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Preparation of Glucosamine from Exoskeleton of Shrimp and Predicting Production Yield by Response Surface Methodology

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Chitin was prepared from Persian Gulf shrimp (*Metapenaeus monoceros*), and then, the obtained chitin was hydrolyzed by hydrochloric acid solutions. The production yield of glucosamine hydrochloride from chitin was optimized, and the effect of three factors (acid concentration, acid to chitin ratio, and reaction time) was investigated. A Box−Behnken design by Minitab software created 12 reactions with different conditions. Each reaction was performed in two replicates. Response surface methodology was used for predicting the glucosamine preparation. The optimum conditions for glucosamine hydrochloride preparation were 30 and 37% hydrochloric acid, 9:1 (v/w) acid solution to solid ratio, and 4 h of reaction time. Time ratio and time acid concentrations were the effective factors on the yield.

KEYWORDS: Chitin; glucosamine; hydrolysis; response surface methodology

INTRODUCTION

Glucosamine (2-amino-2-deoxyglucose, chitosamine) is an amino sugar that occurs in acetylated and polymerized forms in chitin (**Figure 1**). Chitin is mainly produced from cuticles of various crustaceans, principally crabs and shrimps. Glucosamine in the human body participates in the structure of cartilage and works to stimulate joint function and repair. It has been proven effective in numerous scientific trials for easing osteoarthritis pain, aiding in the rehabilitation of cartilage, renewing synovial fluid, and repairing joints that have been damaged from osteoarthritis (*1*, *2*). There are a number of treatment options available to sufferers of arthritis, ranging from simple lifestyle changes to the use of pharmaceuticals to treat pain and inflammation, for example, nonsteroidal antiinflammatory drugs (NSAIDs) and natural products (nutraceuticals) (*1*, *3*, *4*). The reasons for the increased uptake of these products include cost, availability, and a perception of greater safety with the use of natural products (*5*, *6*). This has led to the worldwide consumption of large amounts of a great variety of over-the-counter glucosamine preparations, and reliable optimized methods are needed to prepare glucosamine with high quality and yield. Glucosamine products for arthritis are usually formulated as the hydrochloride salt or glucosamine sulfate and are often combined with chondroitin sulfate (*7*).

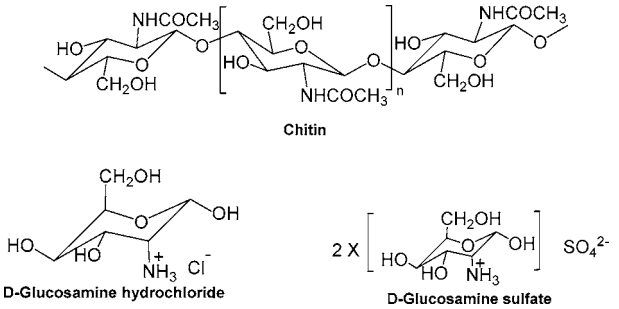

Figure 1. Chemical structures of chitin, glucosamine hydrochloride, and glucosamine sulfate.

Glucosamine sulfate can be prepared by refluxing chitin with a sulfuric acid solution, but this reaction has a low yield. A sulfuric acid solution can oxidize primary and secondary alcoholic groups in chitin or glucosamine. Glucosamine sulfate is very hygroscopic and degrades rapidly (goes from white to off-white to tan to brown) when exposed to moisture. To avoid this problem, glucosamine sulfate is made from glucosamine hydrochloride by adding either potassium or sodium sulfate and cocrystallizing the resulting mixture. Glucosamine sulfate, phosphate, and hydroiodide salts are also prepared by passing glucosamine hydrochloride solution over an anion exchange resin that has been conditioned with sulfuric acid, phosphoric acid, hydroiodic acid, or a metal salt of one of these acids (*8*). The preparation of glucosamine hydrochloride from chitin is a simple hydrolysis reaction. During this reaction, chitin is

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deacetylated and depolymerized to glucosamine hydrochloride in the presence of hydrochloric acid solution. Kamasastri and Prabhu prepared glucosamine from chitin by treating it with a large excess of concentrated hydrochloric acid (*9*). Kocourek et al. hydrolyzed chitin by 37% hydrochloric acid in a boiling water bath (*10*). Inoue proposed 2.5 L of 20% hydrochloric acid for hydrolyzing 594.7 g of chitin, which had been obtained from the exoskeletons of shrimps (*11*). Alphan used 37% hydrochloric acid at 100 °C with an acid solution to solid ratio of 5:1 (v/w) (*12*). Ingle et al. applied 3 parts of 20% hydrochloric acid at 100 °C with 2 h of stirring for hydrolyzing chitin (*13*).

The variable factors that can influence the yield of this reaction are acid concentration, acid solution to solid ratio (v/w), and time. Hydrolysis of amidic and ethereal bonds almost occured in refluxing temperature (about 100 °C), and in the previous works, hydrolysis of chitin was performed at 100 °C (*9*−*13*).

Response surface methodology (RSM) is a very useful statistical technique for optimization of complex chemical, biochemical, and food multifactorial processes (*14*−*16*) and may be used in investigating glucosamine hydrochloride production. Chang and Tsai (*17*) introduced RSM to optimize the deproteinization and demineralization process of chitin from shrimp shell powder. Hwang et al. (*18*) controlled the molecular weight and degree of deacetylation of chitosan by RSM. However, there has been no report on the optimization process for the glucosamine hydrochloride production in relation to the major factors that control the production yield of glucosamine hydrochloride. The aim of this study was the optimization of production of glucosamine with regard to three variables (acid concentration, acid ratio, and reaction time) and achieving an equation to relate variables and production yield of the reaction. This methodology can help us to predict the amount of glucosamine hydrochloride production of the reactions with different conditions.

MATERIALS AND METHODS

Materials. D-(+)-Glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride was purchased from Fluka. Orthophthaldialdehyde (OPA) and 3-mercaptopropionic acid (3-MPA) were purchased from Sigma. Methanol and tetrahydrofuran were purchased from Merck. All chemicals and solvents were analytical grade or high-performance liquid chromatography (HPLC) grade. Deionized water was prepared by an ultrapure water system Absaz (Absaz Company, Tehran, Iran).

Preparation of Chitin. Marine shrimp (*Metapenaeus monoceros*) were obtained from beach of Boshehr port in the Persian Gulf in July, 2005. The shells of the shrimp were scraped free of loose tissue, treated under running hot water to remove soluble organics and adherent proteins, and dried in the sun (25−30 °C) for 3 days. This was done to minimize batch dissimilarities due to the adherent proteins of the shell. To obtain a uniform size product, the dried shell was ground through a Moulinex mill (Moulinex Inc. France) with a 2 mm screen and sieved with a 35 mesh (0.5 mm) sieve. The dried, ground shells were placed in opaque plastic bottles and stored in a refrigerator.

Deproteinization of Shells. The deproteinization of shells involved stirring of the shells in dilute NaOH (3.5%) with a solvent to solid ratio of 10:1 (v/w) for 2 h at 65 °C. The residue was then collected on an 80 mesh (0.177 mm) sieve, washed to neutrality in running tap water, and filtered to remove excess moisture.

Demineralization of Shells. The deproteinized shells were demineralized with 1 N HCl for 0.5 h at ambient temperature with constant stirring and a solvent to solid ratio of 15:1 (v/w). Following demineralization, the decalcified chitin was then washed and filtered as above.

Decolourization of Chitin. Carotenoids and other pigments were extracted by absolute acetone, and the chitin residue was bleached with sodium hypochlorite solution (0.315%) for 5 min at ambient temperature with a solvent to solid ratio of 10:1 (v/w). The white chitin was collected, washed with water, and dried at 60 °C in a forced-air oven for 4 h (*19*−*21*).

Preparation of Glucosamine Hydrochloride. Chitin (0.5 g) was treated with different ratios and concentrations of HCl solutions and stirred at 100 °C for 1−4 h. The acid solution was added stepwise in four portions in predetermined intervals. The liberated HCl gas was absorbed in water, the reaction mixtures were filtered and washed with water, and the filtrate and the washings were mixed and collected in 25 mL volumetric flask. The collected solution was used for determining glucosamine hydrochloride by HPLC.

Characterization of Glucosamine Hydrochloride. *Thin-Layer Chromatography (TLC).* Identification of glucosamine hydrochloride was preformed on 20 cm × 10 cm high-performance silica gel $60F_{254}$ GLP plates (Merck, Darmstadt, Germany). The plates were precleaned using dichloromethane−methanol (1:1) and dried in a fume hood before use. The solutions of standard and obtained glucosamine hydrochloride were applied to the plates. 1-Butanol−glacial acetic acid−deionized water (*3*:1:1) was used as the mobile phase. The development time was about 1.5 h. After development, the mobile phase was evaporated from the plate by drying the plate in a fume hood for 10 min. The plate was then sprayed heavily and evenly with ninhydrin reagent (0.3 g of ninhydrin in 100 mL of 1-butanol plus 3 mL of glacial acetic acid) and dried in the fume hood for ca. 10 min. The plate was then heated on a plate heater at 115 °C for several minutes to produce red zones of glucosamine on a white background. The R_f for obtained and standard glucosamine hydrochloride was 0.4 (*22*).

Melting Point and Fourier Transform Infrared (FT-IR) Spectrum. For identification and comparing purity, the melting points and FT-IR spectra of the obtained and standard glucosamine hydrochloride were compared. The melting point was measured by a Gallenkamp melting point apparatus. The FT-IR spectrum was recorded on a Shimadzu FT-IR 4300 spectrometer (Shimadzu, Kyoto, Japan) in the form of KBr disks. The resolution was 4 cm^{-1}, and the scanning range was 400−5000 cm^{-1}.

HPLC Method. The HPLC system consisted of a pump, online degasser, autosampler, and fluorescence detector (Knauer, Berlin, Germany). The method was based on precolumn derivatization with OPA. A 20 μL aliquot of derivatized sample was injected onto the column, and separation was performed on a 250 mm × 4mm, 5 mm size, Spherimage 80 C18 (ODS2) analytical column (Knauer), employing a C18 precolumn guard cartridge. Samples were eluted with an isocratic system consisting of mobile phase A (12.5 mM phosphate buffer, pH 6.5, and methanol, 90:10 v/v) and mobile phase B (methanol: tetrahydrofuran, 97:3) at a flow rate of 1.0 mL/min. The ratio for A and B was 85:15. Detection was performed by a fluorescence detector at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 450$ nm, respectively. The peak area was determined by integration using Eurochrom 2000 version 2.05 software and used to calculate concentrations by interpolation from a standard curve extracted from glucosamine hydrochloride standard solution. Standards were prepared by using 0.1−20 μg/mL of glucosamine hydrochloride (*23*).

Statistical Design. The experimental design was a modified Box−Behnken design for three variables. The acid concentration % (X_1), acid solution to solid ratio (v/w) (X_2), and reaction time (X_3) were three independent variables considered in the preparation of glucosamine hydrochloride. The actual values and the corresponding values of three variables (X_1, X_2, and X_3) are given in **Table 1**. The complete design consisted of 24 experimental points, which included two replications of 1−12 experiments. The 24 experiments were carried out in random order. Data were analyzed to fit the following polynomial equation to Y (production yield of glucosamine hydrochloride):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where β values are constant regression coefficients and X_i values are independent variables. Minitab version 14 software (Minitab Inc., United States) was used for analysis of variance (ANOVA) and regression coefficient calculations.

Preparation of Glucosamine Sulfate. Glucosamine hydrochloride (5.62 g, 0.026 mol) was dissolved in 15 mL of water and added to 15

Table 1. Experimental Design Results from Various Trials for Preparation of Glucosamine HCl and Internal PE of the Proposed Model for the 24 Experiments

trial	X_1 (%)[a]	X_2 (v/w)[b]	X_3 (h)[c]	OPY (%)[d] ± SD[e]	CPY (%)[f]	PE
1	37	3:1	1	51.5 ± 2.1	51.8	0.6
2	37	9:1	1	68.5 ± 0.7	66.7	2.6
3	30	3:1	1	35.5 ± 2.1	37.1	4.5
4	30	9:1	1	51.0 ± 1.4	51.9	1.8
5	37	3:1	4	71.5 ± 3.5	76.3	6.7
6	37	9:1	4	97.0 ± 1.4	98.2	1.2
7	30	3:1	4	78.5 ± 2.1	71.9	8.4
8	30	9:1	4	97.5 ± 0.7	93.8	3.8
9	20	3:1	1	18.0 ± 1.4	16.1	10.5
10	20	9:1	1	30.0 ± 2.8	30.9	3.0
11	20	3:1	4	64.0 ± 1.4	65.7	2.6
12	20	9:1	4	85.0 ± 2.8	87.5	2.9
average of PE						4.05

[a] X_1 = HCl concentration (%). [b] X_2 = acid solution to chitin ratio (v/w). [c] X_3 = reaction time (h). [d] OPY = obtained production yield. [e] SD = standard deviation. [f] CPY = calculated production yield.

mL of sodium sulfate [1.85 g, 0.013 mol (or potassium sulfate (2.27 g, 0.013 mol)] solution in water. The obtained solutions were dried under vacuum at low temperature to produce glucosamine sulfate 2NaCl or glucosamine sulfate 2KCl salts, respectively.

RESULTS AND DISCUSSION

Statistical Analysis. Preparation of glucosamine hydrochloride can be reasonably optimized by studying three variable factors, including acid concentration, acid solution to solid ratio, and reaction time. Komori (*24*) prepared glucosamine hydrochloride at 110−120 °C with $SnCl_2$ and diluted hydrochloric acid in 4 h. They proposed that hydrolysis in diluted acid solution needs catalysis and high temperature. In other works (*9*−*13*), the hydrolysis of chitin has been carried out at 100 °C and a high concentration of hydrochloric acid solution. In this study, any catalysis such as $SnCl_2$, which could produce heavy metal impurities in the final product, was not used. Therefore, the experiments were conducted using 20−37% hydrochloric acid and 100 °C to enhance the chitin hydrolysis. Three variable factors were investigated at two or three levels. **Table 1** shows independent factors and the levels. On the basis of Box−Behnken RSM, 24 experimental points were performed. The results of the reactions are given in **Table 1**. We used a RSM and ANOVA to investigate the dependence of production yield of glucosamine hydrochloride on the independent factors. **Table 2** shows estimated regression coefficients (β) from RSM and the result of goodness-of-fit tests for the full models using ANOVA. Our models for production yield were found to be significant ($p < 0.05$, $R^2 = 0.98$). However, some regression coefficients shown in **Table 2** are not significant ($p > 0.05$). Therefore, we used a stepwise regression method to optimize the model. The final selected modified model for production yield is given in **Table 3**. The regression coefficients on X_1X_3 and X_2X_3 are all significant ($p < 0.000$ and 0.035, respectively) with $R^2 = 0.983$ (the same as that of the full model). The modified model for production yield is

$$y = -58.675 + 2.597X_1 + 2.0833X_2 + 25.2293X_3 - 0.4926\,X_1X_3 + 0.3889\,X_2X_3 \quad (2)$$

The production yield of glucosamine hydrochloride depends linearly on acid concentration, solids to acid solution ratio, and time and also the cross-product of acid concentration and time

Table 2. ANOVA and Regression Coefficients for the Polynomial Model[a] for Production Yield (Glucosamine Hydrochloride)

term	regression coefficients	*P* value
intercept	−50.9781	0.000
X_1	2.3316	0.000
X_2	0.8005	0.499
X_3	25.2293	0.000
X_1X_2	0.0442	0.239
X_2X_3	0.3889	0.034
X_1X_3	−0.4926	0.000
regression		
$R^2 = 0.984$		
$F = 174.42$		
probability of $p = 0.000$		

[a] Model on which X_1 = HCl concentration (%), X_2 = acid solution to chitin ratio (v/w), and X_3 = reaction time (h) is $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$.

Table 3. ANOVA and Regression Coefficients from the Modified Model for Production Yield (Glucosamine Hydrochloride)

term	regression coefficients	*P* value
intercept	−58.6750	0.000
X_1	2.5970	0.000
X_2	2.0833	0.001
X_3	25.2293	0.000
X_2X_3	0.3889	0.035
X_1X_3	−0.4926	0.000
regression		
$R^2 = 0.983$		
$F = 203.46$		
probability of $p = 0.000$		

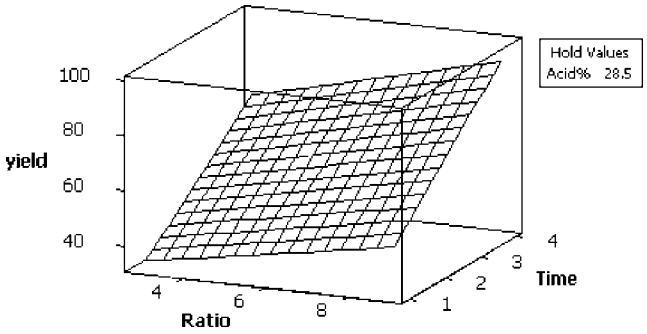

Figure 2. Response surface plot of yield (%) vs time (h); ratio (acid solution to solid v/w).

and the cross-product of solids to acid solution ratio and time. The production yield significantly increased with an increase of acid concentration, acid solution ratio, and reaction time. The production yield is inversely related to the cross-product of acid concentration and time. It means that at high acid concentrations, the longer reaction times give lower production yields. This phenomenon could be attributed to the side reactions, which produce impurities and hence low production yield. Although a higher acid concentration can cause the produced glucosamine to degrade, it promotes chitin hydrolysis. According to our preliminary experiments, stepwise addition of acid solution can decrease side reactions and increase the yield. The predicted model was used to create a response surface plot within the experimental region. Three-dimensional surfaces obtained from the predictive modified model for production yield are shown in **Figures 2** and **3**. RSM is a very useful statistical technique for the investigation of the dependence of the production yield on major independent factors such as acid concentration, acid solution to solids ratio, and reaction time. RSM results show

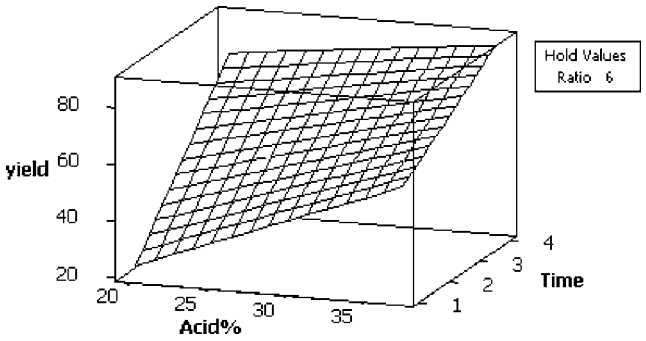

Figure 3. Response surface plot of yield (%) vs time (h); acid (%).

Table 4. Conditions, Obtained and Calculated Yield, and Corresponding PE of the Test Set Reactions for Validation of the Proposed Model

trial	X_1 (%)	X_2 (v/w)	X_3 (h)	OPY (%) ± SD	CPY (%)	PE
1	37	6:1	1	57.5 ± 0.7	59.2	2.9
2	37	6:1	2	64.0 ± 7	68.6	7.2
3	37	6:1	4	86.0 ± 1.4	87.3	1.5
4	30	6:1	1	43.5 ± 0.7	44.5	2.3
5	30	6:1	2	80.0 ± 1.4	57.3	28.4
6	30	6:1	4	86.0 ± 1.4	82.9	3.6
average of PE						7.65

that only linear and interaction components in the proposed model are significant. In this study, glucosamine hydrochloride was obtained in optimal conditions, using 30% HCl and 37% HCl with a 9:1 acid solution to solid ratio (v/w) in 4 h with 96−98% yield.

Validation. The accuracy of the proposed model was validated by conducting other reactions with different conditions and then comparing the obtained results with the model. The internal percent error (PE) of the proposed model can be calculated using eq 2 for the 24 experiments. The average PE for all 24 experiments is 4.05% (see **Table 1**). The CPY is the production yield of glucosamine hydrochloride that was calculated by the use of eq 2, and OPY is the production yield of glucosamine hydrochloride that was obtained in defined conditions for each experiment as shown in **Table 1**.

$$\mathrm{PE} = \frac{|\mathrm{OPY} - \mathrm{CPY}|}{\mathrm{OPY}} \times 100 \qquad (3)$$

To evaluate the external predictive performance of the model, six more experiments were carried out in duplicate as a test set. **Table 4** shows conditions and results of these reactions. In this table, CPY is the amount of glucosamine hydrochloride that was calculated via eq 2. The results revealed that the average PE for these 12 experiments is 7.65%. Considering the low internal (4.05%) and external (7.65%) PE, it might be concluded that the model has a good predictive power in the studied range of variables.

Characterization of the Glucosamine Hydrochloride. High quality of the obtained glucosamine hydrochloride is demonstrated by its melting point, IR spectrum, TLC method, and HPLC determination, which are similar with results of standard glucosamine hydrochloride. The melting point of the obtained glucosamine hydrochloride was 188−189 °C, which is comparable with 190−192 °C for standard glucosamine hydrochloride.

The results of FT-IR spectrometry of chitin and obtained glucosamine hydrochloride are given as follows:

Chitin FT-IR (KBr): cm^{-1} 532 (w), 565 (w), 952 (w), 1024 (m), 1074 (m), 1114 (m), 1157 (m), 1205 (w), 1261 (w), 1314 (m), 1379 (m), 1429 (m), 1559 (m), 1629 (m), 1658 (m), 2890 (m), 2930 (m), 3103 (m), 3254 (m), 3443 (s), 3471 (s).

Obtained glucosamine HCl FT-IR (KBr): cm^{-1} 570 (s), 597 (s), 698 (w), 773 (m), 854 (m), 889(w), 912 (m), 1002 (s), 1034 (s), 1066 (s), 1095 (s), 1137 (s), 1183 (m), 1394 (m), 1421 (s), 1535 (s), 1583 (s), 1614 (s), 2943 (s), 3042 (s), 3105 (s), 3350 (s).

The fingerprint of the FT-IR spectrum of obtained and standard glucosamine hydrochloride does not show any excess peaks. The IR spectrum of the obtained glucosamine hydrochloride shows a deacetylation because the wave number at ~1700 cm^{-1} for C═O, which exists in the IR spectrum of chitin, has disappeared.

The R_f value of spots was 0.4 for the both obtained and the standard glucosamine hydrochloride. The proportion of R_f of obtained glucosamine hydrochloride to R_f of standard is one. TLC did not show any other spots for impurities.

The HPLC method based on precolumn derivatization of glucosamine with OPA was used to identify and quantitize the obtained glucosamine. The retention time and chromatogram of obtained glucosamine hydrochloride compared with the chromatogram and retention time for the standard solution of glucosamine hydrochloride. The retention time and shape of peak for obtained glusosamine was identical with standard solution. Quantization was done according to a standard curve, which was plotted by concentrations ranging from 0.1 to 20 μg/mL of glucosamine hydrochloride (*23*).

Glucosamine Sulfate. Two moles of glucosamine chloride was cocrystallized with 1 mol of potassium or sodium sulfate to produce glucosamine sulfate 2KCl or 2NaCl. The amount of the glucosamine in this salt was determined by the HPLC method that previously was applied for glucosamine hydrochloride. The amount of glucosamine sulfate in cocrystallized salt was determined by the HPLC method to be 75−80%.

ABBREVIATIONS USED

3-MPA, 3-nercaptopropionic acid; ANOVA, analysis of variance; CPY, calculated production yield; NSAIDs, nonsteroidal antiinflammatory drugs; OPA, orthophthaldialdehyde; OPY, obtained production yield; PE, percent error; RSM, response surface methodology; SD, standard deviation.

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