ABUAD Journal of Engineering Research and Development (AJERD)
Volume 1, Issue 1, 8-17

Statistical Optimization of Chitosan Extraction from Shrimp Shells Using Response Surface Methodology

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Date of First Submission: 17/09/2017
Date Accepted: 18/10/2017

Abstract**:** *The determination of optimum input parameters required for chitosan extraction from shrimp shells using response surface methodology (RSM) has been carried out in this study. The chitosan was produced from the shrimp shell waste by chemical method involving demineralization, deproteinization and deacetylation. The extraction was optimized using five input variables, viz. concentration of HCl (mol%), HCl immersion time (hr), concentration of NaOH (N), deacetylation temperature (°C) and deacetylation time (hr). Central composite design methodology was used to design the experiments carried out, with the aid of Minitab version 17. Thereafter, the analysis of results and optimization of the process were accomplished using the same Minitab software. From the results obtained, it was discovered that the extraction process of chitosan from shrimp shell gave 4.883% as the yield of chitosan when the concentration of HCl, the immersion time, the concentration of NaOH, the deacetylation temperature and the deacetylation time were 8 mol%, 48 hr, 3.5 N, 60 °C, and 1.5 hr, respectively. Good correlation was found to exist between the experimental and the predicted yields of chitosan as confirmed by the validation experiment carried out and the values of the square of the correlation coefficient of the developed model, which was estimated to be 0.9433.*

Keywords: Chemical method, central composite design, chitosan extraction process model, chitosan yield, surface optimizer.

1. INTRODUCTION

Synthetic polymers have long been the major daily life resources. Their applications range from dietary to mechanical support. However, these synthetic polymers constitute environmental pollution problems in disposal because they are non-biodegradable and consume large space [1]. In order to eliminate or reduce this environmental challenge, there is increasing demand for biopolymers as a substitute for synthetic polymers due to its biocompatibility, biodegradable and non-toxicity characteristics. Extraction of chitosan (as biopolymer) from shrimp shells can be developed to proffer solutions to these environmental pollutions. Also, chitosan is popular for its vital applications in medical and wastewater management fields due to its antibacterial and adsorptive properties respectively [2-5]. Chitosan is expedient in a wide industrial application such as pharmaceuticals, biochemistry, biotechnology, cosmetics and biomedical industries [6].

Shrimp shells waste generated by the sea food industry in Nigeria is another key problem contributing to environmental and health hazards [7]. They are insoluble in nature and occupy a big portion of land space, creating environmental pollution. Burning is often employed as a means of disposal, and this is ineffective and environmentally costly because of low burning capacity of the shells. However, the possible solution to this challenge is recycling by extraction of commercially viable products such as chitin and chitosan from the shrimp shells. Conventionally, extraction of chitin from raw shrimp shells consists of two steps, viz. demineralization and deproteinization. Then, chitin can be converted into chitosan by n-deacetylation, which partially removes acetyl groups from the polymer chain composition [8-9].

Chitosan extracted with variation in processing parameters were found in different studies such as nitrogen purging, reflux condition, high temperature, long treatment time and high concentration [10-11]. Various authors have studied the extraction of chitin and chitosan from waste shrimp shells using traditional technique: demineralization, deproteinization and deacetylation processes. Ameh *et al.* [12] reported the kinetics of demineralization of shrimp exoskeleton in chitin and chitosan synthesis. Ahing and Wid [13] studied and reported the extraction and characterization of chitosan from shrimp shell waste in Sabah. These extraction methods are laborious and consume time with low chitosan yield. Hence the need to optimize and establish viable operating parameters for chitosan extraction for industrial scale.

Recently, chitosan has been applied practically in dietary supplements, water and wastewater treatment, food preservation, agriculture, cosmetics, pulp, paper, and medical fields [9, 11, 14-16]. These demands create the need for mass production of chitosan. Therefore, understanding the effectiveness and efficiency of chitosan extraction method will improve the quality of the product and definitely give more benefits.

In order to contribute to estimating viable operating conditions for this process of chitosan extraction, this study

was carried out to optimize the conditions required for chitosan extraction process from shrimp shells using the central composite design (CCD) of response surface methodology (RSM) with the aid of Minitab.

2. METHODOLOGY

2.1 Sample Acquisition and Preparation

Fresh shrimp shell bio-wastes were collected from Lagos (Coordinates: 6.455027°N 3.384082°E), Nigeria. The collected shrimp wastes were washed to remove all the dirty particles on them after which they were dried using oven at a temperature of 60 ^{o}C until constant weight was achieved. The dried shell was then crushed with mortar and pestle. The crushed shrimp waste was kept in a polyethylene bag at ambient temperature for 24 hours for partial autolysis to facilitate chemical extraction of chitosan and improve its quality [17-18].

2.2 Experimental Design

In this work, response surface methodology (RSM) was applied with the aid of Minitab software [19] to design the experiments that were carried out. The set of experiments was designed using 5 levels (16 factorial points, 10 axial points, and 6 centre points) and 5 variables (HCl concentration, its immersion time, NaOH concentration, deacetylation temperature and deacetylation time) as shown in Table 1.

Table 1: CCD matrix for chitosan extraction experiment

Run No	A	B	C	D	E
1	8	16.5	2	50	2.5
2	12	16.5	2	50	1.5
3	8	37.5	2	50	1.5
4	12	37.5	2	50	2.5
5	8	16.5	3	50	1.5
6	12	16.5	3	50	2.5
7	8	37.5	3	50	2.5
8	12	37.5	3	50	1.5
9	8	16.5	2	70	1.5
10	12	16.5	2	70	2.5
11	8	37.5	2	70	2.5
12	12	37.5	2	70	1.5
13	8	16.5	3	70	2.5
14	12	16.5	3	60	1.5
15	8	37.5	3	60	1.5
16	12	37.5	3	60	2.5
17	8	27	2.5	60	2
18	12	27	2.5	60	2
19	10	16.5	2.5	60	2
20	10	37.5	2.5	50	2
21	10	27	2	70	2
22	10	27	3	60	2
23	10	27	2.5	60	2
24	10	27	2.5	60	2
25	10	27	2.5	60	1.5
26	10	27	2.5	60	2.5
27	10	27	2.5	60	2
28	10	27	2.5	60	2
29	10	27	2.5	60	2
30	10	27	2.5	60	2
31	10	27	2.5	60	2
32	10	27	2.5	60	2

In Table 1, A = HCl concentration (mol%), B = time of demineralization (hr), C = NaOH concentration (N), D = deacetylation temperature (^{o}C) and E = deacetylation time (hr).

2.3 Extraction of Chitosan

The crushed shrimp shell powder was made to undergo demineralization using hydrochloric acid (HCl), deproteinization process using weak sodium hydroxide (NaOH), and followed by deacetylation process using strong NaOH. In the demineralization process, 50 g of shrimp shell powder was immersed in 1000 ml of HCl (16.5-37.5 mol%) for 8-12 hr. Thereafter, it was treated with 50 ml of NaOH for an hour. The remaining powder was washed with deionized water. For the deproteinization, the demineralized shell was immersed in NaOH solution (2-3.0 N), followed by boiling in water bath for 1 hour to remove protein. The mixture was then cooled at room temperature for 30 min, filtered and washed with distilled water until it became neutral. The deacetylation (removal of acetyl groups from chitin) process was carried out by adding 50 mol% NaOH and then boiling at a temperature of 50-70 °C for 1.5-2.5 hr on a hot plate. The sample was placed under fumed hood and cooled for 30 minutes at room temperature. After that, it was washed continuously with the 50 mol% NaOH solution and filtered in order to retain its solid matter, which was the chitosan. Furthermore, the sample was oven-dried at 110 °C for 6 hr, based on the information obtained from the work of Puvvada *et al.* [2]. The residue was washed with deionized water until neutral pH was attained. The resulting chitosan was then dried in a cabinet dryer for 4 hr at 65 ^{o}C, and the yield was calculated using the expression given in Equation (1).

$$Yield\,(\%) = \frac{Weight\ of\ chitosan\ extracted}{Weight\ of\ sample\ used} 100\% \qquad (1)$$

2.4 Analysis of Variance and Optimization

The analysis of the data obtained from the experiments carried out was performed and a statistical model of the process in the form given in Equation (2) was formulated.

$$\begin{aligned} Y_p &= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_6 A^2 + \cdots \\ &\cdots + \beta_7 B^2 + \beta_8 C^2 + \beta_9 D^2 + \beta_{10} E^2 + \beta_{11} AB + \cdots \\ &\cdots + \beta_{12} AC + \beta_{13} AD + \beta_{14} AE + \beta_{15} BC + \beta_{16} BD + \cdots \\ &\cdots + \beta_{17} BE + \beta_{18} CD + \beta_{19} CE + \beta_{20} DE \end{aligned} \qquad (2)$$

where Y_p is the predicted chitosan yield (%), and β values are the regression coefficients for the variables of the model equation.

The analysis of variance of the developed model gave rise to its modification after considering the values of the probability value based on the confidence level (95%) chosen for the work.

Thereafter, the process was optimized by taking the five factors involved as the manipulated variables and the maximization of the yield of chitosan given as the objective function.

3. RESULTS AND DISCUSSION

The results obtained in this work, which was carried out on optimizing chitosan extraction process using shrimp shells via response surface methodology, are outlined and discussed thus.

3.1 Results of Experimental Design and Analysis

The experimental yields of extracted chitosan obtained together with the values of the factors (input variables) used are given in Table 2.

Table 2: Experimental chitosan yield with the values of the factors used for the experiment

Run No	A	B	C	D	E	Yield (%)
1	8	16.5	2	50	2.5	3.7152
2	12	16.5	2	50	1.5	0.5422
3	8	37.5	2	50	1.5	1.9087
4	12	37.5	2	50	2.5	1.3064
5	8	16.5	3	50	1.5	2.3321
6	12	16.5	3	50	2.5	1.1921
7	8	37.5	3	50	2.5	5.3337
8	12	37.5	3	50	1.5	2.1001
9	8	16.5	2	70	1.5	2.698
10	12	16.5	2	70	2.5	2.6159
11	8	37.5	2	70	2.5	2.9000
12	12	37.5	2	70	1.5	2.4060
13	8	16.5	3	70	2.5	2.3937
14	12	16.5	3	60	1.5	2.3937
15	8	37.5	3	60	1.5	2.8790
16	12	37.5	3	60	2.5	0.7408
17	8	27	2.5	60	2	3.2433
18	12	27	2.5	60	2	1.5100
19	10	16.5	2.5	60	2	2.6220
20	10	37.5	2.5	50	2	2.8071
21	10	27	2	70	2	0.8117
22	10	27	3	60	2	1.6781
23	10	27	2.5	60	2	0.9476
24	10	27	2.5	60	2	0.8881
25	10	27	2.5	60	1.5	2.1899
26	10	27	2.5	60	2.5	3.2177
27	10	27	2.5	60	2	2.0123
28	10	27	2.5	60	2	2.3400
29	10	27	2.5	60	2	2.3320
30	10	27	2.5	60	2	2.0088
31	10	27	2.5	60	2	2.7694
32	10	27	2.5	60	2	2.3368

Based on the results given in Table 2, the highest experimental yield was found to be approximately 5.33% when 8 mol% of HCl, 37.5 hr of demineralization time, 3.0 M of NaOH, 50 ^{o}C of deacetylation temperature and 2.5 hr of deacetylation time were used as the input variables. Furthermore, the lowest value of the yield was obtained to be 0.54 when the concentration of HCl was 12 mol%, the demineralization time was 16.5 hr, the NaOH concentration was 2.0 N, the temperature of deacetylation was 50 ^{o}C and the deacetylation time was 1.5 hr. It can be noticed from these results that the change in the yield of chitosan obtained from the extraction process was caused by the variation in the values of the input variables used. In other words, the output of the process, which was chitosan yield, was responding to the changes in the input variables. That observation was found to be a reason for saying that the chosen input variables were valid ones for the process.

Using the results obtained from the experiments carried out, a model was developed for the process after carrying out its analysis of variance, and it is given in Equation (3). The *p*-value of each variable of the model together with the coefficients of linear, quadratic and interaction of test variables that were estimated are presented in Table 3.

$$\begin{aligned} Y_p &= -32.43 - 0.533A + 0.039B + 9.89C + \cdots \\ &\cdots + 0.556D + 8.37E + 0.0242A^2 + 0.001644B^2 + \cdots \\ &\cdots - 0.745C^2 + -0.002679D^2 + 0.714E^2 + \cdots \\ &\cdots - 0.002679AB - 0.1350AC + 0.01698AD + \cdots \\ &\cdots - 0.3820AE + 0.0451BC - 0.002406BD + \cdots \\ &\cdots - 0.0115BE - 0.0712CD - 0.757CE - 0.00799DE \end{aligned} \quad (3)$$

Table 3: Estimated regression coefficients for chitosan yield model

Variable	Coefficient	P-value
Constant	-32.43	0.000
A	-0.533	0.000
B	0.036	0.303
C	9.89	0.144
D	0.556	0.807
E	8.37	0.024
A^2	0.0242	0.206
B^2	0.001644	0.029
C^2	-0.745	0.025
D^2	-0.002679	0.003
E^2	0.714	0.030
AB	-0.002679	0.210
AC	-0.1350	0.193
AD	0.01698	0.005
AE	-0.3820	0.002

Variable	Coefficient	P-value
BC	0.0451	0.033
BD	-0.002406	0.025
BE	-0.0115	0.549
CD	-0.0712	0.004
CE	-0.757	0.078
DE	-0.0799	0.002
R^2 = 94.33%;	Adj. R^2 = 84.03%	

The values obtained for the *p*-value of each variable showed that some input factors were not significant because their *p*-values were greater than 0.05, which was chosen based on 95% confidence level. The fact is that the lesser the *p*-value for a particular factor than 0.05, the more significant the factor is to the model [20].

Considering the results shown in Table 3, the extraction of chitosan had linear significant influence on A and E (because their *p*-values were less than 0.05), but at 95% confident level, B, C and D were found not to be significant. For the quadratic effect, B^2, C^2, D^2 and E^2 were observed to be significant while A^2 was not. For interaction, based on the estimated values of *p*-values, AB, AC, BE and CE were discovered not to be significant at 95% confident level that was chosen, but other interaction variables had significant effects on the model.

$$\begin{aligned} Y_p &= -32.43 - 0.533A + 8.37E + 0.001644B^2 + \cdots \\ &\cdots - 0.745C^2 - 0.002679D^2 + 0.714E^2 + \cdots \\ &\cdots + 0.01698AD - 0.3820AE + 0.0451BC + \cdots \\ &\cdots - 0.002406BD - 0.0712CD - 0.00799DE \end{aligned} \quad (3)$$

Simulating the developed model equation to obtain the predicted value of the chitosan yield, the results obtained are given in Figure 1. It can be seen from the results shown in Figure 1 that there was a good agreement between the experimental and predicted yield of the chitosan obtained from the extraction process. Therefore, it can be said that the developed model equation was a good representative of the process of chitosan extraction.

Furthermore, the precision and accuracy of the model equation was checked by determining the square of the correlation coefficient (R^2). The value of R^2 was used to determine the adequacy of the model; the higher the R^2 of a model, the better it is [21]. The value of R^2 obtained in this work was found to be approximately 94.33%, indicating that only approximately 5.67% of the total value of variation were not accounted for by the model. Adjusted R^2 is the corrected value for R^2 after the elimination of the unnecessary model terms [22]. The adjusted model of this work is given in Equation (3). The value of adjusted R^2 was estimated to be 84.03%, and this was high enough and close to the normal R^2 value to say that there were enough of significant terms included in the model and, as such, there was a very high correlation between experimental and test variables. The value of R^2 obtained was observed to be supporting the nature of the relationship found between the experimental and predicted yield (see Figure 1).

Further analysis of variance (ANOVA) was done to test the significance of regression towards linear, square and interaction of the parameters of the model. The large value of F obtained indicated that most of the variation in the test variables could be explained by the regression model equation [17, 22-24]. The *F* values (given in Table 4) for regression model, linear, square and interaction were high compared to *p*-values, and this means that the second order polynomial estimated in this work was highly significant and adequate to represent the actual relationship between the response (output variable) and the input parameters (factors).

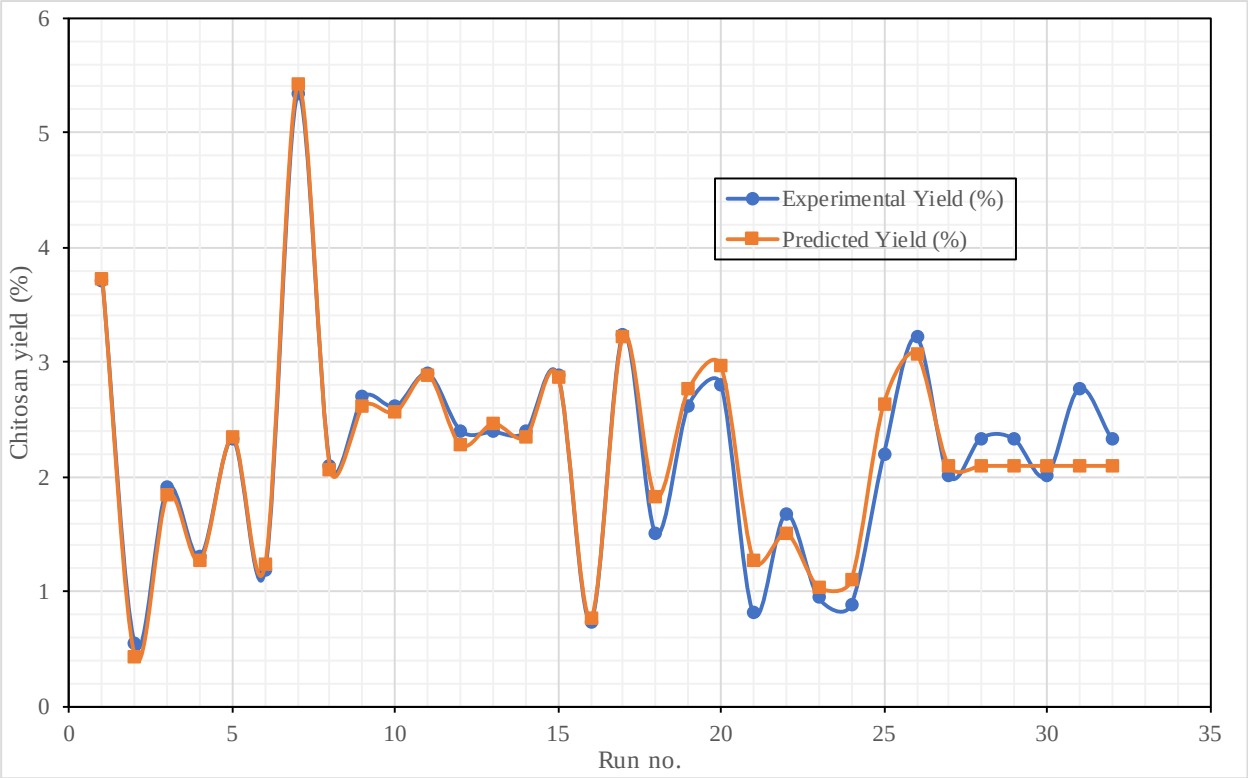

Figure 1. Comparison between experimental and predicted yields of chitosan

Table 4: Analysis of variance (ANOVA) for the chitosan extraction process

Source	DF	Adj SS	Adj MS	F-value	p-value
Regression	20	27.8090	1.3905	9.16	0.000
Linear	5	10.1580	2.0316	13.38	0.000
Square	5	5.7773	1.1555	7.61	0.024
Interaction	10	11.8736	1.1874	7.82	0.001
Residual error	11	1.6701	0.1518		
Lack-of-fit	6	1.2782	0.2130	2.72	0.146
Pure error	5	0.3919	0.0784		
Total	31	29.4791			

It was also found from Table 4 that the *p*-value for the regression model, linear, square and interaction were highly significant ($p<0.05$), which confirmed further that the model was a good representation of the process and that it could fit the experimental data very well.

Moreover, the contour and surface plots of the results were used to explain the relationship between the yield and the input variables of the process. Normally, the shapes of the contour plot, whether circular or elliptical, could be used to indicate the significance of the interactions among the variables of the process. For instance, a circular contour plot would occur when the corresponding input variables are negligible, and if the interactions between the variables are significant, the shape of the contour plot would be elliptical [25-26]. For this case of the chitosan extraction process, the contour and surface plots are shown in Figures 2 and 3 respectively. As can be seen from Figures 2a, b and c, the contour plots of the process were elliptical in shape, and this was found to be an indication that perfect interaction was occurring between the response and the input parameters.

From the surface plots shown in Figures 3a and b, it was observed that the yield of the chitosan extracted was increasing as the concentration of HCl was also increasing. However, it was found to decrease as the concentration was slightly above 10 mol%. Also, the increase in the concentration of NaOH was observed to give rise to increase in the yield of the chitosan obtained from the extraction process. For the temperature of deacetylation, it was found that as the temperature was increasing, the yield of chitosan extracted was also increasing. When a temperature of 70 ^{o}C was attained, the yield was observed to start to decrease. This observation was found to be similar to the one reported in the work of Zainal *et al.* [17].

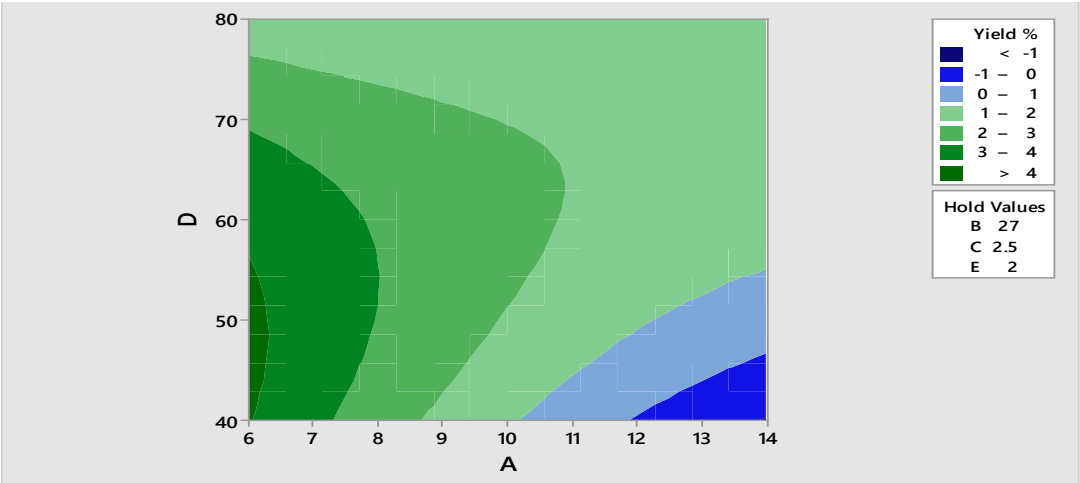

Figure 2a: Contour plot of chitosan extraction process considering factors A and D

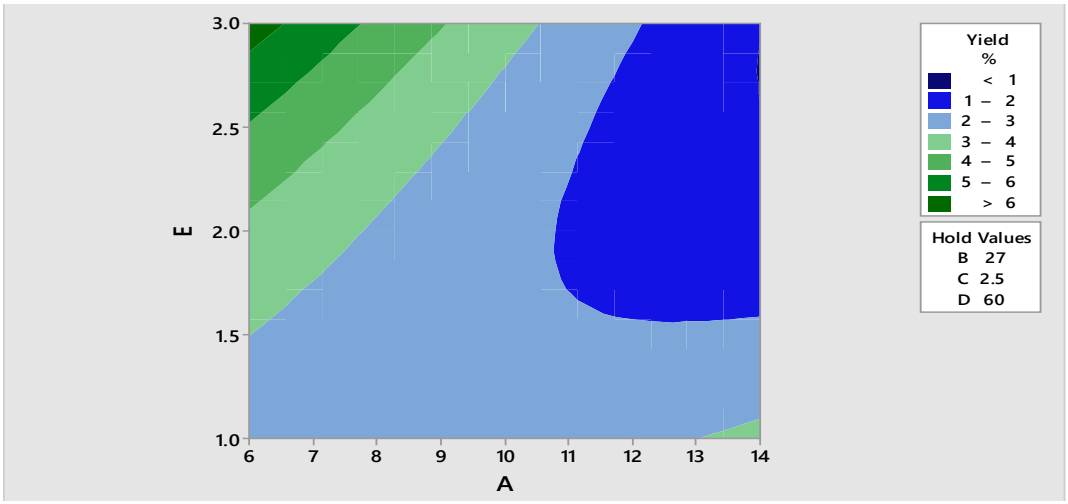

Figure 2b: Contour plot of chitosan extraction process considering factors A and E

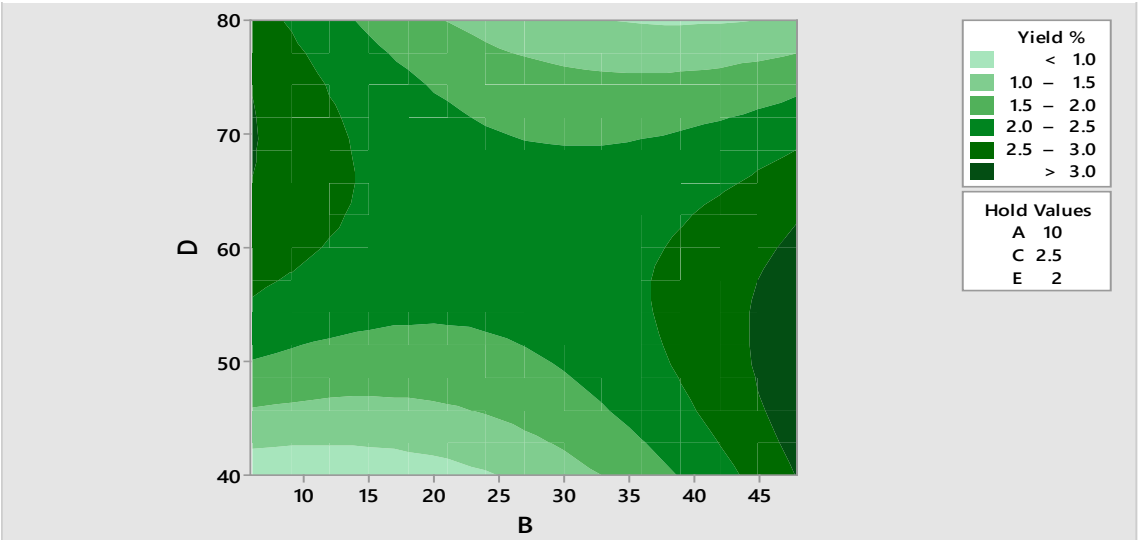

Figure 2c: Contour plot of chitosan extraction process considering factors B and D

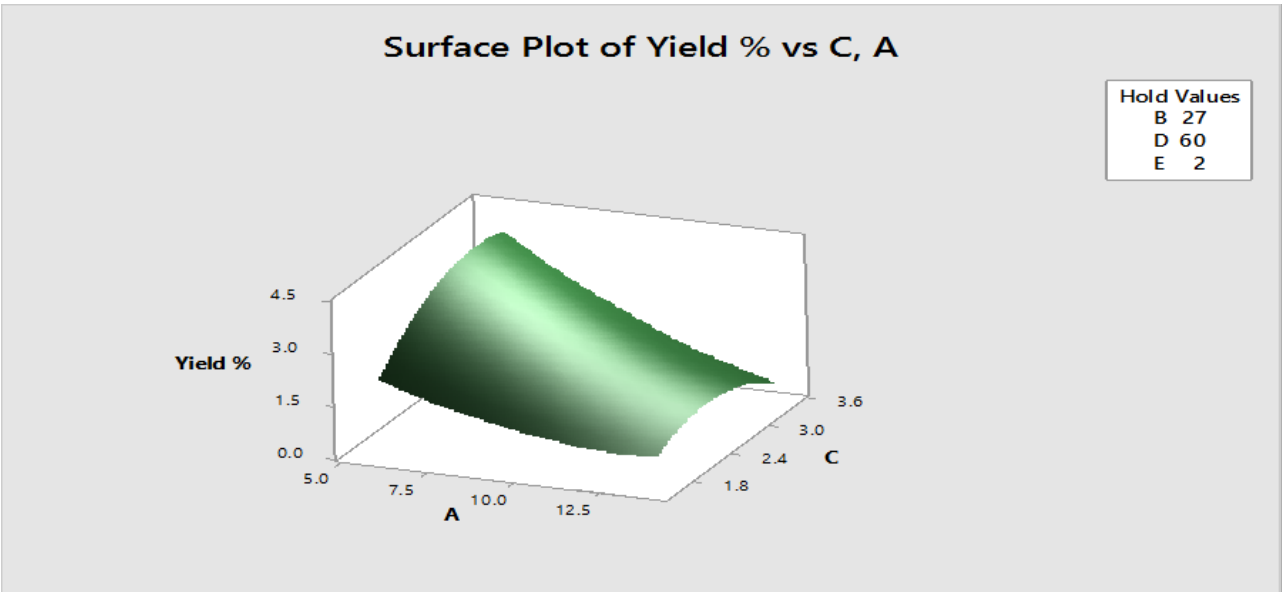

Figure 3a: Surface plot of chitosan extraction process considering factors A and C

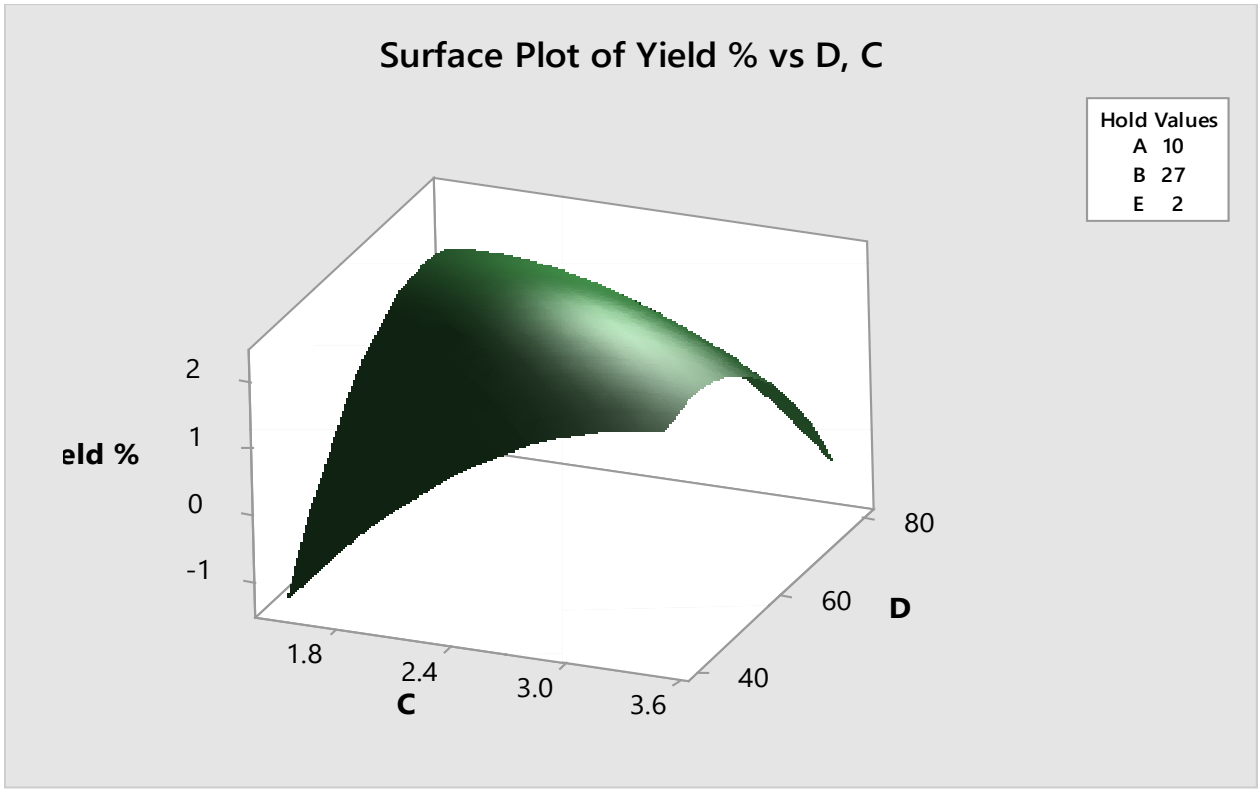

Figure 3b: Surface plot of chitosan extraction process considering factors C and D

3.2 Results of Optimization

The exact optimum parameters which led to response goal was formed and determined by the response optimizer using Minitab software version 17, and the results obtained were as given in Figures 4, 5 and 6.

From the results given in Figure 4, it was clear that to obtain a target yield of 5.0690, 8 mol% concentration of HCl, 48 hr of demineralization time, 2.2 N concentration of NaOH, deacetylation temperature of 50 ^{o}C and 2.5 hr of deacetylation time were required as the input variables.

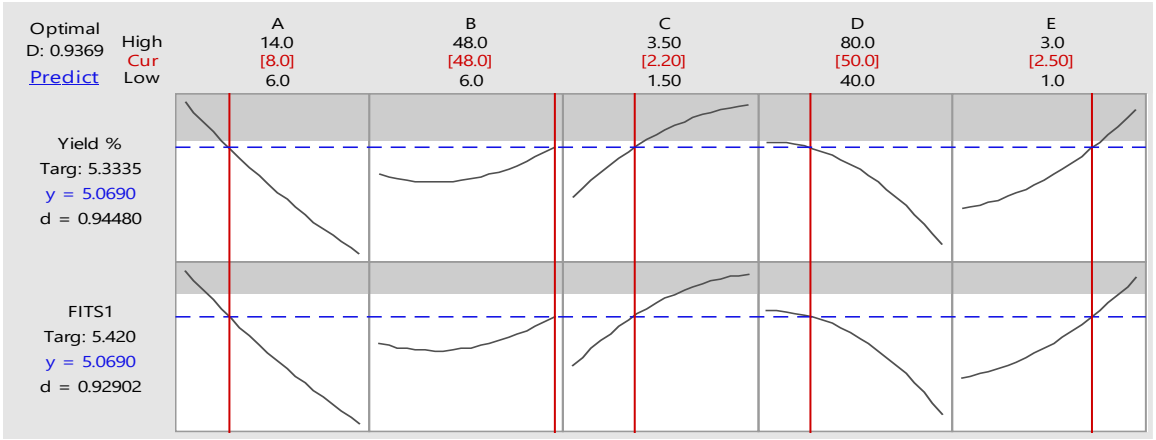

Figure 4: Response optimizer at optimum for the target goal

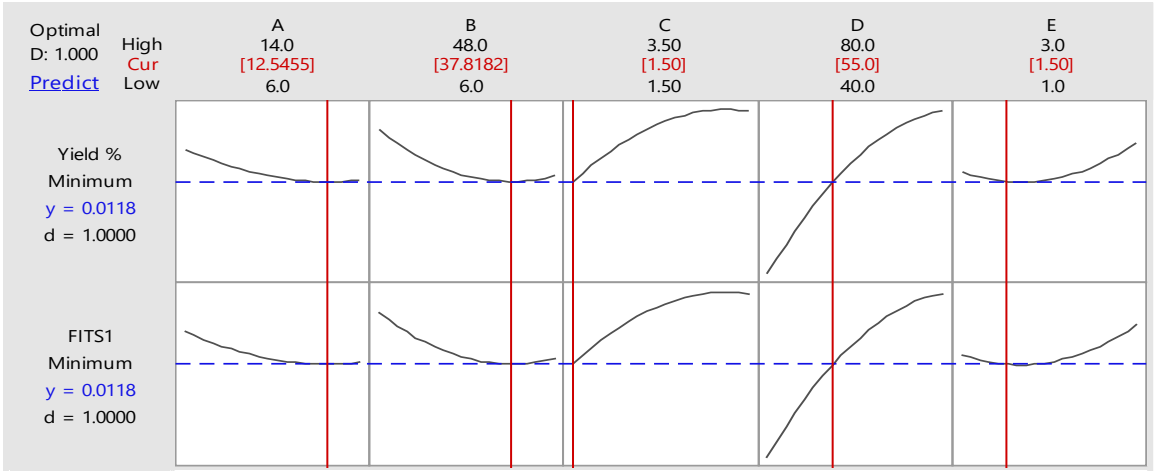

Figure 5: Response optimizer at optimum condition for minimum goal

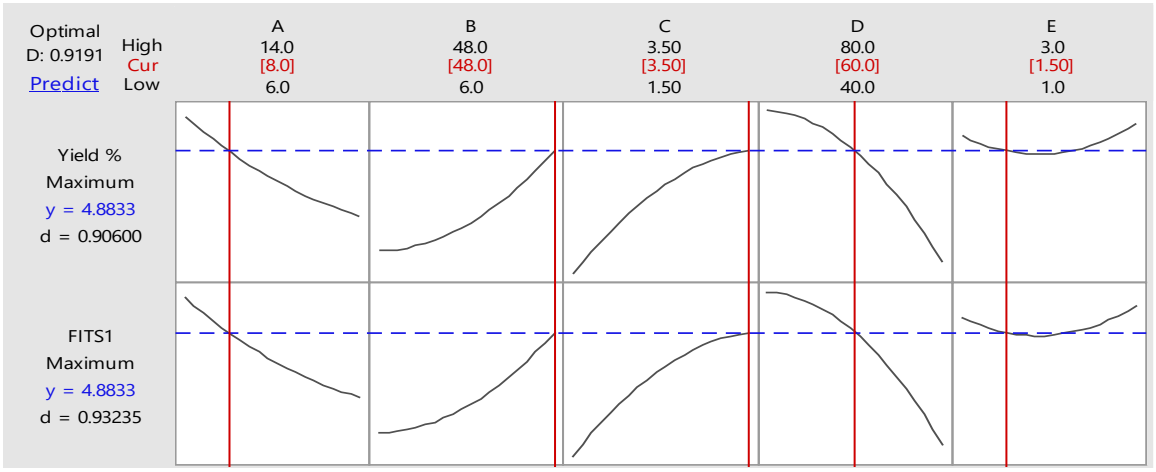

Figure 6: Response optimizer at optimum condition for maximum goal

It was further seen from the result of optimization given in Figure 5 that using HCl concentration of 12 mol%, 37 hr of demineralization time, 1.5 N NaOH, deacetylation temperature of 55 ^{o}C and 1.5 hr deacetylation time as the values of the input variables would give a very low yield of chitosan of about 0.0118%. As such, care should be taken when selecting the values of the input factors to be used for carrying out the extraction process of chitosan.

Furthermore, according to Figure 6, it was observed from the optimization that when HCl concentration of 8 mol% was used with 48 hours of demineralization, 3.5 N of NaOH, deacetylation temperature of 60 ^{o}C and 1.5 hr of deacetylation time, the maximum yield obtained from the process was 4.8830%. This has shown that the value of the chitosan yield obtained from the extraction process was a function of the input variables.

3.3 Validation

In order to validate the results obtained from the optimization carried out, an experiment was run using the optimum values of the variables estimated with the aid of Minitab via the response surface methodology, and it was discovered that the results compared very well because the experimental and the predicted chitosan yield were found to be 4.696 and 4.883 respectively. The closeness of the values, with less than 5% difference, was an indication that the optimum values of the variables estimated with the aid of Minitab were valid and feasible ones.

4. CONCLUSIONS

The results obtained from the optimization carried out on the extraction of chitosan from shrimp shell have revealed that a yield of chitosan of up to 4.883% could be obtained when the concentration of HCl was 8 mol%, the immersion time was 48 hr, the concentration of NaOH was 3.5 N, the deacetylation temperature was 60 ^{o}C and the deacetylation time was 1.5 hr. The results of the experiment carried out using the estimated optimum values also showed that the optimum values estimated were valid ones because there was a good agreement between the predicted and the experimental values of the yield of chitosan.

ACKNOWLEDGMENT

The authors are very grateful to Aare Afe Babalola, LL.B, FFPA, FNIALS, FCIArb, LL.D, SAN, OFR, CON – The Founder and President, and the Management of Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria for providing the necessary materials and conducive environment required for successful completion of this research work.

NOMENCLATURE

A	HCl concentration (mol%)
Adj	Adjusted
ANOVA	Analysis of variance
B	HCl concentration immersion time (hr)
C	NaOH concentration (N)
CCD	Central composite design
D	Deacetylation temperature (^oC)
DF	Degree of freedom
E	Deacetylation time (hr)
p-value	Probability value
R^2	Square of correlation coefficient
RSM	Response surface methodology
Y_p	Predicted yield of chitosan
β	Regression coefficients

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