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STATISTICAL OPTIMIZATION OF PARAMETERS FOR DEMINERALIZATION OF FISH SCALES USING RESPONSE SURFACE METHODOLOGY

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Abstract

Introduction: *Fish processing industries generate 30-40% of waste that is not properly dumped so becoming a pollutant for the global environment. Fish waste tends to get spoiled rapidly by synthetic or microbiological processes so cause diseases through contaminating the environment. Apart from the negative effects of fish waste, it also contains many useful substances such as chitin and collagen.* ***Aim:*** *The aim of this study was to optimize the demineralization conditions for chitin extraction from fish scales (Labeo rohita) using Response Surface Methodology (RSM).* ***Methods:*** *Fish scales were collected from the local market and demineralized using hydrochloric acid (HCl) at different concentrations, treatment times, and substrate-to-solution ratios. The demineralization process was optimized using RSM, and the effects of the independent variables on demineralization were analyzed using analysis of variance (ANOVA) and regression analysis.* ***Results:*** *The optimal conditions for demineralization were found to be 0.8M HCl, 1.5:10 substrate-to-solution ratio, and 4 hours of treatment, resulting in a maximum demineralization of 99.97%. The results showed that the concentration of HCl and treatment time had significant effects on demineralization, while the substrate-to-solution ratio had a lesser effect.* ***Conclusion:*** *This study demonstrated the optimization of demineralization conditions for chitin extraction from fish scales using RSM. The optimal conditions obtained in this study can be applied in*

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various industries, including pharmaceutical, medicinal, and agronomical, for the production of high-quality chitin. The results of this study provide a cost-effective and efficient method for chitin extraction from fish scales.

INTRODUCTION

Demineralization refers to removal of inorganic minerals in the form of mineral ions. Calcification of fish scales occurs continuously throughout the life of the organism [1]. Demineralization is an expeditiously developing and demanding feature of various scientific directions including space-biology, histopathology, paleohistory, paleoclimatology, biogeochemistry, geobiology, biological engineering, and many more. Marine solid waste is not properly dumped so considered as an environmental pollutant globally constituting about 30-40% of solid waste leaving 50-60% as actual harvest [2, 3]. Consequently, a colossal amount of seafood wastes is available as a crude material for various manufacturing units for production of numerous value added life-giving pharmaceutical products [3]. Furthermore, seafood comprises various beneficial chemicals such as proteins especially collagen, minerals mainly calcium and phosphorous and chitin (2nd most abundant polysaccharide) which are in demand due to their use in various disciplines. Therefore, unexploited solid waste produced by seafood industries can be a potential incalculable biological resource, if it is refined by modernized and leading edge biotechnology to prepare highly beneficial products [4].

Seafood waste can be an easily available raw material for the extraction of chitin [3] along with increasing annuity for the ambassadors [5]. Chitin with chemical formula poly (β-(1 → 4)-N-acetyl-D-glucosamine) [6] first identified in 1884 [7] is a most abundant polysaccharide 2nd only to cellulose [3], composed of long polymer chain of N-acetyl glucosamine units linked through -1, 4-glycoside bonds. Chitin is present in the external layer of arthropod's shells, cell wall (a layer outer to plasma membrane) of fungi and in cellular structures of seaweed and yeast [8].

Crustaceans are the most exploited resource for the production of polysaccharides. Fish solid waste is a helpful source of industrially valuable products. Fish is the most diversified group of chordates with about 33000 species occurring in [9]. Chitin, chitosan, and its derived products are extensively used in several industries some are agriculture, pharmaceutical, [10, 11], food, textile, paper, and cosmetic industries. It also has applications as a food additive and semi-permeable membrane [3, 12]. In addition, it also works as a principal primal matter for wastewater purification, stent coatings, sensors, wound dressing, horticultural, and biomedicinal industries [3, 13]. The process of chitin production involves three basic steps in the traditional standard sequence: demineralization (separation of inorganic minerals like (calcium and phosphates), deproteinization (elimination of proteins), and bleaching or discoloration (removal of pigments) [14].

Optimization of demineralization for chitin extraction is now a keen interest for researchers. Inorganic acids such as hydrochloric acid would rather be applied for demineralization [5, 15]. It gives quicker and better results even with a small concentration. Only a little literature is available on the demineralization of fish scales for the extraction of chitin. All the cited demineralization methods in history were based upon uninterrupted treatments employing proportionally more concentrations of 1 to 10M and enhanced time for treatment from 1 to 72h [16-18].

So the concern of this research was to statistically optimize parameters for the degree of demineralization and ash contents were found as a dependent variable to check the degree of demineralization by converting the demineralized sample into ash.

MATERIALS AND METHODS

Materials: Hydrochloric acid fuming 37% of 'EMSURE* ACS, ISO, Reag, Ph, Eur' was purchased and used in this experiment. Other chemical agents used for the experiment were of analytical grade. The commercial chitin (Pure 98%) was brought from

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Sigma-Aldrich Company Limited, Germany. Fish scales of *Labeo rohita* were purchased from the local fish market of Lahore, Pakistan. Samples were collected in Lahore on 15 January 2022 at 1:00 pm. Samples collected were then taken to the microbiology laboratory at the University of Punjab Lahore, Pakistan, and kept in a freezer until further experimental process. The gathered scales were then washed with tap water properly and dried in an oven at 50 °C for 24 hours until constant weight. The dried fish scales were then ground in a blender machine and further cut down into small pieces. The prepared sample was then stored in a bottle for further use.

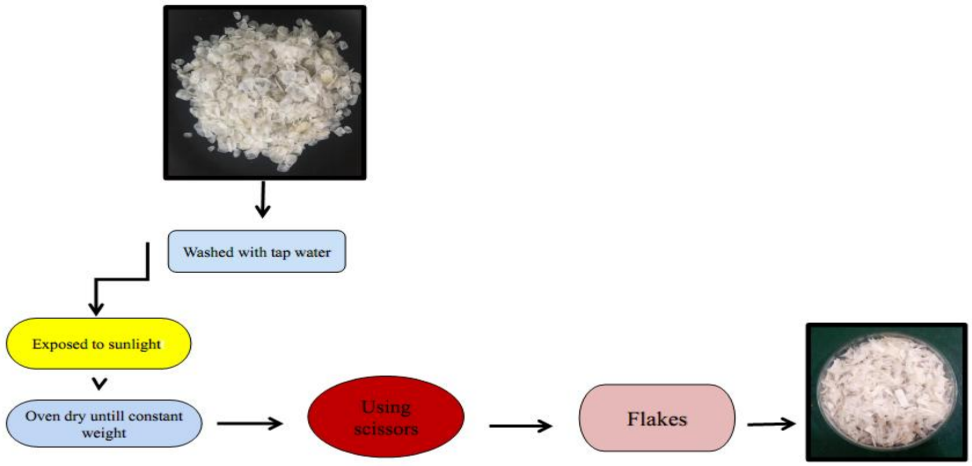

Figure 1: Methods of sample preparation

Methodology: Different concentrations (0.2, 0.4, 0.6, 0.8, 1.0M) of HCl (g/mole) were prepared. The experiment was designed for 17 runs up to five levels (-2, -1, 0, +2, +!) whose encoded values are presented in (Table 1) and performed in triplicates (Table 2). Measured samples of fish scales were taken in culture bottles and different concentrations of HCl were poured separately and incubated in a shaking incubator preset at a temperature of 37 °C with constant stirring of 140 rpm to remove minerals from the fish scales. After treatment, products had a pale white color; treated scales were then washed with distilled water thrice until pH approached zero. The average mass of each concentration was measured. The weight of pre and post-treatment scales was used to find out the percentage yield of demineralization. Ash contents are used to check the percentage of demineralization by converting the demineralized sample into ash [19]. Ash contents of scale were determined through the [20]. Ash contents were measured by using the formula [21].

$$\% \text{ Ash} = W2 - W1 \div Ws \times 100$$

W_2 = weight of crucible and ash

W_1 = weight of crucible

W_s = weight of substrate

Percentage DM was then measured by using the formula given below (Bellali et al., 2017)

(%) of Demineralization = (A-B)/A × 100

A: concentration of ash in the raw material (%).

B: concentration of ash in the demineralized sample

Table 1: Independent and dependent variables and levels for concentration of (HCl) for optimization of demineralization of fish scales for chitin extraction.

Dependent variable	Levels of concentration					
	Independent variables	-2	-1	0	+1	+2
Percentage Demineralization	Concentration of HCl(mole/L)	0.2	0.4	0.6	0.8	1.0
	Time (hours)	2	4	6	8	10
	W/V ratio	10	15	20	25	30

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Table 2: Box Behnken Design (BBD) for optimization of parameters for demineralization of fish scales.

Run no.	Factors		
	X_1	X_2	X_3
1	0	-2	0
2	0	0	-2
3	1	1	1
4	0	2	0
5	2	0	0
6	1	-1	1
7	0	0	0
8	-2	0	0
9	-1	1	-1
10	-1	-1	-1
11	0	0	2
12	1	1	-1
13	-1	1	1
14	-1	-1	1
15	1	-1	-1
16	0	0	0
17	0	0	0

RESULTS

In this study, BBD was used for the estimation of the degree of demineralization. The subsequent observed as well as estimated values for the concentration of hydrochloric acid (HCl), time for demineralization, and w/v ratio applied for the separation of minerals from fish scales are presented in Table 3. Three variables were analyzed at a time to determine the optimum conditions for pretreatment with *Labeo rohita* (Rohu) scales. Full Factorial Design with up to five levels was used to find the optimized concentrations of factors affecting the demineralization of fish scales. The percentage yield for having the maximum degree of demineralization was 49.33 shown in Table 3 which is the maximum ever performed demineralization of crustacean waste such as 18.18 was the yield of demineralization of crab shell waste with the demineralization up to 81.82% [3].

Table 3: Observed and predicted values of percentage yield of demineralization.

Run	Acid (HCl) X1	w/v (g/100ml) X2	Time (hours) X3	%age Yield of Demineralization		
				Observed	Predicted	Residual
1	0.6	1:10	6	60.80000	53.57457	7.2254
2	0.6	2:10	2	78.90000	72.28207	6.6179
3	0.8	2.5:10	8	86.09000	76.45418	9.6358
4	0.6	3:10	6	78.40000	85.72457	-7.3246
5	1.0	2:10	6	60.40000	62.88457	-2.4846
6	0.8	1.5:10	8	64.17000	64.60418	-0.4342
7	0.6	2:10	6	78.10000	73.93304	4.1670
8	0.2	2:10	6	88.80000	86.41457	2.3854

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9	0.4	2.5:10	4	86.56000	86.02668	0.5333
10	0.4	1.5:10	4	56.19000	65.72668	-9.5367
11	0.6	2:10	10	78.40000	85.11707	-6.7171
12	0.8	2.5:10	4	78.88000	78.70668	0.1733
13	0.4	2.5:10	8	92.30000	87.89418	4.4058
14	0.4	1.5:10	8	80.74000	80.81418	-0.0742
15	0.8	1.5:10	4	49.33000	53.63668	-4.3067
16	0.6	2:10	6	60.80000	73.93304	-13.1330
17	0.6	2:10	6	82.80000	73.93304	8.8670

Table 4: Results for regression analysis of percentage yield of demineralization.

Source	SS	DF	MS	F-Value	P-Value
Intercept	**1.3612**	**1**	**1.36121**	**0.013664**	**0.910229**
Linear	528.8239	3	528.82344	1.672699	1.812333
A	14.7839	1	14.78394	0.148398	0.711509
B	498.755	1	498.7548	1.478977	0.263348
C	15.285	1	15.2847	0.045324	0.837476
Square	411.332	3	411.3315	1.219737	1.922874
A*A	47.781	1	47.7809	0.141687	0.717760
B*B	353.035	1	353.0346	1.046867	0.340282
C*C	10.516	1	10.51600	0.031183	0.864832
2-Way interaction	726.54	3	726.5414		
A*B	364.230	1	364.2310	1.080066	0.333242
A*C	97.580	1	97.5804	0.289359	0.607302
B*C	264.730	1	264.7300	0.785014	0.405020
Error	2360.607	7	337.2295		

Significance level is not less than 95%. SS= Sum of Square, MS=mean of square, DF= Degree of Freedom

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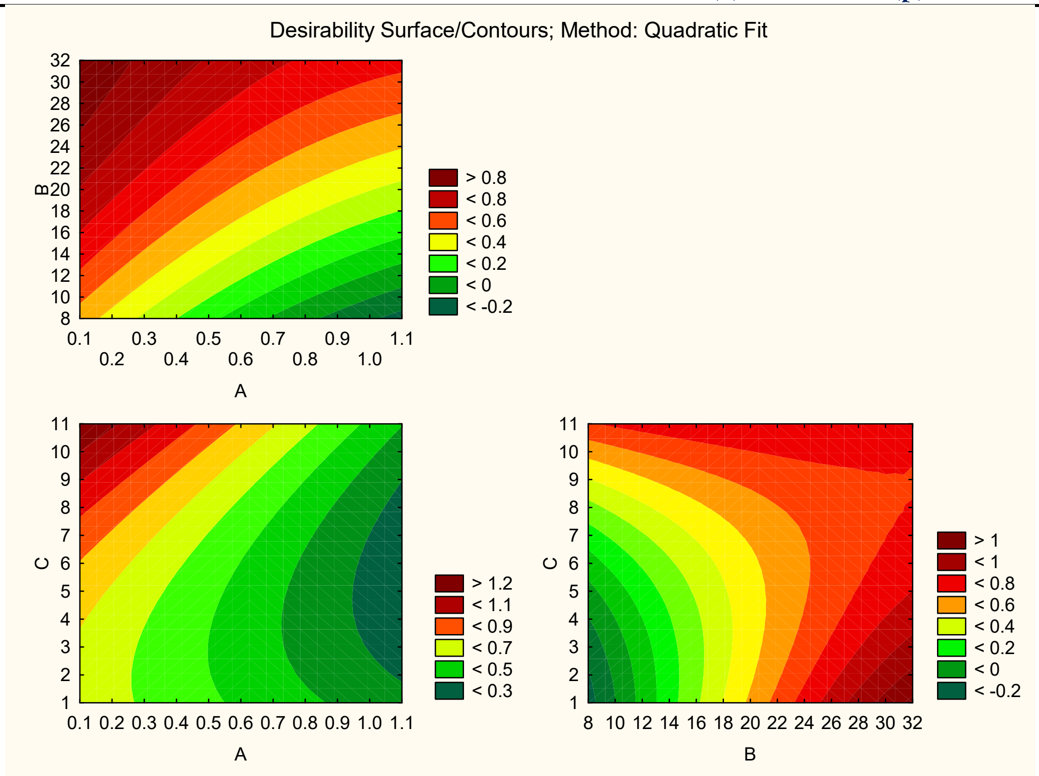

Figure 2: Contour graph showing the interactive relationship of independent factors to percentage yield of demineralization.

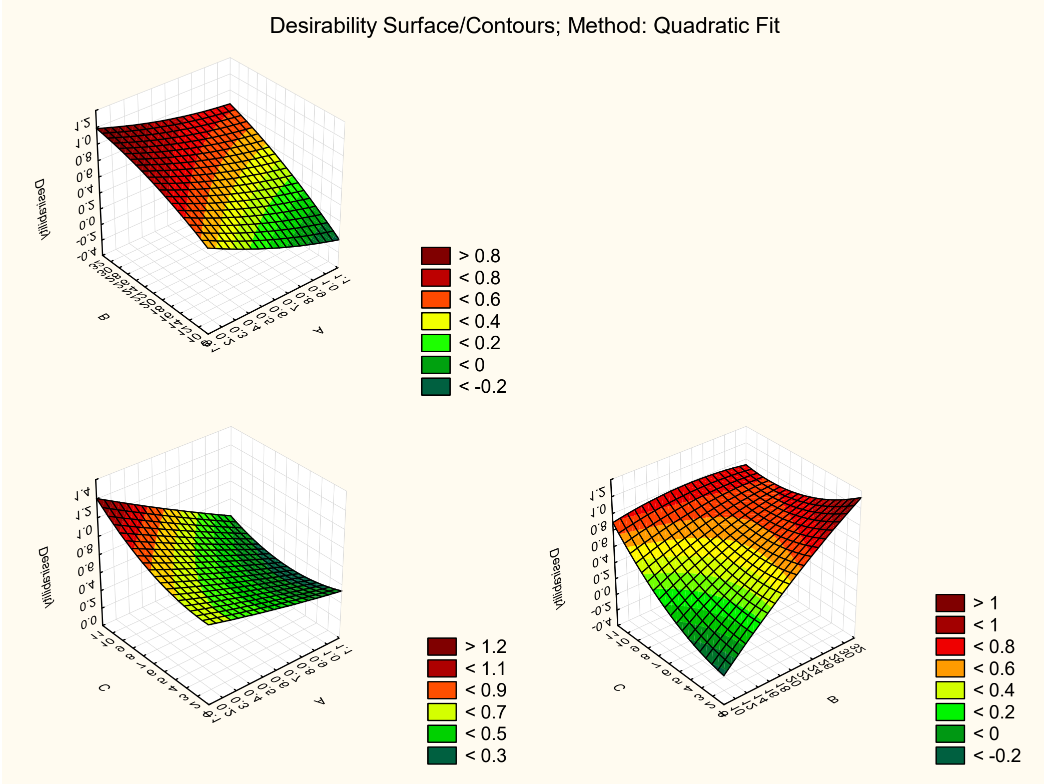

Figure 3: Surface plot showing interactive relationship of independent factors and percentage yield of demineralization.

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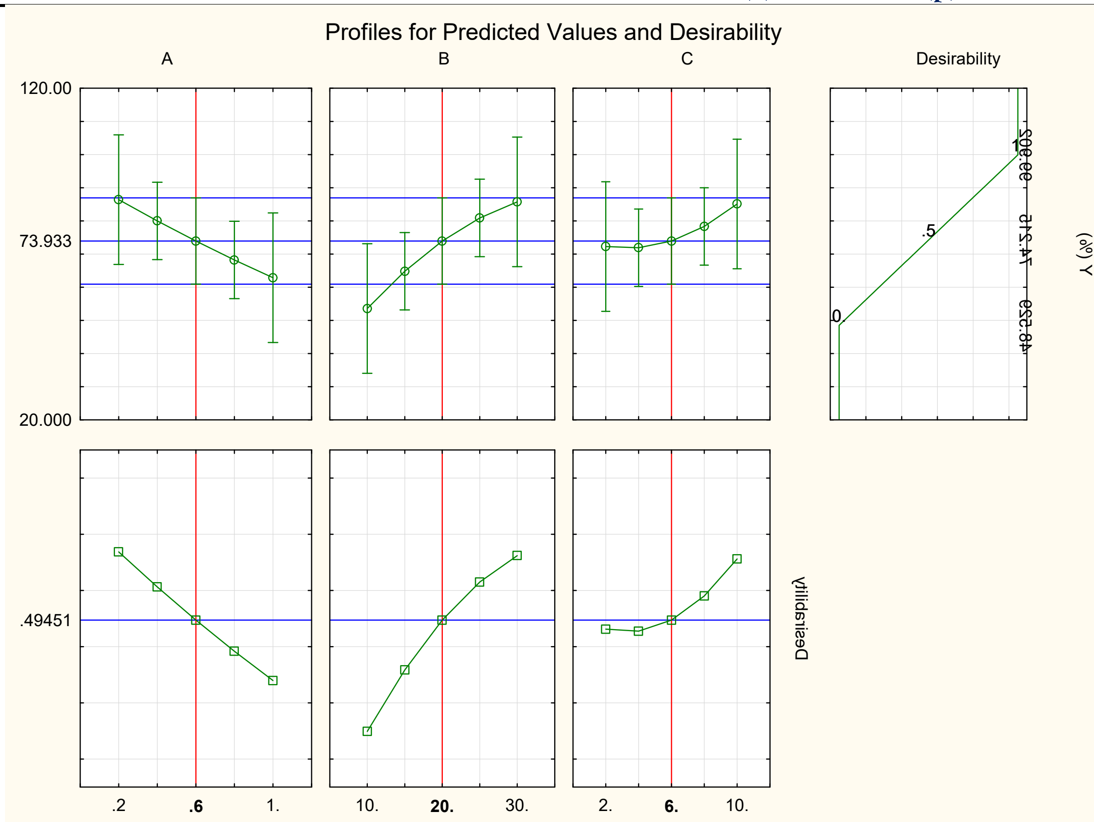

Figure 4: Desirability curve for predicted values of independent factors and percentage yield of DM.

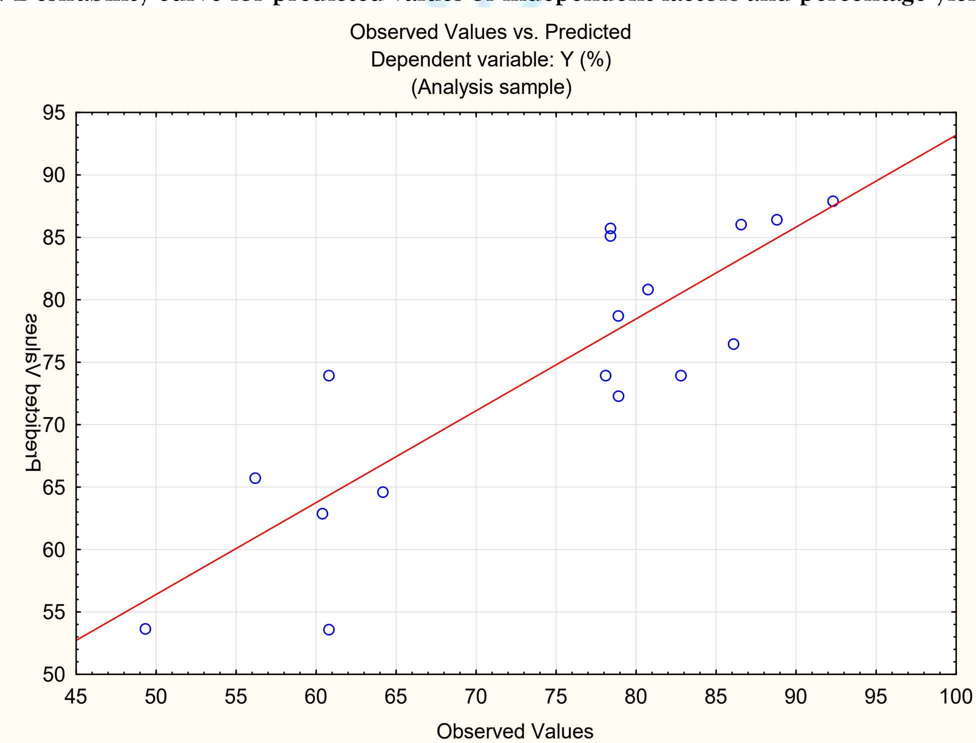

Figure 5: Residual plot for predicted and observed values of percentage yield of demineralization.

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Ash contents: Ash contents are measured to know the minerals that remain. Scales treated under the conditions of 0.6M HCl, 6 hours, and 3:10 as w/v ratio were observed to have maximum ash contents, while minimum ash contents were shown by optimized conditions for demineralization as 0.8M HCl, 4 hours and 1.5:10 as w/v ratio as shown in table 5.

Dried demineralized scales | **Ash of demineralized scales**

Figure 6: Dried demineralized scales and their ash.

Table 5: Observed and estimated values of percentage ash contents of treated scales.

Run	HCl concentration (M)	w/v ratio (g/100ml)	Time (Hours)	%age Ash contents		
				Observed	Predicted	Residual
1	0.6	1:10	6	2.500	6.888	-4.388
2	0.6	2:10	2	16.000	10.138	5.861
3	0.8	2.5:10	8	33.000	27.142	5.857
4	0.6	3:10	6	42.000	41.263	0.736
5	1.0	2:10	6	0.500	0.611	1.111
6	0.8	1.5:10	8	1.000	1.544	2.544
7	0.6	2:10	6	22.000	16.217	5.782
8	0.2	2:10	6	23.000	27.763	-4.763
9	0.4	2.5:10	4	23.500	2.392	1.107
10	0.6	1.5:10	4	14.500	16.705	-2.205
11	0.8	2:10	10	15.500	25.103	-9.513
12	0.8	2.5:10	4	10.000	17.705	-7.705
13	0.4	2.5:10	8	41.500	38.580	2.919
14	0.4	1.5:10	8	33.500	22.142	11.357
15	0.8	1.5:10	4	0.500	0.232	0.732
16	0.6	2:10	6	9.500	16.271	-6.717
17	0.6	2:10	6	13.500	16.217	-2.717

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Table 6: Analysis of variance for Quadratic model of determination of ash contents of treated fish scales in acid HCl

SOURCE	DF	SS	MS	F -Value	P – Value
Intercept	1	1.3612	1.36121	0.013664	0.910229
Linear	3	138.8083	138.80828	1.393331	1.674284
A	1	14.7839	14.78394	0.148398	0.711509
B	1	93.7062	93.70618	0.940605	0.364432
C	1	30.3182	30.31816	0.304328	0.598343
Square	3	50.3353	50.33528	0.505255	2.20594
A^2	1	0.6215	0.62149	0.006238	0.939256
B^2	1	22.2110	22.21096	0.222949	0.651167
C^2	1	27.5028	27.50283	0.276068	0.615517
2–way interaction	3	107.2478	107.24785	1.076534	1.904328
A*B	1	11.3764	11.37645	0.114195	0.745326
A*C	1	8.4872	8.48720	0.085193	0.778840
B*C	1	87.3842	87.38420	0.877146	0.380162
Error	7	697.3633	99.62333		
Total	17	1291.5073			

R=0.912613. R^2=0.832862, adjusted R^2=0.617970. SS stands for Sum of Squares, DF is degree of freedom and MS is Mean of Squares.

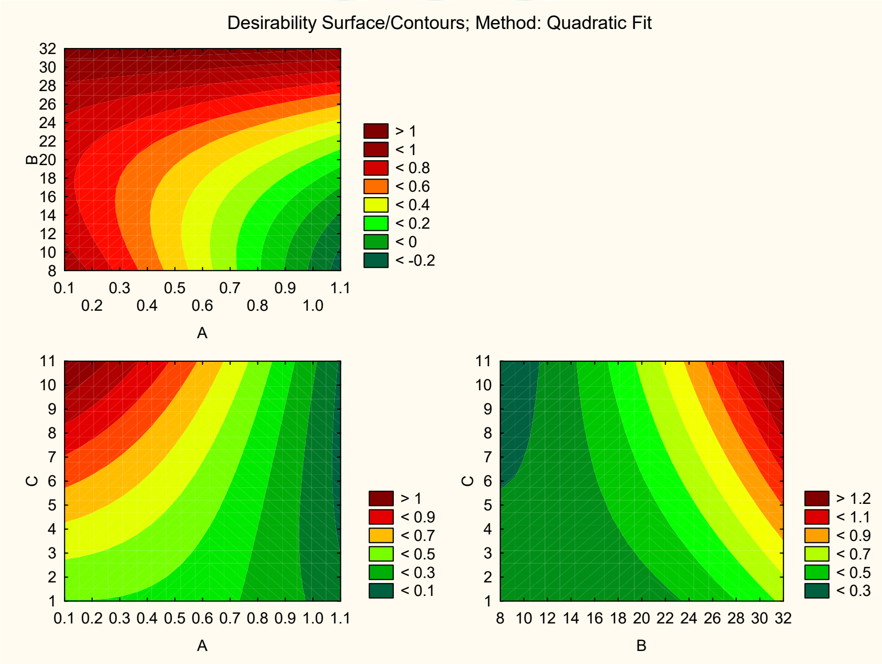

Figure 7: Contour plot showing the interactive relationship of independent factors and percentage ash contents.

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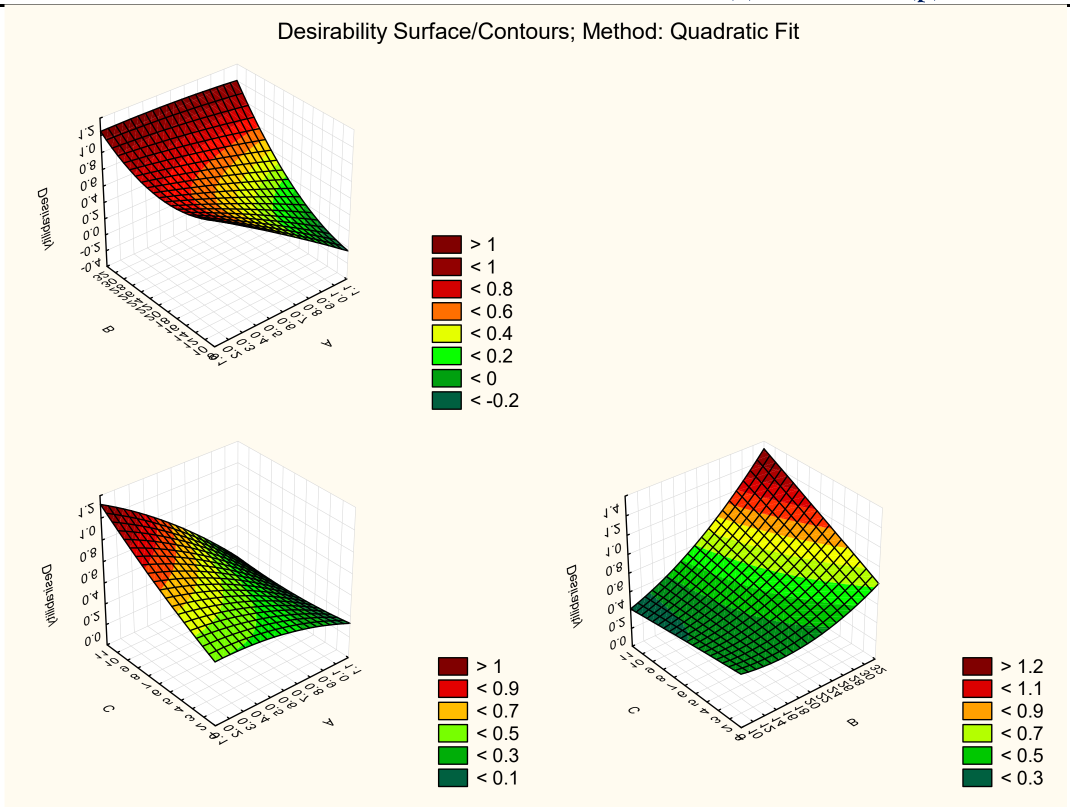

Figure 8: Surface plots depicting the interactive relationship of independent factors and percentage ash contents.

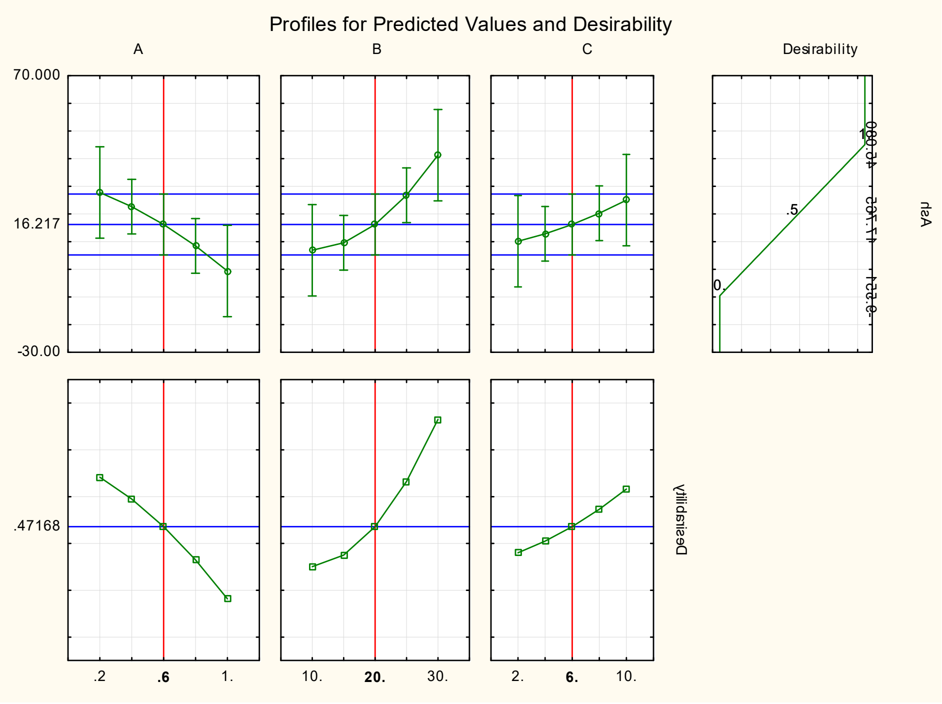

Figure 9: Desirability graph showing interactive relationship of independent factors and percentage ash contents.

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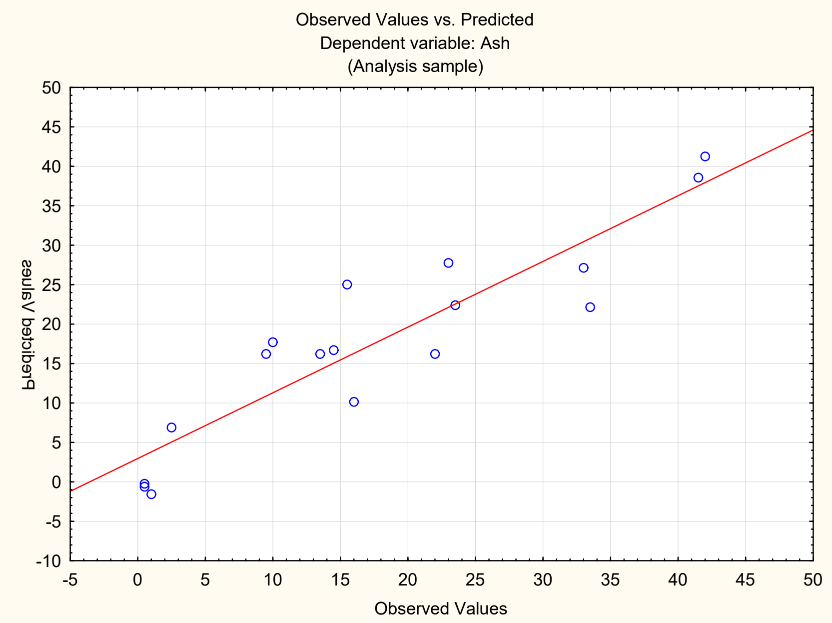

Figure 10: Residual plot for predicted and observed values of percentage ash contents.

Percentage demineralization of fish scales: Percentage demineralization of fish scales was found by finding ash contents using the formula given below [19].

(%) of Demineralization = (A-B)/Ax100

A: concentration of ash in the raw material (%).

B: concentration of ash in the demineralized sample (%)

Maximum percentage of demineralization (99.97%) was achieved with the conditions of 0.8M HCl, 1.5:10 as w/v ratio and 4 hours of time for treatment whereas minimum percentage demineralization was found with the conditions of 0.6M HCl, 3:10 as w/v ratio, and 6 hours for time of treatment as shown in Table 7.

Table 7: Observed and estimated values of percentage demineralization.

Run	Acid (HCl) M	Substrate to solution ratio (w/v)	Time (hours)	Percentage of demineralization		
				Observed	Predicted	Residual
1	0.6	1:10	6	94.560	85.130	9.429
2	0.6	2:10	2	65.210	78.150	-12.940
3	0.8	2.5:10	8	28.260	41.056	-12.796
4	0.6	3:10	6	8.690	10.225	-1.535
5	1.0	2:10	6	99.970	102.320	-2.350
6	0.8	1.5:10	8	97.820	103.508	-5.688
7	0.6	2:10	6	52.170	64.755	-12.585
8	0.2	2:10	6	50.000	39.755	10.245
9	0.4	2.5:10	2	48.910	51.116	-2.206
10	0.4	1.5:10	4	68.470	63.568	4.902
11	0.6	2:10	10	66.300	45.465	20.835
12	0.8	2.5:10	4	78.600	61.918	16.682
13	0.4	2.5:10	8	9.780	16.283	-6.503
14	0.4	1.5:10	8	27.170	51.746	-24.576

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15	0.8	1.5:10	4	99.970	101.361	-1.391
16	0.6	2:10	6	79.340	64.755	14.585
17	0.6	2:10	6	70.650	64.755	5.895

R=913595, R^2=0.834655, Adjusted R^2=0.622069. SS stands for Sum of Squares, DF is degree of freedom, MS is Means of Squares.

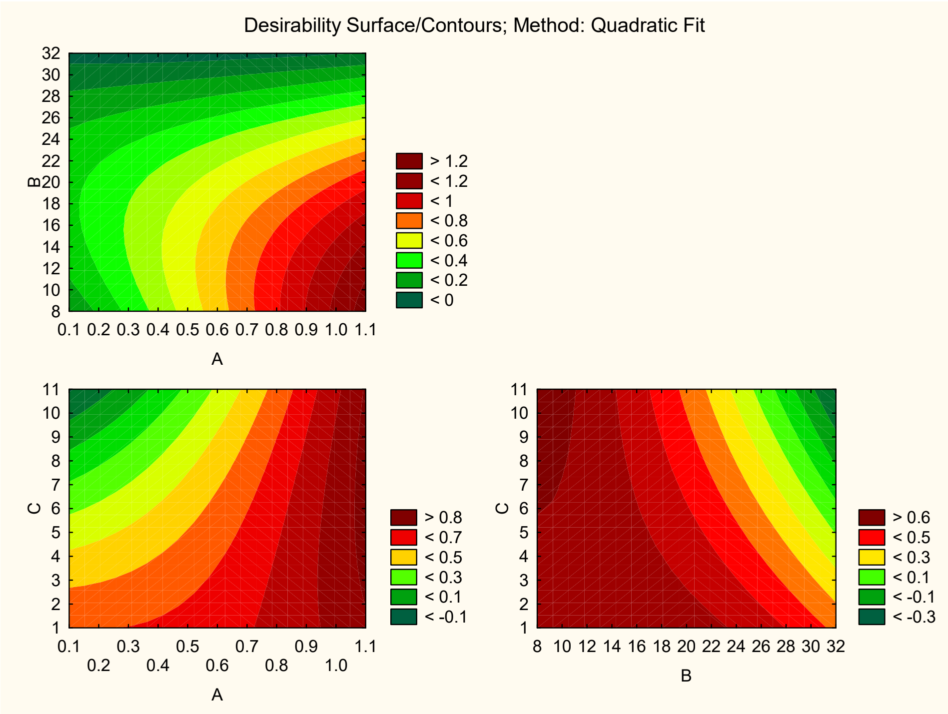

Figure 11: Contour plots showing the interactive relationship of independent factors and percentage demineralization.

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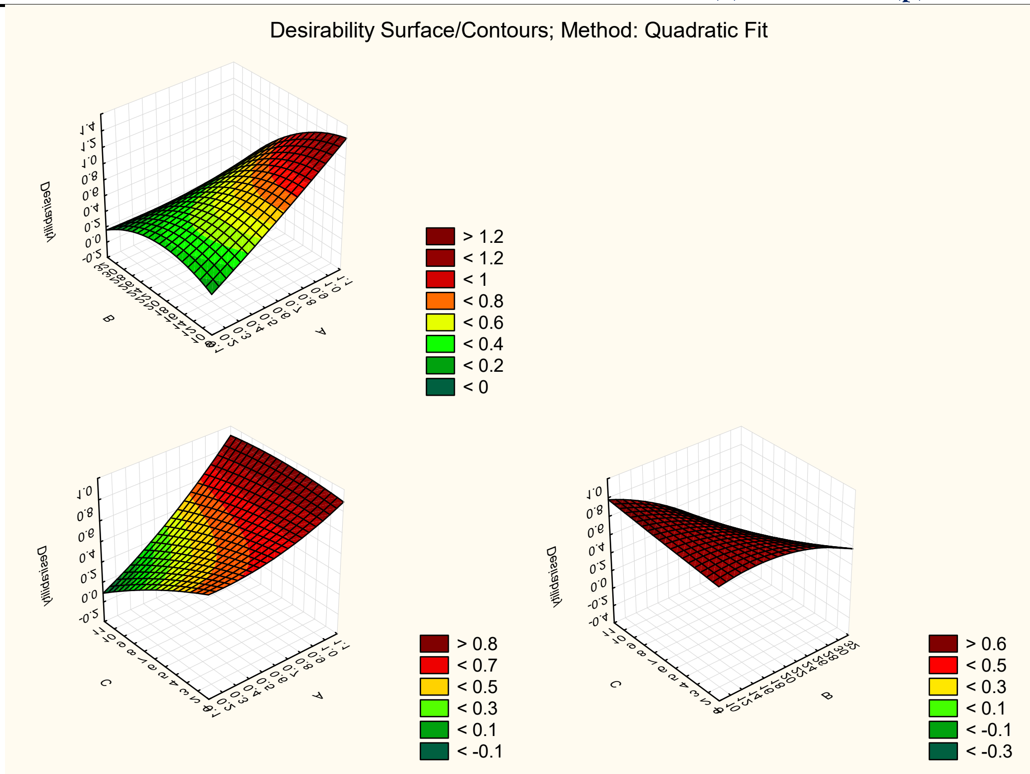

Figure 12: Surface plot showing the interactive relationship of independent factors and percentage demineralization.

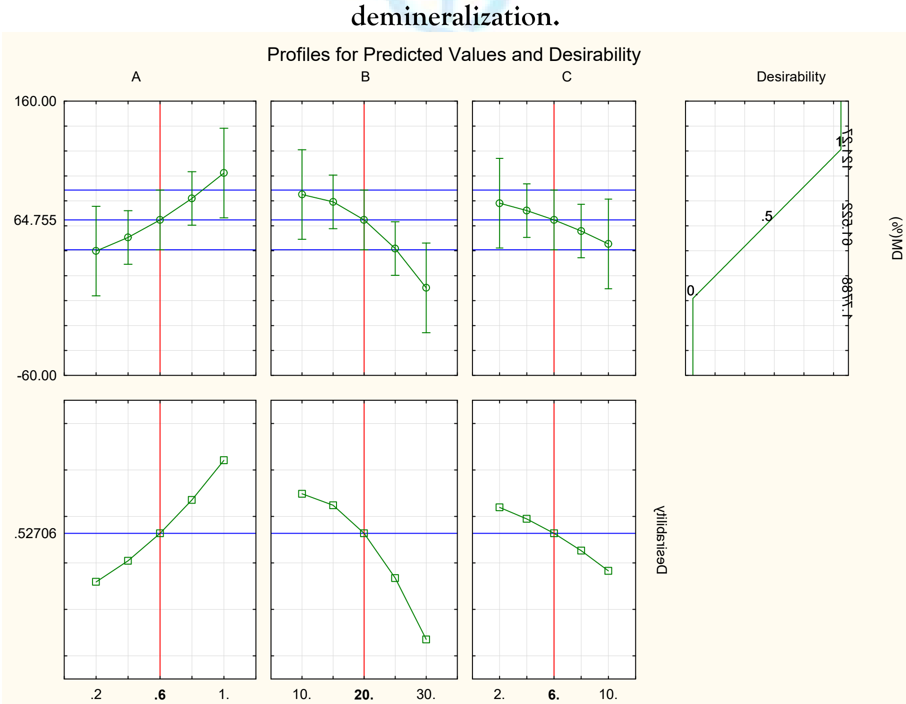

Figure 13: Desirability graph for percentage demineralization.

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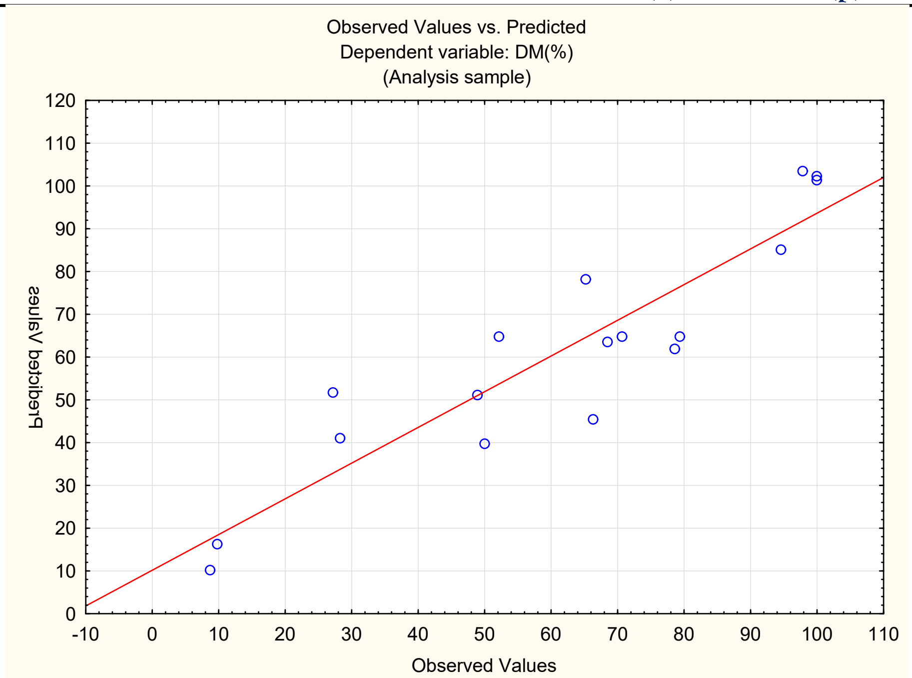

Figure 14: Showing residual plot for predicted and observed values of percentage demineralization.

DISCUSSION

Braconnot first detected chitin in 1811 in mushrooms [22], it is considered the most abundantly found natural polysaccharide second to cellulose only. The present study is intended to statistically optimize conditions for the demineralization of fish scales (*Labeo rohita*) using a chemical method. An experimental study was designed to optimize three parameters HCl concentration (X1), w/v ratio (X2), and time (X3) for acid treatment at a time using RSM. The yield of demineralization was found in the form of a dry mass of fish scales. Maximum yield was found to be 92.30 that of run 13 with 0.4M, HCl, 2.5:10 (w/v), and treatment time of 8 hours; while run (15) considered to have optimized conditions was found to have Y equal to 49.33 (Table 3) that is maximum than ever carried out demineralization process of crustaceans shell waste. This yield is equally profitable for industrial-level demineralization of fish scales. It is reached from the literature that increasing the acid or base concentration for extraction of chitin lowers the yield of chitin/demineralization due to excessive demineralization and deproteinization respectively [4, 23]. The effect of concentration of Hydrochloric acid X1, treatment time X2, and substrate to solution (w/v) ratio X3 to percentage yield of demineralization was determined using RSM. The residual plot for observed and predicted values of percentage yield of demineralization shows the feasibility of the model used in figures (1, 2, 3, and 4). The percentage of demineralization refers to the degree of removal of organic minerals that was calculated by determining the ash contents of treated scales in a muffle furnace at a temperature of 800 ^{o}C for 4 to 5 hours (Table 6). Treated scales showing the lowest ash contents were considered highly demineralized. Maximum percentage of demineralization 99.97% was achieved through conditions of 0.8M, HCl, 1.5:10 (w/v), and 4 hours as time for treatment (Table 7). Box Behnken Design (RSM) was applied for statistical optimization of the parameters for demineralization. F- Value by applying two-way ANOVA comes to be 0.04 (Table 8) the significance of the model. The efficiency of mineral removal increases with increasing the acid concentration, 0.8M brought about 99.97% demineralization with a treatment time of 4h, which is slightly higher than 98.7% demineralization in 0.1M HCl, and 99.7% in 0.3M HCl, time for treatment was 24 hours [19]. In the present case, 1.0M Hydrochloric acid showed approximately similar results at 99.97% acid concentration so there was little to negligible change from 0.8M to 1.0M

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HCl with time for treatment as 6h. Al Shaqsi, Al Hoqani [3] showed 81.82% demineralization of crab shell waste with 2M HCl. Mineral solubility in acid increased with increasing acid concentration, time for treatment also showed the same results but substrate to solution ratio has inverse effects. 1.0M Hydrochloric acid was used with 3:10 w/v ratio and 0.8M was used with 1.5:10 as w/v ratio so both showed approximately similar results. Approximately similar results were shown by many researchers. A literature review showed that the percentage demineralization of fish (sardine) scales was found about 90% with a concentration of hydrochloric acid 1.0M [24]. Matmaroh, Benjakul [15] also showed that the demineralization of sea bream scales with 0.6 M HCl after 24h has removed 90% of the inorganic minerals. Statistical analysis of demineralization of bream scales has shown 79.18% demineralization using 0.43M HCl.

The effect of concentration of HCl, treatment time, and substrate to solution ratio to percentage ash contents and their interactive effect were depicted through RSM as shown in figures (5, 6, 7, and 8). The effect of three parameters (X1, X2, and X3) on percentage demineralization and their interactive effect were shown through contour and surface plots in Figures (9, 10, 11, and 12).

CONCLUSION

The purpose of this study was to optimize parameters for demineralization of fish scales to extract pure chitin with a minimum quantity of minerals remaining. The chemical method of demineralization was applied to achieve approximately 100% demineralization which is the best found. Based on the observed results it is concluded that 0.8M HCl, 1.5:10 and 4 hours of treatment are optimized conditions for demineralization. In conclusion, these optimized conditions for demineralization can be applied to the extraction of purified commercial chitin. It is equally functional for the production of raw materials for medicinal, pharmaceutical, and agronomical industries. It is the first-ever study done for the optimization of demineralization in Pakistan.

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