9	0.40		
Cor Total	88.52	52			

R- Squared	0.9196
Adj R-Squared	0.8678
Pred R- Squared	0.7904

Table 6: Time duration for the hydrogel formation

Sample	**Formaldehyde solution (ml)**	**Time of gel formation (min)**				
		5	**10**	**15**	**20**	**25**
S9	1.5	-	-	-	+	+

Table 7: Swelling test of chitooligosaccharide gel samples

S. No	Sample	pH – 4			pH – 6			pH – 7		
		Weight in dry state (mg)	Weight in swollen state (mg)	Swelling ratio (%)	Weight in dry state (mg)	Weight in swollen state (mg)	Swelling ratio (%)	Weight in dry state (mg)	Weight in swollen state (mg)	Swelling ratio (%)
1	S 9	40	42.25	5.62	40	44.07	10.17	40	42.53	6.32
2	Chitosan	40	42.59	6.47	40	42.30	5.75	40	42.41	6.02

Table 8: Comparative evaluation of irritation scores for test materials, negative control and positive control by HET-CAM test

Materials on CAM	Mean value of endpoint development			Irritation score [b]
	Hm[a]	Hy[a]	Cg[a]	
Sample-1(negative control)	0	0	0	0
Sample-1(positive control)	5.9	6.7	6.3	18.9[c]
S9	0	0	0	0

Hm- hemorrhage, Hy-hyperemia, Cg-coagulation.,a - Mean values of time until development of identified endpoint, b - Irritation score calculated as described by IS [B] analysis,c - Irritation category – severe irritation.

Result and Discussion

The objective of this work was screening and characterizing the chitosanolytic microbial isolates for their potential biomedical importance. We collected soil samples from different places where chicken feathers had decomposed of 1month deposited and 6 months deposited. These samples were screened for presence of chitosanolytic microbes. The soil sample were plated after diluting serially on CDA medium with 0.5% and 1% soluble chitosan (Choi *et al*. 2004). After 3 -5 days of incubation at room temperature, microbial colonies showing zones were observed on CDA plates with both 0.5% and 1% chitosan. Shaida et al. (2012) reported about using 0.5% soluble chitosan for the isolation of chitosan utilising organism. Similarly screening of colonies on CDA revealed presence of noticable halos or clear zones (Shimosaka *et al*. 1995).

The chicken feather decomposed poultry soil considered as a dominant source for isolation of prominent chitosanase producers of the present study. Among the 36-sample collected, it was noticed that only 21 isolates revealed clear zone on CDA plates. Whereas 94 isolates were with absence of clear zone as noticed in the above case. In the group of zones producing colonies, all 20 isolates were obtained from the one-month chicken feather decomposed soil and remaining 1 isolate was obtained from few days of feather deposited soil (Table 1). Among the 21 zone producing colonies the higher zone producers of 15 colonies were selected and named as SB1 to SB15 Among all the isolates, the isolate SB9 (Fig 1) revealed high chitosanase activity and was chosen for further detailed studies.

S9 was identified as *Bacillus tequilensis* by molecular characterization and phylogenetic relationship constructed and submitted to GenBank with accession number KR873427 (https://www.ncbi.nlm.nih.gov/nuccore/KR873427)

Culture media optimization for the effective chitosanase production

S9 medium showed the maximum activity in the shrimp shell media shown in Table 2 and activity was highest on the third day (Gao *et al*. 2008). The present result coincided with the results of Shimosaka *et al*.1995., Chen and Li 2000 that the suitable media for production of chitosanase to be M9 with chitosan (CDA) media. All the isolates show highest chitosanase activity in CDA media but not in shrimp shell medium wastes.

Optimal Conditions for Chitosanase Production-Central Composite Design

Screening significant factors for chitosanase production using Plackett-Burman design (PBD) as shown in Table 3. Shrimp shell powder, Magnesium sulphate and $(NH_4)2SO_4$, exerted positive effects on chitosanase production but NaCl, peptone,yeast extract, KH_2PO_4, initial pH, cultivation temperature exerted negative effects. The factors including shrimp shell powder ($P = 0.035$), magnesium sulphate ($P = 0.046$) and ammonium sulphate ($P = 0.015$) had significant effects on chitosanase production.

The next step of optimizing the medium using CCD is selecting five significant factors ammonium sulphate, shrimp shell powder, magnesium sulphate, pH (5-8) and temperature (27- 37 º C). The corresponding responses with actual values are presented in design model (Table 4)

Statistical analysis of variance (ANOVA) of CCD for S9 Chitosanase production on shrimp shell media

Fisher's statistical test for ANOVA was used to analyse the model for the chitosanase production and the results are tabulated (Table 5). *F* value of 17.73 for chitosanase production signifying the model is significant with "Prob > *F*" values of 0.0001 less than 0.0500 indicating significance of model term. The model's fit was assessed by the coefficient of determination R^2, which was 0.9196, indicating 91.6 %. It indicates that the mathematical model of the experimental value is reliable for chitosanase activity

The coefficients were computed and were expressed in terms of coded factors:

S9 = + 3.66-2.826 E- 0.003*A + 0.26 * B + 0.086 * C -5.607 E- 0.03 * D + 0.11 *E- 0.094 * AB – 0.027 * AC – 0.019 * AD – 0.096 * AE + 0.099 *BC + 0.064 * BD + 0.081* BE – 0.033 * CD – 0.030 * CE + 0.071 * DE – 0.57 * A^2 – 0.46 * B^2 – 0.53 * C^2 -0.62 * D^2 – 0.63 * E^2

3D contour graphs drawn against two variables on the Z axis, while one variable was maintained its level optimum. The contour plot (Figures 2.a-j) display a significant degree of curvature making it easy to identify the optimum level. The S9 isolate, chitosanase production was 4.112 U/ml with specific conditions: ammonium sulphate was 1.5g/l, shrimp shell powder concentration was 10%, magnesium sulphate was 2.0 g/l was 10%, pH 6.5 and temperature 35º C.

Partial purification of chitosanase & SDS PAGE

The concentrated supernatants after ammonium sulphate precipitation exhibited chitosanase activity. Present study revealed expression of crude enzyme is 40 kDa and partially purified 29 kDa of chitosanase in the isolate S9 (Figure 4). The molecular weight of most of the microbial chitosanase ranged from 10-50 kDa (Somashekar and Joseph, 1992). Wang et al. (2008) reported 21kDa chitosanase from *Serratia marcescens* subsp *marcescens*. The molecular weights of the most of the chitosanase of the present study fell into the range of 20–40kDa. 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 S9 enzyme fall into the endo chitosanase class.

Partially purified enzyme –effect of pH and temperature

The pH activity profiles revealed maximum activity for S9 was at pH 5.5. The stable pH was5-7 for S9. The S9 chitosanase was relatively stable at pH 5 - 7.5. Furthermore, it is obvious from the present study, a sharp drop in activity was observed at pH values higher than 7.5 Earlier reports of El Sherbiny et al. 2007. El Sherbiny 2011 noticed chitosanase enzyme with optimal pH 5.0, whereas Choi et al. 2004, Chasanah et al. 2009 noticed the same with pH 7.0. The optimum temperature for the activity of S9 was 45ºC and was stable between 40ºC - 50ºC (Figure 5) whereas El Sherbiny 2011 noticed 40˚C optimal temperature and 60˚C temperature stability.

Preparation of chitooligosaccharides

The portion of the enzyme mixture was taken and mixed to 1% chitosan solution with varied time of incubation. No precipitate revealed the depolymerisation into oligosaccharides (Zhang et al. 1999).

Antibacterial activity of chitooligosaccharides

S9 COS hydrolysates showed higher activity against the foot ulcer pathogens. (Figure 6) The chitooligosacharride hydrolysate showed significantly stronger antimicrobial activities than chitosan and chitosanase.

The present results exactly matched with that of the chitosan hydrolysate obtained by the partial enzymatic hydrolysis (Uchida1989). Sudarshan, Hoover and

Knorr (1992) reported that the water soluable chitosan showed antimicrobial activity for both Gram negative and Gram-positive bacteria. Our investigation has controversial results with that of Jeon, Park and Kim et al. (2001) on the antibacterial effect of the chitooligoosaccharide that they were greater than the chitosan and also disproved the results of Jeon,Park and Kim (2001).Thus the chitooligosaccharide hydrolysate reportedin the present study is novel and has greater potency in biomedical research.

Preparation COS hydrogel

The formaldehyde solution of about 1.5 ml is required for the formation of the gel in the present study is listed in Table 6 after 20 minutes and was able with stand at room temperature for 48 hrs (Figure 7). Among the different cross-linking agents – formaldehyde was used as cross-linking agents because of their cheap and easily available. The aldehyde plays crucial role in water retention efficiency cross linking capacity, hydrophilic properties of hydrogel. The hydroxyl group of the polymers require low pH, high temperature, etc to cross link with aldehydes, on the other hand amine containing polymers such as chitooligosaccharides can be cross linked with the aldehydes in a milder condition due to the formation of Schiff bases (Singh *et al*.2006).

Swelling test of chitooligosaccharide gel Samples

The swelling ratio for the chitooligosaccharide hydrogel calculated as following in the Table 5. In the present study the swelling behavior at various pH levels of 2, 4, 6, 7, 9 and 10 at room temperature was checked. S9 gel.showed higher swelling at pH 6 (Table **7).** The hydrogels kept the shape of integrity after longer exposure time (after 300 hr) increased which synchronized with the results of Shu and Zhu (2002).

Effect of chitoligosaccharide hydrolysate on CAM assay

All the test samples along with chitosan control did not develop any irritant end points revealingthat biocompatiblity (Figure 8). The results were thus compared with both control samples (Figure 8) which showed irritant endpoints. And irritation score (IS) was calculated (Table 8) using the standard formula.

Conclusions

Chitooligosaccharides are widely studied for biomedical applications. It has a significant impact on the material properties essential for tissue engineering applications, as well as biodegradation and biocompatibility. *Bacillus tequilensis* was isolated from the chicken feather decomposed soil sample for the preparation of chitooligosaccharide enzyme. The prepared hydrolysate showed higher antibacterial activity against selected test pathogens. The outcome from the present study it was concluded that the reported chitosanase could be utilized for enzymatic hydrolysis of chitosan and the product chitooligosaccharide could be used in treatment of diabetic foot ulcer. Thus, the decomposed soil sample could be a source of promising novel chitosanase production for preparing a novel bioactive chitooligosaccharide.

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