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Development of a Microbial-Assisted Process for Enhanced Astaxanthin Recovery from Crab Exoskeleton Waste

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Abstract: Astaxanthin is a xanthophyll carotenoid possessing impressive nutraceutical, antioxidant, and bioactive merits. Traditionally, astaxanthin is extracted from crustacean wastes via solvent extraction methods. However, the rigid structure of shells that comprise complex proteins and chitin challenges the extraction process. This investigation addressed an efficient microbial-assisted method to facilitate astaxanthin recovery from crab exoskeleton waste utilizing chitinolytic and proteolytic microorganisms. Herein, we evaluated the effect of pretreatment of the exoskeleton waste with a newly isolated probiotic strain, *Bacillus amyloliquefaciens* CPFD8, showing remarkable protease and chitinase activity and a proteolytic *Saccharomyces cerevisiae* 006-001 before solvent extraction, using acetone/hexane, on astaxanthin recovery. Furthermore, the antioxidant and anti-inflammatory activities of the recovered astaxanthin were inspected. Results revealed that both strains boosted the astaxanthin yield from the crab (*Callinectes sapidus*) exoskeleton compared with solvent extraction using acetone/hexane. Under optimum conditions, astaxanthin yield was 217 and 91 µg/g crab exoskeleton in samples treated with *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001, respectively. Interestingly, pretreatment of crab exoskeleton waste with *B. amyloliquefaciens* CPFD8 yielded more than 6-fold astaxanthin compared with the solvent extraction method that yielded just 35 µg/g. This increase could be attributed to the proteolytic activity of *B. amyloliquefaciens* CPFD8 that rendered deproteinized shell chitin accessible to chitinase, facilitating the penetration of solvents and the recovery of astaxanthin. The recovered astaxanthin exhibited excellent antioxidant activity in scavenging DPPH or ABTS free radicals with IC_{50} values of 50.93 and 17.56 µg/mL, respectively. In addition, the recovered astaxanthin showed a remarkable anti-inflammatory impact on LPS-induced murine macrophage RAW264.7 cells and significantly inhibited the production of nitric oxide, TNF-α, and IL-6 compared with the untreated control. These findings suggest the potential use of the developed microbial-assisted method utilizing chitinolytic and proteolytic *B. amyloliquefaciens* CPFD8 to maximize the recovery of bioactive astaxanthin from crab (*C. sapidus*) exoskeleton waste.

Keywords: astaxanthin extraction; crustacean waste; chitinase activity; antioxidant activity; anti-inflammatory; microbial-assisted extraction

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1. Introduction

Astaxanthin is a fat-soluble xanthophyll carotenoid synthesized by certain bacteria, fungi, and algae and found in crustaceans and various aquatic animals [1]. Recently, astaxanthin has received considerable attention due to its extraordinary merits such as its superior antioxidant and anti-inflammatory activity. It has been reported that global natural astaxanthin production has steadily increased from 44 tons in 2013 to 68 tons in 2014, with an average rate of 11.23% per year [2]. Consequently, astaxanthin has become a highly demanded metabolite, and its global market size was estimated at USD 1943 million in 2022 [3]. The leading driving factor for the market growth of astaxanthin is its demand from various applications such as nutraceuticals, pharmaceuticals, cosmetics, aquaculture and animal feed, and the food industry. It possesses significantly superior antioxidant activity, even more than vitamin E and β-carotene [4]. Hence, astaxanthin is a well-documented nutraceutical for cardiovascular disease prevention [5,6], skin protection [7–9], antidiabetic [10,11], anticancer [12,13], anti-obesity [14,15], anti-inflammatory [16,17], antiaging [18,19], natural food colorant [20], and feed supplement in poultry and aquaculture [21–23]. Structurally, astaxanthin is a 40-carbon tetraterpene consisting of two terminal β-rings joined by an unsaturated polyene chain (Figure 1). The presence of 11 conjugated double bonds determines the pinkish-orange color of astaxanthin and is responsible for its antioxidative potential [24–26]. As a member of the xanthophyll subclass of carotenoids, astaxanthin possesses oxygen-containing hydroxyl and carbonyl groups attached to each ionone ring [27]. The presence of oxygen atoms on both terminals of the terpenoid chain confers a remarkable polarity of the molecule. Owing to this unique polar–nonpolar structure, astaxanthin can fit the hydrophobic polyene chain (lipophilic) inside the bilayer lipid in the cell plasma membrane, and its polar terminal ionone rings (hydrophilic) can be positioned near its surface [28]. Consequently, the dual lipophilic and hydrophilic properties allow astaxanthin to extend into the bilayer of the cell membrane, enhancing cell defense and conferring exceptional beneficial roles.

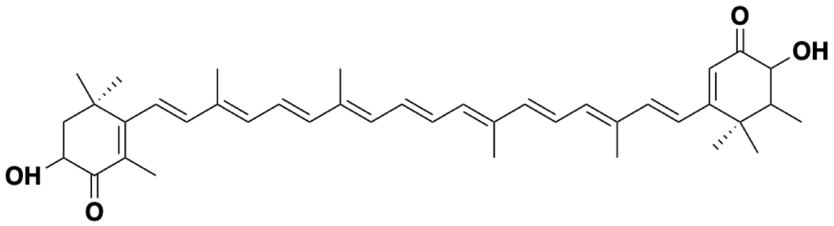

Figure 1. Planner structure of astaxanthin ($C_{40}H_{52}O_4$).

Commercial astaxanthin is obtained mainly from natural sources or by chemical synthesis. So far, synthetic astaxanthin dominates the global market, though the current appeal of natural astaxanthins has grown substantially [29,30]. Despite the low cost of synthetic astaxanthin, natural sources have many advantages over synthetic astaxanthin. In this regard, natural astaxanthin has better pigmentation and antioxidant activities compared with synthetic ones. It has been reported that natural astaxanthin has over 50 times stronger antioxidant activity than synthetic astaxanthin, and the latter may not be suitable as a human nutraceutical supplement due to safety concerns [31]. Moreover, synthetic astaxanthins are poorly soluble due to their crystalline nature, while natural astaxanthins are lipid-soluble, non-crystalline, and exhibit better absorption properties [32]. Thus, natural astaxanthins surpass synthetic ones for food and feed applications. It has believed that algae, yeast, and bacteria constituted the primary natural sources of astaxanthin. Furthermore, several aquatic animals such as salmon, trout, krill, and crustaceans, including shrimp, crab, crayfish, and lobsters, contain considerable levels of astaxanthin, conferring their reddish coloration [33]. Indeed, aquatic animals can not synthesize astaxanthin or other carotenoids by themselves, but they accumulate the digested astaxanthin obtained from their diet. On this point, aquatic zooplanktons fed on marine β-carotene-rich algae convert β-carotene to astaxanthin in their bodies; consequently, they are ingested by aquatic

animals that accumulate astaxanthin [22]. Apart from pigmentation, astaxanthin plays crucial roles in the health and reproduction of aquatic animals, especially crustaceans. The essential role of astaxanthin in reproductive performance, including egg production and quality, has been demonstrated. It has been reported that the accumulated astaxanthin in crustaceans' hepatopancreas migrates to the ovaries in the late stages of maturity. Additionally, astaxanthin boosts the crustaceans' immune system, disease resistance, and resistance to abiotic stressors such as temperature and pH [22,34,35]. Crustaceans accumulate high concentrations of astaxanthin in their exoskeleton in the form of carotenoproteins, such as crustacyanin, that exhibit red, purple, and blue to blue-black [36]. The crustaceans' astaxanthin can be found in complex forms associated with proteins (carotenoproteins) and lipids (carotenolipoprotein) exhibiting blue to green coloration [37]. Industrial crustacean processing generates accumulated crustacean shells waste as by-products, reaching 50–70% of the raw weight [38]. Hence, shell biorefinery may offer an ecological and economical solution to valorize shells as a source of valuable materials such as astaxanthin. Traditionally, solvent extraction is the most common method for astaxanthin extraction from crustaceans' exoskeleton using acetone, isopropanol, hexane, ethyl acetate, and other solvents [39–41]. However, rigid structure and complex composition of crustaceans' exoskeleton constitute a critical bottleneck for astaxanthin recovery from their residues [42]. Crustacean shells ordinarily consist of protein (20–40%), chitin (15–40%), mineral salts (20–50%), and small amounts of lipids varying with species and seasons [43]. The firm exoskeleton, its high chitin content, and the complex forms of astaxanthin in conjugation with lipids and proteins limit the diffusion of the solvent and remain challengeable for astaxanthin extraction. Therefore, several extraction strategies have been proposed to overcome these obstacles. Recently, astaxanthin was extracted from crab shell wastes by integrating microwave pretreatment and supercritical fluid extraction [42]. Additionally, ultrasound-based extractions have been reported as a practical approach for improving the extraction processes by generating microcavities that enhance solvent penetration [44]. It has been suggested that ultrasound-induced cavitation generates fragmentation of the shell matrix, boosting the solubilization of bioactive compounds, and thus enhancing their extraction by solvents [45]. Moreover, the high-pressure extraction (HPE) method improved the yield of extracted astaxanthin from crustaceans due to enhancing the penetration of the solvents and improving the intermolecular physical interactions [46]. In this manner, high-pressure disorders the fiber structure and damages the cellular membranes, leading to the higher diffusion of solvents and improves astaxanthin extraction. In addition, accelerated solvent extraction (ASE) combined with pulsed electric fields (PEF) pretreatments significantly increased astaxanthin recovery [47]. Despite enhancing astaxanthin recovery, these combined chemical and physical pretreatment methods require special equipment and are considered energy-consuming strategies and could affect the structure and activity of astaxanthin. Alternatively, green eco-friendly strategies such as microbial fermentation and pretreatment of crustacean shells with hydrolytic enzymes have recently emerged. In this regard, it has been reported that *Aeromonas hydrophila* is capable of recovering astaxanthin from shrimp shell wastes [48]. Similarly, astaxanthin was recovered from crayfish "*Procambarus clarkii*" using probiotics strains such as *Saccharomyces cerevisiae*, *Candida utilis*, *Lactobacillus lactis* and *Bifidobacterium lactis* [49]. However, the role of these strains in releasing of astaxanthin from crustacean wastes and the mechanism of recovery are not fully understood. Additionally, the application of proteolytic enzymes such as alcalase and bromelain for deproteinization of crustacean waste was found to promote astaxanthin recovery [50]. In a recent study, astaxanthin was easily extracted from shell residue after removal of protein and chitin by treatment with recombinant protease and chitinase [51]. Although this biological approach facilitated astaxanthin recovery, the use of recombinant enzymes is not a cost-effective choice. Here, we addressed a microbial-assisted process for enhanced astaxanthin recovery from the crab exoskeleton involving simultaneous hydrolysis of proteins and chitin in the crab exoskeleton using chitinolytic and/or proteolytic *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001prior to solvent extraction.

2. Materials and Methods

2.1. Microoganisms and Stock Cultures

The proteolytic yeast, *S. cerevisiae* 006-001, was purchased from the culture collection of the Reginal Centre for Mycology and Soil, Al-Azhar University, Cairo, Egypt. *B. amyloliquefaciens* CPFD8 exhibiting a remarkable chitinolytic and proteolytic activity was isolated and identified from fermented milk (Figure S1) [52,53].

2.2. Crab Waste Preparation

Two species of crabs, *Callinectes sapidus* and *Portunus pelagicus*, were bought from the local fish market. All internal organs from the crabs were peeled out, and the crab's exoskeleton, made up of the carapace and legs, was collected as waste. Afterward, the exoskeletons were dried in an oven for 8–10 h at 55–60 °C. Finally, the dried exoskeleton waste was crushed with a home blinder and passed through a sieve to obtain a fine powder (1–3 mm particle size). The prepared powder samples were collected in clean, labelled containers and stored at 4 °C.

2.3. Chemical Extraction of Astaxanthin

To extract astaxanthin, 10 g of the exoskeleton powder of each crab species were combined with 50 mL of a mixture of hexane and acetone at various ratios (1:1, 1:2, and 1:3) in a 250-mL flask, vortexed for 30 s, and then heated at 50 °C for 10 min. Subsequently, the waste materials were removed by centrifugation at 5000× *g* for 10 min, and the supernatants were filtered through 0.45 µm filters. Then, the resulting extracts were evaporated under reduced pressure using a rotary evaporator. The obtained astaxanthin was dissolved in dimethyl sulfoxide (DMSO), and its concentration was determined spectrophotometrically at a wavelength of 476 nm as described elsewhere [54]. A standard curve using astaxanthin (Sigma-Aldrich, St. Louis, MO, USA) was established and the concentration of the recovered astaxanthin was expressed as µg/g exoskeleton powder.

2.4. Microbial-Assisted Extraction of Astaxanthin

In attempts to boost the recovery of astaxanthin, the dried crab exoskeleton powders were pretreated with the selected chitinolytic proteolytic bacterial strain CPFD8 and the proteolytic yeast strain *S. cerevisiae* 006-001, in separate experiments, prior to the chemical extraction process. Practically, 10 g of each dried crab exoskeleton powder were added to 100 mL a fermentation medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl) in 250-mL flasks as a sole carbon and nitrogen source. Then, *B. amyloliquefaciens* CPFD8 was inoculated, and the flasks were incubated in a shaking incubator (150 rpm) at 30 °C for 5 days. In another set, *S. cerevisiae* 006-001 was inoculated into 100 mL of modified Czapek–Dox broth (Condalab, Spain) containing 10 g of the dried crab exoskeleton powder, and the flasks were incubated in a shaking incubator (150 rpm) at 30 °C for 5 days. Afterward, each fermentation culture was lyophilized, and the astaxanthin was extracted from each freeze-dried powder by the above-described chemical method, and quantified.

2.5. Effect of Pretreatment Conditions on Astaxanthin Recovery

The optimum fermentation conditions for astaxanthin recovery from *C. sapidus* exoskeleton waste were determined by the one-factor-at-a-time method, keeping others constant. In brief, the effect of temperature on astaxanthin recovery was studied by cultivating *B. amyloliquefaciens* CPFD8 or *S. cerevisiae* 006-001 for 5 days at different temperatures ranging from 20 to 50 °C as independent treatments. The influence of the initial pH of the fermentation media (at the optimum temperatures) was investigated by adjusting the pH of the fermentation media at various levels ranging from 3 to 10 before sterilization. The optimum incubation period for astaxanthin recovery was determined by inoculating the fermentation media with each microbial strain and cultured at their optimum temperature and pH values. Then, the recovered astaxanthin was quantified daily over 7 days.

Each experiment was repeated three times and the extracted astaxanthin concentration was estimated.

2.6. Enzymatic Activity

The potential role of *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001 in astaxanthin extraction from crab exoskeleton waste was investigated by performing the fermentation processes at their optimum conditions. Afterward, the fermentation cultures were centrifuged at 15,000× *g* for 15 min at 4 °C. The obtained cell-free supernatants were assayed for extracellular chitinase, protease, and lipase activities. The chitinase activity was assayed by the dinitrosalicylic acid (DNS) method using colloidal chitin as a substrate [55]. In brief, the reaction mixture consisted of 1 mL of 1% colloidal chitin suspension in 50 mM phosphate buffer (pH 6.8), and 0.5 mL of the cell-free supernatant as a source of the enzyme was incubated for 30 min at 50 °C. Subsequently, the reaction was terminated by adding 1 mL of DNS reagent and boiling the mixture for 10 min. Afterward, the absorbance of the developed color was measured at 540 nm. A standard curve was plotted using N-acetyl glucosamine. One unit (U) of chitinase activity was defined as the amount of enzyme that released 1 µmol N-acetyl glucosamine from colloidal chitin per min under reaction conditions. Protease activity was estimated by mixing 0.5 mL of the cell-free supernatant with 2 mL of 1% casein in 50 mM phosphate buffer (pH 6.8). After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 2.5 mL of 100 mM trichloroacetic acid (TCA) solution. After centrifugation at 15,000× *g* for 5 min, 2.5 mL of 500 mM sodium carbonate was added to the supernatant, followed by the addition of 1 mL of 0.5 mM Folin–Ciolcalteu reagent. Then, the mixture was incubated for 30 min at room temperature, and the absorbance of the developed color was determined at 660 nm using an EPOCH2 spectrophotometer. One unit of protease activity was defined as the amount of enzyme that catalyzes the conversion of 1 µmol casein per min [56]. Lipase activity was estimated using p-nitrophenyl laurate (pNPL) as substrate [57]. Briefly, 0.5 mL of the cell-free supernatant was added to 2 mL of reaction buffer (0.1 M KH2PO4; 0.1% gum Arabic, 0.2% deoxycholate, pH 8.0). After incubation at 37 °C for 5 min, 200 µL of 8 pNPL "solubilized in isopropanol" was added. After incubation at 37 °C for 15 min, the reaction was terminated by adding 0.5 mL of 3 M HCl and centrifuged at 10,000× *g* for 10 min. Then, 1 mL of NaOH solution (2 M) was added to 0.5 mL of the supernatant, and absorbance was measured at 420 nm. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of p-nitrophenol from pNPL per min.

2.7. ^{13}C-NMR and HPLC Analysis

In this study, ^{13}C NMR spectra analysis was performed on the astaxanthin extracts recovered from crab exoskeletons using the chemical and microbial-assisted techniques. The spectra were acquired by a Bruker Advance III 400 MHz device (Bruker, Rheinstetten, Germany). Based on the residual carbon resonances of the appropriate deuterated solvent, chemical shifts about TMS are reported and referenced. 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 m, Eclipse) was used to estimation the astaxanthin content of the extracts [58,59].

2.8. Antioxidant Activity of Astaxanthin

To investigate the antioxidant activity of astaxanthin, 1,1-diphenyl-2-Picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assays were performed [60–62]. The DPPH solution (0.2 mM) was prepared by dissolving 7.89 mg of DPPH in 100 mL methanol. Various concentrations of astaxanthin solution (0.3 mL) were mixed with 2.7 mL of DPPH solution. The mixtures were shaken vigorously and left at room temperature in the dark for 30 min. Afterwards, the absorbance

was recorded at 517 nm, and the radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging \%} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

where A_0 is the absorbance of the DPPH solution and A_1 is the absorbance of the sample after 30 min.

On the other hand, ABTS stock solution 1 was prepared by dissolving 96.02 mg of ABTS in 25 mL of acetic acid buffer (pH 4.5), while stock solution 2 was prepared by dissolving 66.24 mg of potassium persulfate ($K_2S_2O_8$) in 100 mL acetic acid buffer (pH 4.5). To prepare the ABTS reaction solution, equal volumes of stock solutions 1 and 2 were mixed and kept in the dark at room temperature for 12–16 h. Finally, 2.80 mL of ABTS reaction solution was diluted to 65 mL in acetate buffer (pH 4.5) to obtain the ABTS working solution. The ABTS assay was conducted in 96-well plates. Briefly, 200 µL ABTS working solution was mixed with 10.0 µL astaxanthin solutions of various concentrations and kept in the dark for 7 min. Afterward, the absorbance was measured at 734 nm using an Epoch 2 microplate spectrophotometer (BioTek, Santa Clara, CA, USA). The scavenging effect of the sample was calculated as follows:

$$\text{ABTS radical scavenging \%} = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

where A_0 is the absorbance of the blank solution and A_1 is the absorbance of the sample after 7 min.

The antioxidant activity of astaxanthin and ascorbic acid were determined and their IC_{50} values which represent the concentrations of the samples required to scavenge 50% of the DPPH or ABTS free radicals were calculated by GraphPad Prism software (Version 7.0.0).

2.9. Anti-Inflammatory Activity of Astaxanthin

The anti-inflammatory potential of the extracted astaxanthin was investigated using the murine macrophage RAW264.7 cell line (American Type Culture Collection, ATCC, Rockville, MD, USA). The cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, Cambridge, UK), 2 mM L-glutamine, and antibiotics (100 µg/mL streptomycin and 100 units/mL penicillin). The macrophage RAW264.7 cells were cultured in a water-jacketed incubator (BINDER GmbH, Tuttlingen, Germany) at 37 °C under 5% CO_2 in a high-humidity atmosphere. To assess the anti-inflammatory effect, the Raw 264.7 cells were exposed to various concentrations of astaxanthin for 24 h and then stimulated by lipopolysaccharides (LPS) from *Pseudomonas aeruginosa* (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 1 µg/mL, and the cells were incubated at the same conditions for an additional 24 h. Additionally, untreated cells and cells treated with LPS alone served as control. Afterward, nitric oxide (NO) and immunologically active mediators, including tumor necrosis factor (TNF)-α and interleukin (IL)-6 were determined. In brief, the production of NO by the RAW264.7 cells was measured calorimetrically using the Griess reagent (Promega, Madison, WI, USA). Practically, 100 µL of culture supernatants were incubated with 50 µL of Griess reagent and incubated at room temperature for 15 min. The absorbance was recorded at 540 nm using an Epoch 2 microplate reader. The nitrite concentration of each sample was determined using a freshly prepared sodium nitrite as standard. TNF-α was measured by ELISA using a mouse-specific TNF-α ELISA Kit (Elabscience, Houston, TX, USA), and IL-6 was determined using a mouse-specific IL-6 ELISA kit (Elabscience, Houston, TX, USA) according to the manufacturer's instructions.

2.10. Statistical Analysis

The data presented in each experiment are the mean of triplicate assays. The measured data were subjected to the analysis of variance (ANOVA). The significant differences

between treatments were compared with the critical difference at 5% level of probability by Duncan's test using IBM SPSS software version 22.

3. Results

3.1. Astaxanthin Recovery

Results revealed that astaxanthin recovered from *C. sapidus* was significantly higher than that recovered from *P. pelagicus*, and superior extraction was achieved by using acetone/hexane solvent in a ratio of 1:1, in all cases (Figure 2). In the chemical extraction method, the maximum extraction of astaxanthin was obtained from *C. sapidus* with a yield of 35.26 µg/g of crab exoskeleton waste using acetone/hexane (1:1 *v*/*v*) as a solvent system. On the other hand, only 19.46 µg/g astaxanthin was recovered from *P. pelagicus* exoskeleton waste under the same conditions. Interestingly, pretreatment of *C. sapidus* exoskeleton waste with *B. amyloliquefaciens* CPFD8 boosted the recovery process, yielding 92.2 µg/g astaxanthin. The enhanced recovery of astaxanthin was also observed when crab exoskeleton waste was pretreated with *S. cerevisiae* 006-001 but to a smaller extent. The *C. sapidus* exoskeleton waste yielded 66.26 µg/g astaxanthin upon pretreatment with *S. cerevisiae* 006-001 prior the chemical extraction.

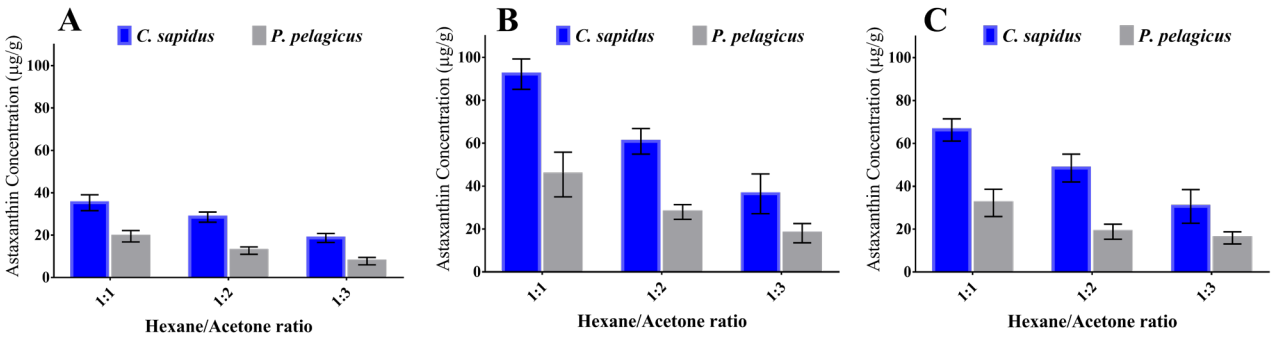

Figure 2. Astaxanthin yield recovered from *C. sapidus* and *P. pelagicus* exoskeleton using various hexane/acetone ratios. Exoskeleton without pretreatment (**A**), exoskeleton pretreated with *B. amyloliquefaciens* CPFD8 before solvent extraction (**B**), and exoskeleton pretreated with *S. cerevisiae* 006-001 before solvent extraction (**C**).

3.2. Effect of Pretreatment Conditions on Astaxanthin Recovery

In this study, the impact of temperature, pH, and incubation period on the astaxanthin yield was investigated. Results revealed that the optimum temperature for maximum recovery of astaxanthin was 30 and 40 °C for *S. cerevisiae* 006-001 and *B. amyloliquefaciens* CPFD8, respectively (Figure 3A). The optimum pH value was observed at 8.0 and 6.0 for *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001, respectively (Figure 3B). The effect of the incubation period was investigated by the cultivation of *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001 on the *C. sapidus* exoskeleton waste for various incubation periods prior to the extraction process. The maximum astaxanthin recovery was attained after 3 and 4 days of incubation with *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001, respectively (Figure 3C). Under the optimum conditions, the astaxanthin yield was 217.96 and 91.23 µg/g when *C. sapidus* exoskeleton waste was exposed to *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001, respectively.

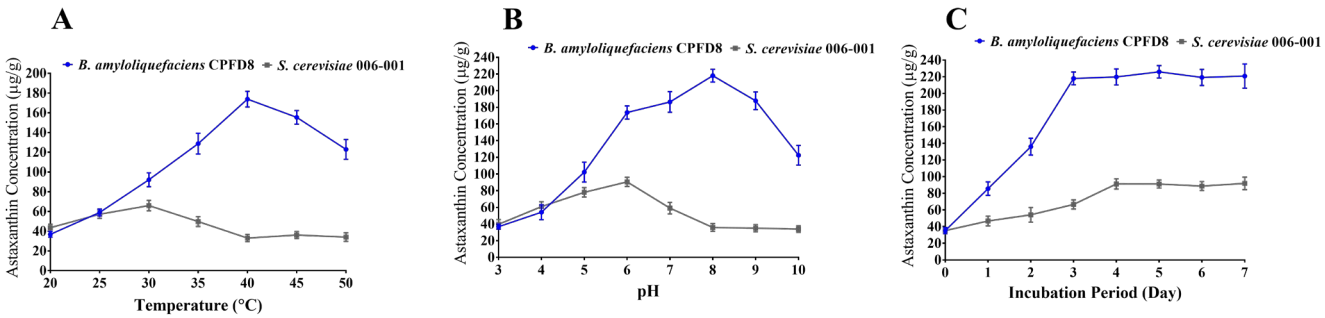

Figure 3. Effect of initial treatment conditions on astaxanthin recovery from *C. sapidus* exoskeleton waste by *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001, prior to solvent extraction. Effect of temperature (**A**), pH (**B**) and incubation period (**C**).

3.3. Enzymatic Activity

Results revealed that *B. amyloliquefaciens* CPFD8 cultivated on *C. sapidus* exoskeleton waste as the sole carbon and nitrogen sources secreted extracellular protease, chitinase, and lipase enzymes while *S. cerevisiae* 006-001 secreted protease only (Table 1).

Table 1. Extracellular enzymatic activity of *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001 cultivated on *C. sapidus* exoskeleton waste.

Enzyme	Enzyme Activity (U/mL)	
	***B. amyloliquefaciens* CPFD8**	***S. cerevisiae* 006-001**
Chitinase	18.6 ± 2.1	-
Protease	127.6 ± 19.2 [(a)]	44.8 ± 5.4 [(b)]
Lipase	7.2 ± 1.9	-

The same letter in each row indicates no significant difference according to Duncan's multiple range test ($p < 0.05$). Symbol: ± represents standard deviation.

3.4. ^{13}C-NMR and HPLC Analysis

^{13}C-NMR analysis identified astaxanthin as the major carotenoid in the samples extracted from *C. sapidus* (Figure S2). The two peaks between 7.004 and 8.557 ppm denoted the presence of methine protons on the main chain of astaxanthin. Because of the symmetry loss, these peaks demonstrated that astaxanthin existed as two sets in the monoesterified molecules. At 2.505 ppm, four protons represented the carbonyl and four pr tons represented the methylene protons. The methyl moieties were represented by the signals at 2.022 and 1.83 ppm. In the region between the 1.307 and 1.593 ppm signals, a signal that represented the methylene protons on the astaxanthin fatty acid moiety could be seen. The protons of the methylene moiety were identified by overlapping peaks appearing between 1.830 and 2.022 ppm. The astaxanthin molecule's OH moiety was represented by a wide signal that was discovered at 3.892 ppm. Furthermore, HPLC verified and quantified the extracted astaxanthin compared with standard astaxanthin. Results of HPLC analysis confirmed the successful recovery of astaxanthin and estimated the concentration of the recovered astaxanthin (Figure S3).

3.5. Antioxidant Activity

Results revealed the remarkable antioxidant potential of astaxanthin extracted from *C. sapidus* with efficient DPPH and ABTS radical scavenging activity. Clearly, the IC_{50} value of astaxanthin and ascorbic acid against DPPH was 50.93 and 39.73 µg/mL, respectively. In addition, the extracted astaxanthin showed superior ABTS radical scavenging activity compared with ascorbic acid. The IC_{50} value of astaxanthin and ascorbic acid against ABTS was 17.56 and 28.46 µg/mL, respectively (Figure 4).

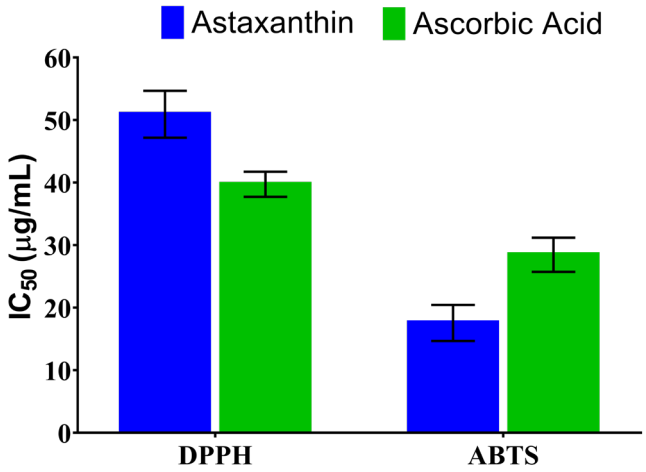

Figure 4. Antioxidant activity of astaxanthin recovered from *C. sapidus*.

3.6. Anti-Inflammatory Activity

Clearly, treatment of the murine macrophage RAW264.7 cells with LPS resulted in a marked increase in NO, TNF-α, and IL-6 production. However, co-treatment of cells with astaxanthin induced significant attenuation of LPS-induced production of NO, TNF-α, and IL-6 production in a dose-dependent manner (Figure 5). The production of NO was reduced by 88% in LPS-induced cells treated with 30 µg/mL of astaxanthin. Results showed no significant difference in TNF-α production by LPS-induced cells treated with astaxanthin up to 15 µg/mL. Further increase in astaxanthin concentration resulted in significant reduction in TNF-α production compared with cells treated with LPS only. More than 68% reduction was recorded in LPS-induced cells treated with 40 µg/mL astaxanthin. Regarding IL-6, about 76% reduction was observed in LPS-induced cells treated with 35 µg/mL astaxanthin.

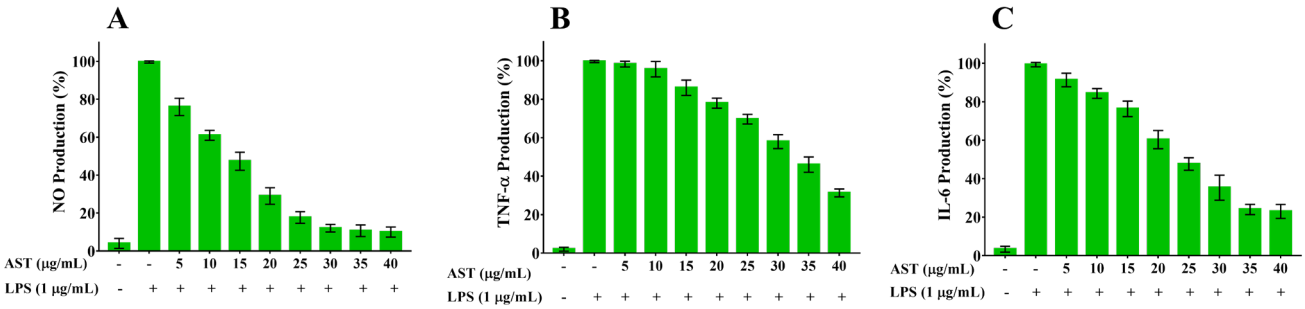

Figure 5. Effect of various concentration of astaxanthin recovered from *C. sapidus* on production of NO (**A**), TNF-α (**B**) and IL-6 (**C**) by murine macrophage RAW264.7 cells.

4. Discussion

In this study, *C. sapidus* yielded considerably more astaxanthin than *P. pelagicus* in all investigated solvent systems. Moreover, the best solvent system for astaxanthin was acetone/hexane (1:1 ratio). Increasing the proportion of acetone (a polar solvent) in the hexane/acetone solvent mixture reduced astaxanthin extraction. This effect could be attributable to increasing the polar solvents, which favored other component extraction while preventing astaxanthin extraction. Accordingly, *C. sapidus* was chosen as an astaxanthin-rich source and acetone/hexane (1:1 ratio) as the most efficient extraction solvent system. Being a lipophilic compound, astaxanthin has been traditionally extracted from its natural sources by solvent extraction. Compared with algal astaxanthin, the extraction of astaxanthin from crustacean wastes by solvents only resulted in low yields due to the complex structures of the crustacean exoskeletons that limit the diffusion of solvents. Principally, crustacean exoskeletons (shells) contain chitin nanofibrils complexed with proteins to

form long chitin-protein fibers embedded in the mineral matrix [63,64]. The presence of chitin and proteins in the crustacean exoskeletons forms a barrier that reduce the ability of solvents to penetrate the firm shell and dissolve astaxanthin. Therefore, the digestion of the chitin and proteins in the exoskeleton could enhance the extraction of astaxanthin. Hence, we investigated the effect of pretreatment of the scrab exoskeletons with microbial strains exhibiting chitinase and/or protease activity prior to the extraction on the extraction efficiency. Consequently, *B. amyloliquefaciens* CPFD8 showing remarkable proteolytic and chitinolytic activity was isolated. In this regard, *B. amyloliquefaciens* is a probiotic strain that has been isolated from various sources including fermented food products [65–67]. Similarly, various probiotic strains belonging to the genus *Bacillus* with beneficial enzymatic activity and generally regarded as safe (GRAS) have been reported [68–72]. In the present study, the pretreatment of *C. sapidus* exoskeleton waste with *B. amyloliquefaciens* CPFD8 (before optimization) resulted in a more than 2.6-fold increase in astaxanthin recovery compared with the chemical extraction method using acetone/hexane (1:1 *v*/*v*) as a solvent system. As mentioned above, crustacean exoskeletons are firm structures consisting of protein, chitin, and mineral salts, in which astaxanthin is found in conjugation with lipids and proteins. Obviously, pretreatment of crab exoskeleton waste with *B. amyloliquefaciens* CPFD8 enhanced the efficacy of astaxanthin recovery compared with *S. cerevisiae* 006-001. Hence, the potentiation of astaxanthin recovery could be attributed to the secretion of chitinase, protease, and lipase. On the other hand, *S. cerevisiae* 006-001 secretes protease only. These findings highlighted the enzymatic power of *B. amyloliquefaciens* CPFD8 compared with that of *S. cerevisiae* 006-001 which correlated with the enhanced recovery of astaxanthin. Unlike *B. amyloliquefaciens* CPFD8, *S. cerevisiae* 006-001 lacks chitinase and lipase activity and secreted less than half of the protease activity, thus resulting in lower astaxanthin recovery. In the case of *B. amyloliquefaciens* CPFD8, the proteolytic activity rendered shell chitin accessible to chitinase. Therefore, astaxanthin was easily extracted from exoskeleton residue after pretreatment with *B. amyloliquefaciens* CPFD8 that could be attributed to protein and chitin hydrolysis. It has been believed that astaxanthin in shrimp shell and crustaceans is mostly bound to proteins, forming carotenoprotein or carotenolipoprotein complexes that appear as different colors in living organisms [29]. So, the proteolytic and lipolytic activity could enhance the release of astaxanthin. It has been reported that β-crustacyanin is the responsible pigment for the blue color in *C. sapidus* [73,74]. The binding of the carotenoid astaxanthin in the protein multimacromolecular complex crustacyanin is responsible for the blue coloration [75]. β-Crustacyanin is formed by two closely positioned astaxanthin molecules encapsulated in protein [76]. Thus, protease-producing *B. amyloliquefaciens* CPFD8 and *S. cerevisiae* 006-001 facilitate astaxanthin recovery by dual action. Their proteases deproteinize the shell allowing the penetration of solvents and degrade the protein molecules to which astaxanthin is bounded. In this context, various powerful extracellular enzymes from *B. amyloliquefaciens* strains such as protease, chitinase and lipase have been extensively reported [77–83]. In a similar study, astaxanthin was recovered from shrimp wastes using *Lactobacillus acidophilus* DSM 29,979 and *Streptococcus thermophilus* over submerged fermentation [84]. Likewise, astaxanthin was extracted from a shrimp waste using *Lactobacillus plantarum* and *L. acidophilus* [85]. In a recent study, enhanced astaxanthin extraction process from shrimp shell waste was conducted using ethyl acetate after the treatment with recombinant chitinase and protease [51]. Our study highlighted the role of chitinolytic and proteolytic microorganisms as potential candidates in microbial-assisted extraction of astaxanthin. It is worth to mention that the optimum conditions (40 °C, pH 8.0 and 3 days of incubation), more than 6-fold increase in astaxanthin yield was attained by pretreatment of the exoskeleton waste with *B. amyloliquefaciens* CPFD8 compared with that obtained by the traditional solvent extraction method.

In this study, the biological activity of the *B. amyloliquefaciens* CPFD8-mediated astaxanthin extract was investigated. The recovered astaxanthin exhibited remarkable antioxidant activity with feasible DPPH and ABST radical scavenging potential. It is worth mentioning that the recovered astaxanthin exhibited higher ABST radical scavenging potential

compared with ascorbic acid. These findings agree with previous literature reporting on the antioxidant activity of astaxanthin [30,86–90]. Several studies elucidated the unusual antioxidant activities of astaxanthin in vitro and in vivo. Astaxanthin is well-known powerful carotenoid antioxidant that efficiently removes active oxygen and inhibits the production of lipid peroxide caused by ultraviolet light [91]. It has been suggested that the strong antioxidant efficacy of astaxanthin is due to its unique chemical structure with long conjugated double bonds (polyene chain) [92]. It has been thought that the polyene chain of astaxanthin accomplishes the antioxidant activities by quenching singlet oxygen and scavenging radicals to terminate chain reactions [26]. In a recent study, astaxanthin exerted a potent protective effect against oxidative stress and exhibited obvious ability to counteract lipid peroxidation and ferroptotic cell death in neuroblastoma cell model [93]. In addition to its superior antioxidant merits, our investigation demonstrated the anti-inflammatory action of astaxanthin extracted from *C. sapidus* by the described microbial-assisted method. We observed impressively protective effects of astaxanthin on LPS-induced macrophage RAW264.7 cells with reduced NO, TNF-α, and IL-6 production compared with untreated LPS-induced cells. These findings concord with previous literature that showed a decrease in NO, TNF-α, and IL-6 levels upon treatment with astaxanthin [94–97]. Inflammation is a defense mechanism in response to harmful stimuli such as pathogens, damaged cells, and certain compounds to remove the injurious stimuli [98]. Unfortunately, chronic inflammation activated by the imbalance between the excessive production of proinflammatory mediators and the low production of anti-inflammatory mediators leads to several self-destructive conditions [99]. The intracellular messenger "NO" regulates some cellular functions, such as inflammation and pathogen elimination [100]. Nonetheless, excess NO reacts with superoxide (O_2^-) to produce a strong oxidant peroxynitrite ($ONOO^-$) that attributes to much of the cytotoxicity of NO [101]. Additionally, TNF-α and IL-6 are proinflammatory cytokines responsible for a broad spectrum of functions, including cytotoxic and cytostatic effects [102]. Thus, anti-inflammatory natural products such as astaxanthin could be used to alleviate the undesired inflammatory responses.

5. Conclusions

The findings of this investigation indicated the higher astaxanthin content of exoskeleton waste of *C. sapidus* compared with that of *P. pelagicus*. Among various investigated solvent systems, acetone/hexane (1:1 *v*/*v*) showed superior astaxanthin extraction efficacy. The developed microbial-assisted process involving the pretreatment of crab exoskeleton waste with chitinolytic and/or proteolytic microorganisms significantly enhanced the recovery of astaxanthin from the shells. Particularly, pretreatment of the waste with *B. amyloliquefaciens* CPFD8 yielded more than 6-fold astaxanthin compared with the traditional solvent extraction method. We showed the secretion of extracellular chitinase, protease and lipase *B. amyloliquefaciens* CPFD8 cultivated on crab exoskeleton waste as the sole carbon and nitrogen sources, which may be attributed to the digestion of chitin and proteins in the exoskeleton, facilitating the release and recovery of astaxanthin. The inferior activity of *S. cerevisiae* 006-001 in terms of astaxanthin recovery could be due to the lack of chitinase and lipase activity. The developed process recovered astaxanthin in an active form, possessing antimicrobial, antioxidant, and anti-inflammatory activity. These findings suggest the potential use of the developed microbial-assisted method utilizing chitinolytic and proteolytic microorganisms to maximize the recovery of bioactive astaxanthin from crustacean exoskeleton waste.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9060505/s1, Figure S1: Isolation and identification of chitinolytic and proteolytic *B. amyloliquefaciens* CPFD8. Figure S2: ^{13}C-NMR spectra of astaxanthin extracted from crab exoskeleton; Figure S3: HPLC chromatograms of astaxanthin extracted from crab exoskeleton.

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