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Parametric optimization of yield percent of chitosan extracted from fish scale (*Labeo rohita*) through central composite design approach

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Fish scales have been extensively reported as waste material that takes a lot of time to degrade, causing environmental pollution. This work has been substantiated to summarize a sequence of chemical processes (demineralization, deproteinization, and deacetylation) used for the extraction of useful product like chitosan from fish scale (*Labeo rohita)*. The obtained chitosan have been efficiently characterized by SEM, FTIR, XRD, TGA, XRF, and proximate analysis. The small particle size (3.3748 µm) and the high surface area (4.046 m^2/g) of the produced cost-effective chitosan (0.26 USD/g) have justified its applicability as an antimicrobial filler. The degree of deacetylation have been reported to be 52.11% along with a high capacity of water binding (160%) and fat binding (457%), indicating its biodegradable nature. The individual effect of the essential parameters like deacetylation time, deacetylation temperature, and amount of NaOH added, influencing the yield percent have been studied using the central composite design approach of response surface methodology. The maximum yield percent have been reported to be 29.63% for the optimized conditions of 4.48% of NaOH content, 6.624 hr deacetylation time, and 58.2°C deacetylation temperature.

Keywords: Chitosan, Demineralization, Deproteinization, Deacetylation, Biodegradability; Central composite design; Optimization

1 Introduction

The generation of waste from food remains has been the cause of a substantial volumetric addition of garbage in the environment. Fish is used as an enormous source of food in many tropical countries. The total fish production in 2016 reached an all-time high of 171 million tones, of which 88% was utilized for direct human consumption according to the state of world fisheries and aquaculture 2018[1]. The wastes of fish like scale, skin, and head have low economic value or almost having no cost[2]. Indian local market produces a huge amount of fish waste each day, approximately 30-40% of raw fish. They have been repeatedly thrown in landfills, ponds, and sea, which pollute water and soil. However, these wastes are used for the utilization of value-added products like chitosan[3-4]. The utilization of fish scale waste for the extraction of biopolymer have not been only a waste treatment process but also a waste utilization technique. After cellulose, chitin have been reported as the second most abundantly found biopolymer in nature[5]. It can easily be obtained by simple extraction from marine waste[6]. Chitin and chitosan are biopolymers having excellent non-toxic biodegradability, biocompatibility, and absorption properties[7]. Both chitin and chitosan have been excellent chelating agents[8]. Chitin structure is compact and does not allow it to be soluble in the most solvent. Thus it needs to be converted to chitosan by deacetylation process to make it easily soluble in the solvent[9]. Being derivatives of chitin, chitosan is soluble in many solvents, making it preferable to chitin. The crystallinity and orientation arrangement of polysassharide strands of mud crabs have been investigated by Naudin *et al.*[10]. Chitosan possesses various unique properties like film-forming ability, absorption capacity, bioresorbable degradation, hydrophilicity, biocompatibility, cellular binding capability[11,12] making it to be a very important value-added product. Chitosan has further shown commendable application in the process of purification of water and has an extensive wound healing capacity[13]. Removal of Eriochrome black T (EBT) dye from aqueous solutions have been efficiently done using chitosan extracted from shrimp cells[14]. Additionally, its ability for conversion into fibers, powders, and beds provides diversity in its application in several fields[15,16].

Chitosan can be extracted from various sources like shrimp waste, razor calm[17], crab, lobster, krill etc[18]. Still, fish being one of the primary food sources, its waste serves as the major source for chitosan. Fish scales are initially demineralized and then deproteinized to produce chitin[3]. Demineralization and deproteinization techniques help in the removal of inorganic mineral contaminants and unwanted proteins[7,18,19] from the fish scale. Thus after removal of them, the extracted chitin can be utilized for chitosan extraction process. Various proteolytic enzymes have been used for the removal of protein. Still, its complete replacement have not been found using enzymatic approach[20] instead, the use of chemical solutions like Na_2CO_3, K_2CO_3, Na_2SO_3, and KOH was found to be more effective[18]. The primary purpose of the conduction of demineralization has been to remove calcium carbonate as calcium chloride by the addition of hydrochloric acid[18-21]. The chitin formed can be further deacytalized from chitosan using an aqueous alkali solution[19]. The removal of N-acetamide groups is the primary concern for the deacetylation of chitin, which makes the formed chitosan to be soluble in various solvents, and this further leads to the increase in the applicability range of chitosan.

For the last few decades, various study regarding the factors affecting the process of production of chitosan has been elaborately studied[22-23]. Not only the selection of proper chemicals but also factors like deproteinization, demineralization and deacetylation time, and temperature have been observed to play an important role in the quality and the yield of the product[24]. Standardization of all these parameters is quite essential for the optimization of the process. Classical techniques of optimization involve the study of one parameter keeping the other constant, which gives a little understanding of the overall effect of various parameters on the quality of the product[25]. Moreover,it requires more time and less accuracy. Computed standardization using response surface methodology can be a suitable solution to study the multi-parametric effect on a single or multiple response[26]. The optimization of the degree of deacetylation (DDA) of pink shrimp (*Penaeus notialis*) has been elaborately studied by Amoo *et al.*[27] by BoxBehnken design using response surface methodology (RSM), showing the highest value of 89.73% of DDA at 97.2°C and 90 min of deacetylation temperature and time. Comparative investigation using RSM and ANN of chitosan derived from *Archachatina marginata* shell were discussed by Bello *et al.*[28] and the results obtained portray that ANN has a better modelling abilities.Statistical optimization of the yield percent of the shrimp cell is also carried out using the central composite design of RSM[29,] and the optimized result of 4.833% was obtained at 8 mol% HCl concentration for a deacetylation temperature and time of 60℃ and 1.5 hr. Though various works are reported for the optimization of properties of shrimp waste, but work is related to the optimization of the yield percent of chitosan extracted from fish scale waste combining the various parameters like deacetylation time, deacetylation temperature, and NaOH content (%) has not been reported yet. Besides this, the cost estimation of the produced chitosan has also not explored earlier.

In the present work, the extraction of chitosan has been carried out from waste fish scales of *labeo rohita*. The obtained chitosan is characterized by BET, XRD, FTIR, SEM, XRF, and TGA analysis. The fixed carbon content and the particle size are determined by proximate and particle size analyzer, respectively, to justify its properties for a specific application. The various parameters like deacetylation time, deacetylation temperature, and NaOH percent has been varied to obtain the optimum yield percent. The yield percentage of chitosan was optimized through response surface methodology using the central composite design method. A second-order regression model equation has been formulated to check the significance of each parameter. Analysis of variance (ANOVA) has been used to statistically analyze the universal relation of various parametric conditions among itself. Thus the obtained optimized parametric conditions will help to produce the highest yield percent of a value-added product(chitosan) from waste.

2 Materials and Method

2.1 Materials

Fishscales (*labeo rohita*) freshly removed were obtained from the local fish market of Bhubaneswar, Odisha. Laboratory quality distilled water was prepared from the steam distillation unit was used for the purpose. Hydrochloric acid and sodium hydroxide were purchased from E. Merck, MumbaiIndia Pvt. Ltd.

2.2 Methods

The process of extraction of chitosan from the fish scale is shown in Fig. 1. The method includes the following steps. In the first step,isolation of chitin was

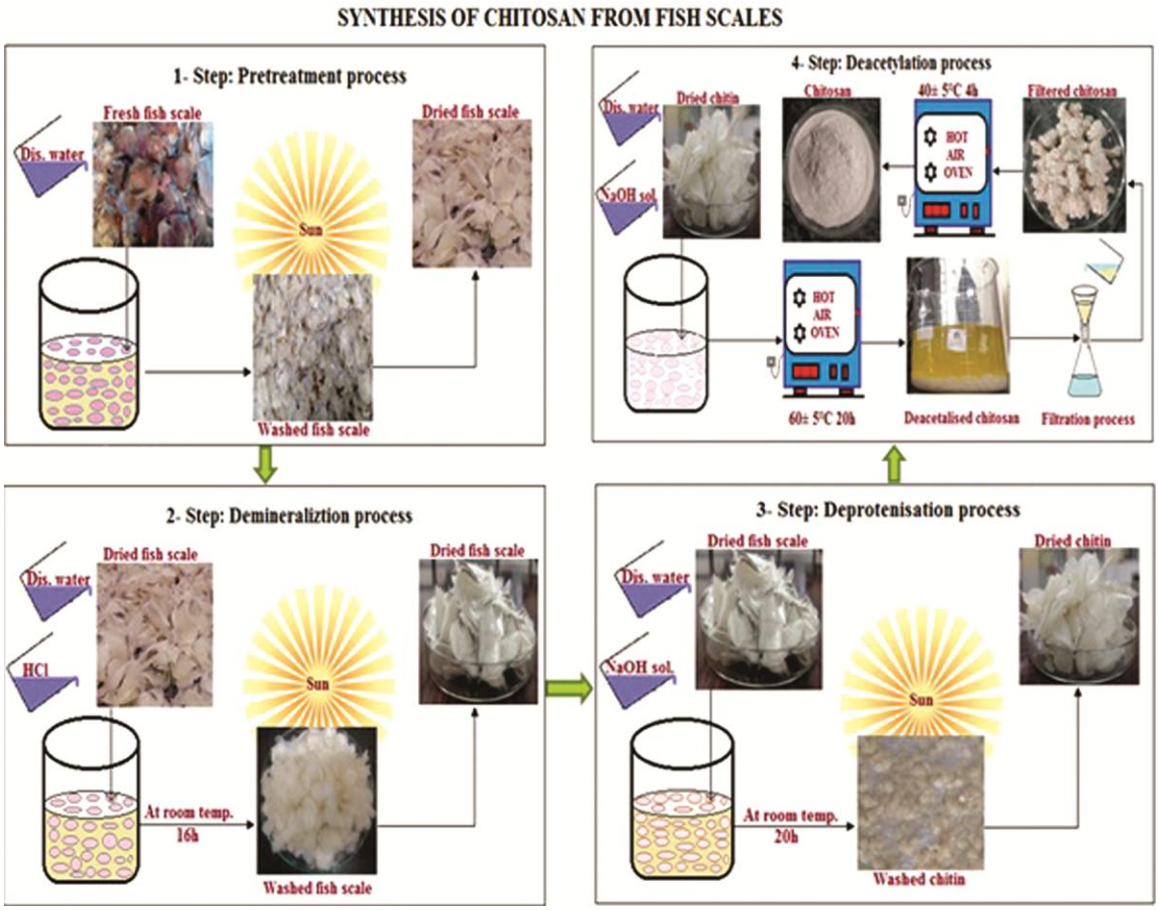

Fig. 1 — Flow diagram for the preparation of chitosan from fish scales.

done from raw fish scales, and in the second step, deacetylation of chitin was done to produce chitosan.

2.2.1 Pre-treatment process

The waste fish scales were initially pretreated by washing them with clean water thoroughly, followed by washing with distilled water. All the impurities like dust particles, other fish parts, extra oils, and other contaminants were removed. Then the clean fish scales were sundried and kept for one to two days in a polyethylene bag for autolysis. This step removes some unnecessary components and lessens the bad smell.

2.2.2 Demineralization process

The pre-treated fish scales were then demineralized. The dried sample wascorrectly weighed and added to 2% of hydrochloricacid (HCl) with a weight to volume ratio of 1:5 and kept for 16 hours. In this process, the natural mineral content of the fish scale was removed, making the scales more transparent and thin. After 16 hours, the sample was washed thoroughly until a neutral pH is achieved. Then the sample was sundried to measure the exact weight. The possible chemical reactions involved during the process [(1) – (8)] are as follows:

$$MgO(s) + 2HCl(aq) = H_2O(l) + MgCl_2(aq) \quad \ldots (1)$$

$$Al_2O_3(s) + 6HCl(aq) = 2AlCl_3(aq) + 3H_2O(l) \quad \ldots (2)$$

$$K_2O(s) + 2HCl(aq) = 2KCl(aq) + H_2O(l) \quad \ldots (3)$$

$$CaO(s) + 2HCl(aq) = CaCl_2(aq) + H_2O(l) \quad \ldots (4)$$

$$Fe_2O_3(s) + 6HCl(aq) = 2FeCl_3(aq) + 3H_2O(l) \quad \ldots (5)$$

$$Na_2O(s) + 2HCl(aq) = 2NaCl(aq) + H_2O(l) \quad \ldots (6)$$

$$P_2O_5(s) + 6HCl(aq) = 2POCl_3 + 3H_2O(l) \quad \ldots (7)$$

$$SO_3(s) + 2HCl(aq) = 2Cl(aq) + H_2O(l) + SO_2(aq) \quad \ldots (8)$$

2.2.3 Deproteinization process

The sample after demineralization was subjected to the deproteinization process. The fish scales were weighed and added to a 4% NaOH solution in the ratio of weight to volume of 1:5 and kept for

20 hours. Here the protein content of the scales was eliminated along with the remaining lipid and inorganic constituents like silica[30]. The washing procedure was again repeated until neutral pH is achieved. Then the sample was sundried. The chitin production is now completed. The removal of silica present in the fish scales was obtained according to reaction (9), as stated below.

$$SiO_2(s) + 2NaOH(aq) = Na_2SiO_3(aq) + H_2O(l) \quad \ldots (9)$$

2.2.4 Deacetylation process

The chitin extracted was then subjected to the deacetylation process[22]. The extracted chitin was put into a jar containing 4% NaOH in the ratio of weight: volume of 1:5 for 20 hours at a temperature of 60°C. The solution was then filtered, and the filtrate was washed till neutral pH is obtained. Then the filtrate was dried in a petri dish at 60°C until it was completely dehydrated. The extracted dried powder sample is chitosan.

3 Results and Discussion

3.1 Determination of the properties of extracted chitosan

The extracted chitosan was extensively characterised by X-ray Fluorescence technology (Rigaku RIX3000, Rigaku technology Inc., Austin) to determine the chemical composition so as to justify the extraction process and to confirm the elimination of all the inorganic constituents after its treatment. The surface area, pore-volume, and pore radius of the extracted chitosan were determined by Brunauer-Emmett-Teller (BET) analysis (Mastersizer 2000 E Ver. 5.60, Malven Instruments Ltd., Melven, UK). Particle size plays a vital role in the applicability of a material as a filler, and hence to determine the particle size of the chitosan,a Particle Size Analyser (LA-96OS, Horiba Scientific Instruments, Japan) was used. Proximate analysis of chitosan was carried out to determine moisture content, ash content, volatile matter and fixed carbon content (wt.%) using Laboratory Analytical procedures LAP-001[(31)] and LAP-005[(32)] respectively of National Renewable Energy Laboratory (NREL). The micrograph of the obtained chitosan was determined using SEM (EVO MA 15, Carl Zeiss SMT, Germany) in order to visualize the dispersion, agglomeration tendency, and homogeneity of the particles for its further application. The amorphousity of the produced chitosan was predicted by X-ray diffraction analysis (Shimadzu, XRD-7000L, Japan). The purity of the obtained product was also justified by the determination of functional groups by Fourier transform infrared spectroscopy (Nicolet 6700, USA). Thermogravimetric analysis (TGA Q50, TA Instruments, USA) was carried out to know the thermal behavior of the chitosan sample, which shows the change in weight of the sample upon heating at a temperature range from ambient to 860°C with the heating rate range of 10°C/min at inert nitrogen atmosphere.

3.1.1 X-Ray fluorescence analysis

The produced chitosan was subjected to X-Ray fluorescence analysis to find out the composition of the elements present in its oxides. The results revealed the maximum presence of the inorganic constituent, CaO (39.7%) in chitosan (Table 1), which helps in providing an antibacterial effect[33] on the products made by it. Hence chitosan can be effectively used for making food-grade products. The second most eminent inorganic constituent visible in chitosan was potassium oxide (21.5%). This indicates that chitosan can be well utilized for making nursery bags as the potassium oxide present in it will help in increasing soil fertility[34]. XRF analysis also indicates the presence of 19% SiO_2. The presence of silica helps in its increasing application as a filler as silica gives an excellent stiffness and rigidity[21]. The other inorganic constituents present are MgO, Al_2O_3, Fe_2O_3, Na_2O, SiO_2, P_2O_5, and SO_3, which sums upto about 19.76% of the total inorganic components present in it.

3.1.2 BET Analysis of chitosan

The BET analysis is carried out for the chitosan sample at a temperature of -195.8° C by measuring nitrogen adsorption isotherm. The BET surface area, total pore volume, and average pore radius of chitosan were obtained to be 4.096 m^2/g, 7.814 A°, and 8.449 cc/g, respectively. The high surface area of chitosan also defines its ability to be used as a good filler and as an excellent adsorbing material[35]. The

Table 1 — XRF analysis of raw fish scale

Component	Fish scales Weight (%)
MgO	7.50
Al_2O_3	0.61
K_2O	21.50
CaO	39.70
Fe_2O_3	0.40
Na_2O	4.36
SiO_2	19.00
P_2O_5	4.70
SO_3	2.19

pores were having a diameter of less than 2nm are referred to as micropores. In contrast, the pores having a diameter between 2 nm and 50 nm are mesopores, and the pores having a diameter greater than 50nm are macropores. As the pore diameter of chitosan was found to be 7.814 A°, the sample consists of micropores with a total pore volume of 8.449 cc/g.

3.1.3 Particle Size Analysis

Particle size analysis helps to measure the crystal size of the chitosan sample. The particle size of the chitosan sample ranges from 0.131 µm to 13.246 µm, as shown in Fig. 2. The reduction in particle size is mainly affected by sonication. The breaking down of the aggregates, reduction in size, and reduction in the polydispersity of a particle is mainly achieved by ultrasonic sonicator vibration (Frequency 20 kHz, speed -150 rpm, time -5 hr). The mean particle size and the geometric particle size range were obtained to be 3.3748 µm and 2.7914 µm, respectively. Thus the reduced size clearly justifies its applicability as a good filler, which can be easily dispersed into any polymer matrix[36] to produce biodegradable packaging polymers. A decrease in size enhances the homogeneous dispersion of the biodegradable filler into the synthetic polymer matrix, thereby inducing the biodegradability of the packaging film. Thus this developed environmentally friendly packaging film can act as a suitable substitute to mitigate the ever-growing threat imposed due to the accumulation of huge non-degradable film into the environment.

3.1.4 Proximate analysis

From the proximate analysis of the chitosan sample, we can find that there is a high ash content of about 29.96%, as shown in Table 2. The high amount of silica present in the extracted polysaccharide increases the ash content[25]. The fixed carbon content of the biomaterial was found to be very high (35.94%), signifying that it can be used as a suitable carbon source and can be well mixed with low-quality coal to improve its calorific value. The high moisture content of 17.67% indicates its hygroscopic nature[37]. The presence of non-water gases in chitosan is low, and hence the volatile matter content is found to be just 16.45%.

3.1.5 SEM analysis of chitosan

Chitosan extracted from fish scales are subjected to SEM analysis at different magnification ranges. The magnification at 4KX and 15KX is shown in Fig. 3. SEM analysis represented that the particles are non-homogeneous and are not of equal size. No particular geometry of the shape of the particles could be observed. The surface of the chitosan sample is rough as a result of which it can easily adhere to other materials, thereby forming a good biodegradable filler[38]. The sample size is observed on the microscale, and conversion of it into nanoscale can improvise the surface area. The particles were found to agglomerate, and a large number of visible pores could be observed on the surface. The high porosity defines its capability to be used as an adsorber[39].

3.1.6 XRD analysis of chitosan

The X-ray diffraction pattern of chitosan obtained from the fish scale is shown in Fig 4. Low intensity

Table 2 — Proximate analysis of fish scales chitosan (dry wt. basis)

Sample name	Moisture content (%)	Ash content (%)	Volatile content (%)	Fixed carbon (%)
Chitosan	17.67 %	29.96 %	16.45 %	35.94 %

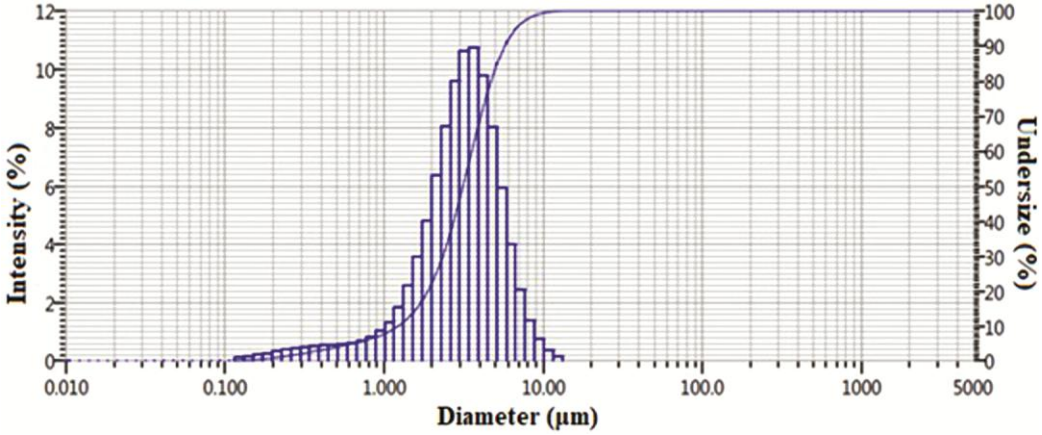

Fig. 2 — Particle size analysis of chitosan.

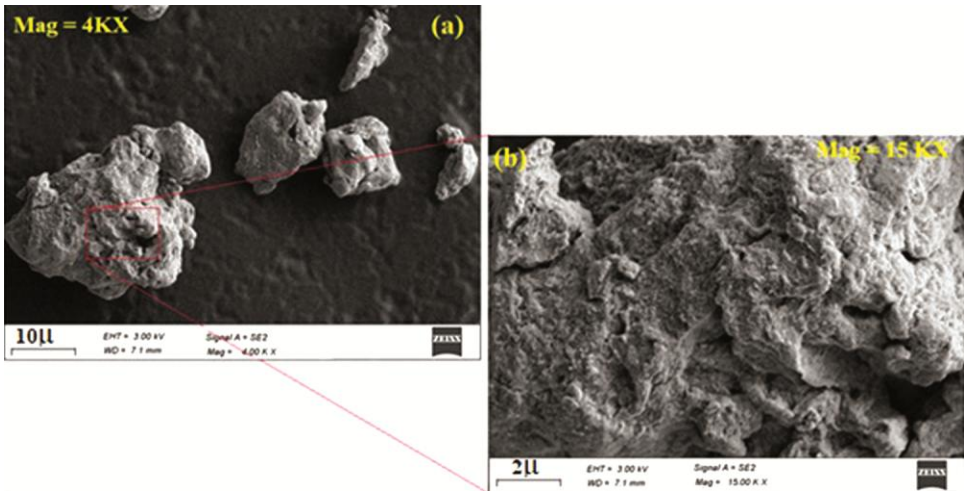

Fig. 3 — SEM analysis of chitosan at a magnification of (a) 4KX, and (b) 15KX.

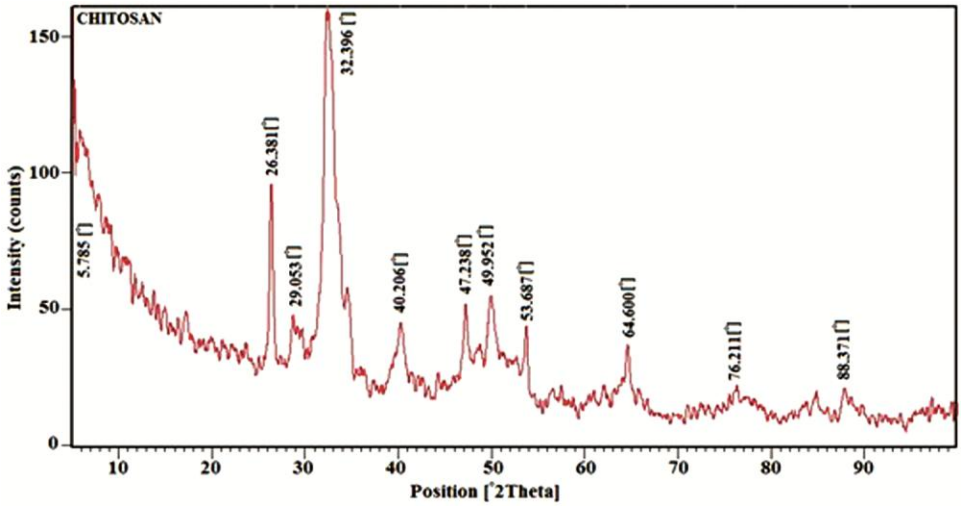

Fig. 4 — XRD analysis of chitosan.

and expressive width peak observed in XRD signifies the sample under observation is amorphous in nature, whereas a high intensity indicates crystalline nature. Various characteristics peak were observed at 5.785°, 26.381°, 32.396°, 40.206°, 47.238°, 49.952°, 53.687°, 64.6°, 76.21°, and 88.37°. No specific broad peak could be observed. Various sharp narrow peaks of high intensities were observed at 32.396°, 26.381°, and 49.95° justifying the extracted biomaterial is crystalline in nature[40].

3.1.7 FTIR analysis of chitosan

FTIR spectroscope shows the major absorption bands for the identification of characteristic functional groups present in the obtained product in the range of 4000 cm^{-1} to 500 cm^{-1}. Figure 5 shows the infrared

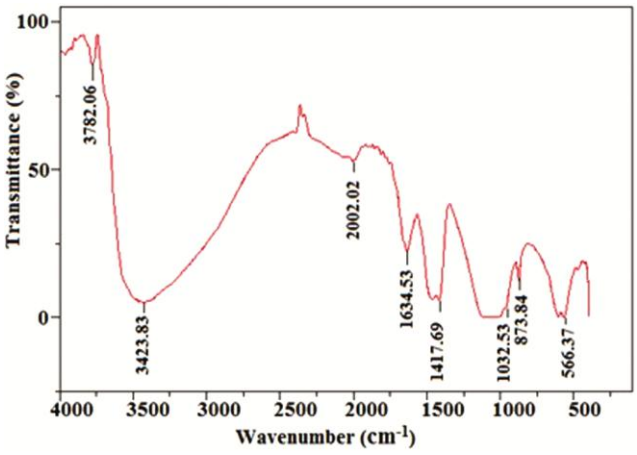

Fig. 5 — FTIR analysis of chitosan.

spectra of the chitosan sample that has been extracted from the fish scale. The characteristic peak at 3423.82 cm^{-1} for the OH stretching band signifies the hygroscopic nature of the extracted polysaccharide. The presence of C=O in chitosan can be highly justified by the visible peak at 2002.02 cm^{-1}. The absorption peaks for the N-H bond present in the amide II band have bending vibration is at 1634.53 cm^{-1}. The presence of CH-OH and CH_2-OH bond[41] can be identified by the bending vibration found at 1417.69 cm^{-1} and 1032.53 cm^{-1}, respectively. The absorption peak at 873.84 cm^{-1} is categorized by the presence of C-H in the chitosan. Thus the above peaks substantiate that the obtained material is chitosan with approximately negligible impurity.

3.1.8 Thermo-gravimetric analysis of chitosan

The thermogravimetric curve was obtained at a heating rate of 10°C/min under a dynamic nitrogen atmosphere having a range of temperature from ambient to 860°C. Figure 6 shows the thermal decomposition of the obtained chitosan sample. From the thermogram, it could be observed that there is a weight loss of chitosan sample at the temperature of about 100°C due to the removal of the adsorbed moisture from the surface. Chitosan is a polysaccharide composed of β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine[42]. The melting point greatly depends on the molecular weight of the polymer. A continuous weight loss could be observed due to the continuous breaking of the internal bonds of the high molecular weight polymer. The presence of high inorganic constituents as silica also acts as an influencing parameter for increasing the melting point.

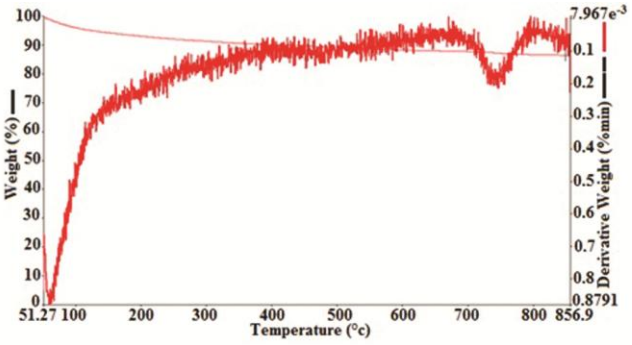

Fig. 6 — TGA analysis of chitosan.

3.1.9 Degree of deacetylation (DD)

The degree of deacetylation determines the removal of the acetyl group from the chain, which can be determined by the process of potentiometric titration.The homogenous solution of chitosan was prepared using diluted HCl (0.010 mol/L), which was titrated against 0.1M NaOH (w/v). The inflection of the pH values is an indication of the endpoint. Neutralization of HCl followed by the neutralization of ammonium ions from chitosan were the two major inflections considered. The number of amino groups thus can be calculated by finding the difference between two points,which is also referred to as the degree of deacetylation (DA)[43].The degree of deacetylation can be calculated by equation.

$$DD\% = 100 - DA\% \quad \ldots (10)$$

DD = Degree of Deacetylation

The degree of deacetylation of the obtained chitosan was found to be 52.11%

3.1.10 Solubility Determination

The results of chitosan solubility are shown in Fig. 7, which reveals the high solubility nature of chitosan in a 1% acetic acid aqueous solution. The applicability of new material is greatly affected by its ability to be compatible with different solvents. Various factors are involved, like temperature, alkali percentage, time of deacetylation, the ratio of chitin to alkali solution, prior treatments during chitin production in determining the solubility of chitosan. A proportional increase in solubility was observed

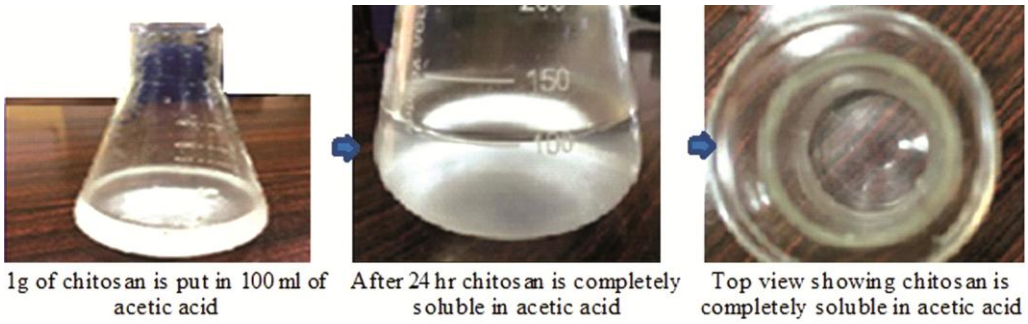

Fig. 7 — Solubility test of the extracted chitosan initially in 100 ml of acetic acid and then after 24 hours followed by the top view showing the complete solubility of chitosan in acetic acid.

with an increasing deacetylation degree. Complete removal of acetyl group and protein is highly necessary for achieving a good solubility of chitosan[44]. The presence of high content of protonated free amino group has a strong affinity for attracting ionic compounds, and this increases the solubility of chitosan in mild organic acids[45].

3.1.11 Water Binding Capacity (WBC)

The water-binding capacity is directly relevant to the physiological properties of the polymer. Minute variation in polymer structure may result in different strengths of the structural binding ability of the polymer with water. There are several methods like filtration and centrifugation that determine the water holding and binding capacity[46]. Water binding capacity was calculated by Eq. (11) as follows.

$$\text{WBC}(\%) = \frac{\text{Water bound (g)}}{\text{Initial sample weight (g)}} \times 100 \qquad \ldots (11)$$

The water-binding capacity of the extracted fish scale chitosan was found to be 160%. Work done by various researchers was also found to tally with the obtained result. Water binding capacity for five commercial chitosan from shrimp and crab shells is repeatedly reported to be found in the range from 458% to 805%[47]. An average of 702% of water binding capacity of chitosan was stated by Brine and Austin[44], however, by reversing the sequence of the steps (demineralization and deproteinization) effective increases, the water binding capacity of the chitosan[48] can be observed.

3.1.12 Fat binding capacity (FBC)

The fat binding capacity(FBC) is closely related to the study of the amount of oil entrapped within the polymer molecules, which asa result increases the bulk density of the polymer. Basically, FBC is defined as the physical oil entrapment within polymer molecule[49]. The fat binding capacity was calculated by equation (12) as follows.

$$\text{FBC}(\%) = \frac{\text{Fat bound (g)}}{\text{Initial sample weight (g)}} \times 100 \qquad \ldots (12)$$

The fat binding capacity of fish scales extracted chitosan was measured using soybean oil[50]. Extracted chitosan samples showed 457% of fat binding capacity.

3.2 Effect of various parameters on yield %

3.2.1 Effect of autolysis time on yield %

The process of autolysis helps in removing the proteinaceous matter from the biomass and thus produces a biomaterial that can be used for various applications. Proteases help in the process of enzymatic degradation by autolysis of the biomass during its incubation at various temperatures[51], producingdifferent valuable compounds[52]. Thus the incubation time during autolysis plays a significant role in the extraction of useful products from the biomass. It is observed that with a change in autolysis time from 20 hr to 48 hr, there is an increase in the yield percent of chitosan from 5.82% to 22.4%; however, the yield percent remains almost constant after 20 hr as shown in Fig. 8.

3.2.2 Effect of change in the ratio of HCl to fish scale

Demineralization is an essential step during the extraction of chitin. The removal of various inorganic constituents like calcium, magnesium, aluminum, potassium, iron, sodium, and phosphorous are necessarily required to obtain good quality and quantity of chitosan. Hydrochloric acid can be effectively used as an important reagent for the process of demineralization. Different ratios of the HCl to fish scale were considered for finding out the variation in the amount of chitosan produced during demineralization. It was observed that the maximum yield of chitosan of 22.4% could be observed for the ratio of 1:5 of fish scale to HCl, as shown in Fig. 9.

3.3 Optimization of chitosan extraction

The main parameters affecting the yield percent of chitosan during its extraction process are percentage of NaOH, deacetylation time, deacetylation temperature, and solid to liquid ratio (dry fish scale to alkaline solution). Percentage and the type of alkali used during the process of deacetylation and deproteinization also play an essential role in the yield percent of chitosan. Among different types of alkaline

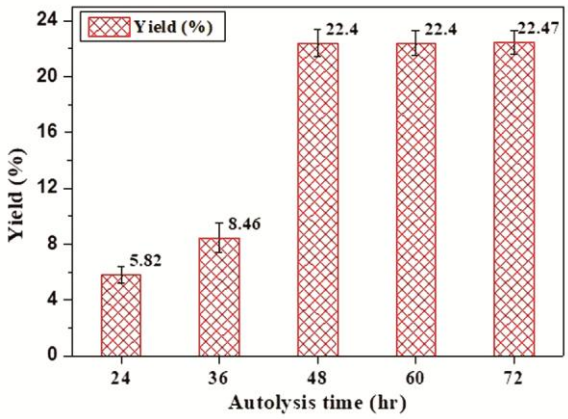

Fig. 8 — Effect of autolysis time on yield percent.

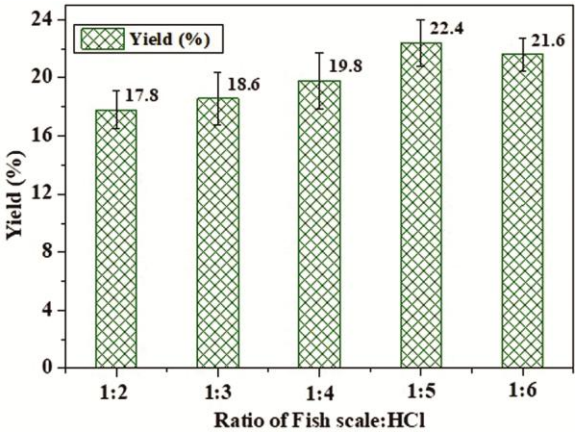

Fig. 9 — Effect of ratio of fish scale and HCl on yield %.

solutions like sodium hydroxide (NaOH), lithium hydroxide (LiOH), potassium hydroxide (KOH), and calcium hydroxide ($Ca(OH)_2$, the effectiveness of NaOH is higher[53] as the quality and quantity of chitosan produced by it are found to be better than the other alkaline solutions. In this study the three vital parameters, i.e., NaOH percentage, deacetylation time, and deacetylation temperature, are considered, and the solid-to-liquid ratio is fixed. The central composite design (CCD) approach of response surface methodology (RSM) was used to study the combined effect of the four parameters on the yield percent. The method makes permutations of different experimental conditions. The parameters used for the study along with their range are NaOH percentage: 4-7% (w/v), deacetylation time: 3-7 hrs; deacetylation temperature: 30-60°C and the solid to liquid ratio was fixed at 1:10 (w/v).

3.4 Experimental extraction design for the chitosan process

In the extraction process of chitosan from fish scale, computed and the mathematical method was used to solve the multiple variable calculations from the measurable data of experimentation. Parametric conditions were optimized with the help of this process utilizing various benefits like simplification of the experimental procedure, estimation of relative dependency of the different variables with each other, consumption of less time, and computation of optimum condition. Central composite design, known as CCD, Box-Behnken design, known as BBD, and two-level full factorial proposal, is the design processes present in RSM. Out of these, central composite design is used as it includes a description of the process by using three important steps. The steps include the process of experimentation, which is designed statistically, coefficient assessment using a model of mathematics through regression as well as response prediction accompanying the verification of the model. The final tentative conclusions include the figures obtained from the parametric response,which are carried out in this process examination and the relationship among the parameters and axial, factorial as well as replicate trials as described in Eq. (13).

$$N = 2^n + 2n + n_c \quad \ldots (13)$$

where, n = the number of dependent factors, n_c = the number of replicates

Thus in Eq. (13), n denotes the number of independent parameters, whereas n_c shows the number of replicating parameters. In the present study, there are three distinct parameters, viz sodium hydroxide (NaOH) percentage, deacetylation time, and deacetylation temperature. Therefore, an experimental matrix is established with 20 experimental runs containing eightfactorial points, 6 axial points, and sixreplicate points. During experimentation, +1 is the coding sign for a high level of factors, and -1 is a coding sign for a low level of elements. With the help of mathematical expression, the value of coding is represented in Eq. (14).

$$y_a = \frac{Y_{ac} - Y_{avg}}{(Y_h - Y_l)/2} \quad \ldots (14)$$

Here Y_{ac} is responsible for representing the true value of i^{th} factor, Y_{avg} is responsible for representing an average of high and low values for i^{th} factor, Y_h, as well as Yl, are the utmost values for i^{th} factor. The above equation is responsible for producingthe design of experiments, which contains twenty experimental runs in the chitosan extraction process. The independent variables and response can be functionally associated using a mathematical relation which is given by Eq. (15) as stated below:

$$Z = \alpha_0 + \sum_{i=1}^{n} \alpha_i y_i + \sum_{i=1}^{n} \alpha^2{}_{ii}\, y_i \sum_{i=1}^{n} \alpha_{ii}\, y_i{}^2 + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \alpha_{ij}\, y_i \quad \ldots (15)$$

where α_0 is the coefficient of constant; α_i is the linear coefficient; α_{ii} is the quadratic coefficient, and α_{ij} is the interactive coefficient.

The following quadratic Eq. (16) is further developed for three separate variables as:

$$Z = \alpha_0 + \alpha_1 y_1 + \alpha_2 y_2 + \alpha_3 y_3 + \alpha_{12} y_1 y_3 + \alpha_{13} y_2 y_3 + \alpha_{23} y_2 y_3 + \alpha_{11} y_1^2 + \alpha_{22} y_2^2 + \alpha_{33} y_3^2 \quad \ldots (16)$$

The help of the ANOVA technique can further check more accuracy of the developed computed model.

3.5 Combined parametric interaction towards chitosan extraction from fish scale

The RSM software establishes response plots in three dimensions by connecting multiple factors that participate in the process. These three-dimensional plots simultaneously correlate with two other variables by changing the response. When the independent factors mutually interact, then with the help of these response plots, the effect of a single parameter on those independent factors can be understood, hence the structure of the response plots is very much important.

The interaction among the various parameters can be stated as a combination of alkali percentage with deacetylation time, alkali percentage with deacetylation temperature and deacetylation time with deacetylation temperature. The individual effect of each parameter in yield % is shown in Fig. 10. With an increase in NaOH percentage, the yield % is found to increase from 26.50 to 28.58, then again it decreases to 25.29 at 7.99% NaOH content as shown in Fig. 10(a). The yield percentage of chitosan also varied appreciably with changes in the deacetylation time and deacetylation temperature. As can be observed from Fig. 10(b) that there is a continuous increase in yield% upto 27.55 at adeacetylation temperature of 45.04°C. Then it continues to drop upto 24.58% at a deacetylation temperature of 70.35°C. The deacetylation time also plays a significant role in the yield % of chitosan, and it could be observed from Fig.10(c) that for a deacetylation time of 5.03 hrs, the maximum yield % of 28.2 could be observed.

The interaction between the parameters over a varied range on the yield % is clearly described in Fig. 11. The 3D plots are shown in Fig. 11(a, c and e), whereas the contour plots are shown in Fig. 11 (b, d and f). The combined effect of NaOH percentage and the deacetylation time could be observed from Fig. 11 (a) and (b). The dark red region indicates the maximum yield percent, whereas the dark green and further dark blue regions indicate a lower value of yield percent. With the increase in NaOH concentration at a lower deacetylation time between 3 to 4 hours, there is an increase in yield % as the colour changes from dark green to pale green and the region covers within the encircled portion confining to a yield % value in between 27.5 to 28.58% whereas within the range of 52 to 6.4% of NaOH content if the deacetylation time is increased to 5 to 6.5 hrs, the colour significantly changes from green to yellow and then to dark red indicating the attainment of maximum yield percent at this condition. The combined effect of NaOH percent and deacetylation temperature, however, shows a different result as shown in Fig. 11(c and d). There is a continuous increase in yield percent with an increase in both the

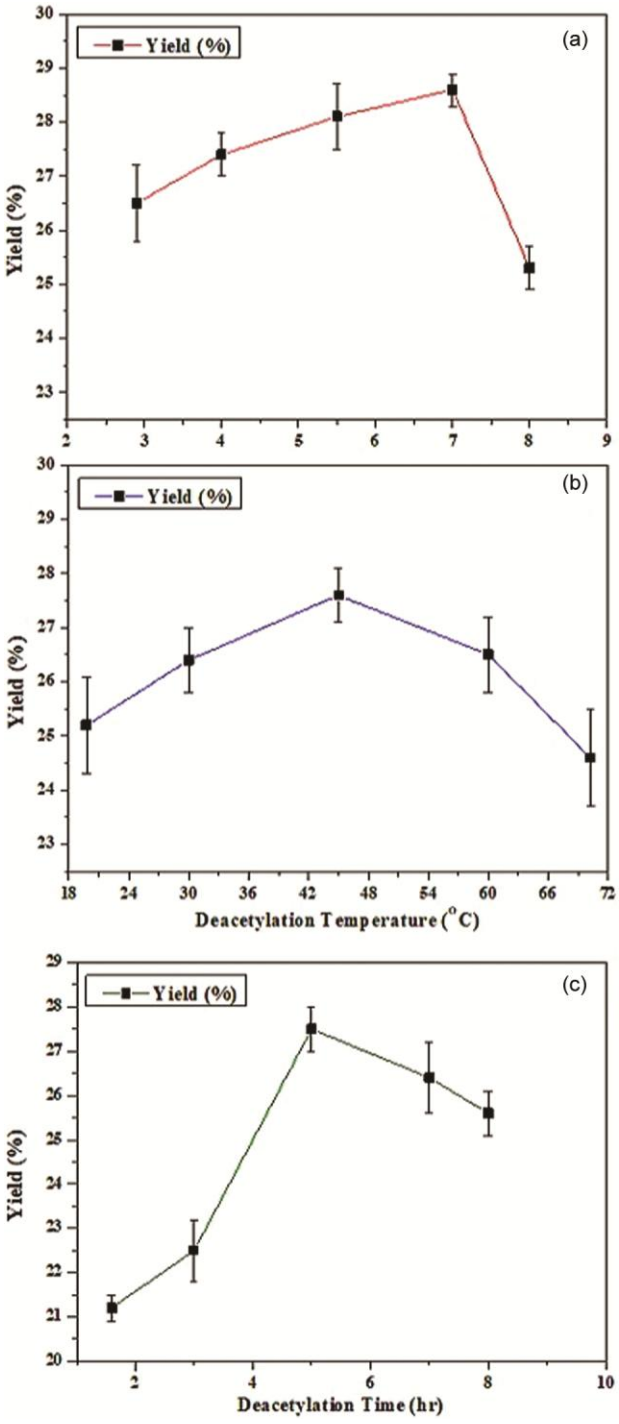

Fig. 10 — Effect of (a) NaOH %, (b) Deacetylation temperature, (c) Deacetylation time on yield %.

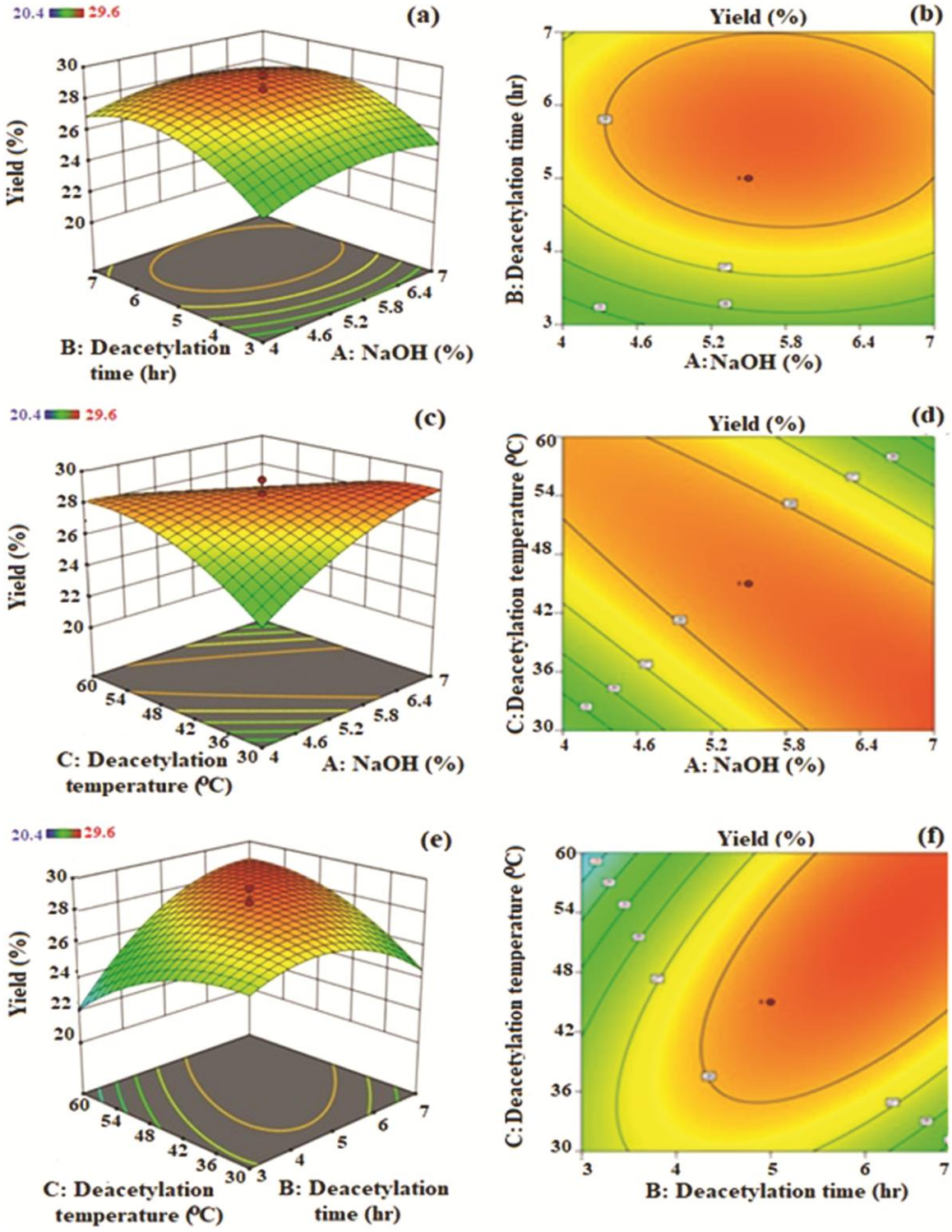

Fig. 11 — 3D plots of the combined effect of (a) NaOH(%) and deacetylation time, (c) NaOH (%) and deacetylation temperature, (e) deacetylation time and deacetylation temperature on the yield %. Contour plots of the combined effect of, (b) NaOH(%) and deacetylation time, (d) NaOH (%) and deacetylation temperature, (f) deacetylation time and deacetylation temperature on the yield % of chitosan obtained from central composite design.

parameters when one parameter is fixed at a lower value, and the other parameter is increased within the range considered. This is well justified by the change in colour observed from green to yellow and then to dark red. However, when both the parameters are considered together at a deacetylation temperature between 30 to 48°C, for a NaOH percent of more than 5.8%, the maximum yield percent of more than 28% could be achieved, whereas for NaOH percent, 4 – 5.2%, if the value of deacetylation temperature is increased to more than 50°C, the maximum yield percent can be observed. From Fig. 11(e and f) it could be observed that at a lower deacetylation time (around 3 hrs), the deacetylation temperature plays an adverse effect on the yield percent as the colour changes from yellow to blue when the temperature increases from 30 - 60°C. The optimum value of 29.6% of yield %, indicated by the dark red region in the contour plot,can be observed when the deacetylation time is in between 6-7 hrs for a deacetylation temperature of more than 50°C.

3.6 Regression Model Equation for extraction of Chitosan

Response surface methodology (RSM) gives the relationship between mathematical association and responses, which can be represented as yield %, and with the help of the regression technique, the effect of three independent parameters on the chitosan extraction process can be easily studied. Through the quadratic equation, the relationship between the factors and their responses can be given. In the process of chitosan extraction from the fish scale, the statistical relationship is established with the help of three separate parameters, which are the alkali percentage, deacetylation time, and deacetylation temperature, which are correlated with one dependent response. The model equation developed to assess the extraction of chitosan from its respective process parameters can be given by Eq. (17)

$$Yield\% = +28.56 + 0.3428 \times A + 1.21 \times B - 0.0080 \times C - 0.1625 \times AB - 2.14 \times AC + 2.16 \times BC - 0.9140 \times A^2 - 1.80 \times B^2 - 1.27 \times C^2 \quad \ldots (17)$$

where, A = Sodium hydroxide (%), B = Deacetylation time (hr), C = Deacetylation temperature (°C)

3.7 Examination of statistical data and model authentication of the chitosan extraction process

The importance of variables and the usefulness of the quadratic regression model, which is part of response surface modeling (RSM), can be determined using analysis of variance. The testing of classifying as well as cross-classifying values of statistics is carried out with the help of Fisher's test (F-test), which is included in ANOVA. Statistical analysis of chitosan extraction from fish scale study was carried out using ANOVA mainly to analyse the effect of different parameters with regard to the authentication of the regression model. Based on Fisher's test (F-test) and the Probability test (P-test), the statistical analysis is categorized. F value can be defined as the value obtained by dividing the mean square of regression with the mean error. A small P-value of the Lack of fit (LOF) test indicates the lack of fitting of the data in the model is less, which further signifies that the actual experimental data and the model predicted data have a good match. Significant value can be obtained when F-value is greater than the corresponding coefficient of F-value and smaller the P-value more significant will be the model. Apart from this, the sum of squares is also an important factor because the high value of the summation of squares indicates the greater signification of respective parameters. In the present study in Table 3, it is found that the deacetylation time having the maximum F value (28.33) than NaOH percent (26.13) and deacetylation temperature (0.0012) plays the major role in the quantity of chitosan extraction from the fish scale. The P-value should be less than 0.05 inorder to make the parameters significant. Though the model was found to be significant, having a P-value to be less than 0.0001, the individual parameters (deacetylation temperature and NaOH percent) showed a higher P- value (>0.05), making them insignificant. The maximum output of 19.93 was observed from the sum of squares data for the parameter deacetylation time followed by NaOH percent (1.61) and then deacetylation temperature (0.0009). The lack of fit is found to be 0.1352, indicating the non-significance of the lack of the data fit, signifying a good fit of the quadratic model on the response generated for the specified parameters. A value of 5.22 for the lack of fit indicates the occurrence of error due to noise.

The relation between actual values and predicted values is shown in Fig. 12. The generation of estimated data is done by computer simulation, whereas the experimental data provides the actual or real values obtained during experimentation. The R^2 value is found to be 0.9592, indicating a good fit between the actual and predicted values. Thus the model suggests its applicability for its utilization process for chitosan extraction. The value of predicted

Table 3 — Statistical analysis for computed extracted chitosan yield % ANOVA for quadratic equation model developed for chitosan yield (%)

Source	Sum of Squares	df	Mean Squares	F-value	P-value	
Model	165.51	9	18.39	26.13	<0.0001	Significant
A- NaOH	1.61	1	1.61	26.13	0.1619	
B- Deacetylation time	19.93	1	19.93	28.33	0.0003	
C- Deacetylation temp.	0.0009	1	0.0009	0.0012	0.9726	
AB	0.2113	1	0.2113	0.3002	0.5958	
AC	36.55	1	36.55	51.94	<0.0001	
BC	37.41	1	37.41	53.16	<0.0001	
A^2	12.04	1	12.04	17.11	0.0020	
B^2	46.58	1	46.48	66.19	<0.0001	
C^2	23.15	1	23.15	32.90	0.0002	
Residual	7.04	10	0.7037			
Lack of Fit	5.22	5	1.04	2.88	0.1352	not significant
Pure Error	1.18	5	0.3627			
Cor Total	172.5519	19				

Other Statistical Parameters						
Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
0.8389	25.85	3.25	0.9592	0.9225	0.7543	17.4157

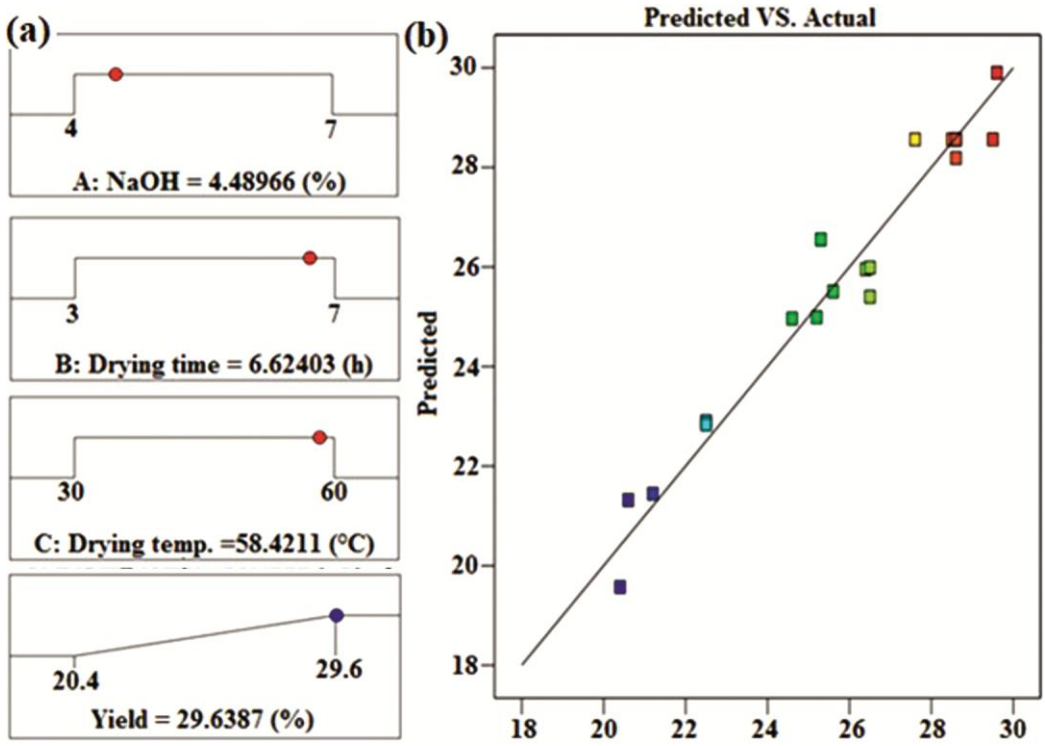

Fig. 12 — (a) Optimum yield (%) conditions derived from computed parametric optimization of chitosan via central composite design; (b) Graphical representation of actual versus predicted yield (%) obtained from the central composite design.

R^2 is 0.7543, whereas the value of Adjusted R^2 is 0.9225. There is a difference of 0.1682 in between the adjusted R^2 and predicted R^2, which is less than 0.2, describing the significance of the model. The Adequate Precision value is the ratio between signal and noise, and its value of more than 4 is highly desirable. In the present study, the adequate precision value is found to be 17.4157, which is more than 4, and hence the model prediction can be justified to be significant.

3.8 Optimal validation of parametric conditions

The present study is focused on the optimization of the parametric condition (NaOH percent, deacetylation time, and deacetylation temperature) under the influence of which the maximum amount of

chitosan can be extracted. The development of the most favorable condition is done by the numerical ranges present in the software. The system offered an array of different options, including the maximum, the targeted one, the minimum, within range, and the null. From the options provided by the system, optimum parameter selection for achieving the highest yield percent of chitosan was obtained by the numerical optimization method. The maximum amount of chitosan extracted from the fish scale was obtained at NaOH percentage (w/v) of 4.44% with a deacetylation time of 6.62 hr and deacetylation temperature of 58.42°C as shown in Fig. 12(a). In this condition, the yield percent for chitosan extraction was found to be 29.06%, with the desirability of 1.00. Investigations were further carried out in triplicates, as shown in Table 4, and the observed yield percentage was found to be 24.02, which shows a good match with the system predicted value.

3.9 Cost estimation for chitosan preparation

About 164 g of the fish scale was used to obtain 128.60 g of chitin, which further produced 29.1 g of chitosan. Thus the cost of 1 g of chitosan in United States Dollar (USD was estimated in accordance to Mondal *et al.*, as follows[54]:

I. The fish scale was used as raw material for the production of chitosan, which was obtained free of cost from the fish market, and hence the cost of fish scale (CFS) = 0.0 USD.
II. Reduction of size was not necessary; hence cost of size reduction (CSR) = 0.0 USD.
III. Tap water was initially used for cleaning of the fish and then it was rinsed using distilled water and hence cost of cleaning fish scale (CCFS) = CW + CDW = 0.0 +0.04902 = 0.04902 USD. As CW= Cost of water = 0.0 USD and CDW = Cost of distilled water = electricity consumption for distillation of 1 L of water in units × Cost of each unit = 0.5 × 0.09804 = 0.04902 USD.
IV. Cost of demineralization of fish scale (CDMFS) = CCU + CDW = 0.05280 + 0.04019 = 3.26902 USD. CCU = Cost of Chemicals used (16.4 ml of 1N HCl) = 0.05280 USD and CDW = cost of distilled water used (820 ml) = 0.04019 USD.
V. Cost of deproteinization fish scale (CDPFS) = CCU + CDW = 0.3142 + 0.03529 = 0.34949 USD.

CCU = Cost of Chemicals used (28 ml of 1N NaOH) = 0.3142 and CDW = cost of distilled water used (700 ml) = 0.03529

VI. Cost of chitin formation by deacetylation process (CDAFS) = CEC + CCU + CDW = 0.98048 + 0.56732 + 0.063726 = 1.611526 USD.

Where, CEC = Cost of electric consumption for hot air oven = hours × units × per unit cost = 20 × 0.5 × 0.09804 = 0.98048 USD, CCU = Cost of chemicals used (52 g NaOH) = 0.56732 USD and CDW = Cost of distilled water used (1300 ml) = 0.063726 USD.

VII. Cost of deacetylation of chitosan obtained (CDC) = hours × units × per unit cost = 4 × 0.5 × 0.09804 = 0.19608 USD.

(A) Total cost of chitosan production = CFS + CRS + CCFS + CDMFS + CDPFS + CDAFS + CDC = 0.0 + 0.0 + 0.049021 + 3.26902 + 0.34949 + 1.611526 + 0.19608 = 5.475137 USD.

Overhead charge = 10% of overall cost = 0.1 × 5.475137= 0.5475137 USD

Net cost of 29.1 of chitosan production = 5.475137 + 0.5475137 = 6.0226507 USD

Thus the cost of 1 g of chitosan = 0.20696 USD

Thus the extracted chitosan (3.7 μm, surface area - 4.096 m^2/g) is found to be a cost-effective product compared to the cost of analytical grade chitosan available in Sigma Aldrich of the same specification (Product Code 448877, 3.7μm, Cost - 1.75USD per gram, April 2020). Thus the commercialization of this work will help in the development of a value-added product from the waste fish scale.

Table 4 — Experimental validation of experimental parameters

	Optimum parameters			
	NaOH (%)	Deacetylation time (hr)	Deacetylation temperature (°C)	Yield (%)
Predicted	4.44	6.62	58.42	29.06
Replica 1	4.44	6.62	58.42	25.02
Replica 2	4.44	6.62	58.42	25.02
Replica 3	4.44	6.62	58.42	25.02

4 Conclusion

The waste fish scale from *labeo rohita* have been effectively utilized for the production of chitosan, which has a suitable characteristics to be effectively used as a biofiller and adsorbent for various applications. This value-added product, having a high surface area of 4.046 m^2/g and a very low particle size of 3.3748 μm, can be effectively used as a filler for various applications. The SEM images justified the high surface roughness and large visible pores having a pore volume of 8.449 cc/g. The chemical reaction justifies the elimination of the inorganic constituents identified by the XRF analysis from the fish scale.

The obtained material has a crystalline nature with a high fixed carbon content of 35.94%. The degree of deacetylation has been found to be 52.11%. The water binding and fat binding capacity have been determined to be 160% and 457%, respectively, signifying the biocompatibility of the film. The effect of autolysis time on yield % over a time range of 24 to 72 hr has been determined, and the maximum yield % of 22.47was observed at an autolysis time of 72 hr. An optimization study has been reported using the Central composite design approach of response surface methodology, and a maximum yield percent of 29.63% was determined at an optimized condition of 4.48% of NaOH content, 6.624 hrdeacetylation time, and 58.2°C deacetylation temperature. ANOVA regression equation suggests the value of determination coefficient R^2 to be 0.9592, indicating the high precision of the predicted model. The cost of the produced chitosan has been found to be 0.26 USD/g. Hence the produced chitosan from the waste fish scale can successfully minimize the environmental pollutants and can serve as a value-added product for its utilization in various applications.

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