Treatment surface water using a novel pumpkin seed-based natural bio-coagulant: optimization by CCD and toxicity evaluation

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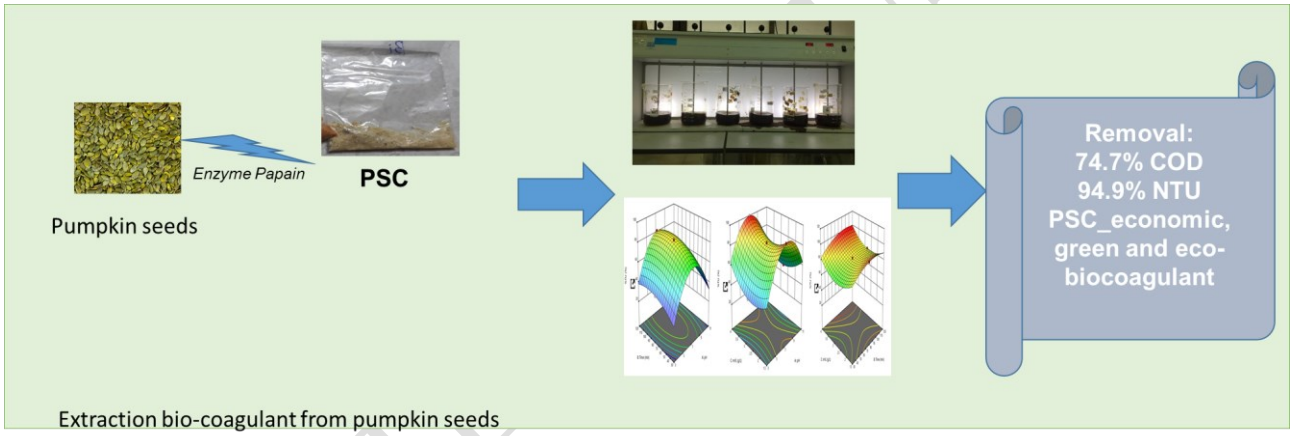
GRAPHICAL ABSTRACT



ABSTRACT

This study aimed to evaluate the effectiveness of a new coagulant extract from pumpkin seeds (PSC) for removing turbidity and organic compounds from different natural surface waters. Two design experiment methods, namely central composite design (CCD) and response surface methodology (RSM), were used to optimize PSC for removal of turbidity and organic matter via the chemical oxygen demand (COD) of surface water. The CCD results indicated that more than 74.7% of COD and 94.4% of nephelometric turbidity units (NTUs) were removed under optimized conditions (pH = 7.4; mPSC = 1.5 g/L; time: 88 min). They allowed for final effluent with significantly higher quality in terms of organic content and safe to *Vibrio fischeri* to be obtained

Keywords: Bio-coagulant; pumpkin seeds; central composite design (CCD); surface water pollution.

1. Introduction

Population growth has increased global consumption and water availability in recent years. Water bodies contain enormous amounts of fine particles and dissolved pollutants, both of which are undesirable for human consumption, especially in tropical countries (Azamzam *et al*., 2022). Therefore, surface water must be treated and purified to meet standard human consumption limits. Coagulants are chemicals (aluminum sulfate, aluminum chloride, ferrous sulfate and ferric chloride) or plant-derived coagulants, which are substances used to remove water pollutants, such as color, turbidity and organic matter from "raw" (unfiltered) water by forming large aggregates that finally settle to the bottom of the container (Nguyen *et al.*., 2020).

Recently, many research insisted that plant-based coagulants (likely *Moringa oleifera*, *Cicer arietinum*, *Dolichos lablab* and *pinecones*) are the suitable and safer treatment alternatives for these chemicals (Abujazar, *et al*., 2022; Hoa and Hue, 2018; Nath *et al*., 2021). These bio-coagulants have been found to contain various efficient proteins in charge of coagulation treatment in which combinations for creating larger agglomerates called flocs are present (Abu Amr *et al.*, 2023). Bio-coagulants are generally considered safe for human health. Their advantages are the generated sludge is typically biodegradable, practically non-toxic, composed of low-cost materials, and locally available (Ugwu *et al.*., 2017). However, some natural coagulants are valuable, expensive, and not widely available, so their use is limited (Abujazar *et al.*, 2022). There are several techniques that can be used to extract protein from plant seeds, including mechanical, chemical, and enzymatic methods. Enzyme-assisted methods are a gentle and efficient way to extract protein from plant seeds. These methods use enzymes to specifically target certain bonds, breaking down carbohydrates and other non-protein components, resulting in a purer protein extract. The use of enzymes allows for selective extraction of desired proteins, as well as improving the functional properties of the protein. (Kumar *et al*., 2021). Prado *et al.*, 2021 have shown that different proteolytic enzymes can be used to extract protein from a variety of seeds, including Corn, Sorghum, and Sunflower. Previous research investigated enzyme-assisted extraction methods for *Moriga oleifera* seed to apply in coagulation

process (Cao *et al.*, 2021). Enzyme-assisted extraction can also be more cost-effective than other methods and is considered to be more environmentally friendly.

Pumpkin (*Cucurbita sp.*) are gourd squashes that are grown commonly in tropical and sub-tropical countries. During pumpkin processing, a number of by-products are generated in the form of seed and peel. The seeds of pumpkin plants contain nutrient-rich compounds with a high content of protein, peptides, dietary fiber, and micro-nutrients (Das *et al.*., 2022). Pumpkins seed (PSC) husks have been used to treat dye-based wastewater (Kowalkowska and Jóźwiak, 2019); however, to the best of the author's knowledge, protein extraction of PSC for treating surface water has never been studied before especially extracted protein assisted by enzyme. In this research, we used protein extracts from PSC powder as a natural coagulant to treat surface water.

Response surface methodology (RSM) is a statistical method that is used to extract and model influence parameters, obtain optimal conditions, and confirm these parameters. One of the quadratic models was designed using the Design–Expert software (version 13) to determine the optimal influence value settings. We used a central composite design (CCD) according to RSM to examine the influence of three factors: (1) initial natural coagulant dosage, (2) pH, and (3) reaction time and to obtain the optimal state of coagulation process (Jayan *et al.*., 2021). In addition, luminescent microorganisms, namely the bioluminescent marine bacterium, *Vibrio fischeri*, were used more often than not in numerous toxicity tests of instruments used for testing the new bio-coagulant extracts from pumpkin seeds.

2. Materials and methods

2.1 Materials and reagents

2.1.1 Materials

The pumpkin seeds were purchased in Muong La district, Lao Cai province, Viet Nam. The seeds were peeled, washed, dried, and then crushed

2.1.2. Reagents

n-hexane; K_2CrO_7; Ag_2SO_4; H_2SO_4; $FeSO_4.7H_2O$; 1-10 phenanthroline were purchased from Merck, Germany with purity of 99%

2.1.3. Surface water samples

The lake water samples were collected at Ban Nguyet Lake, Ha Dong district, Hanoi (20°59'14"N 105°47'36" E) three times during September 2022. Table 1 shows the water quality of lake water samples. All samples were stored in a pharmaceutical refrigerator (Panasonic MPR-S313, Japan) at 4 ºC for 24 h.

Table 1. Water quality of Ban Nguyet Lake.

Characteristics	**Unit**	**Initial values**
pH		7.53 ± 0.02
Turbidity	NTU	37.5 ± 0.01
COD	mg/L	82.1 ± 0.03

2.2 Extraction of protein from pumpkin seeds

PSC flour was acquired by grinding all seeds using a 1 mm mesh mill and a 10-xx mesh sieve. n-hexane was used to defeat wheat flour. The crushed seeds were ultrasonically mixed with n-hexane at a ratio of 1:3 (w/v) for 15 min at 45 °C. This procedure was repeated three times. Delipidated samples were then dried at 50 °C in an oven (Blinder, FD 115, USA). After that step, protein extraction using the papain enzyme was used in this research. The extraction procedure was executed at a temperature of 60 °C. for 1 h at a substance-to-distilled water ratio of 1:3 (w/v) with an enzyme concentration to liquid phase of 1 ml/100 ml. After extraction, the solution was centrifuged at 4000 g for 60 min at room temperature. The supernatant (PSC) was collected and stored.

2.3 Coagulation-flocculation experiments

The coagulation process was conducted following the protocol of Hoa and Hue (Hoa and Hue, 2018) and was conducted using a six-place jar test equipment (VELP, JLT6, Italia). PSC was added to a 1000 mL sample of lake water at six different concentrations ranging from 1.5 to 4 g/L. Firstly, the solution was mixed for 5 min at 200 rpm, after that being swirled gently for 20 min at 40 rpm and

finally, the solution was allowed to settle for 30 min. The effluent was tested for turbidity and chemical oxygen demand (COD) decrease after settling. The percentage of removed turbidity and COD were determined using equation Eq. (1):

$$H = \frac{C_o - C_i}{C_o} x\ 100\% \qquad (1)$$

in which C_o and C_i are the initial and after-treatment concentrations for turbidity or COD.

2.4 Analysis methods

2.4.1 Chemical composition of the PSC.

Using the Association of Official Analytical Chemists technique and a conversion factor of 6.25, the moisture, ash, crude protein, total lipids, and mineral contents of the PSC and protein extracts were calculated (AOAC, 2016). Using Microsoft Excel 2016, mean data with standard deviations (SD) were reported for each experiment in triplicate (Microsoft, USA)

Fourier Transform Infrared Spectroscopy (FTIR) of the bio-coagulant PSC was carried out to determine the chemical characteristics on an FTIR Bruker Tensor II FTIR, Germany spectrophotometer. The FTIR spectra were measured in the range of 400–4000 cm^{-1}.

2.4.2 Water quality characteristics and toxicity analysis

Turbidity was measured by the portable turbidimeter (Hach 2100Q, USA). pH values of samples were adjusted by the pH meter MW101 (Milwaukee, Poland) and COD was calculated by the open reflux method SMEWW 5220.C:2012 using the COD reactor (Hach, DRB 200, USA).

2.4.3 *V. fischeri* acute toxicity

ISO 1348-3 standard 2007 (International Organization for Standardization, 2007) was used for the luminescence inhibition test with V. fischeri before and after the coagulation process for 88 min using different concentrations of PSC (1.5, 2, 3, and 4g/L) with the Microtox bacteria in Microtox SOLO kit. Bacteria were stored at –20 °C and activated by hydration according to the standard operating procedure of the kit. All samples were examined in triplicate and maintained at 15 °C on a thermostatic plate during the evaluation. A solution of 20 g/L NaCl (Acros, USA) was used as a

control sample. After 30 min, bioluminescence was consistent with a Delta ATP luminometer (Modern Water, USA). The bioluminescence inhibition ratio (BR %) was calculated using Eq. (2):

$$BIR = \frac{L_{Blank} - L_{sample}}{L_{Blank}}$$

In which L_{Blank} and L_{sample} are the bioluminescence signals after 30 min of exposure for the sample before and after the coagulation process by PSC, respectively.

2.5 Model design and optimization by response surface methodology (RSM)

A statistical technique called central composite design (CCD) is based on a multivariate nonlinear model and has been frequently utilized to optimize the coagulation of process variables as well as to extract the regression model equations and operating conditions from the suitable trials *(Khettaf et al.*, 2021). The CCD was applied in this present study to determine the optimum process variables for removing turbidity (NTU) and organic matter (COD) using Design experiment ver. 13. The experimental design model was a CCD model based on three factors (Table 2): (1) bio-coagulant (PSC) concentration (1.5–4 g/L), (2). time (30–120 min), and (3) pH (5–9).

Table 2. Variables and levels of the coagulation process using PSC

Parameter	**Unit**	**Range and levels**		
		-1	**0**	**+1**
pH (A)		3	7	11
Time (B)	min	30	75	120
PSC dosage (C)	g/L	1.5	2.75	4

CCD often appears as a series of statistical techniques that require a polynomial function:

$$\mathbf{Y = b_0 + \sum_{i=1}^{n} b_i \times X_i + \sum_{i=1}^{n} b_{ii} \times X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} \times X_i \times X_j} \quad (3)$$

in which Y is the predicted response, b_0 is the constant coefficient, b_i is the linear coefficient, b_{ij} is the interaction coefficient, b_{ii} is the quadratic coefficient, and X_i, X_j is the coded values.

3. Results and Discussion

3.1 Composition of pumpkin seeds and protein extracts (PSC)

The chemical composition of the PSC protein extract is shown in Table 3. Protein content in the PSC samples was high at 74.90% ± 1.71% when extracted with the enzyme papain. The protein extracts exhibited minimal ash and moisture levels. Papain is a cysteine protease enzyme found in the latex of papaya fruit that is commonly used in protein extraction due to its specific catalytic activity. One of the main advantages of using papain in protein extraction is its specificity for cleaving peptide bonds involving the carboxyl group of cysteine, which makes it ideal for breaking down proteins with low molecular weight. Papain has a high catalytic activity, which means it can break down proteins quickly and efficiently and operates under mild conditions such as neutral pH and moderate temperature, which helps to preserve the integrity of the proteins being extracted. Papain is also a relatively inexpensive enzyme compared to other proteases, making it a cost-effective option for protein extraction(do Prado *et al.*, 2021) . The lack of fat in PSC suggests that eliminating lipids with n-hexane is efficient. Simply removing the fat from seed flour is a crucial step in protein extraction because fat would lead to an increase in organic contaminants by adding fatty acids into the water.

Table 3. Chemical composition of protein extracts from pumpkin seed (PSC)

N	Chemical composition (%)	PSC
1	Moisture	9.87 ± 0.31
2	Ash	3.51 ± 0.12
3	Protein	74.90 ± 1.71
4	Carbohydrate	11.22 ± 0.50
5	Lipid	0.50 ± 0.01

2 Fourier transform infrared spectroscopy (FT-IR) of BCM

FTIR was used to measure the amount and type of functional groups in the mucilage to find the active components or groups that help the coagulation and flocculation process (Harfouchi *et al*., 2016). The FTIR spectra of the protein of pumpkin seeds can be found displayed in Fig. 1. The sharp peak around 3418.20 cm^{-1}, which can be seen in the graph, is related to the hydroxyl groups (OH–)

of acid pyranose ring in polysaccharide chain. This demonstrates that pumpkin seeds contain protein. The CH– stretching vibration of the aromatic rings is responsible for the peaks that were observed at approximately 2926.71 cm^{-1} and 2855.68 cm^{-1}. The presence of O–H linkages and CH– linkages, both of which are related to the protein content of pumpkin seeds, provides a large number of adsorption sites, which in turn leads to the inter-particle bridging effect (Mirbahoush *et al.,* 2019). Because of the strong interaction of this natural coagulant with the colloids found in bilge water, the presence of these functional groups justifies the high coagulation ability of PSC. The bridging and adsorption mechanism is assisted by the absorptions that occur at 1649.51 cm^{-1}. These absorptions are associated with the carboxylic acid carbonyl group (C– –O) and the carboxylic -COO- double linkage of deprotonated carboxylate found in uronic acid. The presence of an amide II band is indicated by the band at a frequency of 1405 cm^{-1}, which corresponds to a pumpkin seed wavelength of 12 cm^{-1}. There is a correlation between the CO– bond-stretching vibration in C-OH bands of carbohydrates and the presence of a band at 1243.35 cm^{-1} and 1082.76 cm^{-1}. In addition, the bands in the region between 950 and 1200 cm 1 correspond to the presence of C O linkage in C – OH bands of aromatic compounds of galactose, rhamnose, and galacturonic acid and OH of polysaccharide(Fard *et al.*, 2021).

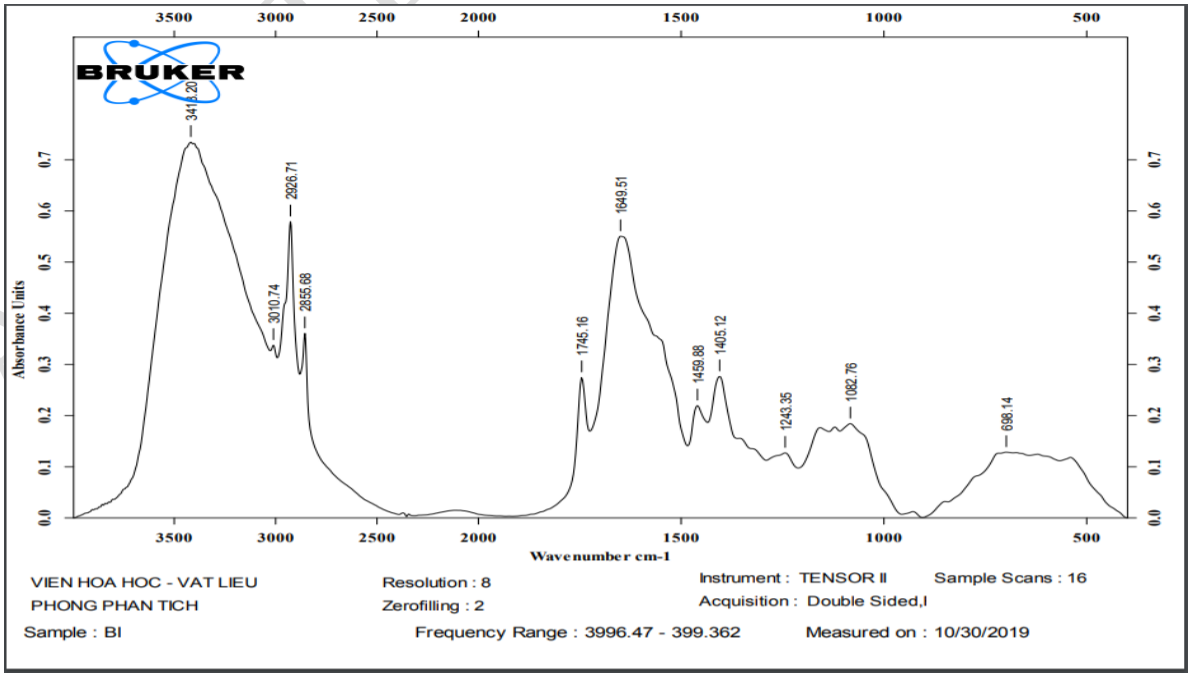


Fig 1. FTIR of protein extracted from pumpkin seeds

3.2 Optimization study of operating parameters in coagulation treatment by PSC

Table 2 shows the experimental design and findings for optimizing water turbidity as a function of three variables: (1) pH (A), (2) reaction time (B), and (3) PSC dose (C). The experimental design findings show that the best conditions for pH, time of coagulation process, and bio-coagulant concentration were 7.4, 87.963 min, and 1.5 g/L, respectively. The expected (theoretical) efficiency of turbidity (%TU) and %COD under this situation were 94.4% and 74.7%, respectively. The models' quadratic and interaction regression coefficients were also assessed. The impacts of the variables and the system's response behavior were described by the complete regression equation shown below ignoring the coefficients of non-significant terms, as illustrated in equations (4) and (5). The regression model for optimizing turbidity (%TU) and COD reduction (%COD) are a function of pH, reaction time, and bio-coagulant dosage

$$\%TU = +82.49 + 3.78\ A + 6.54\ B + 3.11\ C + 1.12\ AB - 4.46\ AC + 1.99\ BC - 37.71\ A^2 - 8.23\ B^2 + 13.91\ C^2$$

$$\%COD = +63.11 + 2.28\ A + 5.04\ B + 7.49\ C + 2.42\ AB - 3.28\ AC + 1.49\ BC - 39.31\ A^2 - 6.43\ B^2 + 18.36\ C^2$$

in which A, B, C are the pH, reaction time, and bio-coagulant dosage, respectively. The coefficients in front of A, B, and C represent the linear coefficient, while the coefficients in front of AB, AC, and BC represent the interaction between factors and that of A^2, B^2, and C^2 represent the quadratic effect, respectively.

The analysis of variance (ANOVA) in Table 3 tests for remaining turbidity optimization and demonstrates that the quadratic model has significance at 95% confidence level ($p = 0.05$). Furthermore, all parameters, pH (A), time (B), PSC dose (C), and AB, AC, BC, A^2, B^2, C^2 were all important determinants in the optimization of residual turbidity, while in terms of COD removal, only BC were negligible interactions to the response situation. The statistics of both responses %TU and

%COD provide several comparison metrics for model evaluation, and the quadratic model has a significant contribution with a smaller standard deviation and a greater coefficient of regression. In terms of a pure mistake, the lack of fit is insignificant. It is important to highlight that a minor lack of fit is preferable for the adequacy of all model terms. It demonstrates that the model well describes the observed values.

Table 4 Experimental design and results obtained from optimization

Nº	**pH (A)**	**Time (B)**	**mPSC (C)**	**%TU**	**%COD**
		min	**g/L**	**%**	**%**
1	11	30	1.5	50.07	27.7
2	7	120	2.75	81.13	62.22
3	7	75	2.75	82.61	61.7
4	3	120	4	61.55	48.54
5	11	75	2.75	48.59	25.91
6	11	30	4	43.27	33.83
7	3	30	1.5	35.72	20.81
8	7	75	2.75	82.61	62.17
9	7	75	2.75	82	62.17
10	7	30	2.75	67.36	50.76
11	11	120	1.5	61.09	38.69
12	3	30	4	47.03	41.28
13	7	75	1.5	93.32	76.38
14	3	75	2.75	40.93	21.31
15	7	75	2.75	82.61	61.7
16	7	75	4	99.44	86.19

17	7	75	2.75	82.61	63.37
18	11	120	4	62.52	51.99
19	7	75	2.75	82.61	68.33
20	3	120	1.5	42.52	23.33

208 Table 5. Variance analysis for a quadratic response surface model of turbidity and organic
209 matter reduction.

	Sum of squares	**df**	**Mean square**	**F value**	**p value**	
Turbidity reduction						
Model	7311.68	9	812.41	12230.93	< 0.0001	significant
A-pH	142.81	1	142.81	2150.00	< 0.0001	
B-Time	427.19	1	427.19	6431.45	< 0.0001	
C-mPSC	96.66	1	96.66	1455.21	< 0.0001	
AB	10.01	1	10.01	150.74	< 0.0001	
AC	159.40	1	159.40	2399.80	< 0.0001	
BC	31.80	1	31.80	478.76	< 0.0001	
A^2	3910.81	1	3910.81	58877.77	< 0.0001	
B^2	186.08	1	186.08	2801.46	< 0.0001	
C^2	532.02	1	532.02	8009.67	< 0.0001	
Lack of fit	0.6642	10	0.0664			not significant
Pure error	0.3541	5	0.0708	1.14	0.4438	
Cor total	0.3101	5	0.0620			
SD	7312.35	19				

Mean	0.2577		**R^2**	0.9999		
C.V.%	66.48		**Adjusted R^2**	0.9998		
PRESS	0.3877		**Predicted R^2**	0.9995		
			Adeq Precision	350.2042		
COD reduction						
Model	7117.16	9	790.80	152.22	< 0.0001	significant
A-pH	52.21	1	52.21	10.05	0.0100	
B-Time	253.92	1	253.92	48.88	< 0.0001	
C-mPSC	561.30	1	561.30	108.05	< 0.0001	
AB	46.90	1	46.90	9.03	0.0132	
AC	86.13	1	86.13	16.58	0.0022	
BC	17.73	1	17.73	3.41	0.0944	
A^2	4250.59	1	4250.59	818.22	< 0.0001	
B^2	113.88	1	113.88	21.92	0.0009	
C^2	927.00	1	927.00	178.44	< 0.0001	
Lack of fit	51.95	10	5.19			
Pure error	18.99	5	3.80	0.5762	0.7201	not significant
Cor total	32.96	5	6.59			
SD	7169.11	19				
Mean	2.28		**R^2**	0.9928		
C.V.%	49.42		**Adjusted R^2**	0.9862		
PRESS	4.61		**Predicted R^2**	0.9721		
			Adeq Precision	41.8524		

The three-dimensional (3D) response surface graphs represent diagrams of the regression equations that are utilized to show the relationship between each factor's responses and experimental

degrees. The 3D response surface plots are shown in Fig 2 and 3. The contour of the plots reflects the interactions of two variables such that a curved contour line represents a modest interaction of two factors, and a deformed contour line indicates a substantial interaction of two factors. The contours in the graphs are deformed, indicating a strong interaction between pH and time of coagulation process, pH and bio-coagulant (PSC) dosage, bio-coagulant and reaction time in turbidity, and COD treatment.

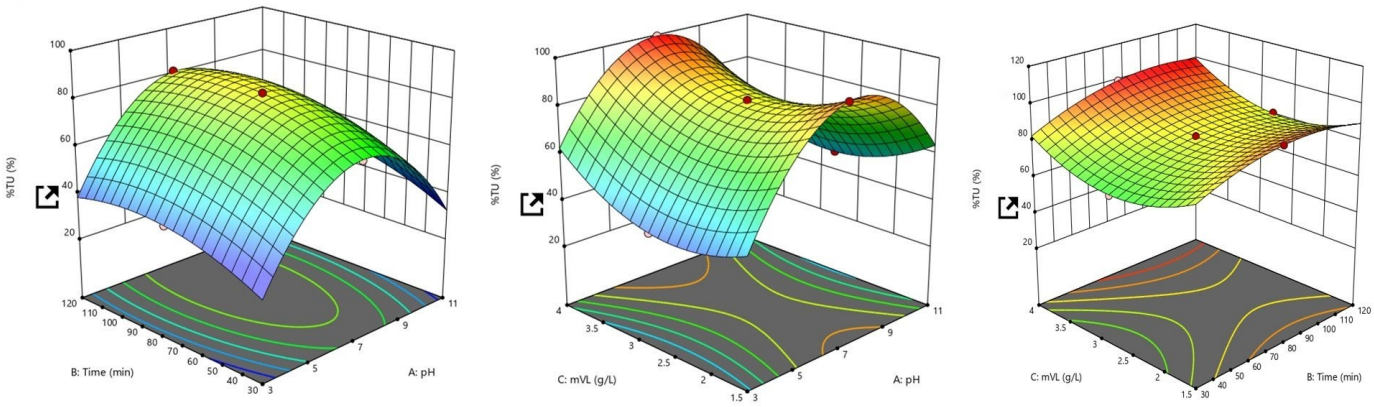


Fig 2. Response surface plots of the effects of pH versus time, pH versus PSC dosage, and time versus PSC dosage for turbidity removal.

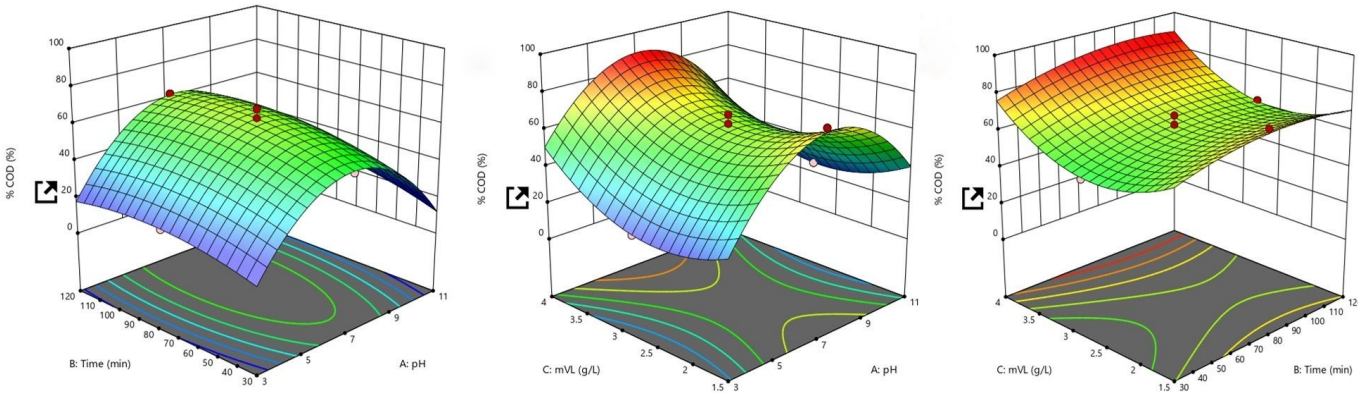


Fig 3. Response surface plots of the effects of pH versus time, pH versus PSC dosage, and time versus PSC dosage for COD removal.

Mechanism of bio-coagulation process: During the coagulation process, a PSC can be a coagulant consisting of extremely tiny particles and organic and inorganic substances dissolve in

water by increasing adsorption, polymer bridging, and charge neutralization processes (Kurniawan *et al*., 2020). The key properties of PSC in coagulation procedures are major protein chains. The high protein content of pumpkin seed extract may favor floc formation because its effectiveness is linked to charge neutralization through the relationship of two particles with oppositely charged ions and to bridge pathways via the creation of particle–polymer particle complexes during particle adsorption onto polymer chains. Other processes that may assist floc production include electrostatic patch and sweeping, adsorption, complexation, chelation, entrapment, and precipitation (Lichtfouse *et al*.., 2019).

3.3 Toxicity evaluation

The results of the toxicity test with PSC showed that the fluorescence of *V. fischeri* was not inhibited. After coagulation treatment, effluent is no longer toxic to V. *fischeri*. The natural coagulants addressed in this article (PSC) are potential biological coagulants that would be useful for environmental preservation and purification, both economically and environmentally. The primary benefits of natural coagulants generated from plants over chemical coagulants are plant sustainability and availability, which likely makes them less economically expensive (sludge handling and coagulant cost). Regardless of the fact that the identified natural coagulants' performance and advantages have been demonstrated on a lab and/or bench scale, considerable skepticism about their cost-effectiveness and consistency of performance in actual treatment procedures still exists (El Bouaidi *et al*., 2022).

3.4 Cost evaluation of PSC bio-coagulant

Bio-coagulant (PSC) pricing should be competitive with chemical coagulant prices (Kurniawan *et al*., 2020) (Table 6)

250 Table 6. A cost-breakdown of production PSC bio-coagulant per 1 kg

Activity	**Sub-section**	**Break-down**	**Cost (USD)**
Processing of raw material	Raw material cost:	Pumpkin seed	0
	Raw material drying cost	Dried under the sun	0
Extract protein from pumpkin seeds	Chemical activation (n-Hexane and enzyme Papain)	n-Hexane (L) x cost of n-Hexane: 0.5 (L) x 0.6 enzyme	0.03 0.005
	Washing cost	Distilled water received from the laboratory setup	0
	Drying cost	Power of oven (kW) x run time (h) x cost per kW/h 1.2 kW x 5 h x 0.0008	0.0048
	Labor cost	Hour wage (USD/h) x time (h) 0.1 x 1h	0.001
Net cost			0.0408
Another overhead cost		10% Net cost	0.00408
Total cost			**0.04408 USD/kg**

251 The prices of potable water and wastewater treatment with bio-coagulant (PSC) 0.0045 in USD
252 per cubic meter of tap water, respectively, are shown in Table 6. Chemical coagulants, on the other
253 hand, have water treatment costs of about USD 1.50 and USD 0.15–1.80, respectively. Although bio-
254 coagulants are more expensive than chemical coagulants, the cost can be decreased if the feedstock
255 is trash and the cost of sludge processing is included. The drawback of aluminum coagulants is that
256 they produce a less dense floc than iron coagulants. Iron coagulants, on the other hand, have the
257 drawback of increasing the weight of sludge by up to 40% when compared with aluminum coagulants.
258 To effectively remove turbidity, COD, and TSS from ceramic industrial effluent, a large dosage (800

mg/L) of ferric sulfate is necessary. As a result, one process to consider is the production of additional sludge (Lichtfouse *et al*., 2019). When ferric sulfate is combined with a bio-coagulant, the amount of sludge produced may be decreased. The amount of sludge produced was not quantified during the jar testing; hence, it is recommended that it be assessed in the future. Moreover, using bio-coagulation treatment in lake water, proper disposal of the created sludge for landfill. However, this waste might be used as a fill material in the construction or as a feedstock in the creation of sanitary gear (Silva *et al*., 2022).

4. Conclusion

The current study demonstrates that the PSC is an excellent natural coagulant (efficient, safe, and economical coagulant) for the treatment of surface water. Moreover, it demonstrates that the response surface methodology (RSM), in conjunction with a central composite design, represents one of the best ways to optimize treatment conditions for COD, turbidity removal. In terms of optimization, it can be shown that at a pH of 7.54, a concentration of 1.5 g/L, and a stirring time of 88 min, the chronic toxicity data carried out on the specific living organisms *V. fischeri* that concentrations below 4 g/L can be considered as safe.

Conflict of Interest

The authors declare no conflict of interest.

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