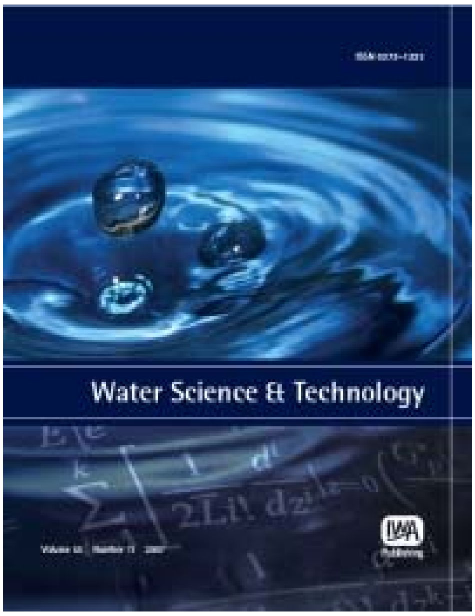
Water Science & Technology

Organics removal and protein recovery from wastewater discharged during the production of chondroitin sulfate

Yanqing Sheng and Li Xing

ABSTRACT

Bentonite, chitosan and polyaluminum chloride (PAC) were applied to treat wastewater discharged during the production of chondroitin sulfate and recover protein dissolved in the wastewater. The results showed that the combination of pH 9.00, 3–4 mL chitosan solution, 2 g of bentonite and 5 mL of 8% PAC solution per 100 mL of wastewater with a 4.0 h flocculation time were the optimal conditions for the recovery of protein and removal of total organic carbon (TOC) from wastewater. A pilot-scale test also was conducted, and 130 kg (dry weight) of sediment was obtained from 1.1 m^3 of discharged wastewater. This sediment contained abundant amino acids (proteins comprised 61% of the total sediment), after the recovery of protein, the dissolved TOC concentration in wastewater was decreased by approximately 80% and the residual wastewater could be readily disposed using a traditional activated sludge process.

Key words | chitosan and bentonite, chondroitin sulfate, polyaluminum chloride, protein recovery, wastewater treatment

Yanqing Sheng (corresponding author)
Research Center for Coastal Environmental Engineering Technology of Shandong Province,
Yantai Institute of Coastal Zone Research,
Chinese Academy of Sciences,
Yantai, 264003,
China
E-mail: *yqsheng@yic.ac.cn*

Li Xing
Ningxia Qiyuan Pharmaceutical Co., Ltd,
Yinchuan, 750100,
China

INTRODUCTION

Chondroitin sulfate is a polysaccharide found in bone, cartilage, and connective tissue and is composed of alternately linked *n*-acetylgalactosamine and glucuronic acid residues. Chondroitin sulfate has received considerable attention recently due to its biocompatibility and strong medicinal benefits (Garnjanagoonchorn *et al.* 2007). Furthermore, chondroitin sulfate has a number of biological properties that are useful for cartilage engineering including anti-inflammatory activity, water and nutrient absorption, wound healing, and biological activity at the cellular level that helps restore arthritic joint function (Hashiguchi *et al.* 2011). Typically, the cartilage used as raw material for chondroitin sulfate production is produced as a by-product from slaughter houses and fishery industries. Chondroitin sulfate is widely consumed by humans and non-humans because it is believed to be beneficial to those with joint-related pathologies. Chondroitin sulfate is commonly extracted from chicken, bovine and shark cartilages by digestion of tissues with exogenous proteinase (Srichamroen *et al.* 2013). However, the wastewater produced during the production of chondroitin sulfate is usually very difficult to treat. In recent years, the process of enzymatic hydrolysis followed by NaCl addition and ethanol crystallization has been widely used in China to produce chondroitin sulfate from chicken keel and shark fin cartilage. During production, many other components derived from cartilage and blood, such as proteins, are transferred into wastewater. This wastewater contains a wide range of organic compounds and has a high concentration of total organic carbon (TOC), and consequently cannot be readily treated by traditional processes (Laridi *et al.* 2005). Therefore, removal and recovery of compounds from wastewater, such as protein, that may be reused has received considerable attention.

Proteins, especially those extracted from animal bodies, are important ingredients in livestock feed. The recovery of these valuable protein components from industrial wastewater would have significant economic and environmental benefits (Sochindra & Mahendrakar 2005; Chi & Cheng 2006). Several substances, including chitosan-alginate, chitosan and $FeCl_3$ (Chen *et al.* 2008; Zeng *et al.* 2008), have been used for protein recovery from wastewater, and many existing technologies, such as resin adsorption, polyethylene glycol–polyacrylic acid, foam separation and spray drying (Kappler *et al.* 2008; Rao & Nair 2011), combined chemical coagulation/flocculation and physical sorption/adsorption have been used in this field (Dumay *et al.* 2008; Jiang *et al.*

doi: 10.2166/wst.2013.405

2011). Bentonite, because of its low cost, low density, and negatively charged surface, is widely used in food industries (Sun *et al.* 2007). The ions or groups (i.e. amino acid or protein) with positive charge can be adsorbed onto the surface of bentonite owing to the interaction between the negative and positive charges. Chitosan is derived from the deacetylation of chitin, which is soluble and positively charged in acidic media and may therefore be used as an eco-friendly coagulant and flocculant (Chatterjee *et al.* 2011). Chitosan has good adsorption properties and can participate in chemical reactions with protein molecules and amino acids through the properties of sorption reactions, bridging mechanisms and hydrophobic attraction (Singgih *et al.* 2007; Chen & Chung 2011) and can be used as a coagulant for bentonite (Renault *et al.* 2009). In solution, chitosan amino groups are protonated resulting in a positively charged polymer providing chitosan with adsorption properties such as ion exchange interactions and hydrophobic attraction (Chen *et al.* 2008). In this paper, we present the preliminary results of protein recovery and wastewater treatment experiments that use the sequential application of bentonite, chitosan and polyaluminum chloride (PAC).

METHODS AND MATERIALS

Description of wastewater and production of chondroitin sulfate

The wastewater was obtained from a local chondroitin sulfate factory. The daily output of chondroitin sulfate at this facility is approximately 400 kg, and nearly 8 tons of wastewater is discharged (Table 1). The raw materials used for chondroitin sulfate production is chicken keels, the wastewater characteristics are often not constant. Details regarding the process of chondroitin sulfate production are illustrated in Figure 1. Briefly, chondroitin sulfate was made by hydrolyzing chicken keels using enzymatic catalysis followed by sodium chloride addition and ethanol crystallization. As a result, a considerable amount of wastewater containing protein was generated. To satisfy the *State Wastewater Discharging Standard (China)*, this company discharged the wastewater after dilution with a large volume of cooling water.

Table 1 | Characteristics of wastewater discharged

Discharged volume (t d^{-1})	Temperature (°C)	pH	Total organic carbon (TOC, g L^{-1})	Total nitrogen (TN, g L^{-1})
6–10	60–90	6.5–7.5	70–150	20–60

Reagents and analysis procedures

Bentonite was purchased from Qingdao Yuzhou Chemical Co., Ltd (Qingdao, China) and had a composition of Al_2O_3 15.89%, SiO_2 64.14%, Na_2O 0.96%, K_2O 0.83%, CaO 2.92%, MgO 3.48%, Fe_2O_3 3.93%, TiO_2 0.19%, MnO 0.38%, and P_2O_5 0.16% and an ignition loss of 8.16%. Prior to this test, the bentonite was acidified with 10% H_2SO_4 and washed to $pH > 2$ with purified water; the material was then dried and subjected to comminution in a series. Chitosan (deacetylation degree of ≥85%) was purchased from Qingdao Hepe Biotechnology Co., Ltd (Qingdao, China). PAC was purchased from Zibo Sanrui Gongmao Co., Ltd (≥27% Al_2O_3, Zibo, China). All other reagents were analytical grade and purchased from chemical companies in China. TOC and total nitrogen (TN) levels in the raw wastewater and upper clarified solutions of treated water (after flocculation and sedimentation) were measured using a TOC-Vcph/SSM-5000A (TNM1) analyzer (Shimazu, Japan) after dilution. All tests were run in three replicates and data presented were averages of duplicate analysis, standard deviation ≤7%. The concentrations of protein dissolved in discharged wastewater were calculated as the TN concentration multiplied by 6.25 (Greenfield & Southgate 2003; Hall & Schonfeldt 2012). Temperature and pH were measured using a pH/temperature meter (HI8424 NEW Portable pH/mV/Temperature Meter, Kernco Instruments Co., Inc., Japan). The contents of proteins in wastewater were analyzed by LCQ Fleet ion-trap mass spectrometer (Thermo Fisher Scientific, Xcalibur software, USA).

EXPERIMENTAL DESIGN

The effect of wastewater pH on flocculation

Two replicate wastewater samples were prepared at a volume of 100 mL at room temperature (25 °C). One sample was titrated with hydrochloric acid (1 mol L^{-1}), and the other sample was titrated with sodium hydroxide (1 mol L^{-1}). During the titration process, the characteristics of the solution (i.e. the production of sediment) were observed carefully.

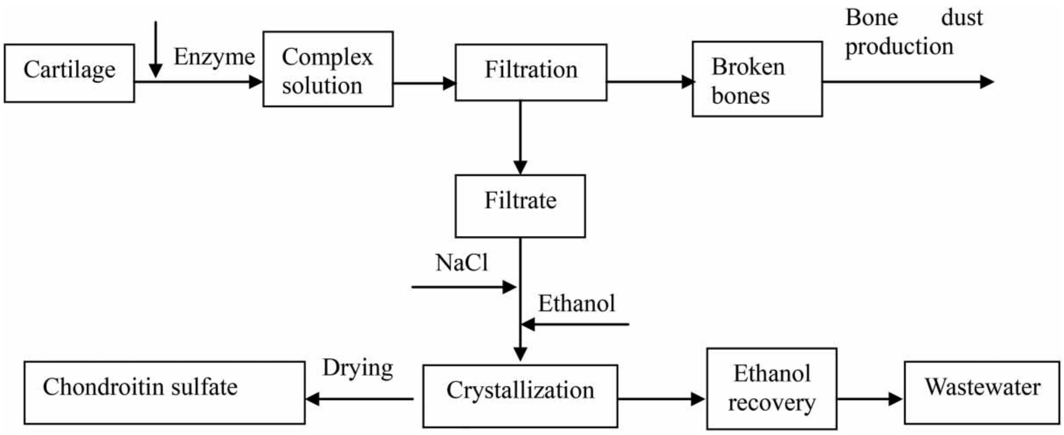

Figure 1 | Process of chondroitin sulfate production.

Comparison of the optimal dosages of bentonite, chitosan and PAC for protein recovery

Five groups of bentonite powder with masses of 1.0, 1.5, 2.0, 2.5, and 3.0 g, a 2% (by mass) chitosan solution in 1% (v/v) acetic acid aqueous solution and an 8% (by mass) PAC aqueous solution were prepared. Three groups of tests, each with five replicates of 100 mL of wastewater (with an unaltered pH), to which a different mass or volume of bentonite, chitosan and PAC were added separately. After flocculation and sedimentation for 2 h, the TOC and TN concentrations of the upper clarified solutions were determined. The recovery rate of protein in wastewater was calculated as follows:

$$\text{Recovery rate (\%)} = [(C_0 - C_t)/C_0] \times 100 \quad (1)$$

In the above formula, C_t is the concentration of protein in the tested wastewater (after treatment), and C_0 is the concentration of protein in the original wastewater (before treatment). The rate of TOC removal was calculated using the same method as for the protein recovery rate.

The comparison of different reactant combinations for protein recovery

Four groups of 100 mL of wastewater were prepared with an unaltered pH (6.82) at room temperature; each group had five replicates. A combination of bentonite and chitosan was added to the first group of replicates. A combination of bentonite and PAC was used in the second group, and chitosan and PAC were added together in the third group. The last group contained bentonite, chitosan and PAC. Each group was conducted using the single factor screening method to choose the optimal conditions. The reagent addition sequence was modulated to investigate the flocculation effects.

Determination of the optimal flocculation temperature and duration

Two groups of tests with five replicates were prepared. The temperature of one group was adjusted to 10, 20, 30, 40, and 50 °C with an ambient pH (6.82). The optimal doses of bentonite, chitosan and PAC were then added separately. The other group was held at room temperature (25 °C), chitosan, bentonite and PAC were added to the replicates, and TN and TOC were analyzed every 2, 3, 4, 5 and 6 h after agitation.

Experiment design and statistical analysis

Based on the above experiments, an optimization study was carried out using an orthogonal test design. The following conditions for optimizing the TOC removal rate and the recovery of protein were tested: pH of the wastewater (X_1), temperature of the wastewater (X_2), the added mass of bentonite (X_3), the added volume of chitosan (X_4), the added volume of PAC (X_5) and the flocculation time (X_6). The experimental design involved six factors (X_1, X_2, X_3, X_4, X_5 and X_6) each at five equidistant levels (1, 2, 3, 4 and 5). The response variables were defined as the recovery of protein (Y_1) and the TOC removal rate (Y_2). The factors, their levels and level codes are listed in Table 2. The experiment was carried out by L_{25} (5^6) (Liu *et al.* 2005) as shown in Table 3. All determinations were carried out in triplicate.

Table 2 | Factors and levels of the orthogonal test design

Factors	Codes	Levels 1	2	3	4	5
pH of wastewater	X_1	3.00	5.00	7.00	9.00	11.00
Temperature of wastewater (°C)	X_2	10.0	20.0	30.0	40.0	50.0
Added volume of bentonite (g)	X_3	0.1	0.5	1.0	1.5	2.0
Added volume of chitosan (mL)	X_4	1.0	2.0	3.0	4.0	5.0
Added volume of PAC (mL)	X_5	1.0	2.0	3.0	4.0	5.0
Flocculation time (h)	X_6	2.0	3.0	4.0	5.0	6.0

Table 3 | Conditions for the removal of TOC and the recovery of protein

Number of experiment	Codes X_1	X_2 (°C)	X_3 (g)	X_4 (mL)	X_5 (mL)	X_6 (h)
1	3.00	20.0	0.1	1.0	1.0	2.0
2	3.00	30.0	0.5	2.0	2.0	3.0
3	3.00	40.0	1.0	3.0	3.0	4.0
4	3.00	50.0	1.5	4.0	4.0	5.0
5	3.00	60.0	2.0	5.0	5.0	6.0
6	5.00	20.0	0.5	3.0	4.0	6.0
7	5.00	30.0	1.0	4.0	5.0	2.0
8	5.00	40.0	1.5	5.0	1.0	3.0
9	5.00	50.0	2.0	1.0	2.0	4.0
10	5.00	60.0	0.1	2.0	3.0	5.0
11	7.00	20.0	1.0	5.0	2.0	5.0
12	7.00	30.0	1.5	1.0	3.0	6.0
13	7.00	40.0	2.0	2.0	4.0	2.0
14	7.00	50.0	0.1	3.0	5.0	3.0
15	7.00	60.0	0.5	4.0	1.0	4.0
16	9.00	20.0	1.5	2.0	5.0	4.0
17	9.00	30.0	2.0	3.0	1.0	5.0
18	9.00	40.0	0.1	4.0	2.0	6.0
19	9.00	50.0	0.5	5.0	3.0	2.0
20	9.00	60.0	1.0	1.0	4.0	3.0
21	11.00	20.0	2.0	4.0	3.0	3.0
22	11.00	30.0	0.1	5.0	4.0	4.0
23	11.00	40.0	0.5	1.0	5.0	5.0
24	11.00	50.0	1.0	2.0	1.0	6.0
25	11.00	60.0	1.5	3.0	2.0	2.0

Pilot-scale test

Based on the results of the above experiments, a pilot-scale test was carried out in a workshop of the local chondroitin sulfate factory. The volume of discharged wastewater (pH of 6.29) was 1,100 L with a temperature of approximately 30 °C (after natural convection cooling). Under manual agitation, specific dosages of modified bentonite, chitosan and PAC were added successively. The protein content within the dried sediment was measured using the Kjeldahl method (Owusu-Apenten 2002).

RESULTS AND DISCUSSION

Detailed water quality parameters of wastewater are listed in Table 1. There was a large amount of amino acids in wastewater, such as threonine, isoleucine, leucine, methionine, histidine and phenylalanine. Prior to the bentonite/chitosan/PAC reagent mix applied below, the potential of a variety of other reagents was assessed. Chen *et al*. (2008) successfully applied chitosan and $FeCl_3$ to recover protein from wastewater discharged during the production of chitin. However, in this work, we found that the addition of $FeCl_3$ did not increase the quantity of produced sediment regardless of the chosen dosage and physical conditions (e.g. pH and temperature). In addition, various innocuous inorganic or organic flocculating agents and their combination, such as diatomite, polyaluminum ferric sulfate, polyferric sulfate, polyferric chloride, modified starch, seaweed glue and konjak gum, were also tested, and all were significantly less effective than the bentonite/chitosan/PAC reagent mix.

The effect of wastewater pH on protein flocculation

The titration experiments results showed that the pH of the wastewater had only a slight effect on the recovery of protein. Wastewater was titrated from its initial value with both acid and alkali. A small quantity of sediment formation was observed when the wastewater pH was adjusted downward to 4.6 and upward to 9.3. There was no distinct further formation of sediment when the pH was adjusted beyond this range to either 3 or 11. Therefore, if other affected factors were neglected, the optimal pH for protein flocculation was between 4.6 and 9.3. Chen *et al*. (2008) reported that the significant effect of wastewater pH on protein flocculation may have been attributed to the different isoelectric points of various proteins in the wastewater.

When the pH of wastewater is changed and reaches the isoelectric points of different proteins, the proteins become flocculated and are deposited (Chen *et al.* 2008). However, in this study, a distinct protein isoelectric point did not appear to exist. This may be explained by the following possible reasons: (1) the protein contained in the raw materials has been enzymatically hydrolyzed during the production of chondroitin sulfate, a lot of proteins would be transferred to peptides or amino acid during this process, such that the bulk protein, peptide, or amino acid dissolved in wastewater does not present a single isoelectric point; (2) there was a lot of NaCl and ethanol added during the chondroitin sulfate production (including residual polysaccharide), which may have influenced the distinct single isoelectric point of protein in wastewater.

Individual comparisons and the optimal dosages of different reagents

Bentonite, chitosan and PAC were added individually at different dosages in separate tests. The original composition and variations in corresponding values for discharged wastewater are shown in Figure 2. These data demonstrate high values of TOC and TN at up to 116 and 46 g L^{-1}, respectively. Because no other nitrogen-containing compounds were added during the chondroitin sulfate production process, and the concentrations of inorganic nitrogen (mainly ammonia, nitrate and nitrite) were close to zero, we assumed that the original concentration of protein dissolved in wastewater was approximately 292 g L^{-1} (TN $\times$ 6.25), which was nearly 30% of the total mass of the solution. Protein recovery (in sediment) thus corresponds to the reduction of TN levels in wastewater and can be calculated by formula (1).

Addition (10 mL) of PAC resulted in TOC decrease from 116 to 61 g L^{-1} (a removal rate of 47%) and a maximum protein recovery rate of 52% (i.e. protein concentrations decreased from 288 to 138 g L^{-1} (calculated from the variation of TN concentration); Figure 2). These results indicate that the optimal dosage of PAC is 8 mL (8% solution) for 100 mL of solution. Maximum protein recovery rates were 52% for chitosan and 59% for bentonite, and the optimal doses of chitosan and bentonite were 6 mL and 6 g, respectively, for a 100 mL solution. At this dosage, the highest TOC removal rates were 38 and 44% for chitosan and bentonite, respectively.

The comparison of various combinations and addition sequences of reagents for wastewater treatment and protein recovery

During the laboratory-scale tests, different waste disposal approaches were investigated using combinations of two or three different reagents (either chitosan, bentonite or

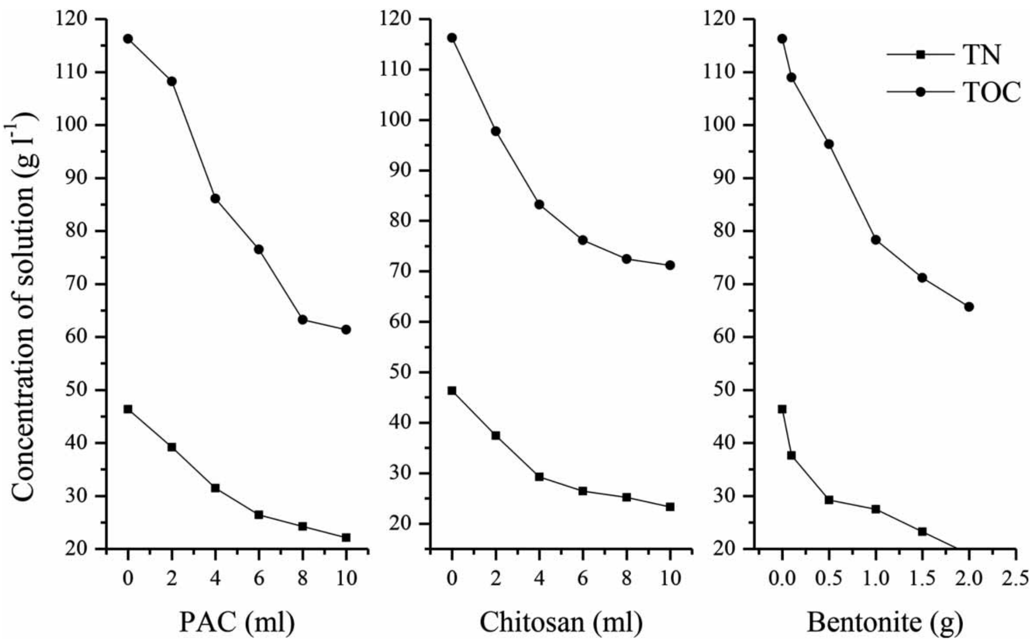

Figure 2 | Variations in TN and TOC concentration with different added doses of PAC, chitosan and bentonite.

PAC) at their respective optimal dosages (protein recovery rates ~50%). The results indicate that protein recovery (Y_1, Table 4) and TOC removal rates (Y_2, Table 4) greater than 50% cannot be achieved by the simultaneous addition of only two reagents. However, most of the protein can be recovered, and the TOC removal rate approaches 80% if the three reagents are applied simultaneously. Therefore, the simultaneous application of chitosan, bentonite and PAC could achieve optimal recovery of protein and removal of TOC. Furthermore, the addition order and different combinations of PAC, chitosan and bentonite for sedimentation were evaluated. Regardless of the chosen combination,

Table 4 | Results of all experiments and statistical analysis

Number of experiment		X_1	X_2 (°C)	X_3 (g)	X_4 (mL)	X_5 (mL)	X_6 (h)	Y_1	Y_2
1		3.00	20.0	0.1	1.0	1.0	2.0	68.87	76.92
2		3.00	30.0	0.5	2.0	2.0	3.0	70.32	77.59
3		3.00	40.0	1.0	3.0	3.0	4.0	70.91	78.51
4		3.00	50.0	1.5	4.0	4.0	5.0	68.41	75.21
5		3.00	60.0	2.0	5.0	5.0	6.0	71.92	77.82
6		5.00	20.0	0.5	3.0	4.0	6.0	69.59	79.16
7		5.00	30.0	1.0	4.0	5.0	2.0	70.78	80.44
8		5.00	40.0	1.5	5.0	1.0	3.0	69.06	75.82
9		5.00	50.0	2.0	1.0	2.0	4.0	70.04	76.71
10		5.00	60.0	0.1	2.0	3.0	5.0	69.26	76.41
11		7.00	20.0	1.0	5.0	2.0	5.0	67.61	76.16
12		7.00	30.0	1.5	1.0	3.0	6.0	68.89	76.19
13		7.00	40.0	2.0	2.0	4.0	2.0	70.99	77.27
14		7.00	50.0	0.1	3.0	5.0	3.0	70.15	78.82
15		7.00	60.0	0.5	4.0	1.0	4.0	67.47	77.12
16		9.00	20.0	1.5	2.0	5.0	4.0	74.34	81.72
17		9.00	30.0	2.0	3.0	1.0	5.0	70.73	81.31
18		9.00	40.0	0.1	4.0	2.0	6.0	70.96	78.57
19		9.00	50.0	0.5	5.0	3.0	2.0	71.66	81.95
20		9.00	60.0	1.0	1.0	4.0	3.0	71.21	77.68
21		11.00	20.0	2.0	4.0	3.0	3.0	72.29	81.88
22		11.00	30.0	0.1	5.0	4.0	4.0	69.62	78.57
23		11.00	40.0	0.5	1.0	5.0	5.0	70.05	78.71
24		11.00	50.0	1.0	2.0	1.0	6.0	63.84	77.72
25		11.00	60.0	1.5	3.0	2.0	2.0	62.59	77.99
Recovery rate of protein (Y_1)	K_1	350.43	352.60	348.86	349.06	339.97	344.89		
	K_2	348.73	350.34	349.09	348.75	341.52	353.03		
	K_3	345.11	351.97	344.35	343.97	353.01	352.38		
	K_4	358.90	344.10	343.29	349.91	349.82	346.06		
	K_5	338.39	342.45	355.97	349.87	357.24	345.20		
	R_1	20.51	10.25	12.68	5.94	17.27	8.14		
Removal rate of TOC (Y_2)	k_1	386.05	395.84	389.29	386.21	388.89	394.57		
	k_2	388.54	394.10	394.53	390.71	387.02	391.79		
	k_3	385.56	388.88	390.51	395.79	394.94	392.63		
	k_4	401.23	390.41	386.93	393.22	387.89	387.80		
	k_5	394.87	387.02	394.99	390.32	397.51	389.46		
	R_2	15.67	8.82	8.06	9.58	10.49	6.77		

K_i (resp. k_i) = sum of all Y_1 (resp. Y_2) values for the level i of the considered factor (same column); R_i (range) = $K_{(\text{highest value})}$–$K_{(\text{lowest value})}$ (same column).

addition of bentonite first always achieved the highest protein recovery rate. However, for PAC and chitosan, there was no clear influence of addition order. Moreover, due to the strong adsorption capacity of bentonite (i.e. negative charge, swelling and layer separation), adsorption processes would reduce the stability of dissolved protein and could help to accelerate the protein molecules entering into its structure (to the interlayer of bentonite) and be convenient for protein adsorption; bigger conglomerations are formed for sedimentation in wastewater. Therefore, the optimal order of reagent addition should be chosen according to the characteristics and function of bentonite, chitosan and PAC in the wastewater remediation process. The reason that the addition order is important is that when bentonite was added first, except for physical or mechanical absorption, protein or amino acid with positive charge would also be adsorbed onto the bentonite; subsequently, chitosan would absorb residual proteins or amino acid (i.e. threonine, isoleucine, leucine, methionine, histidine and phenylalanine) by association of ion-exchange interactions, hydrophobic attraction and physical adsorption between chitosan and protein (Chen *et al*. 2008). Furthermore, chitosan appears to have a high molecular weight, and this might cause enhancement of bentonite and protein coagulation through its properties of electrostatic attraction, sorption and bridging (Chatterjee *et al*. 2011). In the final step during flocculation by PAC, the greatest amount of compounds dissolved in wastewater was transferred to the sediment. In this study, bentonite was used as an adsorbent, chitosan was used as adsorbent and coagulant because bentonite can adsorb proteins and form heavy floccules, and chitosan can destroy the aqueous film around the protein to enhance adsorption. As a flocculant, PAC can form large floccules due its strong flocculation capacity (Lin *et al*. 2008). Thus, when bentonite, chitosan and PAC were used simultaneously, the protein recovery rate and the wastewater treatment efficiency were higher than when the reagents were used separately.

Determination of the optimal flocculation temperature and duration

Because the wastewater was boiled during the production of chondroitin sulfate, its discharge temperature was usually approximately 80 °C (Table 1). After natural cooling, adding bentonite (6 g), PAC (8 mL) and chitosan (6 mL) synchronously, then the different flocculation temperatures and durations were tested. The results indicate that the protein recovery rate and the TOC removal rate were both greatly affected by the flocculation time and were only slightly influenced by temperature. When the flocculation time was 1.0 h, many small particles remained in suspension and sedimentation was not complete. When the flocculation time was greater than 2.0 h, the protein recovery rate and TOC removal rate were approximately the same as the highest value obtained for simultaneous reagent addition. Therefore, the optimal flocculation time was 2.0 h. It was possible that the flocculation process was the result of a combination of physical adsorption of bentonite, hydrophobic attraction of chitosan and flocculation of PAC for the removal of organic compounds and protein in wastewater; therefore, this process may require many hours to equilibrate.

Optimization of conditions for the recovery of protein and removal of TOC

Twenty five experimental trials were implemented according to the matrix shown in Table 4. The responses of each trial for the protein recovery rate (Y_1) and the TOC removal rate (Y_2) were calculated according to Equation (1) and are listed in Table 4. The sum of responses at each level was computed and listed as K_i and k_i, R_i is the highest K_i against lowest K_i (same column), these parameters were listed in Table 4. In orthogonal test, higher K value represents higher rank of influence, higher R value represents more significant effect, so the group with the highest K value will be chosen as the optimal conditions. According to the scores of R_1 (20.55, 10.3, 12.7, 6.0, 17.3 and 8.1) and R_2 (15. 7, 8.8, 8.1, 9.6, 10.5 and 6.8), we concluded that all six factors influenced the recovery of protein and removal of TOC. The pH and added volume of PAC (X_1 and X_5) had significant effects on both Y_1 (R values are 20.5 and 17.3) and Y_2 (R values are 15.7 and 10.5), whereas temperature and flocculation time (X_2 and X_6) had a smaller effect (R values are 10.3 and 8.1). The effect of the other factors (X_2 and X_3) on both Y_1 and Y_2 were less than that of pH and the added volume of PAC but had a stronger effect compared to the flocculation time. Based on the principle of orthogonal test, for the protein recovery rate, the six factors had the following rank of influence (R_1 values order): $X_1 > X_5 > X_3 > X_2 > X_6 > X_4$. Therefore, the optimized conditions for the recovery of protein from wastewater discharged during the production of chondroitin sulfate were the following: a pH of 9.00, a temperature of 20 °C, an addition of 2.0 g of bentonite per 100 mL of wastewater, the addition of 4.0 mL of 1% chitosan per 100 mL wastewater, 5.0 mL of an 8% PAC aqueous solution per 100 mL wastewater and a flocculation time of 4 h. For the removal of TOC, the order of influence was as follows (R_2 values order): $X_1 > X_5 > X_4 > X_2 > X_3 > X_6$. Only the influences of X_3 and X_4 were different when compared to the protein recovery rate, and only the added volume of 2% chitosan was changed from 4.0 to 3.0 mL in the optimized conditions. However, although different operation conditions were conducted, the values of protein recovery rates and TOC removal rates are all close to each other (around 71 ± 5 and 79 ± 5% for Y_1 and Y_2, respectively), except for pH 11 (62.6–72.3%). This phenomenon indicates high values of Y_1 and Y_2 relay on the combined application of bentonite, chitosan and PAC, pH should be controlled strictly during the remediation process. Among six factors, on behalf of K values, the influence on Y_1 and Y_2, applicative conditions and cost, temperature (X_2) and flocculation time (X_6) should be chosen at room temperature (~20 °C) and 4 h (natural sedimentation). Addition quantity of bentonite, chitosan and PAC were selected by corresponding K values. Therefore, the optimized conditions for the recovery of protein and the removal of TOC were as follows: pH of 9.00, temperature of 20 °C, added mass of 2.0 g bentonite per 100 mL of wastewater; 3.0–4.0 mL of 2% chitosan, 5.0 mL of an 8% PAC aqueous solution and a flocculation time of 4 h.

In all of the experimental processes, the TOC removal rates were greater than the protein recovery rates by approximately 10%. This phenomenon may have occurred because there were abundant non-protein organic compounds (carbon-containing compounds) that is more easily swept out by the treatment than proteins while the wastewater was being flocculated and deposited; these compounds or organic matter contributed to a large portion of the TOC in the wastewater. Overall, after proteins recovery, approximately 80% TOC was removed from wastewater, residual wastewater can be readily disposed using a traditional activated sludge process or other remediation techniques.

Pilot-scale test

Based on the results of the orthogonal experiment, for the treatment of 1,100-L discharged wastewater, the optimal dosages of bentonite, chitosan and PAC were 20 kg, 30 and 50 L, respectively. Prior to the test, the pH of the wastewater was adjusted to approximately 9 using a sodium hydroxide solution. After agitation, sedimentation (4 h) and drying at 60 °C for 10 h, 130 kg of solid sediment was obtained. In this dried sediment, the total protein content was 61%. Consequently, this protein sediment can subsequently be used as a protein supplement in feedstuff. Furthermore, compared to other traditional protein feed additives, such as feather powder, the protein in this sediment was easier to ingest because it has been hydrolyzed by enzymes during the chondroitin sulfate production process.

CONCLUSIONS

A combination of bentonite plus chitosan and PAC was identified as an effective approach for protein recovery and TOC removal from wastewater produced during chondroitin sulfate manufacture. Optimal protein recovery (74%) occurred when bentonite flocculant was added first under conditions of: pH of 9.00, temperature of 20 °C, 2 g of bentonite per 100 mL wastewater, 3–4 mL of a 2% chitosan solution in 1% acetic acid per 100 mL wastewater, 5 mL of an 8% PAC aqueous solution per 100 mL wastewater, and a flocculation time of 4.0 h. After protein recovery, the TOC of the wastewater decreased by approximately 80%, and this wastewater was easier to treat using traditional methods. The approach described here can thus be used to produce significant environmental benefits and yield a saleable by-product.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (Grant No.: 40906045). Thanks to Dr Weiwei Zhang, Dr Ying Liu and Dr Chengli Qu (Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences) for their kind help in discrimination of proteins. We acknowledge the contribution of Professor Simon Bottrell (School of Earth and Environment, University of Leeds, UK) for assistance with presentation in English. We are also grateful to anonymous reviewers for their critical and constructive comments, especially in the revision process, which greatly helped to improve the manuscript.

REFERENCES

Chatterjee, S., Chatterjee, T., Lim, S. R. & Woo, S. H. 2011 Adsorption of a cationic dye, methylene blue, on to chitosan hydrogel beads generated by anionic surfactant gelation. *Environmental Technology* **32**, 1503–1514.

Chen, C.-Y. & Chung, Y.-C. 2011 Comparison of acid-soluble and water-soluble chitosan as coagulants in removing bentonite suspensions. *Water, Air, and Soil Pollution* **217**, 603–610.

Chen, X., Li, C., Ji, X., Zhong, Z. & Li, P. 2008 Recovery of protein from discharged wastewater during the production of chitin. *Bioresource Technology* **99**, 570–574.

Chi, F. H. & Cheng, W. P. 2006 Use of chitosan as coagulant to treat wastewater from milk processing plant. *Journal of Polymers and the Environment* **14**, 411–417.

Dumay, J., Radier, S., Barnathan, G., Bergé, J. P. & Jaouen, P. 2008 Recovery of valuable soluble compounds from washing waters generated during small fatty pelagic surimi processing by membrane processes. *Environmental Technology* **29** (4), 451–461.

Garnjanagoonchorn, W., Wongekalak, L. & Engkagul, A. 2007 Determination of chondroitin sulfate from different sources of cartilage. *Chemical Engineering and Processing* **46**, 465–471.

Greenfield, H. & Southgate, D. A. T. 2003 *Food Composition Data. Production, Management and Use* (2nd edn.). Food and Agriculture Organization of the United Nations, Rome.

Hall, N. G. & Schonfeldt, H. C. 2012 Total nitrogen vs. amino-acid profile as indicator of protein content of beef. *Food Chemistry* **140** (3), 608–612.

Hashiguchi, T., Kobayashi, T., Fongmoon, D., Shetty, A. K., Mizumoto, S., Miyamoto, N., Nakamura, T., Yamada, S. & Sugahara, K. 2011 Demonstration of the hepatocyte growth factor signaling pathway in the *in vitro* neuritogenic activity of chondroitin sulfate from ray fish cartilage. *Biochimica et Biophysica Acta* **1810**, 406–413.

Jiang, C., Wu, Z., Li, R. & Liu, Q. 2011 Technology of protein separation from whey wastewater by two-stage foam separation. *Biochemical Engineering Journal* **55**, 43–48.

Kappler, T. E., Hickstein, B., Peuker, U. A. & Posten, C. 2008 Characterization of magnetic ion-exchange composites for protein separation from biosuspensions. *Journal of Bioscience and Bioengineering* **105**, 579–585.

Laridi, R., Drogui, P., Benmoussa, H., Blais, J. & Auclair, J. C. 2005 Removal of refractory organic compounds in liquid swine manure obtained from a biofiltration process using an electrochemical treatment. *Journal of Environment Engineering* **131**, 1302–1310.

Lin, J. L., Huang, C., Pan, J. R. & Wang, D. 2008 Effect of Al (III) speciation on coagulation of highly turbid water. *Chemosphere* **72**, 189–196.

Liu, Z., Huang, R. & Tian, A. 2005 *Experiment Design and Data Processing (in Chinese)*. Chemical Industry Press, Chemical Industry Press Publishing Company, Beijing, pp. 33–76.

Owusu-Apenten, R. K. 2002 *Food Protein Analysis: Quantitative Effects on Processing*. Marcel Dekker, New York.

Rao, J. R. & Nair, B. U. 2011 Novel approach towards recovery of glycosaminoglycans from tannery wastewater. *Bioresource Technology* **102**, 872–878.

Renault, F., Sancey, B., Badot, P. M. & Crini, G. 2009 Chitosan for coagulation/flocculation processes – an eco-friendly approach. *European Polymer Journal* **45** (5), 1337–1348.

Singgih, W., Gonzalo, V., Vivek, S. & Antonio, T. J. 2007 Effect of chitosan type on protein and water recovery efficiency from surimi wash water treated with chitosan-alginate complexes. *Bioresource Technology* **98**, 539–545.

Sochindra, N. M. & Mahendrakar, N. S. 2005 Process optimization for extraction of carotenoids from shrimp waste with vegetable oils. *Bioresource Technology* **96**, 1195–1200.

Srichamroen, A., Nakano, T., Pietrasik, Z., Ozimek, L. & Betti, M. 2013 Chondroitin sulfate extraction from broiler chicken cartilage by tissue autolysis. *LWT – Food Science and Technology* **50**, 607–612.

Sun, X., Li, C., Wu, Z., Xu, X., Ren, L. & Zhao, H. 2007 Adsorption of protein from model wine solution by different bentonites, chin. *Journal Chemical Engineering* **15**, 632–638.

Zeng, D., Wu, J. & Kennedy, J. F. 2008 Application of a chitosan flocculant to water treatment. *Carbohydrate Polymers* **71**, 135–139.

First received 30 September 2012; accepted in revised form 3 June 2013