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Comparison and Optimization of Biological and Antibacterial Activity of Indian White Shrimp Waste Extracted by Biosynthesis Methods through Response Surface Methodology

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Indian white shrimp waste extracted by biosynthesis methods involving demineralization, deproteinization and deacetylation. In this study chitosan with high functionality was produced using mild conditions (temperature (60, 80 and 100 °C), concentration of alkaline (30, 40 and 50 %) and time (90, 195, 300 mins) of reaction in chemical method and power of microwave (300, 600 and 900 W), concentration of alkaline (30, 40 and 50 %) and irradiation time (20, 100, 180 S) in microwave method). Results showed that significant differences (P<0.05) were observed in decreasing of the viscosity and molecular weight of the chitosan samples with increasing concentration of alkali solution (30 to 50 %). DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activities of chitosan samples (15.26 to 17.77%) were observed and samples had low antioxidant activity compared with BHT (Butylated hydroxyltoluene). The antibacterial activities were examined against bacteria (*Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitosan showed the inhibition of the growth of various bacteria tested although it depends on the molecular weight of samples and the species of bacteria.

Key words: Indian white shrimp, Chitosan, Biosynthesis, Antimicrobial activity, Response surface methodology (RSM).

A technological point of view, it would be economic to converting the by-products of seafood processing because of its richness in high value compounds added such as (30-40%) protein, (30-50%) calcium carbonate and (20-30%) chitin on a dry basis[1]. Chitin a homopolymer of N-acetyl-D-glucosamine, ß- (1 - 4) N-acetyl-D-glucosamine, is the most widespread renewable natural resource following cellulose and the major component of the exoskeleton animals like crustaceans, shrimps, insects and fungal cell walls (2). Chitosan, ß- (1 - 4) D-glucosamine, is a cationic amino polysaccharide which is a partly deacetylated (more than 50%) form of chitin[3].

Traditional chemical method for the commercial isolating of chitin from shrimp shell involves alkali and acidic treatments[4]. In recent studies much more interested has been observed in the electromagnetic irradiation because it can accelerate the reaction of conventional heating treatment compared to traditional methods[5]. Some of the past studies reported the roles of **various**

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factors, such as different extraction methods of chitosan, granularity and particle size of raw chitin, temperature and time of reaction, concentration and rate of reagents to chitin and atmospheric qualification on molecular weight and deacetylation reaction of chitosan[6, 7].

Chitosan has been interested in medicine, pharmaceutical, biomedical, biological, agriculture, environment and in food technology such as, food formulations, binding, thickening, gelling, stabilizing, clarifying and used as antimicrobial biopackaging due to its effectiveness of inhibiting the growth of not only bacteria (gram-positive and gram-negative bacteria) but also yeasts and moulds[8, 9]. To prevent lipid oxidation of food products can be added synthetic chelating agents and antioxidants but in new days searchers focused on new natural preservatives. Until now, several sources of natural antioxidants, antibacterial and antifungal were recognized, both biopolymer chitin and chitosan have been shown to activate the antioxidants, antifungal and antibacterial which are affected by its molecular weight and concentration[8, 10] Response surface methodology (RSM) is a very useful statistical technique for complicated chemical, physical and food processes optimally[6]. The aim of present study is to evaluate the effect of chemical and microwave technique at different conditions on functional, antioxidant and antibacterial properties of the chitosan extract exclusively from Indian white shrimp waste by response surface methodology.

MATERIALS AND METHODS

In present article, Chitosan was isolated from waste of species of Indian white shrimp (*Penaeus indicus*). The species is mainly cultured in Saudi Arabia, Vietnam, India and Islamic republic of Iran[9]. Shells of Indian white shrimp are a source of hazardous **contamination** because shells exhibit a very low rate of decomposition[11]. Indian white shrimps were procured from a local region in Persian Gulf. The shrimp shells were washed, dried and grained. Other ingredients like sodium hydroxide, acetic acid, ethanol and acetone were purchased from Merck.

The process of extraction involved deproteinization (2 % (w/w) sodium hydroxide solution 30:1 (w/v) for 2 h at 80 ºC) and demineralized (10% (w/w) acetic acid 40:1 (w/v) for 4 h at 50 ºC). After each step separation done by centrifugation (4,000 g, 15 min) and the solution from centrifuge was used in next steps (Deacetylation of chitin). The deacetylation of chitin was produced by precipitation of sodium hydroxide solution. The parameters employed at chemical and physical methods ,all reaction under microwave irradiation was carried out in the microwave device Butane microwave (M 245, Iran), are listed in Table 1.

Determination Intrinsic viscosity and molecular weight (Mw) of chitosan

The intrinsic viscosity was measured according to Weska *et al* (2007) by some alteration. The viscosity of samples was measured by flowing through a capillary in a Cannon-Fenske capillary viscometer (model 9721-B53, USA) at 25 ºC. The molecular weight of the chitosan was estimated by applying the Mark Houwinke Sakurada equation[12, 13].

Apparent Viscosity of chitosan

The apparent viscosity of chitosan was determined using a Brookfield viscometer (model RV-DVIII, Brookfield programming Rheometery, Inc., USA). Chitosan solution was prepared in 1% acetic acid at 1 % concentration on dry basis[14]. Measurements were carried out by using spindle 2 RV at 40 rpm at 20-±-1 ºC and results were recorded in cP.

Antioxidant activity of chitosan

The antioxidant/ free radical-scavenging activity of chitosan was evaluated using DDPH (2, 2-diphenyl-1-picrylhydrazyl) according to the method of Blois (1958) with some modification[15]. The chitosan sample of various concentrations (2.5, 5, 7.5 and 10 µg/mL) were prepared. The absorbance was then measured at 517 nm using a spectrophotometer (CECIL 2502-Instruments Cambridge England Serial no 125- 624). Butylated hydroxyltoluene (BHT) was used to compare the DPPH free radical-scavenging activity was calculated by following equation 1:

Scavenging activity (%)=[1-((absorbance sample)/(absorbance control)]×100) ...(1)

Determination of minimal inhibitory concentrations for antibacterial activity

The bacterial activity of samples was measured by enumeration of viable organisms.

gram- positive bacteria: *Staphylococcus aureus* (PPCC 1431) and gram- negative bacteria: *Esherichia coli* (PPCC 1399) and *Salmonella typhimurium* (ATCC 14028) were grown in Mueller Hinton Broth (MHB) incubated overnight at 37 ºC. Minimum inhibitory concentrations (MICs) are important to determine resistance of microorganisms to antimicrobial agents and antibacterial activities of samples were examined as inhibitory effects against the growth of bacterial by a turbidimetric method. Briefly, in this method, a number of test tubes each containing 5.0mL of MHB were autoclaved. Chitosan powder was added to 0.25% acetic acid. To the first tube, 5.0 mL of chitosan solution (1mg/mL) suspension was added. After mixing, 5.0 mL of the mixture was transferred to the second tube, and similar transformations were repeated. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The tubes were inoculated with the freshly prepared for each bacteria suspension (50 µL). The positive control was given with doxycycline, and the blank control tubes only contained MHB and 0.25% acetic acid. After mixing, the tubes were incubated at 37 ºC for 24 h. The tubes were then studied for the visible signs of turbidity. The lowest concentration of chitosan that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC. The minimum bactericidal concentrations (MBCs) are determined by assaying for group of the organisms in those tubes from the MIC test that showed no growth. A loopful from each of those tubes was examined for signs of growth on EMB (Eosin–Methylene Blue) agar[16].

Experimental design and statistical analysis

Box–Behnken Design (BBD), which is well suited for accommodating a quadratic surface and action well for the process optimization, was offered for the experimental design. The quadratic regression models for predicting the response variable are obtained from BBD (equation 2):

$$Y=Ck_0+ Ck_i x_i+ Ck_{ii} x_i^2+ Ck_{ij} x_i x_j \quad ...(2)$$

Where Y is the predicted response variable; C_{k0}, C_{ki}, C_{kii} and C_{kij} represent regression coefficients; and, X_i, and X_j are the coded. The quality of the model was expressed by the coefficient of determination (R^2), adjusted-R^2 (R^2 adj), and predicted-R^2 (R^2pred). The regression analyses, graphical analyses and analyses of response surfaces were carried out using Design Expert software 7.1.6 (Stat-Ease Corporation, USA) to examine the effects of parameters and their combination on extraction levels.

RESULTS AND DISCUSSION

Optimisation of molecular weight and viscosity of chitosan

The molecular weight of chitosan, biopolymer with high molecular weight, depends on the raw material sources and the method of extraction. The reduced viscosity of each treatment with Huggins equation was used for the determination of intrinsic viscosity and graph of each sample was indicated the reduced viscosity (η_{sp}/C) in relation to four solution concentration for regression analyses. The viscosity average molecular weight of chitosan for each sample was calculated by taking the values of $K= 1.81\times10^{-3}$ Ml/g and $\alpha= 0.93$ [2]. The molecular weights of samples were ranged from 1105032 to 806931 Da (Table 2).

These values showed a good agreement between the actual and predicted values (Figure 1_{a-b}). Good adjustments to these models were for Mw of chitosan extraction of chemical (Y_1) and microwave (Y_2) designs by the high determination coefficient (in coded form), (3 and 4):

$$Y_1 = 983398-42436 X_1-27151 X_2 +32026 X_3 +15795 X_1^2+29928 X_{22}+ 10770 X_3^2+7760 X_1 X_2- 17959 X_1 X_3-17542 X_2 X_3 \quad ..(3)$$

$$Y_2=860577-45349 X_1-25300 X_4 +26326 X_5 + 41544 X_4^2 \quad ...(4)$$

The viscosity of chitosan is an important factor in the conventional determination of molecular weight and samples varied considerably from 220.32 to 428.40 cP depending on the different extraction conditions (Table 2). The two regression models for predicting the optimal viscosity of chitosan of chemical (Y_3) and microwave (Y_4) techniques were obtained from BBD, these models were following equations (5 and 6):

$$Y_3=353.91-25.93 X_1-14.08 X_2 +14.40 X_3 +17.77 X_2^2 \quad ...(5)$$

$$Y_4=251.40-30.39 X_1-18.29 X_4+16.90 X_5 +5.98$$

X_1^2+27.76 X_4^2-7.55 $X_1 X_5$ ... (6)

It is noticed that molecular weight were significantly correlated with viscosity of chitosan (. Data revealed that the Mw and viscosity of chitosan decreased by increasing of both NaOH concentration (from 30 to 50 %) and temperature of mechanism, however with higher reaction time (more than 180 min in chemical and 30 S in microwave methods) changed the behavior of Mw and viscosity of samples to the worst. Results are related to deacetylation of chitosan did not completely occur at low concentration of NaOH, time and temperature because the acetyl groups cannot be separated at ambient condition, the reaction need high temperature and time to achieve a suitable deacetylation which it is leading to dismissal of acetyl groups thus increasing number of NH_2 groups in fragment structure, but if these factors explosion a lot, these will have a negative effect on Mw and viscosity of chitosan.

Table 1. Levels of various independent variables at coded values of RSM experimental design

Chemical method (Model 1) Run order	Variables and Units		
	NaOH concentration: X_1 (%) by weight	Reaction temperature: X_2 Celsius (ºC)	Reaction time: X_3 Minutes (min)
1	-1 (30)	-1 (60)	0 (195)
2	1 (50)	-1 (60)	0 (195)
3	-1 (30)	1 (100)	0 (195)
4	1 (50)	1 (100)	0 (195)
5	-1 (30)	0 (80)	-1 (90)
6	1 (50)	0 (80)	-1 (90)
7	-1 (30)	0 (80)	1 (300)
8	1 (50)	0 (80)	1 (300)
9	0 (40)	-1 (60)	-1 (90)
10	0 (40)	1 (100)	-1 (90)
11	0 (40)	-1 (60)	1 (300)
12	0 (40)	1 (100)	1 (300)
13	0 (40)	0 (80)	0 (195)
14	0 (40)	0 (80)	0 (195)
15	0 (40)	0 (80)	0 (195)
Microwave method (Model 2) Run order	Variables and Units		
	NaOH concentration: X_1 (%) by weight	Power of microwave: X_4 Watt (W)	Irradiation time: X_5 Second (S)
16	-1 (30)	-1 (300)	0 (100)
17	1 (50)	-1 (300)	0 (100)
18	-1 (30)	1(900)	0 (100)
19	1 (50)	1(900)	0 (100)
20	-1 (30)	0 (600)	-1 (20)
21	1 (50)	0 (600)	-1 (20)
22	-1 (30)	0 (600)	1 (180)
23	1 (50)	0 (600)	1 (180)
24	0 (40)	-1 (300)	-1 (20)
25	0 (40)	1(900)	-1 (20)
26	0 (40)	-1 (300)	1 (180)
27	0 (40)	1(900)	1 (180)
28	0 (40)	0 (600)	0 (100)
29	0 (40)	0 (600)	0 (100)
30	0 (40)	0 (600)	0 (100)

Table 2. The optimum conditions by the BBD design on molecular weight (M_w) and viscosity of chitosan extraction

Run order	Mw of chitosan (Da)		Viscosity (Cp)		Run order	Mw of chitosan (Da)		Viscosity (Cp)	
	Experimental value [a]	Predicted value	Experimental value [a]	Predicted Value		Experimental value [a]	Predicted value	Experimental value [a]	Predicted value
1	1105032±510	1106467	428.40±20	419.72	16	969832±321	974370	332.21±09	331.44
2	1005032±870	1006076	370.24±14	364.42	17	914320±560	903161	274.90±11	275.44
3	1037690±632	1036645	382.32±09	388.13	18	932100±1392	943258	300.21±13	299.66
4	968730±170	967294	331.01±12	339.68	19	837612±460	833073	233.31±06	234.07
5	1004876±233	1002413	364.71±17	370.99	20	911237±987	896330	264.71±15	266.28
6	955531±1228	953460	312.61±06	316.03	21	806931±863	807721	220.32±10	220.58
7	1100314±965	1102384	400.14±23	396.71	22	951862±981	951071	315.45±21	315.18
8	979132±767	981594	354.22±24	347.93	23	843380±1178	858286	240.86±05	239.29
9	1000653±980	1001679	374.58±19	376.97	24	900320±485	910688	283.48±15	282.67
10	978954±1674	982460	345.11±07	333.01	25	850012±910	853760	248.77±06	247.74
11	1104321±551	1100814	377.89±05	389.98	26	960761±1445	957013	317.11±09	318.13
12	1012456±867	1011429	380.01±21	377.61	27	923109±387	912741	279.09±16	279.89
13	983765±1989	983398	353.81±23	353.91	28	867900±662	860577	251.33±19	251.40
14	984320±953	983398	357.42±09	353.91	29	863510±1973	860577	248.73±07	251.40
15	982109±541	983398	350.51±16	353.91	30	850321±650	860577	254.15±10	251.40

[a] Mean ± standard deviation (n = 3)

Table 3. MIC (µg/mL) and MBC (µg/mL) of chitosan solution samples against various microorganisms in 0.25% acetic acid

Run order	MIC (µg/mL) and MBC (µg/mL) of chitosan solution							Microorganisms
	3.90	7.81	15.62	31.25	62.50	125	250	
1			×	+	××	++		*E. coli*
				+	×	++	××	*S.typhimurium*
16			+	×			++ ××	*S. aureus*
2		×	+	××	++			*E. coli*
			×	+	××	++		*S.typhimurium*
17	×		+		××		++	*S. aureus*
3			+	×	++ ××			E. coli
				+	×	++	××	S.typhimurium
18		+	×			++	××	S. aureus
4	×	+		++	××			E. coli
			+	×	++	××		S.typhimurium
19		+	×			++	××	S. aureus
5		×	+	××	++			E. coli
			+	×	++	××		S.typhimurium
20			+	×	××	++		S. aureus
6	×	+		++	××			E. coli
		×	+	××		++		S.typhimurium
21	×	+				++	××	S. aureus
7			×	+	××	++		E. coli
				+	×	++	××	S.typhimurium
22		×	+			++	××	S. aureus
8	×	+		++	××			E. coli
		×	+	××	++			S.typhimurium
23	×	+			××	++		S. aureus
9	×	+		++	××			E. coli
			+	×	++	××		S.typhimurium
24		+ ×			××	++		S. aureus
10	×	+		++	××			E. coli
		×	+	××		++		S.typhimurium
25		+ ×				++	××	S. aureus
11			×	+	××	++		E. coli
			×	+	××	++		S.typhimurium
26		×	+			++	××	S. aureus
12		×	+		++ ××			E. coli
		×		+	××	++		S.typhimurium
27			+ ×			++	××	S. aureus
13	×	+		++	××			E. coli
		×	+	××	++			S.typhimurium
28	×	+			××	++		S. aureus
14	×	+		++	××			E. coli
		×	+	××	++			S.typhimurium
29	×	+			××	++		S. aureus
15	×	+		++	××			E. coli
		×	+	××	++			S.typhimurium
30	×	+			××	++		S. aureus
Doxycycline				++			××	E. coli
				++			××	S.typhimurium
Control					++		××	S. aureus

× = MIC (µg/mL) of Chitosan produced by chemical method; ××= MBC (µg/mL) of Chitosan produced by chemical method and Doxycline; + = MIC (µg/mL) of Chitosan produced by microwave method; + += MBC (µg/mL) of Chitosan produced by microwave method and control

Commercial chitosan usually has a molecular weight ranging from 50 to 2000 kDa and viscosity from 60 to 5110 cP depending on the species and the extraction methods[2, 8, 12] the results of these factors in this study were in the range of above.

Antioxidant activity of chitosan

The results show that maximum antioxidant activity of different concentration of

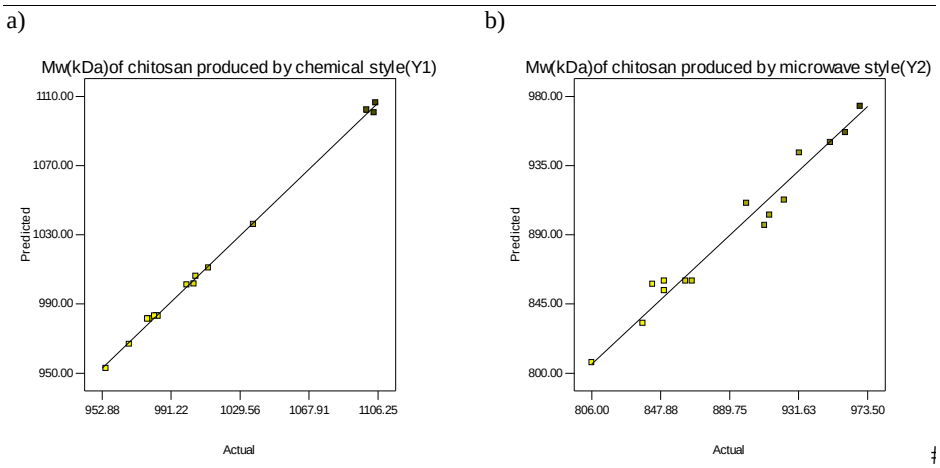

Fig. 1. (a-b)Comparison between predicted and actual values of the Mw of chitosan extraction from Indian white shrimp waste

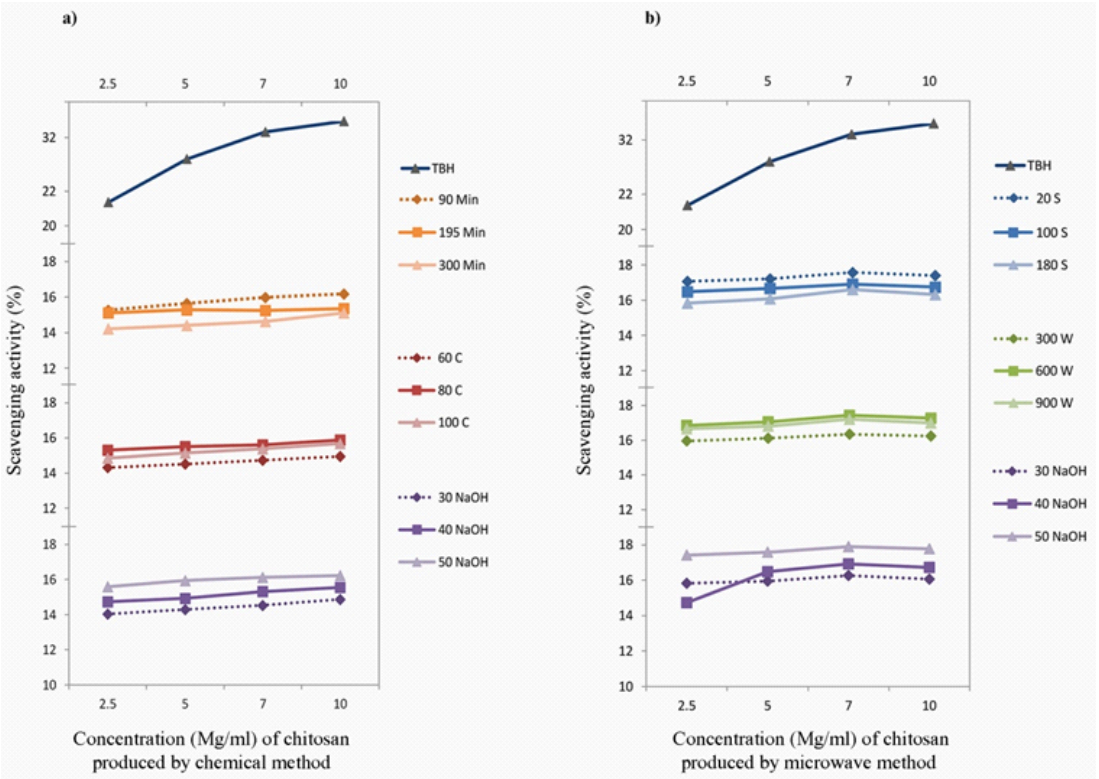

Fig. 2. (a-b)The optimum conditions, NaOH Concentration (30 to 50%), reaction temperature (60 to 100 °C) and reaction time (90 to 300 mins) in chemical style (Figure 1a) and NaOH Concentration (30 to 50%), power of microwave (300 to 900 W) and irradiation time (20 to 180 S) in microwave method (Figure 1b), on scavenging activity (%) of chitosan sample of various concentrations (2.5, 5, 7.5 and 10 µg/mL).

chitosan was observed at 50 % NaOH concentration (15.59 to 16.22 %), 80 ºC reaction temperature (15.29 to 15.87 %) and 90 Min reaction time (15.26 to 16.17 %) in chemical method (Figure 2_a). in microwave method at 50 % NaOH concentration (17.41 to 17.77 %), at 600 W power of microwave (16.84 to 17.26 %) and 20 S reaction time (17.06 to 17.39 %) was received the maximum antioxidant activity (Figure 2_b).

Scavenging activity of the samples were produced by chemical method increased with increase in concentration of chitosan (from 2.5 until 10 µg/mL) however activity for samples were extracted by microwave method reached their maximum activity at 7.5 µg/mL. This circumstance may be due to the fact that the activity of microwave samples did not contribute to increasing of concentration more than 7.5 µg/mL and a similar result has been reported by Ocloo *et al*., (2011). BHT had higher scavenging activity than the chitosan samples. Zhu *et al* reported that chitosan has no antioxidant activity and this finding was explained because of strong intra- and inter-molecular hydrogen bond[17]. Rao *et al* reported that 80% scavenging activity of 30 kGy chitooligosaccharide sample in a solution form of chitosan[18]. However our results showed that chitosan had a low antioxidant activity. The scavenging activity of samples may be due to the reaction between free radicals with the hydrogen ion form the ammonium ion (NH_3^+) to form a stable molecule[4, 8, 10]. The lower molecular weight or viscosity of chitosan demonstrated increase mobility and the chance of exposure of their residual free amino group and inhibition of lipid peroxidation[4].

Antibacterial assessment of chitosan

The MICs and MBCs of chitosan samples, doxycycline and the blank control are compared in Table 3. Results of MICs and MBCs of global microorganism were found, due to the use of different condition of production, to be 3.90 to 31.25 µg/mL and 31.25 to 250 µg/mL respectively.

It seemed the MICs and MBCs values of chitosan samples produced by microwave method are lower than chitosan samples produced by chemical method, which indicate higher antibacterial activity. According to these data, higher antimicrobial effect of samples was observed by increasing concentration of NaOH it may be because of more deacetylation and mobility of chitosan because the large amount of amino group enhances antibacterial activity of chitosan. Results concerning molecular weight were significantly correlated with MIC of *E.coli* ($Y_{Mw} = 8188\,X_{MIC\ of\ E.coli} + 861281,\ R^2 = 0.90$) and it is observed that increasing chitosan Mw led to decreasing activity against *E.coli*. However, the mechanism of the bactericidal effect of chitosan is not yet completely understood but according to the literature the most important mechanism is that bacteria can adhere to the surface of chitosan significantly in short time, thus it exhibit antimicrobial activity against a various number of bacteria. Some studies have reported that, the positive charge on the polycationic structure of chitosan (at acidic environment, pH=6.3, 6.5) is crucial for its antimicrobial activity through the electrostatic attractions between the negatively charged cell membrane of microorganisms.

A similar inhibition effect (MICs) was observed for both types of S. aureus and E. coli a similar result has been reported by Katas *et al*., (2011). The MICs and MBCs are applied to determine the antimicrobial activity of chitosan by many researchers. These factors are specified differently in diverse conditions and this makes the act of comparison between two elements difficult in different studies. In the nearly conditions Qi *et al*., (2004) reported MICs and MBCs of chitosan in the range of 8 to 16 µg/mL and 32 to 64 µg/mL respectively, the results of these factors in this study were in the nearly the range of above.

CONCLUSION

We focused on further development of the previous procedures of chitosan extraction and specifically on using waste of Indian white shrimp to produce potential chitosan by altering structure of shrimp waste. We demonstrated that the RSM was the most advantageous statistical technique for investigating the effect of major independent factors such as temperature, NaOH concentration, power of irradiation and time of reaction on the biological, antioxidant and antibacterial properties of chitosan for choosing the best biosynthesis treatment with the highest inhibitory influence of the growth of organism and low molecular weight

and viscosity. In the present study the microwave process has a considerable amount of attention because it is more advantageous over that of using the alkaline way. Significant correlations were observed between molecular weight and viscosity as well as between antibacterial activity and molecular weight. Further studies are needed to focus on size of chitosan extracted in different conditions to determine the properties such as antioxidant and antibacterial activity.

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