



Bioprocess of astaxanthin extraction from shrimp waste via the common microorganisms *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* in comparison to the chemical method

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Received: 22 March 2022 / Revised: 20 June 2022 / Accepted: 21 June 2022 / Published online: 1 July 2022

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Abstract

This study compared microbiological and chemical methods used in astaxanthin extraction from the exoskeleton of the shrimp species *Penaeus japonicus* and *Penaeus semisulcatus*. The microbiological method was performed using *Saccharomyces cerevisiae* (bakery yeast) or *Lactobacillus acidophilus* (from yogurt), followed by solvent extraction with hexane and acetone at different ratios (1:1, 1:2, and 1:3). The chemical method was performed traditionally using hexane. The highest astaxanthin yield from *P. japonicus* exoskeleton was obtained using either *S. cerevisiae* or *L. acidophilus* followed by solvent extraction with hexane and acetone at a ratio of 1:1 (8.5 and 8.1 mg/g waste, respectively) as well as by the chemical method (8.4 mg/g waste). Likewise, the highest astaxanthin yield from *P. semisulcatus* exoskeleton was obtained using either *S. cerevisiae* or *L. acidophilus* followed by solvent extraction with hexane and acetone at a ratio of 1:1 (3.0 and 4.1 mg/g waste, respectively) as well as by the chemical method (3.2 mg/g waste). The values obtained from *P. semisulcatus* exoskeleton were considerably lower than those attained from *P. japonicus* exoskeleton. In addition, the nuclear magnetic resonance (C-NMR) analysis confirmed that astaxanthin was the main carotenoid present in the extract. In **conclusion**, the pretreatment of exoskeleton wastes of *P. japonicus* using *S. cerevisiae* followed by solvent extraction with hexane and acetone at a ratio of 1:1 as well as the classical chemical treatment led to the highest astaxanthin content.

Keywords Astaxanthin · Shrimp waste · Carotenoid, *Saccharomyces cerevisiae* · *Lactobacillus acidophilus*

1 Introduction

Crustaceans are one of the oldest and the most diverse group of arthropods. They are also considered one of the most successful groups of invertebrates on Earth [1]. Crustaceans are regarded as an enriched source of many bioactive substances, such as carbohydrates, proteins, amino acids, fatty acids, vitamins, and minerals, especially the *Decapoda* order within the *Malacostraca* class. This order includes many familiar groups such as shrimps, prawns, crabs, lobsters, and crayfish, which have well-known nutritional function and importance. They are also delicious and easily digestible. In addition, their nutritional function depends on the biochemical composition of their bodies, which consists of high protein, low fat, and carbohydrate contents similar to those in fish flesh. Moreover, crustaceans are considered a source of omega-3 fatty acids, and vitamins, including A, B, especially B3 (niacin) and B12 (cobalamin). Hence, they are among the most valuable components of the human diet [2].

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One of the major problems in modern food production is the generation of large quantities of underused by-products. These food wastes may contain substances of high value and important health benefits. Thus, the seafood processing industry, for example, produces a tremendous quantity of by-products and wastes, such as heads, tails, skins, scales, viscera, backbones, and shells that may be an amazing source of proteins, lipids, and pigments [3]. In addition, shells may be a source of chitinous materials and carotenoids. Therefore, the waste generated during food processing should be utilized as it is considered a wasted fortune [4]. This would also alleviate the problems generated by their disposal [5].

Carotenoids such as astaxanthin, beta-carotene, lutein, and others are extracted from crustacean exoskeletons. They are responsible for the pigmentation of most aquatic organisms. Some carotenoids are precursors of vitamin A. They can also act as antioxidants in the biological systems [6], and exhibit protective action against cancer [7]. In the carapace of crustaceans, carotenoids exist as free and esterified

forms. Many crustaceans can produce astaxanthin from beta-carotene ingested from dietary algae via echinenone, 3-hydroxyechinenone, canthaxanthin, and adonirubin, as shown in Fig. 1 [8]. Thus, astaxanthin has been reported to be 10 times larger than that of any other carotenoids such as zeaxanthin, lutein, and canthaxanthin [9].

Currently, astaxanthin is a renowned compound for its commercial application in various industries, comprising aquaculture, food, cosmetics, nutraceutical, and pharmaceutical [10]. Moreover, astaxanthin effectively suppresses cell damage caused by free radicals, and induction of matrix metalloproteinases (MMPs) in skin after UV irradiation [11]. It repairs DNA damage caused by skin exposure to UV radiation, which can lead to oncogenic mutations. Studies showed that astaxanthin inhibited the UV-induced DNA damage and increased the expression of oxidative stress-responsive enzymes [12]. Astaxanthin is also reported to be an inhibitor of matrix metalloproteinases (MMPs) in different cells, including macrophages and chondrocytes [13].

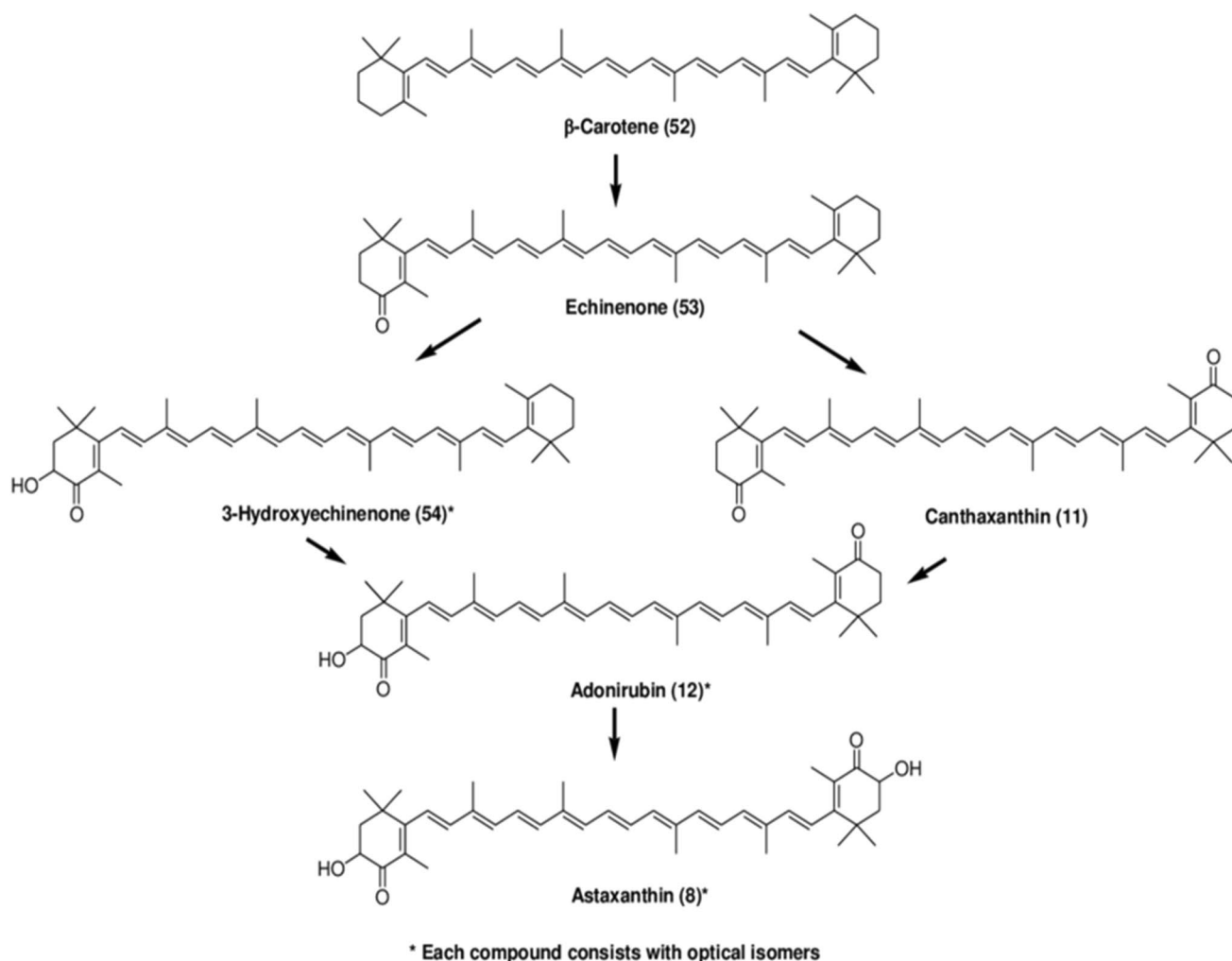


Fig. 1 Metabolism of β -carotene in crustaceans

It has anti-inflammatory properties as it inhibits the gene expression of several proinflammatory biomarkers, such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) in Alzheimer disease (AD), animal model [14]. Furthermore, astaxanthin has anti-aging effects such as hyper-pigmentation suppression, melanin synthesis, photoaging inhibition, and wrinkle formation reduction [15]. Astaxanthin has antioxidant activity since it inhibits reactive oxygen species (ROS) formation and modulates the expression of oxidative stress-responsive enzymes such as heme oxygenase-1 (HO-1) [12, 16]. Astaxanthin has immune-enhancing effects as it increases natural killer (NK) cell cytotoxic activity [17], suggesting that it may regulate NK cells that serve as an immunosurveillance system against tumors and virus-infected cells [18]. In addition, astaxanthin supplementation was proven to be useful to heart failure patients with left ventricular systolic dysfunction (LVSD). It was found that after 3 months of astaxanthin supplementation in patients with LVSD, the levels of the oxidative stress markers decreased, and both cardiac contractility and exercise tolerance improved [19]. The recommended dose of astaxanthin for adult patients is 2–4 mg/day. In addition, astaxanthin is safe and has no side effects when consumed with food [20].

Khanafari et al. [20] used the *Lactobacillus* species *Lactobacillus plantarum* and *Lactobacillus acidophilus* for the extraction of astaxanthin from wastes of the shrimp species *Penaeus semisulcatus*. Also, Hamdi et al. [21] used *Lactobacillus* and *Saccharomyces* for the extraction of astaxanthin from byproducts of the crayfish *Procambarus clarkia*. Consequently, in the present research, *Lactobacillus* and *Saccharomyces* species were assessed as pretreatment for the extraction of astaxanthin from shrimp wastes as an eco-friendly alternative to the classical chemical method. The shrimp species selected, namely, *Penaeus japonicus* and *Penaeus semisulcatus*, are two commercially used shrimps in Egypt.

2 Materials and methods

2.1 Sample preparation

Fresh shrimps of two different species, *Penaeus japonicus* and *Penaeus semisulcatus*, were purchased from a local fish market. The shrimps were peeled, and all the internal organs were removed in order to obtain the exoskeleton (waste). Shrimp waste was then dried in an oven for 8–10 h at 55–60 °C [22]. The dried waste was ground to obtain a fine powder (1–3 mm particle size). The process was performed separately for each species. The powder samples were collected in sterilized containers, labeled, and stored in a fridge at 4 °C.

2.2 Microorganisms and culture media

Lactobacillus acidophilus was obtained from a commercial yogurt and identified biochemically according to Pyar and Peh [23]. *Saccharomyces cerevisiae* was obtained from a commercial bakery yeast in Egypt and identified biochemically and microbiologically. Czapek-Dox medium consisted of sucrose 20.0 g/L, sodium nitrate 2.0 g/L, dipotassium phosphate 1.0 g/L, magnesium sulfate 0.50 g/L, potassium chloride 0.50 g/L, ferrous sulfate 0.01 g/L, and agar 15.0 g/L. De Man, Rogosa, and Sharpe (MRS) broth medium was composed of peptone from casein 10.0 g/L, yeast extract 4.0 g/L, meat extract 8.0 g/L, D (+) glucose 20.0 g/L, Tween 80 1.0 g/L, di-ammonium hydrogen citrate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, manganese sulfate 0.04 g/L, and agar 15 g/L. The media were sterilized by autoclaving at 121 °C and 1.5 bars for 15 min. Streptomycin (30 mg/mL) that was previously sterilized by filtration (0.2 μ m) was added after cooling down in order to prevent bacterial contamination. All the used flasks were cleaned by soaking them overnight in diluted H₂SO₄, followed by ethanol, and distilled water washing. Flasks (250 mL) containing 100 mL of Czapek-Dox medium were inoculated with 5 g of instant dry yeast and incubated for 5 days at 35 °C [24]. Flasks (250 mL) containing 100 mL of MRS medium were inoculated with 1 mL of *L. acidophilus* and incubated for 3 days at 30 °C [22].

2.3 Shrimp waste bioprocess

5 mL of previously cultured MRS broth containing *L. acidophilus*, and 5 mL of Czapek-Dox medium containing *S. cerevisiae* were added to a fermentative medium (100 mL distilled water + 10 g of the ground exoskeletons) and incubated for 5 days at 30 °C in the presence of 5% CO₂ [25]. The fermentative medium was filtered with Whatman filter paper no. 41 and centrifuged at 3075 g for 5 min. 25 mL of the filtered fermentation medium was added to 25 mL of a mixture of hexane and acetone at different ratios (1:1, 1:2, and 1:3) to determine the one leading to the highest astaxanthin yield. The entire extraction process was performed separately for each species of shrimp. The obtained extracts were used for astaxanthin determination.

2.4 Chemical extraction

Astaxanthin was extracted by mixing 10 g of the ground exoskeletons of each shrimp species with 50 mL of hexane in a 100-mL flask, vortexed for 30 s, and placed in a 50 °C water bath for 10 min. Aqueous and organic layers were separated by centrifugation at 1008 g for 5 min. In the final step, 6 mL of dimethyl sulfoxide (DMSO) were added to the tube, vortexed vigorously, placed in a water bath for 10 min,

and then vortexed again. The obtained extracts from each shrimp species were used for astaxanthin determination.

2.5 Determination of astaxanthin concentration

The astaxanthin contained in the extracts from each shrimp species obtained via all microbial and chemical methods was determined by UV–VIS spectrometry (Jenway 6300 spectrophotometer) at a wavelength of 476 nm [26]. In the blank, the extract was replaced by distilled water [27].

2.6 HPLC analysis

The astaxanthin content of the extracts was determined by high-performance liquid chromatography (HPLC) (Agilent 1260 series) equipped with a diode array detector (DAD) and an Eclipse C18 column (250 mm × 4.6 mm, 5 μm, Eclipse). The mobile phase consisted in water (A) and 0.05% of trifluoroacetic acid in acetonitrile (B), and the samples were eluted at a flow rate of 1 mL/min for 8 min with an isocratic gradient. The injection volume was 5 μL for both samples and standard. The column temperature was maintained at 40 °C. The UV detection of the elute was performed at 480 nm. Astaxanthin was qualitatively analyzed by comparing the retention time of the standard, and its quantification was done by using a calibration curve. This work was conducted at the National Research Centre, Cairo, Egypt.

2.7 C-NMR studies

The extracts from shrimp exoskeletons via the microbial and chemical methods were subjected to C-NMR analysis. The results were compared to the standard astaxanthin C-NMR analysis [28]. Dimethyl sulfoxide (DMSO) was used as a solvent. All NMR spectra were recorded on a Bruker Advance III 400 MHz. Chemical shifts were reported relative to TMS and referenced via residual carbon resonances of the appropriate deuterated solvent.

2.8 Statistical analysis

The matrix of the factorial experimental design is presented in Supplementary Table 2. The statistical analysis and the ANOVA of the experimental design were performed using the SPSS statistics software version 25 (Supplementary Tables 3 and 4). All determinations were carried out in triplicate. All data were expressed as mean ± SD, at ($p < 0.001$). The standard deviation of the samples was calculated using IBM SPSS statistics software version 25.

3 Results

3.1 Astaxanthin concentration in the extracts

3.1.1 UV–VIS spectroscopy

Astaxanthin concentrations in the extracts of *P. japonicus* exoskeleton were higher than 8.0 mg/g waste using either *S. cerevisiae* or *L. acidophilus* followed by solvent extraction with hexane and acetone at a ratio of 1:1 as well as by the chemical method (Fig. 2A). However, astaxanthin from the extracts of *P. semisulcatus* exoskeleton led to much lower values (3.0 mg/g waste with *S. cerevisiae* followed by solvent extraction with hexane and acetone at a ratio of 1:1, 4.1 mg/g waste with *L. acidophilus* followed by solvent extraction with hexane and acetone at a ratio of 1:1 and 3.2 mg/g waste with the chemical method) (Fig. 2B). It is noteworthy that the microbiological method followed by solvent extraction with hexane and acetone at a ratio of 1:2, and especially at 1:3, acutely decreased the extracted astaxanthin (Fig. 2).

3.1.2 HPLC

HPLC chromatographs of the extracts are presented in Supplementary Fig. 3. The obtained results (Table 1) were much lower than those determined by UV–VIS spectroscopy shown in Fig. 2. The reason for this is that the UV–Vis method does not enable the distinction between carotenoids in a given mixture and the most common protocols use a mean absorption coefficient and a mean absorption wavelength. Therefore, more accurate results are attained by the HPLC method for specific carotenoids.

3.1.3 C-NMR studies

The C-NMR analysis (Supplementary Fig. 4) of astaxanthin extracted from *P. japonicus* and *P. semisulcatus* by chemical and microbiological methods confirmed that astaxanthin was the main carotenoid present in the samples. The two peaks, between 7.004 and 8.557 ppm, symbolized the presence of protons of methine on the astaxanthin main chain [29]. These peaks proved the presence of astaxanthin as two sets in the monoesterified compounds due to the loss of symmetry. At 2.505 ppm, four protons corresponded to the methylene protons, and α to the carbonyl. Signals at 2.022 and 1.83 ppm corresponded to the methyl moieties [28]. A signal that represented the methylene protons on the astaxanthin fatty acid moiety was shown in the middle of 1.307 and 1.593 ppm signals.

Fig. 2 Concentration of astaxanthin extracted from *Penaeus japonicus* (A) and *Penaeus semisulcatus* (B) using *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* followed by solvent extraction with hexane and acetone at a ratio of 1:1, 1:2, and 1:3

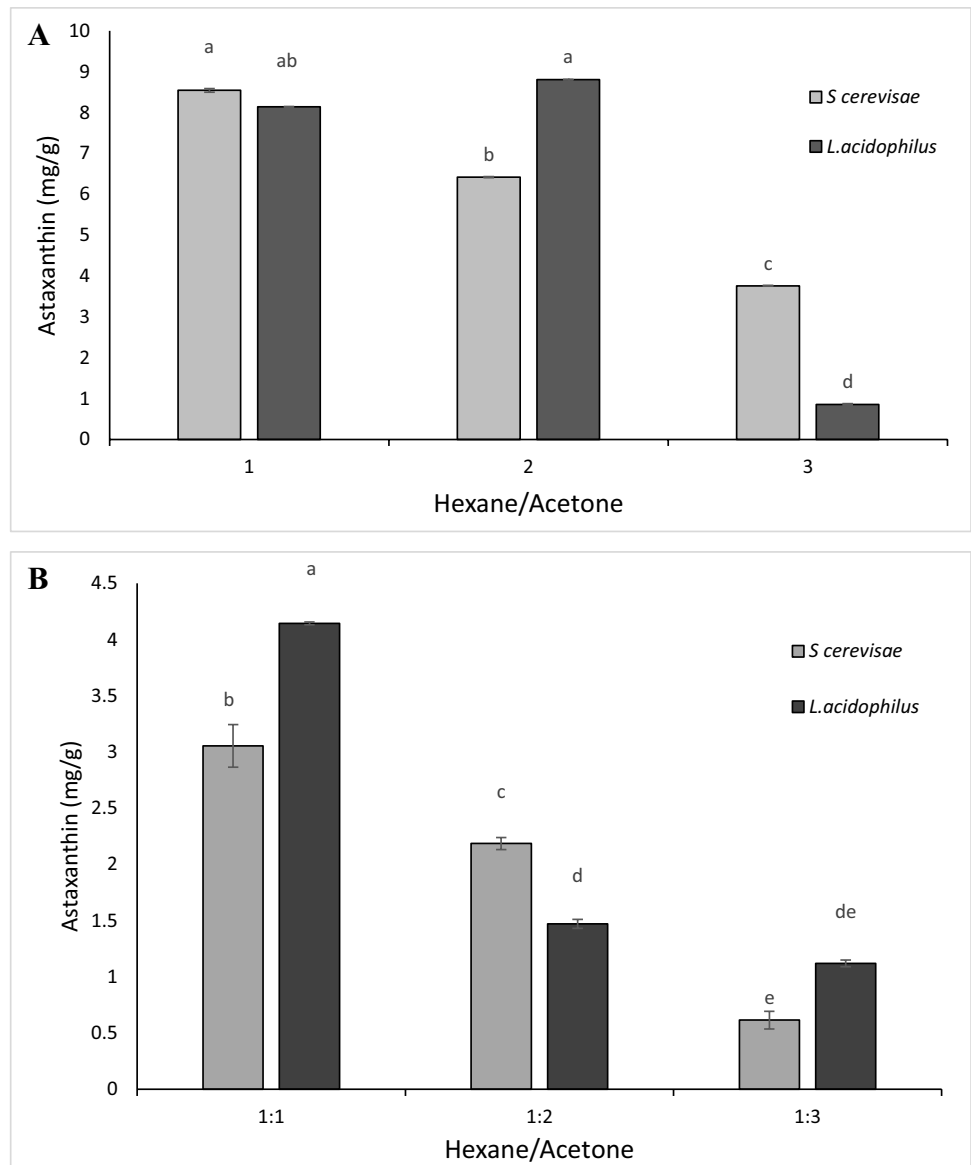


Table 1 Astaxanthin concentrations obtained for the different extracts by using the HPLC method

Shrimp species	Extraction method	Average peak area	[Astaxanthin] (µg/mL)	[Astaxanthin] (mg/g waste)
<i>Penaeus semisulcatus</i>	<i>Lactobacillus acidophilus</i> + solvent extraction with hexane and acetone at 1:1	307.55	4.88	0.488
	Chemical method	253.77	3.774	0.3774
	<i>Saccharomyces cerevisiae</i> + solvent extraction with hexane and acetone at 1:1	168.09	2.0119	0.2012
<i>Penaeus japonicus</i>	<i>Lactobacillus acidophilus</i> + solvent extraction with hexane and acetone at 1:1	443.83	7.68	0.768
	Chemical method	501.61	8.87	0.887
	<i>Saccharomyces cerevisiae</i> + solvent extraction with hexane and acetone at 1:1	517.05	9.192	0.9192

Overlapping peaks presented around 1.830 and 2.022 ppm referred to protons of the methylene moiety. A broad signal found at 3.892 ppm corresponded to the OH moiety on the astaxanthin molecule [29].

4 Discussion

In the marine crustaceans, astaxanthin is considered as the main carotenoid [30]. Traditionally, chemical extraction of astaxanthin was being used in the industry. In this study, the astaxanthin extracted from *P. japonicus* and *P. semisulcatus* via the chemical method were 8.4 and 3.2 mg/g waste, respectively. These values are comparable to those attained using the microbiological method, followed by solvent extraction with hexane and acetone (1:1). However, Khanafari et al. [20] found that the microbiological method led to higher astaxanthin yields than the chemical method for shrimp waste of *P. semisulcatus*.

The results obtained indicated that astaxanthin extraction via the microbiological method depended on the microbiological strain used, as well as the solvent system used afterwards. Thus, in the present study, *P. japonicus* led to a markedly higher astaxanthin yield than *P. semisulcatus*. Similarly, Lim et al. [31] elucidated that *P. japonicus* was a good source of astaxanthin. As for the solvent system used, increasing the amount of acetone (polar solvent) in the hexane/acetone solvent mixture decreased astaxanthin extraction. This might be due to an increase in the polar solvent, which favored the extraction of other components, thus hampering astaxanthin extraction.

According to the sustainable development goals (SDGs), combining microbial with chemical extraction is better for the environment since it reduces the use of chemicals [32]. In this sense, green technologies, including fermentation via probiotic bacteria, have recently been developed [33]. Microbial extraction is a humble and eco-friendly method for the extraction of extremely unstable pigments such as carotenoids [25] due to the action of the extracellular proteolytic enzymes secreted by the microorganisms [34]. In addition, shrimp waste is extremely perishable. The carotenoid-rich broth obtained by the microbiological process can be stored for a long-time span under normal storage conditions, which is not possible with other extraction methods [33].

The presence of astaxanthin was confirmed by NMR analysis. In the same way, Azizan et al. [28] confirmed the presence of astaxanthin in the hexane extract of *Chaetoceros calcitrans* at 1.34 ppm, which represented the methylene protons on the astaxanthin fatty acid moiety, shown in the middle of 1.307 and 1.593 ppm in the NMR chart of the present study (Supplementary Fig. 4).

5 Conclusion

According to the obtained results, *P. japonicus* led to a significantly higher astaxanthin yield than *P. semisulcatus*, which means that the former was a better source of astaxanthin. In addition, the extraction of astaxanthin from shrimp wastes via the microbial method led to equal or slightly higher yields than those extracted via the conventional chemical method. Therefore, the usage of chemicals in extraction can be reduced and replaced by edible microorganisms. This paves the way for the exchange of some chemicals with useful microorganisms commonly found in nature.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13399-022-02984-2>.

Author contribution Salwa A. H. Hamdi: conceptualization, result analysis, methodology, project administration, writing the original draft, and supervision; Ghadeer M Ghonaim: methodology, visualization, result analysis, and writing the original draft; Rana R. El Sayed: visualization, methodology, result analysis, and writing the original draft; Susana Rodríguez-Couto: reviewing and editing manuscript; Mohamed N. Abd El-Ghany: conceptualization, data curation, formal analysis, investigation, methodology, result analysis, project administration, writing the original draft, resources, software, supervision, validation, visualization, writing, review and editing.

Funding Open Access funding provided by LUT University (previously Lappeenranta University of Technology (LUT)).

Declarations

Conflict of interest The authors declare no competing interests.

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