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ORIGINAL ARTICLE

Optimization and identification of astaxanthin esters from shrimp waste using microbial fermentation method

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Abstract

Astaxanthin is a ketocarotenoid that is the primary pigment in shrimp and has powerful biological effects. This molecule comprises a 40-carbon chain with multiple double bonds at the end that connects to ketone and hydroxyl groups. The terminal hydroxyl groups can react with fatty acids and form monoester and diester of astaxanthin. Shrimp waste is an inexpensive source of this compound, whose extraction and purification can improve the economy of fishery production and processing in addition to solving the problem of shrimp waste disposal. The efficiency of astaxanthin extraction from *Penaeus semisulcatus* and *Penaeus merguensis* wastes was compared for the first time in the present study, employing the fermentation process with two strains of *Lactobacillus acidophilus* and *Lactobacillus plantarum* bacteria. The extraction conditions and effects of various treatments on astaxanthin yield were then adjusted using a Box-Benken design with three parameters: fermentation temperature (30–40 °C), fermentation time (12–72 h), and specifically by modifying the waste particle size (40–60 mesh). Following purification of the pigment, H-NMR and FT-IR spectroscopies, as well as Rf values, were clearly utilized to identify the astaxanthin present in the waste. The findings demonstrated that *P. semisulcatus* shrimp waste had considerably greater extraction yield with both bacterial strains than *P. merguiensis* shrimp ($p<0.05$). Also, *L. plantarum* bacteria had a higher ability to create better fermentation conditions and, as a result, higher extraction of astaxanthin from both shrimp species. Optimum conditions for this bacterium include a fermentation temperature of 35°C, a time of 72 h, and a particle size of 80 mesh. The Rf value for mono (0.59) and diester (0.76) of astaxanthin obtained were in accordance with international standards. FTIR analysis determined the recognizable peaks of mono and diester of astaxanthin, including hydroxyl functional groups and the vibrational mode related to its ester at the end of the polyene chain of astaxanthin. Also, an examination of H-NMR data showed the presence of indicative protons related to astaxanthin and fatty acids attached to it in the chemical shift regions of the spectra of extracted astaxanthin esters.

Keywords *Penaeus semisulcatus* · *Penaeus merguiensis* · Astaxanthin · NMR · FTIR · Box-Behnken design

1 Introduction

Astaxanthin is a fat-soluble red pigment that plays basic physiological and photochemical roles, such as antioxidant activity in plants, animals, and microorganisms [1–5]. In addition, the presence of astaxanthin is necessary for staining, the growth process, and light protection for numerous organisms [6]. Astaxanthin is known for its unique antioxidant capacity, which is likely due in large part to its potent free radical scavenging properties that account for its diverse protective activity against inflammation and reduction of oxidative damage in the cell [7–10]. In addition, this pigment is used as a feed additive and supplement in fish and shrimp farms to improve the growth and health of aquatic animals [11]. In short, astaxanthin has many uses in various

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industries, including pharmaceuticals, cosmetics, and human and animal food industries [12].

Ketocarotenoid astaxanthin is a member of the xanthophyll carotenoid subgroup, which is characterized by the presence of an oxygen molecule in its complex structure in addition to carbon and hydrogen; this molecule has both hydroxyl and ketone functional groups in its two end rings [1, 3, 13, 14]. Also, depending on the origin of astaxanthin, one or both of the terminal hydroxyl groups may react with saturated or unsaturated fatty acids and create monoester and diester, respectively [3, 15, 16].

This carotenoid pigment is abundant in marine and aquatic animals, such as most crustaceans [17]. Economically, shrimps are one of the most important commercial crustaceans that have non-edible by-products. Various studies have shown that the wastes of crustaceans, including shrimp, possess valuable biological resources and functional compounds, such as chitin, protein, and carotenoids, such as astaxanthin, minerals, and enzymes [18–20].

Throwing away these wastes, in addition to losing a valuable resource, causes a significant ecological impact on the environment and creates a serious environmental problem [3, 21]. Recovery of valuable components from non-edible shrimp by-products improves the economy of fisheries and its processing industries. Since astaxanthin is the main carotenoid found in shrimp, using these wastes to obtain astaxanthin will be a cheaper natural alternative to synthetic carotenoids [22, 23].

In this sense, in recent decades, researchers have paid more attention to extracting and optimizing its methods as well as identifying this compound [16, 24–27]. Several extraction methods have been used to become more efficient and increase the yield of astaxanthin extraction, including solvent extraction [28–33]. Among these, fermentation is an effective and environmentally friendly process that can increase extraction yield [34]. Previously, researchers have studied the recovery of pigment and chitin from marine crustacean waste using lactic acid fermentation [25, 31, 35–38]. However, this process can be affected by various [28]. Therefore, it is necessary and important to determine the best conditions for extraction to obtain higher astaxanthin yields.

Penaeus semisulcatus and *Penaeus merguensis* shrimps are two commercial and important shrimp species of the Persian Gulf coast in southern Iran, which include almost 70% of the total shrimp catch of these coasts. According to the reports, the wastes of these two shrimp species are not effectively recovered [39, 40].

According to this issue, the recovery of astaxanthin in these wastes can improve environmental and economic conditions. Therefore, it is important to extract and optimize its conditions and understand the molecular structure of astaxanthin in these lesions to increase insight into the mechanisms of using this pigmentation. For the first time, this research compared the extraction yield of astaxanthin in the wastes of these two shrimp species using the lactic acid fermentation method. It also determined the optimal fermentation conditions for extraction with the highest yield and different updated methods, identified its astaxanthin using FT-IR and NMR methods, and calculated the Rf value.

2 Materials and methods

2.1 Materials

This study was carried out at the Department of Biomedical Sciences, Alzahra University, Tehran, Iran. Freshly caught shrimps *P. semisulcatus* and *P. merguensis* were purchased from the central seafood market of Bushehr and Bandar Abbas in the south of Iran, respectively. The samples were transferred to the laboratory under freezing conditions (−4°C). The shrimps were peeled manually, and the shrimp scraps, including head and carapace, were saved for use in the next experiments. This study used *Lactobacillus plantarum* (PTCC1058) and *Lactobacillus acidophilus* (PTCC1643) bacteria to ferment shrimp wastes. The bacteria used were obtained from the Collection Center of Industrial Microorganisms of Iran.

2.2 Experimental design

The present study was conducted to extract astaxanthin from shrimp shells and was divided into three stages (Fig. 1). The first stage consisted of comparing the performance of astaxanthin extraction from two freshly caught shrimp species of the Penaeus genus using two strains of Lactobacillus bacteria. The second stage was experimental design to optimize the extraction conditions of astaxanthin using the Box-Behnken design. The third step was to purify and identify the extracted astaxanthin using TLC, NMR, and FTIR.

2.3 Preparation of shrimp waste

Shrimp by-products (including head and carapace) were dried in an oven at 40°C for 24 h (Fig. 2A and B) and then ground into fine powder in a mixer (Depose, Moulinex, Italy), and the powders obtained in 3 sizes (mesh 40, 60, and 80) were sieved (Fig. 2C) and stored in a freezer at −20°C until use.

2.4 Bacterial culture and preparation of microbial suspension

In order to obtain the highest extraction yield and to identify the most efficient bacteria, two strains of bacteria, *L.*

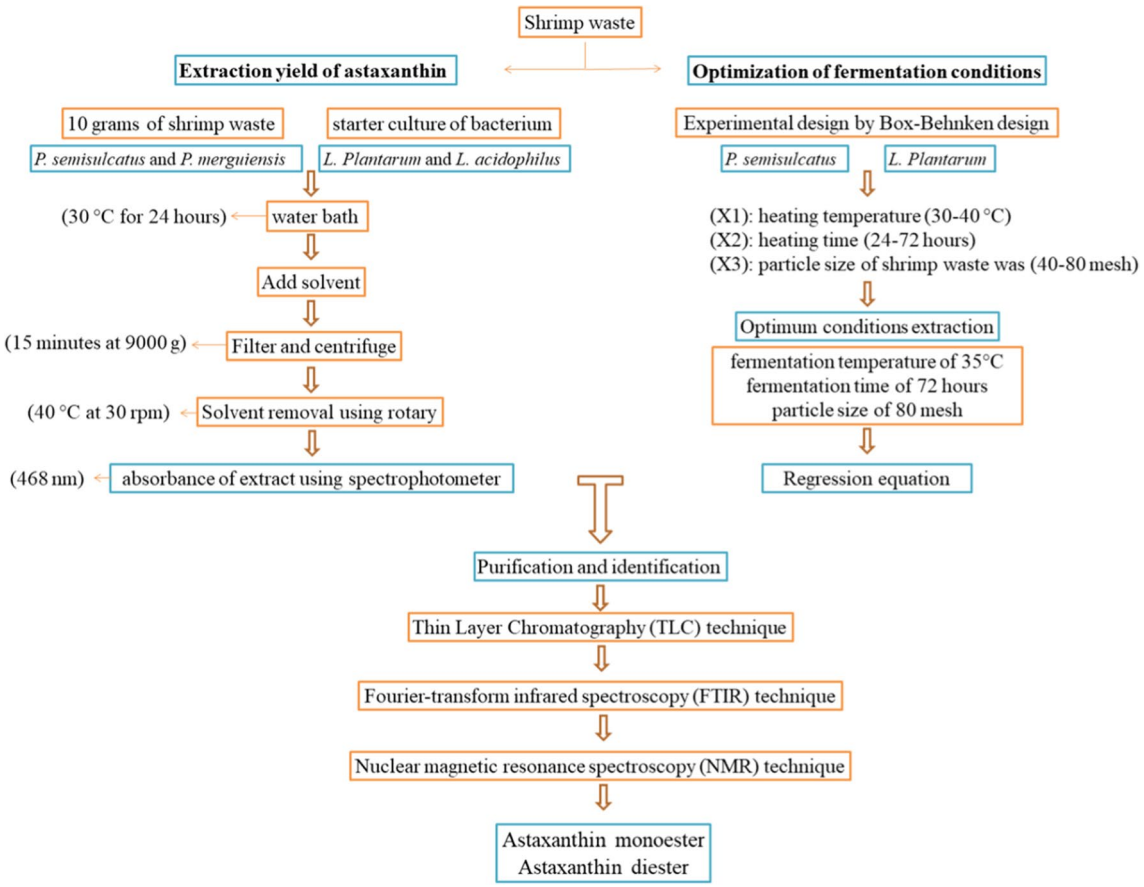


Fig. 1 Flowchart of extraction, purification, and identification protocol for the analysis of astaxanthin in shrimp waste

plantarum and *L. acidophilus* were used for the semisolid fermentations of shrimp waste [38, 41]. Purchased lyophilized bacteria were cultured in Man Rogosa Sharpe (MRS) liquid culture medium for 24 h at 30°C. Then, from the prepared inoculum, a starter culture with a cell concentration of about 1.5×10^8 bacterial colony units per ml was considered to continue the extraction process.

2.5 Fermentation process to extract astaxanthin from shrimp waste

In this assay, 10 grams of waste from each shrimp with a size of 60 mesh was placed separately in 250-ml Erlenmeyer flasks that were covered with aluminum foil and inoculated with 10% glucose (w/w), and 5% cell suspension (v/w) of each bacterium were mixed. The flasks were placed in a hot water bath at 30 °C for 24 h to carry out the fermentation (32).

2.6 Extraction yield of astaxanthin from fermented shrimp waste

In order to extract astaxanthin, 20 ml of n-hexane was added to the fermented wastes in Erlenmeyer flasks separately, and they were kept for 30 min at 20 °C in a shaker incubator until the extraction was complete. The obtained extract was washed with solvent several times using filter paper no. 41 until the color of the waste was colorless; the solvent extracts were combined, and then this solution was centrifuged for 15 min at 9000 g to separate the impurities from the solution. The solvent was removed from the pigment using a rotary at a temperature of 40 °C at 30 rpm. The resulting carotenoid extract was diluted in a balloon using hexane, and the absorbance of the diluted extract was measured at 468 nm using a spectrophotometer [37]. The yield of carotenoid extraction (as astaxanthin) was expressed as micrograms of astaxanthin per gram of residual waste, which was calculated with the following equation (Eq. 1):

$$Y = \frac{\text{A468 nm} \times \text{V extract} \times \text{Dilution factor}}{0.2 \times \text{W sample}} \quad (1)$$

where A is the absorbance at 468 nm, V is the volume of extract, 0.2 µg/ml absorbance of standard astaxanthin solution at 468 nm, and W is sample weight in grams.

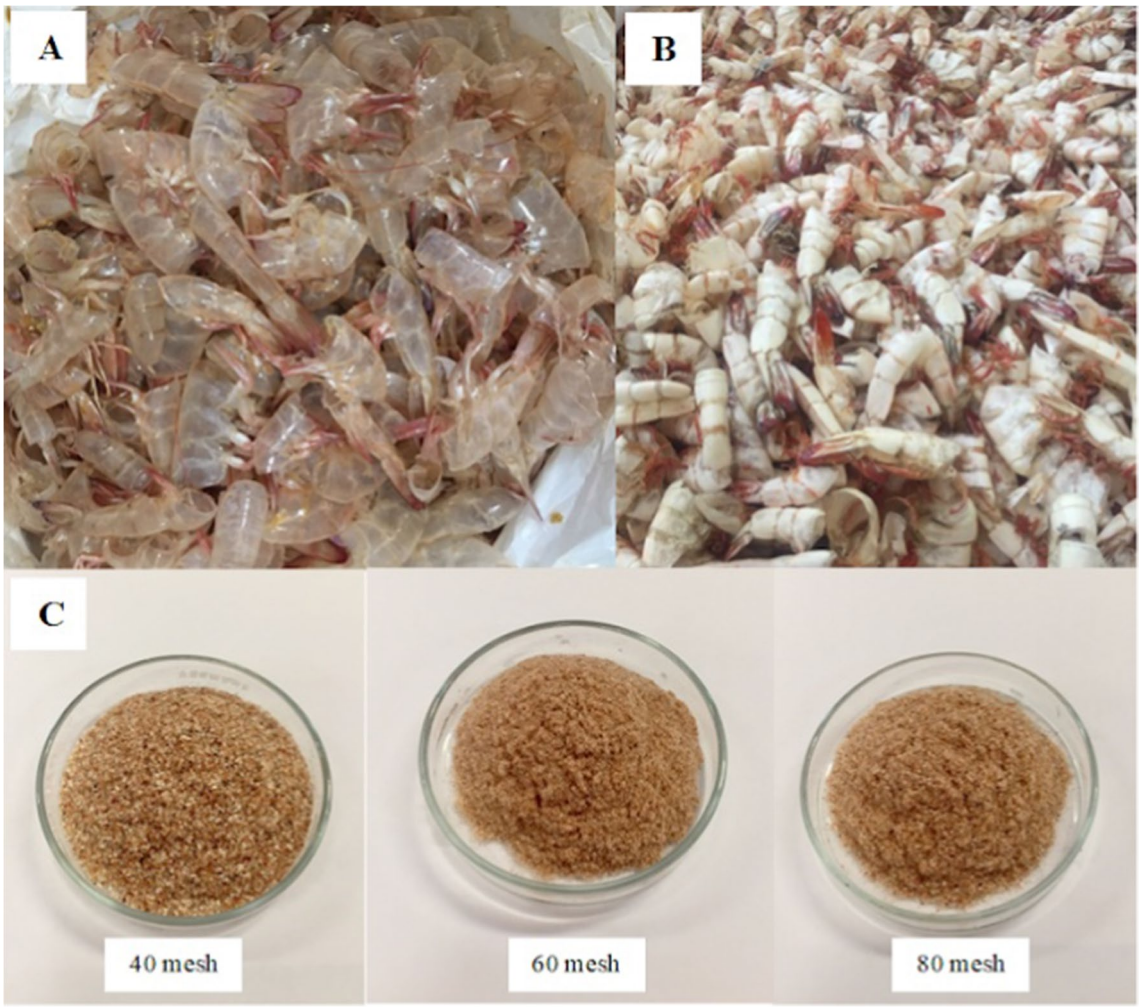


Fig. 2 Shrimp waste preparation steps. Fresh shrimp waste (**A**). Dried shrimp waste (**B**). Shrimp waste powder (**C**)

2.7 Designing an experiment to optimize fermentation conditions for extracting astaxanthin from shrimp waste by Box-Behnken-design

In this research, in order to optimize the extraction conditions of astaxanthin using the fermentation method from shrimp waste, the Box-Benken scheme was used. Optimization studies using *L. plantarum* bacteria on *P. semisulcatus* shrimp wastes were continued due to higher extraction yield in the previous stage. For this purpose, to create an optimized model of extraction conditions, three factors affecting this procedure were examined, which included 15 runs and three repetitions at the central point. The response pattern was determined based on single factor tests of three independent variables X1, X2, and X3 and coded with −1, 0, and +1, which include fermentation temperature (30–40 °C) (X1), fermentation time (24–72 h) (X2), and the particle size of shrimp waste was (40–80 mesh) (X3), while the response value (Y) was the extraction yield.

The factors, their levels, and the level codes are listed in Table 1. A regression model to optimize the dependent variables that include the linear effect and quadratic factors, as well as the linear effect of interactions to describe the relationships between the response (Y) and the experimental factors (X1, X2, and X3), is considered based on Eq. 2 as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2)$$

In each equation, Y is the extractable carotenoid yield. Xi and Xj are the coded values of the independent variables, while β0, βi, βii, and βij are the regression coefficients of the width from the origin, linear, quadratic, and interaction, respectively.

Table 1 The factors and their levels (−1, 0, and 1+), as well as the codes related to each factor, are depicted

Factors	Code	Level		
		−1	0	+1
Temperature of fermentation (℃)	X1	30	35	40
Time of fermentation (h)	X2	12	24	72
Particle sizes of shrimp waste (mesh(	X4	40	60	80

2.8 Purification of astaxanthin using thin-layer chromatography

The analysis of pigments in shrimp by-product extracts was performed using thin-layer chromatography according to a method established by Sindhu and Sherief [42]. For this purpose, a proper volume of the extract obtained in the extraction step was spotted on the silica gel plate. A mixture of hexane and acetone solvents with a ratio of 7 to 3 was used for separation and mobile phase. Then, the pigments were identified by obtaining the Rf value of the extracted pigment and comparing it with the internationally accepted Rf values. In order to carry out further tests, the main parts of the band containing pigments were carefully cut (Fig. 3A), and the pigments were quickly extracted using hexane and filtered with filter paper number 41 and centrifuged at 9000 g for 15 min with pressure of 100 mbar (Fig. 3B). Then the solvent was removed using a rotary instrument at 20°C at 30 g, and the pigments were stored in separate containers covered with foil in a freezer at −80°C. In addition to the Rf value, H-NMR and FTIR methods were used to identify their structure.

2.9 Fourier-transform infrared spectroscopy technique

In order to determine the functional groups of pure bands separated by TLC, the FTIR spectroscopy method was used. For this purpose, the purified pigment was mixed with dried potassium bromide (KBr) completely at a ratio of 1:100 to obtain a uniform and completely soft powder. Then, using a tablet press machine, a transparent KBr disk with a diameter of 15 mm and a thickness of 1 mm was made for measurement. Before scanning the samples, a blank scan of the transparent disk (KBr) was performed, and absorption spectra were recorded in the frequency range of 500–4000 cm^{-1}.

2.10 Nuclear magnetic resonance spectroscopy

In this experiment, 10 mg of pigments obtained from TLC was used for H-NMR analysis. Proton nuclear magnetic resonance spectrum of astaxanthin from shrimp wastes in CDCl3 solvent was recorded with a Bruker device (Germany) at a frequency of 500 MHz at a temperature of 25 °C. Data analysis was performed using MestReNova-6.1.1NMR software.

Fig. 3 Bands separated from thin layer chromatography plate. Silica gel bands containing purified pigments (**A**). Pigments purified and separated from TLC bands with solvent (**B**)

2.11 Statistical analysis

Statistical analysis was performed to compare the mean difference between groups using a two-way analysis of variance using statistical software (GraphPad Prism Version-8.4.3. 686.x64). All tests were performed in three repetitions. Also, the data were expressed as the mean value of astaxanthin extraction yield ± SD. Statistically, a p-value <0.05 was considered significant. Also, the optimized data were analyzed for the effect of each factor and their interaction on astaxanthin extraction performance using analysis of variance (ANOVA) by Design-Expert software (version v11.0.4.64). The optimization data were analyzed to determine the regression coefficients to achieve a regression equation. A regression model containing the obtained coefficients, including the linear and quadratic effect of the factors as well as the linear effect of interactions, was considered to describe the relationships between the response (Y) and experimental factors (X1, X2, and X3).

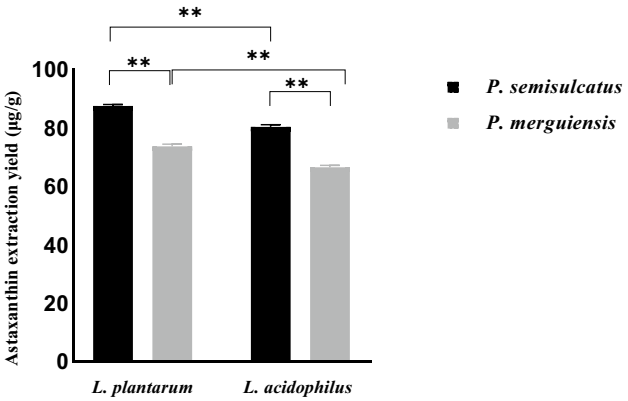


Fig. 4 The results of statistical analysis comparing the yield of astaxanthin extraction from *P. semisulcatus* and *P. merguiensis* shrimp wastes using two bacteria strains ($n = 3$). The asterisk indicates a significant difference in the extraction yield of astaxanthin from *P. semisulcatus* and *P. merguiensis* shrimp wastes by each bacterial strain ($p < 0.001$). * and ** indicate $p < 0.05$ and $p < 0.001$, respectively

3 Results and discussion

3.1 Comparison of the extraction yield of astaxanthin from fermented shrimp wastes

Lactic fermentation of shrimp waste is a simple and environmentally friendly way to obtain astaxanthin compared to other extraction methods, such as chemical methods [43]. In this study, for the first time, the efficacy of astaxanthin extraction from the wastes of two shrimp species, *P. semisulcatus* and *P. merguiensis*, was compared using the bacterial fermentation method with *L. plantarum* and *L. acidophilus* strains. The data of this research showed that *L. plantarum* bacteria have better performance than *L. acidophilus* bacteria in terms of extracting pigment from the wastes of both shrimp species. The statistical analysis of data related to astaxanthin extraction, as shown in Fig. 4, showed that the yield of astaxanthin extraction using *L. plantarum* bacteria from wastes of both species of shrimps has a significant difference (p<0.001) in comparison with *L. acidophilus*. Also, the yield of pigment extraction in *P. semisulcatus* shrimp waste compared to *P. merguiensis* shrimp waste using both strains of bacteria showed a significant difference (p<0.001).

Research has shown that shrimp shell waste has relatively different compositions according to the season, species, and other factors. The decrease in pH during the fermentation of these wastes using bacteria can be due to the ability of each strain to use the available primary resources for growth and acid production. This produced acid can increase the yield and availability of enzymes and, consequently, the extraction yield [44, 45]. Also, by producing proteolytic enzymes, bacteria hydrolyze shrimp shell proteins and cause the release of astaxanthin from the protein-chitin complex, thus increasing the extraction yield [25]. Researchers have reported that during the fermentation of shrimp by-products, microorganisms produce acid and deproteinize with the activity of proteases [46]. These acid and enzyme produced in each strain of bacteria are different from another strain. Prameela et al. reported that among several bacteria, the *L. plantarum* strain is the strongest producer of lactic acid, and for shrimp waste fermentation, it causes more recovery of chitin and carotenoids at the end of the fermentation period [38]. Also, Bellaaj Ghorbel and colleagues showed during their research that the deproteinization rate is higher in some strains that produce lower proteases, which can show the importance of the availability and yield of the enzymes of each bacterial strain in fermentation. Therefore, deproteinization and, as a result, extraction are related to the amount of protease activity produced and the yield and availability of enzymes by each strain [44]. The results of this research showed that the *L. plantarum* bacterium achieves a higher extraction yield than the *L. acidophilus* bacterium from the wastes of both shrimp species, thus confirming previous studies regarding the better fermentation of the *L. plantarum* strain. As a result, the extraction of astaxanthin using fermentation depends on the strain of bacteria used in addition to other factors. In addition, the examination of the data of this experiment showed that *P. semisulcatus* shrimp wastes have a higher extraction yield than *P. merguiensis* wastes using both bacterial strains. Gildberg and Stenberg stated during their research that the difference in carotenoid extraction yield between two species could stem from species type, environmental conditions, and

carotenoid content of food consumed [47]. Irna et al. also showed during their research among six shrimp species that the total carotenoid obtained from a part of the waste of each species is different from another species, which is caused by diet and seasonal and physicochemical factors such as food composition and salinity [48]. Therefore, since the species investigated in this study were in different habitats and with different ecological conditions, the difference in their pigment extraction yield can be caused by these factors in addition to the type of species.

3.2 Optimization of fermentation conditions to extract astaxanthin from shrimp wastes

Optimizing the extraction conditions of astagrantin from *P. semisulcatus* shrimp waste using bacteria considering different conditions such as particle size was performed for the first time. The experiments designed by the Box-Behnken design and the results of the statistical analysis related to it are shown in Tables 2, 3, and 4. Table 2 shows that the experimental astaxanthin extraction yield is between 59.2 and 95.34 µg/g according to different conditions.

Table 2 Experimental and observed values, as well as predicted values of astaxanthin extraction yield in different conditions, are shown using the Box-Behnken design

Std	Run	Factor 1 A: temperature of heating (°C)	Factor 2 B: time of heating (h)	Factor 3 C: waste size (mesh)	Response Y-observed (µg/g)	Y-predicted (µg/g)
11	1	35	24	80	78.5	79.3
3	2	30	72	60	88.6	89.6
10	3	35	72	40	90.1	89.3
1	4	30	24	60	70.3	70.1
8	5	40	48	80	81.1	81.2
2	6	40	24	60	62	61.0
15	7	35	48	60	87.1	87.4
5	8	30	48	40	74.5	74.4
12	9	35	72	80	95.34	95.0
4	10	40	72	60	86.7	86.9
6	11	40	48	40	65.4	66.0
13	12	35	48	60	87.4	87.4
9	13	35	24	40	59.2	59.6
14	14	35	48	60	87.8	87.4
7	15	30	48	80	85.2	84.6

Table 3 Analysis of variance (ANOVA) results for the Box-Behnken design. All three factors and the interaction between them had a significant effect on extraction yield. The extraction model was significant ($p < 0.05$), while the lack of fit test was not statistically significant ($p > 0.05$). The R^2 value of the extraction model was around 0.99, the standard deviation was 0.96, and the coefficient of variation (CV) was 1.2. (* and ** indicate $p < 0.05$ and $p < 0.001$, respectively)

Source	Sum of squares	df	Mean Square	*F*-value	*p*-value	
Model	1742.17	9	193.57	209.83	< 0.0001	Significant
A-temperature of heating	68.45	1	68.45	74.19	0.0003	
B-time of heating	1029.22	1	1029.22	1115.67	< 0.0001	
C-waste size	324.36	1	324.36	351.61	< 0.0001	
AB	10.24	1	10.24	11.10	0.0207	
AC	6.25	1	6.25	6.77	0.0481	
BC	49.42	1	49.42	53.57	0.0007	
A^2	201.33	1	201.33	218.24	< 0.0001	
B^2	36.62	1	36.62	39.69	0.0015	
C^2	45.21	1	45.21	49.01	0.0009	
Residual	4.61	5	0.9225			
Lack of fit	4.37	3	1.46	11.80	0.0791	Not significant
Pure error	0.2467	2	0.1233			
Cor total	1746.79	14				

Std. Dev. = 0.9605, $R^2 = 0.9974$, Adeq Precision = 45.1656

C.V. %= 1.20, predicted R^2= 0.9597, adjusted R^2= 0.9926

Table 4 The results of constant, linear, and quadratic regression coefficients of variables and linear regression coefficients of the interaction of variables are shown

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	87.43	1	0.5545	86.01	88.86	
A-temperature of heating	−2.93	1	0.3396	−3.80	−2.05	1.0000
B-time of heating	11.34	1	0.3396	10.47	12.22	1.0000
C-waste size	6.37	1	0.3396	5.49	7.24	1.0000
AB	1.60	1	0.4802	0.3655	2.83	1.0000
AC	1.25	1	0.4802	0.0155	2.48	1.0000
BC	−3.51	1	0.4802	−4.75	−2.28	1.0000
A^2	−7.38	1	0.4998	−8.67	−6.10	1.01
B^2	−3.15	1	0.4998	−4.43	−1.86	1.01
C^2	−3.50	1	0.4998	−4.78	−2.21	1.01

The results showed that a second-order polynomial regression model is able to interpret the experimental data accurately. The polynomial regression model includes the factors of fermentation temperature (X1), fermentation time (X2), and shrimp waste particle size (X3). It was found that all three factors and their interaction significantly affect the extraction yield of astaxanthin (Table 3). In addition, as shown in Table 3, the analysis results showed that the astaxanthin extraction model was significant ($p < 0.05$), whereas the lack of fit test was not statistically significant ($p > 0.05$). R^2 and Adeq Precision are 0.99 and 45.16, respectively, and the coefficient of variation is very low (1.20), and the standard deviation is about 0.96, showing the ability of this model to predict data. Also, the extraction yield of astaxanthin predicted by the model using the regression equation was close to the observed ones, as shown in Table 2. The regression equation using the constant, linear, and quadratic regression coefficients of the main variables and the linear equation using the linear regression coefficients of the interaction of the variables shown in Table 4 for the extraction yield of astaxanthin (Y) as an equation of three independent variables (X1, X2, X3) and their interaction was obtained as follows (Eq. 3):

$$\begin{aligned} Y = & 87.43 - 2.93\,X1 + 11.34\,X2 \\ & + 6.37\,X3 - 7.38\,X1^2 - 3.15\,X2^2 - 3.50\,X3^2 \\ & + 1.60\,X1X2 + 1.25\,X1X3 - 3.51X2X3 \end{aligned} \quad (3)$$

Figure 5 shows 2-D and 3-D response surface plots of the effects of two independent variables on the response variable while holding the other factor constant. As the data analysis showed, it was found that all three factors of fermentation temperature, fermentation time, and particle size of shrimp waste significantly affect the extraction yield. The response level (Y) reached its maximum when the fermentation temperature and time were approximately 35°C and 72 h, respectively, and the shrimp waste particle size was 80 mesh (Fig. 5). Also, the extraction yield fluctuated with the increase in fermentation temperature from 30 to 40°C, so the extraction yields first increased with the increase in temperature to 35°C and then decreased with the increase in temperature (Fig. 5A–F). It is also well shown that the pigment extraction yield increases with increasing fermentation time (Fig. 5A–D) and increasing particle size (Fig. 5C–F).

According to the obtained results, the optimal conditions included a fermentation temperature of 35 °C, a fermentation time of 72 h, and a waste particle size of 80 mesh. The higher yield of astaxanthin extraction during 72 h can be due to the higher growth of *L. plantarum* bacteria, increased acidity of the environment, and further hydrolysis of wastes, facilitating the extraction yields. Raw shrimp waste has alkaline pH values (7.5~8.0), and these conditions increase the growth of spoilage bacteria. Biological fermentation of shrimp waste to increase the yield of astaxanthin extraction using lactic acid bacteria creates acidic conditions and lowers the pH of the environment; at the same time as better fermentation, it also slows down the growth of spoilage microorganisms [38]. Sachindra et al., during the optimization of fermentation conditions for extracting astaxanthin from *Penaeus indicus* shrimp waste using *L. plantarum* bacteria, showed that as the fermentation time increases due to the increase in acidity, the extraction yield of astaxanthin increases [37]. Other reports showed that the *L. plantarum* bacterium gradually decreases the pH from 8.0 to 4.173 with the passage of time within 72 h, and simultaneously with the increase in the acidity of the environment, the yield of pigment extraction from shrimp waste also increases [38]. The yield of astaxanthin extraction increased first and then decreased during the optimization of fermentation temperature so that the extraction yield increased along with the temperature increase to 35°C and decreased when the temperature reached 40°C. Based on the experiments of Wardani et al., who investigated the effect of temperature between 30 and 40 °C on the growth of *L. plantarum* bacteria during fermentation, it was found that the intensity of bacterial growth and acid production by it increases up to a temperature of 37°C, and then with an

increase in temperature up to 40°C, the growth of bacteria slows down and leads to a sharp decrease in acid production [49]. Studies have confirmed the structural changes and isomerization and, as a result, the reduction of carotenoid yield when exposed to high temperatures [50, 51]. Getachew et al. (2022) also reported that increasing the temperature can facilitate extraction performance by increasing the solubility and breaking the carotenoid-protein complex; however, high temperatures can reduce extraction yield with a higher degree of hydrolysis [52]. Based on this, it can be said that bacterial growth slows down during exposure to higher temperatures, and the possibility of pigment structural degradation increases. The results of this experiment show that the hydrolysis of shrimp waste to obtain higher astaxanthin extraction yield is performed using *L. plantarum* bacteria at an optimal temperature of 35°C. In addition, the results of the present study showed that the size of the waste particles has a significant effect on the extraction performance, and a positive relationship was found between the size of the waste particles and the extraction yield. Handayani et al. showed that the length of the diffusion paths decreases with decreasing particle size, and therefore, the transport speed of astaxanthin particles increases [53]. Also, reducing the size of the particles facilitates the reaction of enzymes with the substance and changes the characteristics of the particles to a higher level of solubility [54]. Therefore, reducing the particle size of shrimp waste can increase the access of bacteria to the primary sources and cause more growth of bacteria and increase the acidity as well as increase the effect of the produced enzymes, and as a result, facilitate the fermentation process and improve the extraction of astaxanthin.

3.3 Identification of chemical structures

3.3.1 Thin-layer chromatography

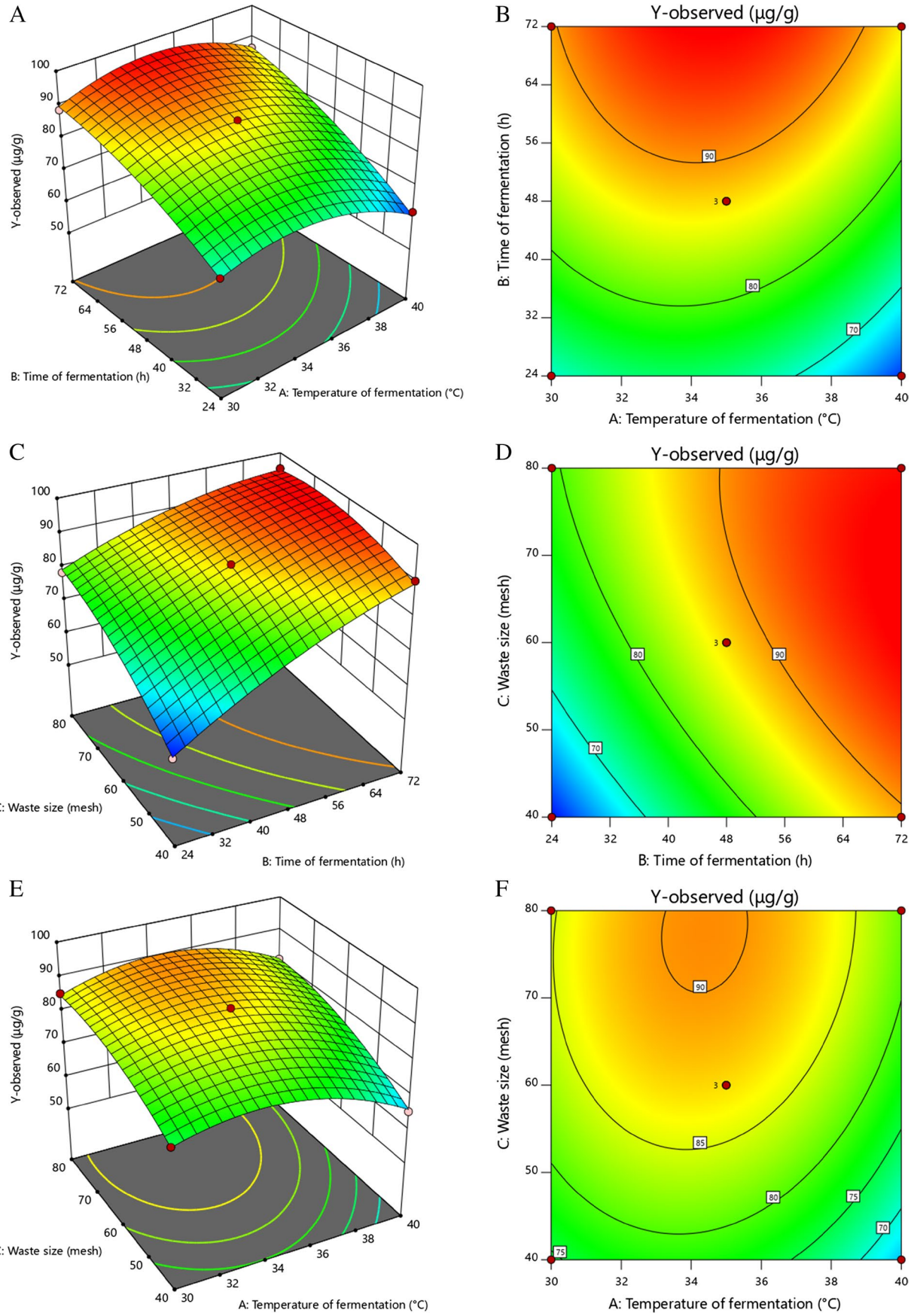
The carotenoid analysis in *P. semisulcatus* shrimp waste was performed using thin-layer chromatography, and the mobile phase of which was acetone and hexane solvents at a ratio of 7:3 (v/v). The Rf value obtained for the isolated bands was compared with the internationally accepted Rf values for carotenoid pigments, and the presence of astaxanthin monoester and astaxanthin diester was confirmed for each band.

The relative value of Rf for isolated astaxanthin monoester and astaxanthin diester was recorded as 0.59 and 0.76, respectively (Table 5). The Rf values obtained for astaxanthin monoester and astaxanthin diester are consistent with the results reported by Kobayashi and Sakamoto, which included Rf values of 0.6 for astaxanthin monoester and 0.75–0.85 for astaxanthin diester extracted from microalgae [55]. Dalei and Sahoo obtained the Rf value for astaxanthin, and its esters extracted from shrimp waste with different solvents. According to their study, the Rf values of astaxanthin and monoester and diester of astaxanthin extracted were reported as 0.36, 0.60, and 0.75, respectively [56]. The Rf value of astaxanthin obtained in the present study is in accordance with the Rf value obtained by Dalei and Sahoo and also in agreement with the Rf value standards for astaxanthin esters reported by Lorenz [57]. There are several reports that astaxanthin and its esters are the main pigments in marine crustaceans [21, 22]. Sachindra et al. showed during their research on two important species of Indian sea deep water shrimp that the main pigments of these two species are astaxanthin and its mono and diesters [21]. Sindhu and Sherief extracted pigments from Aristeus alcocki shrimp waste and showed that astaxanthin, monoester, and diester of astaxanthin with Rf values of 0.33, 0.60, and 0.78, respectively are the dominant pigments of this species [22].

3.3.2 Fourier-transform infrared spectroscopy

Structural analysis of this molecule demonstrated that the ester and hydroxyl groups located on the hexa-atomic rings at both ends of the molecule are functional groups associated with astaxanthin esters. These two groups allow the astaxanthin molecule to perform its functional abilities, for example, antioxidant activity and solubility in polar and non-polar solvents [58]. The analysis of the peaks obtained from the FTIR analysis of the pigments extracted from shrimp waste confirms the presence of functional groups related to astaxanthin esters, according to other research. Figure 6 shows multiple absorption peaks in a range of 500 to 4000 cm^{-1} based on molecular vibrations. Peaks in the region of 3449 cm^{-1} in the absorption spectrum of Fig. 6A correspond to the presence of the hydroxyl group. In both spectra, there are peaks in a range of 2850 and 2970 cm^{-1} for symmetric and asymmetric stretching vibrations of methyl groups (CH_2), respectively. Also, the absorption peaks in the ranges of 1733 and 1734 cm^{-1} are assigned to the existence of an ester functional group (R'COOR) in the structure of both compounds, showing the characteristic spectrum of the structure of astaxanthin esters [59]. Peaks in the regions of 1465 and 1461 cm^{-1} represent C-CH_3 stretching bonds in both spectra of Figs. 6 A and B, respectively [60].

The peaks in the range between 1370 and 1380 cm^{-1} in both spectra demonstrate the symmetric deformation of the CH functional group. Also, a peak shown in the region of 1291 cm^{-1} is assigned to C–OH stretching vibrations in spectrum A. A peak in the region of 969 cm^{-1} corresponds to the existence of C-H stretching vibration in the C=C conjugate system in the absorption spectrum of B [61]. Also, the presence of peaks in the region of 720 to 860 cm^{-1} of the bending bonds in the aromatic rings at both ends of the aliphatic chain confirms the structure of astaxanthin [62].


A
Y-observed (µg/g)
B: Time of fermentation (h)
A: Temperature of fermentation (°C)
B
Y-observed (µg/g)
B: Time of fermentation (h)
A: Temperature of fermentation (°C)
C
Y-observed (µg/g)
C: Waste size (mesh)
B: Time of fermentation (h)
D
Y-observed (µg/g)
C: Waste size (mesh)
B: Time of fermentation (h)
E
Y-observed (µg/g)
C: Waste size (mesh)
A: Temperature of fermentation (°C)
F
Y-observed (µg/g)
C: Waste size (mesh)
A: Temperature of fermentation (°C)

◂**Fig. 5** Two-dimensional and three-dimensional response surface plots of the effects of two independent variables on the response variable when the other variable is constant; **A** and **B** two-dimensional and three-dimensional graphs of simultaneous effect of fermentation time and temperature on extraction yield; **B** and **C** two-dimensional and three-dimensional graphs of the simultaneous effect of fermentation time and waste particle size on extraction yield; **E** and **F** two-dimensional and three-dimensional graphs of the simultaneous effect of fermentation temperature and waste particle size on extraction yield

Functional groups such as hydroxyl (OH) and ester (R'COOR) are the main groups for the identification of astaxanthin esters. The analysis of the peaks from both bands obtained from thin-layer chromatography confirms the functional groups related to the presence of monoester and diester of astaxanthin in accordance with other reports. These results were in line with the results of Rf obtained from thin layer chromatography for each compound in such a way that in the compound of astaxanthin monoester, there is a fatty acid on one side of the structure and a hydroxyl group on the other side. The results of infrared spectroscopy also show the presence of a hydroxyl group in the region of 3449 cm^{-1} in addition to the presence of the peak of the ester state, which is consistent with the results obtained from thin-layer chromatography, which has an Rf value of 0.59 for astaxanthin monoester. In the astaxanthin diester structure, there is a hydroxyl functional group instead of hydrogen on both sides of the structure. The absence of the hydroxyl group peak in the spectrum related to the FTIR results for this band indicates the astaxanthin diester, which is consistent with the results obtained from the Rf value (0.76) obtained from TLC for this compound. Also, a sharper peak can be seen in the astaxanthin diester molecule in the range of 1773 cm^{-1} compared to the astaxanthin monoester, which can indicate the presence of more esters in this compound. Subramanian et al. identified the monoester and diester of astaxanthin in shrimp oil. They stated that in the case of the monoester, the hydroxyl peak, which indicates the difference from its diester state, can be seen in the spectral regions of 3300–3500 cm^{-1}; also, the intensity of esterification in the region of 1660–1900 cm^{-1} is higher in the case of astaxanthin diester than in the monoester condition [16].

3.3.3 Nuclear magnetic resonance spectroscopy

In order to identify the type of astaxanthin, the ratio of H-NMR resonances of astaxanthin extracted from shrimp wastes was determined. Since other studies using H-NMR with similar conditions have been conducted on astaxanthin, and its esters and the peaks related to the protons of these compounds have been shown with high clarity, the presence of astaxanthin and its esters can be checked based on this [63]. Figure 7 is related to the structure of astaxanthin, mono, and diester of astaxanthin. Figure 8 shows the H-NMR spectrum with deuterated chloroform solvent and in environmental conditions at a temperature of 25 °C of the sample of astaxanthin esters obtained from shrimp waste. Spectrum A corresponds to the monoester state of astaxanthin, while spectrum B corresponds to the diester state of astaxanthin. As depicted in Fig. 8, aromatic and aliphatic protons overlap slightly, and some minor impurities can be observed in the spectrum. The peak observed in the chemical shift region of 7.26 ppm in both spectra is related to the deuterated chloroform solvent.

In both spectra, the presence of peaks appearing in the region between 0.86 and 1.1 ppm and the region of 2.80 ppm is related to CH3 and CH2 hydrogens in fatty acids attached to the astaxanthin molecule, respectively. The sharp peaks appearing in the chemical shift region between 1.24 and 1.30 ppm indicate the presence of protons 16/16′ and 17/17′ on the carbons of the CH3 group attached to the aromatic ring at both ends of the aliphatic chain [63]. The area between 1.95 and 2.8 ppm corresponds to hydrogens 18/18′, 19/19′, and 20/20′, which overlap each other. The peak appearing in the range of 5.35 ppm is related to proton on asymmetric carbons 3 in the structure of astaxanthin esters. The peak shown in the spectrum of monoester astaxanthin in the range of 3.6 ppm shows the presence of OH in its monoester state, which is the main difference from its diester state [63, 64].

Examining the results of H-NMR spectra, the presence of hydroxyl functional group in the chemical shift range of 3.5 ppm for monoester state and peaks in the range of 0.8 to 1.1 ppm are related to CH_3 and CH_2 group hydrogens in fatty acids, which can be seen in both mono and di-stereo modes [64]. These results were in line with the results of FTIR and Rf value obtained from TLC, which confirms the existence of mono and diester of astaxanthin in *P. semisulcatus* shrimp wastes. Other researchers also identified astaxanthin and its esters in crustacean wastes and showed that these molecules are the dominant carotenoids in crustaceans [16, 65, 66]. Montoya et al. investigated the shrimp *L. vannamei* and showed that in this species, the astaxanthin molecule is normally found in the form of esterified fatty acids [67]. Breithaupt and colleagues showed the presence of free astaxanthin and its esters by examining the extract of commercial shrimp *Pandulus*. *Borealis* stated that the main components of astaxanthin in this shrimp are in the form of mono and diester, and its free state appears as a minor compound

Table 5 The value of Rf obtained from different pigments in *P. semisulcatus* shrimp waste extract using the mobile phase of hexane/acetone at a ratio of 7 to 3 (average of three repetitions)

Carotenoids	Rf value
Astaxanthin monoesters	0.59
Astaxanthin diesters	0.76

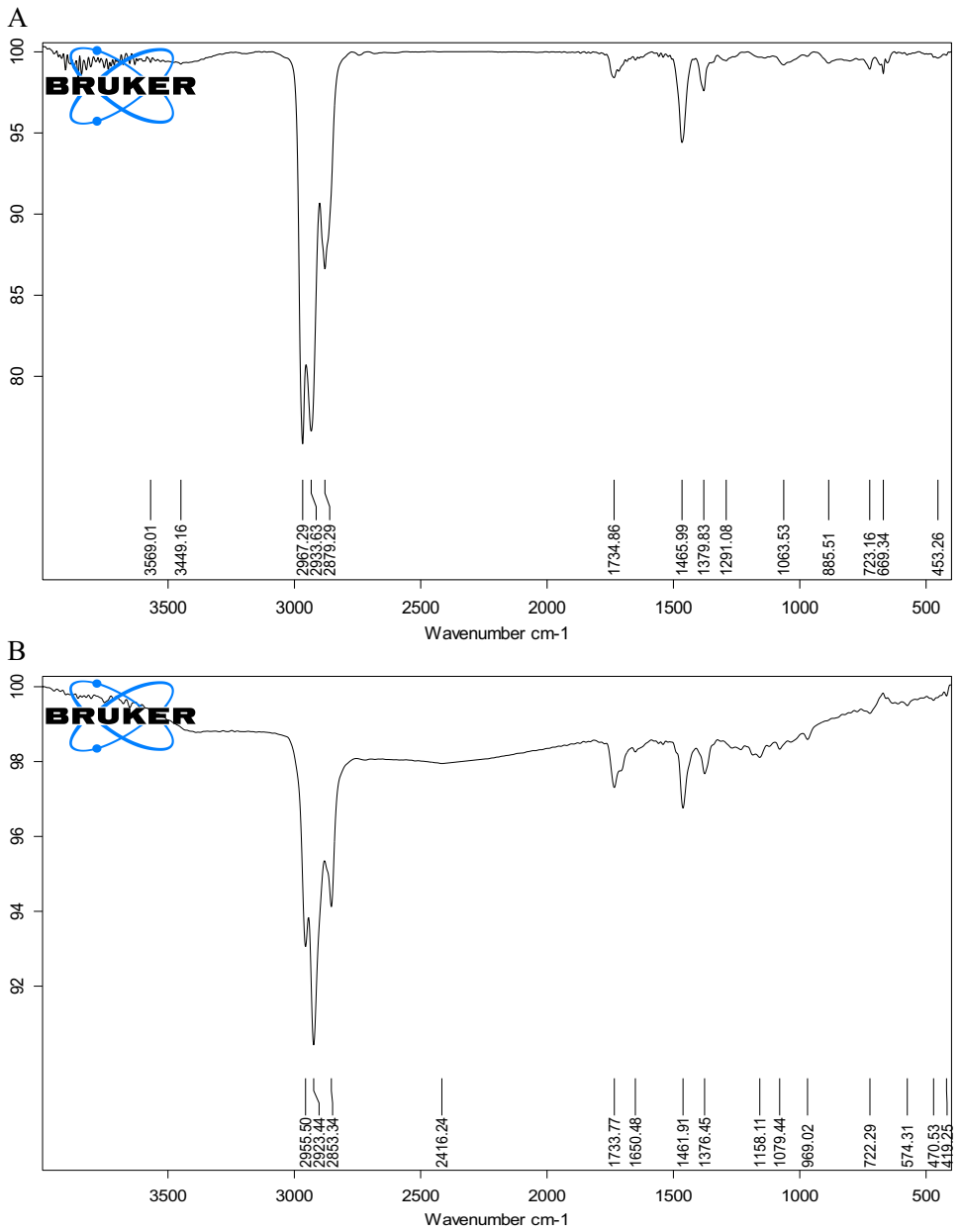
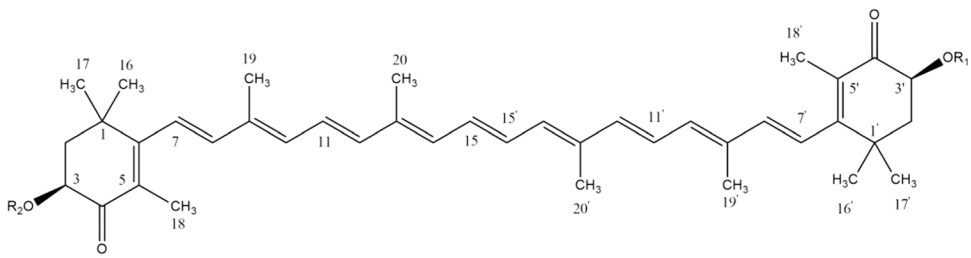


Fig. 6 FTIR absorption spectrum of pigments obtained from fermented *P. semisulcatus* shrimp waste; ***A*** absorption spectrum of monoester astaxanthin; ***B*** absorption spectrum of diester astaxanthin



Astaxanthin free: R1 and R2 = H
Astaxanthin fatty acid monoester: R1 or R2 = fatty acid
Astaxanthin fatty acid diester: R1 and R2 = fatty acid

Fig. 7 Chemical structure of astaxanthin monoester and diester forms of astaxanthin

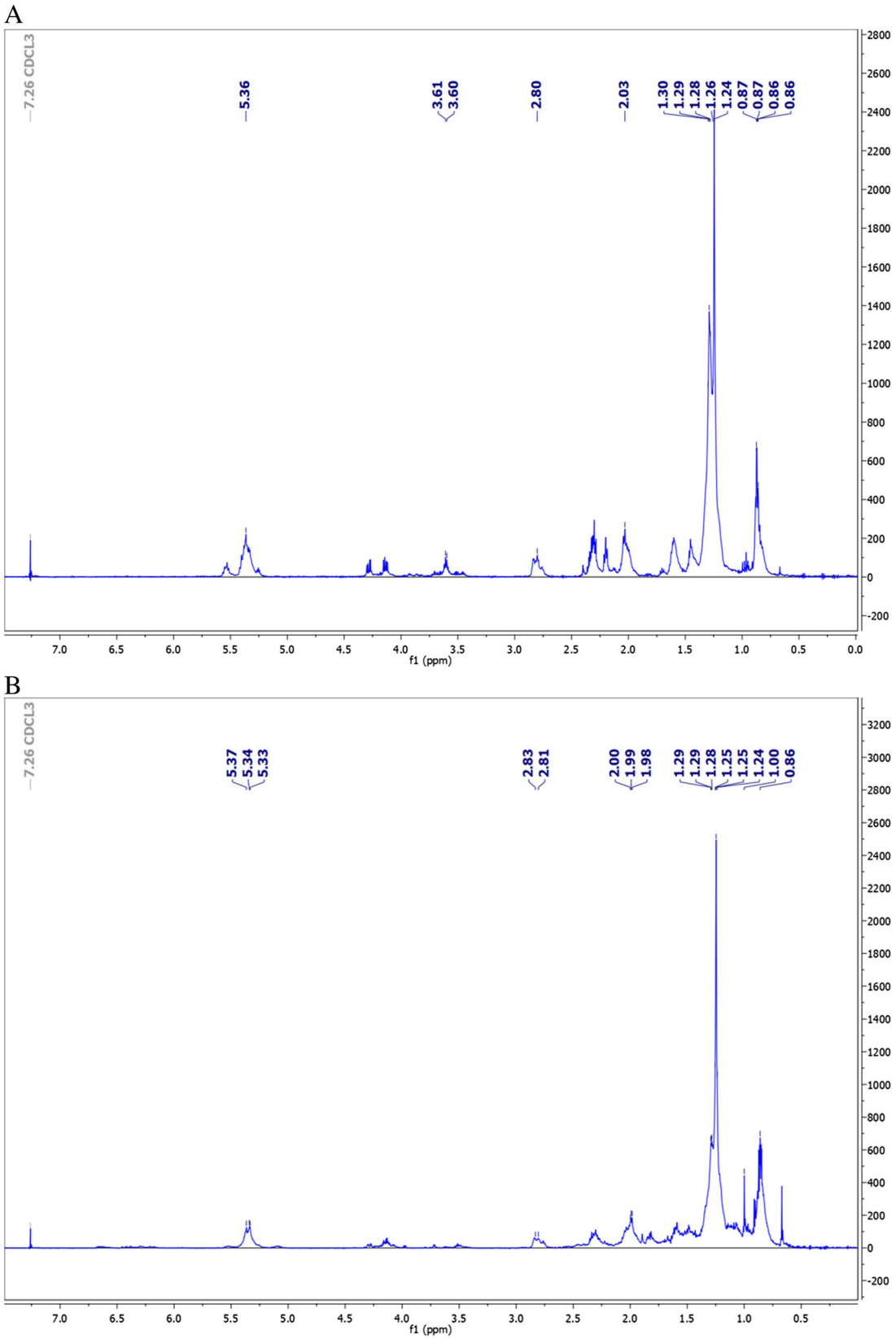


Fig. 8 H-NMR spectrum of pigments extracted from *P. semisulcatus* shrimp waste; ***A*** spectrum of the monoester of astaxanthin; ***B*** spectrum of diester astaxanthin

[68]. Coral-Hinostroza and Bjerkeng isolated three types of diesters and one type of monoester of astaxanthin from *Pleuroncodes planipes* using TLC and stated that the diesters and monoester of astaxanthin were approximately 70% and 12%, respectively. Free astaxanthin constitutes approximately 10% of the total carotenoids of this species [69]. Also, research has shown that astaxanthin in shrimp oil is 100% esterified, and specifically, about 70% is in the form of astaxanthin diester [16].

4 Conclusions

Since fermentation is an environmentally friendly method to improve pigment extraction, in this study, *L. plantarum* and *L. acidophilus* bacteria were used as pretreatment of *P. semisulcatus* and *P. merguiensis* shrimp biological wastes to obtain astaxanthin extraction yield. The *L. plantarum* strain in the fermentation of *P. semisulcatus* shrimp biological waste led to a higher recovery of astaxanthin with a significant difference ($p < 0.05$). Then the fermentation conditions were designed using BBD statistical test with three variables. Optimum levels and experimental conditions to achieve higher extraction yield (95.34 µg/g), including fermentation temperature of 35°C and fermentation time of 72 h, and particle size of 80 mesh, were obtained. As a result, the use of fermentation pretreatment for shrimp waste using *L. plantarum* bacteria can be a beneficial method to increase the yield of astaxanthin extraction. After purification and performing H-NMR and FT-IR spectroscopy, and calculating the Rf value obtained from TLC, the extracted astaxanthin was identified. Examining these results proved the presence of monoester and diester of astaxanthin in shrimp lesions. In addition, spectroscopic studies showed that monoester and diester of astaxanthin are the main carotenoid present in *P. semisulcatus* shrimp wastes.

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Author contribution Ahmad Homaei and Ali-Reza Ahmadi conceived and designed the research. Robabeh Jafari conducted the experiments. Robabeh Jafari, Ahmad Homaei, Ali-Reza Ahmadi, and Ehsan Kamrani analyzed the data. Robabeh Jafari, Ahmad Homaei, Ali-Reza Ahmadi, and Ehsan Kamrani are also major contributors in writing the manuscript. All authors read and approved the final manuscript.

Data availability All data were included in the manuscript.

Declarations

Ethical approval The study was in accordance with the ethics standards of the "Helsinki Protocol of Laboratory Animal Care."

Competing interests The authors declare no competing interests.

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