

EXTRACTION OF ASTAXANTHIN ESTERS FROM SHRIMP WASTE BY CHEMICAL AND MICROBIAL METHODS

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ABSTRACT

The carotenoid pigments specifically astaxanthin has many significant applications in food, pharmaceutical and cosmetic industries. The goal of this research was the extraction of Astaxanthin from a certain Persian Gulf shrimp species waste (*Penaeus semisulcatus*), purification and identification of the pigment by chemical and microbial methods. Microbial fermentation was obtained by inoculation of two *Lactobacillus* species *Lb. plantarum* and *Lb. acidophilus* in the medium culture containing shrimp waste powder by the intervention of lactose sugar, yeast extract, the composition of Both and the coolage (-20°C). The carotenoids were extracted by an organic solvent system. After purification of astaxanthin with the thin layer chromatography method by spectrophotometer, NMR and IR analysis the presence of astaxanthin esters was recognized in this specific species of Persian Gulf shrimp. Results obtained from this study showed that the coolage at -20 °C not only does not have an amplifying effect on the production of astaxanthin but also slightly reduces this effect. Also the effect of intervention of lactose sugar showed more effectiveness in producing astaxanthin than yeast extract or more than with the presence of both. The results also indicated that there is not much difference in the ability of producing the pigment by comparing both *Lb. plantarum* and *Lb. acidophilus*. Also results showed the microbial method of extraction of astaxanthin is more effective than chemical method. The pigment extracted from certain amount of shrimp powder, 23.128 mg/g, was calculated.

Key words: Astaxanthin, extraction, shrimp waste, *Lactobacillus*

INTRODUCTION

Carotenoid pigments are the most important and numerous pigments that are found in nature. These compounds soluble in lipids are the factors that produce yellow-red color in plant and animal products. In this group of pigments astaxanthin has important applications in human and animal food industries specifically nutraceutical pharmaceutical and cosmetic industries. The Food and Drug Administration of the United States has permitted it for use in the aqua-cultural industry (Golkhoo, 2006). The main sources for this pigment can be found in many favorable sea foods, for example salmon, *Oncorhynchus mykiss*, sea bream, lobster and caviar. Also in birds like flamingo and quails, in microorganisms, insects, crustaceans and

micro-green alga (*Haematococcus pluvialis*) it can be found (Guerin, 2003). Astaxanthin has effects on many of these creatures' main body function like prevention from oxidation of essential unsaturated fatty acids, prevention from effects of ultraviolet light, Immunological reactions, pigmentation, communication, and reproduction. There is currently considerable interest in the role carotenoids in delaying or preventing degenerative diseases such as atherosclerosis, cancer, aging and eye diseases. The protective effect of astaxanthin was even more pronounced compared to β -carotene. Furthermore, a significant ($P < 0.001$) decrease in the incidence of induced colon cancer in rats fed with astaxanthin versus those administered only the carcinogen, was found. Dietary astaxanthin is also effective in fighting

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mammary tumors by > 50%, more than β -carotene and cantaxanthin. Astaxanthin inhibits the enzyme 5- α -reductase responsible for prostate growth and eventually prostate cancer. Astaxanthin's anti-cancer activity might be related to the carotenoid's role in cell communications at gap junctions, which might be involved with slowing cancer cell growth, the induction of xenobiotic-metabolizing enzymes or by modulating immune responses against tumor cells (Guerin, 2003). Among them, xanthophylls including astaxanthin and cantaxanthin have been shown to inhibit effectively bladder carcinogenesis without any toxicity through their inhibition of cell proliferation (Tanaka, 1997). The antioxidant activity of astaxanthin has been reported to be 10 times stronger than that of other carotenoids, namely zeaxanthin, lutein, canthaxanthin, and-carotene (Naguib, 2000). Carotenoids with no pro-vitamin A property are of interest for use as chemo preventives. Today with the use of methods like infrared spectroscopy-IR., mass spectroscopy- MS and optical rotary dispersion - ORD also nuclear magnetic resonance-NMR the number of carotenoids discovered are about 563 kinds (Martelli and Dasilva, 1993). An alternative method to de-proteinase crustacean's residues is using fermentation with Lactic acid bacteria. Lactic fermentation can also be a process to obtain carotenoids especially astaxanthin (Armenta-Lopez, 2002).

The objective of this study was the extraction of astaxanthin esters pigment by chemical and microbial methods and comparing them with each other regarding their effectiveness and also studying the effects of intervening factors on microbial extraction.

MATERIALS AND METHODS

All data reported in this study are from triplicate measurements.

Microorganism and culture media

Two species of *Lactobacillus* named *Lactobacillus plantarum* (PTTC 1058) and *Lactobacillus acidophilus* (PTTC 1643) were kindly provided from the microbiology laboratory of North-Tehran branch of Islamic Azad University. They were sub-cultured on MRS broth and MRS agar media (peptone from casein 10.0 g, yeast extract 4.0 g, meat extract 8.0 g, D (+)

glucose 20.0 g, tween 80 1.0 g, di-ammonium hydrogen citrate 2.0 g, sodium acetate 5.0 g, magnesium sulfate 0.2 g, manganese sulfate 0.04 g (MERCK). MRS Broth was mixed with 15 g/L agar to solidify the medium and incubated at 35-37°C in the presence of 5% CO₂ for 48-72h.

Preparation of shrimp waste

Shrimp waste from processing of *Penaeus semisulcatus*, comprising of head and carapace, was collected from a shrimp processing landing centers situated at Persian Gulf in south of Iran and transported to the laboratory under iced condition. The yield of dried shell was determined by weighing after dried at 50°C in oven for 24h. Samples were stored at two temperatures, of 25°C and -20°C until use. The material was thawed in running water before use and homogenized in a laboratory mixer.

Chemical extraction of astaxanthin

Astaxanthin was extracted by mixing 5g shrimp waste powder homogenate, 50 mL of hexane and 5 mg of glass beads and vortexed for 30 seconds, place in the 50°C water bath for 10 minutes. Aqueous and organic layers were separated by 3000 rpm for 5 minutes. This step repeat until the hexane is colorless. At the final step 6 mL of di-methyl sulfoxide (DMSO) was added to the tube and vortex vigorously and place in the water bath for 10 minutes and vortex again. Concentrated carotenoid was subjected to Thin Layer Chromatography (TLC) using silica gel 60 F MERCK TLC paper (Thomason, 1998).

Microbial extraction of astaxanthin

After adding 5 mL of MRS broth containing each of the two *Lactobacillus* sp. to the fermentative medium culture (100 mL distilled water + 10 g of shrimp waste powder and incubating for 3 days at 30°C, in the presence of 5% CO₂). The fermentative culture medium was filtered with Wattman filter paper No.41 and centrifuged at 3000 rpm for 5 minutes. Three groups of solvents (Group (1): petroleum ether: Acetone: Di-ethylamine (10:4:1), Group (2): Hexane: Acetone (3:1), Group (3): benzene: ethyl acetate (1:1)) were added to extraction astaxanthin. Concentrated carotenoid was subjected to TLC using silica gel 60 F MERCK TLC paper.

Optimization of conditions for carotenoid extraction

The conditions for extraction were optimized with the effect of a combination of process variables such as lactose sugar 1%, yeast extract 1%. The composition of lactose sugar 1%, yeast extract coolage (-20°C) and were added to fermentation medium and their interactions on the response variable was determined. Each at three equidistant levels, and the response variable was the carotenoid yield. In total, combinations of factors were used. The extraction of carotenoid and the determination of their concentration were carried out as explained earlier.

Purification of Astaxanthin by TLC

Concentrated carotenoid extract by chemical and microbial methods was subjected to TLC using silica gel 60 F MERCK TLC paper. Concentrated carotenoid extract was spotted on TLC plates along with standard astaxanthin and eluted with a mobile phase of petroleum ether: acetone: diethylamine (10:4:1), hexane: acetone (3:1), benzene: ethyl acetate (1:1) (Lorenz Todd, 1998).

Spectrophotometer assay

The synthetic and concentrated astaxanthin was dissolved separately in acetone and hexane. λ_{Max} was estimated with UV-VIS scanning spectrophotometer, UV 2101 pc, SHIMADZU by measuring the absorbance at 200 - 500 nm.

Infrared analysis

The fraction of TLC paper which had a fluorescence characteristic stronger than others and its Rf value being around astaxanthin Rf value was scrapped and dissolved in acetone and the peaks were obtained by IR assay (Nageswara Rao, 2005).

NMR analysis

The fluorescence fraction was obtained from the TLC was dissolved in acetone and the NMR spectrum was obtained. The NMR equipment had V: 300 MHz, B 0:7 T (Bruker) (Negewara Rao, 2005).

Statistical analysis

All determinations were carried out in triplicate. All data are expressed as mean \pm SD. Data were analyzed by an analysis of variance ($P < 0.05$)

and the means separated.

RESULTS

Extraction of astaxanthin esters from a certain Persian Gulf shrimp species waste (*Penaeus Semisulcatus*), purification and identification of this pigment by chemical and microbial methods was investigated. The total carotenoid and fractions were compared with synthetic astaxanthin by TLC. The solvent extracted carotenoid was in the form of a paste with an orange-red color. Results of TLC indicated that in the chemical method extract, the orange-colored astaxanthin forms the major band which migrates slowly. In microbial extraction method, the highest number of carotenoid fractions (in TLC method) from waste was obtained when the carotenoids were extracted with hexane, followed by solvent Group (2): hexane: acetone (3:1) (Table 1).

Table 1: Extraction of astaxanthin by 3 groups of solvents

Rf value	Group 1	Group 2	Group 3
Band 1	0.53	0.65	0.95
Band 2	-	0.75	-
Band 3	-	0.95	-

Group (1): petroleum ether: Acetone: Diethyl amine (10:4:1), Group (2): Hexane: Acetone (3:1), Group (3): benzene: ethyl acetate (1:1)). The highest number of carotenoid fractions (in TLC method) was obtained followed by solvent Group (2): Hexane: Acetone (3:1)

The lowest carotenoid yield was obtained with two other solvents, Groups 1 and 3. Rf values varied slightly using standards to confirm the carotenoids (Table 2) (Lorenz Todd, 1998). Results of TLC analysis indicated that in microbial extraction of astaxanthin nine to eleven different carotenoids bands were produced. These fractions were fluorescence property under UV cabinet at 366 nm. Results showed that microbial method of extraction of astaxanthin was more effective than chemical method. *Lactobacillus plantarum* (PTTC 1058) had ability to determined fractions in TLC method like *Lactobacillus acidophilus* (PTTC 1643). No significant difference was observed in carotenoid content between astaxanthin extraction by *Lactobacillus plantarum* (PTTC 1058) and *Lactobacillus acidophilus* (PTTC 1643) ($P < 0.05$) (Table 3).

Table 2: Rf values using standards to confirm the carotenoids (Lorenz, Todd, 1998)

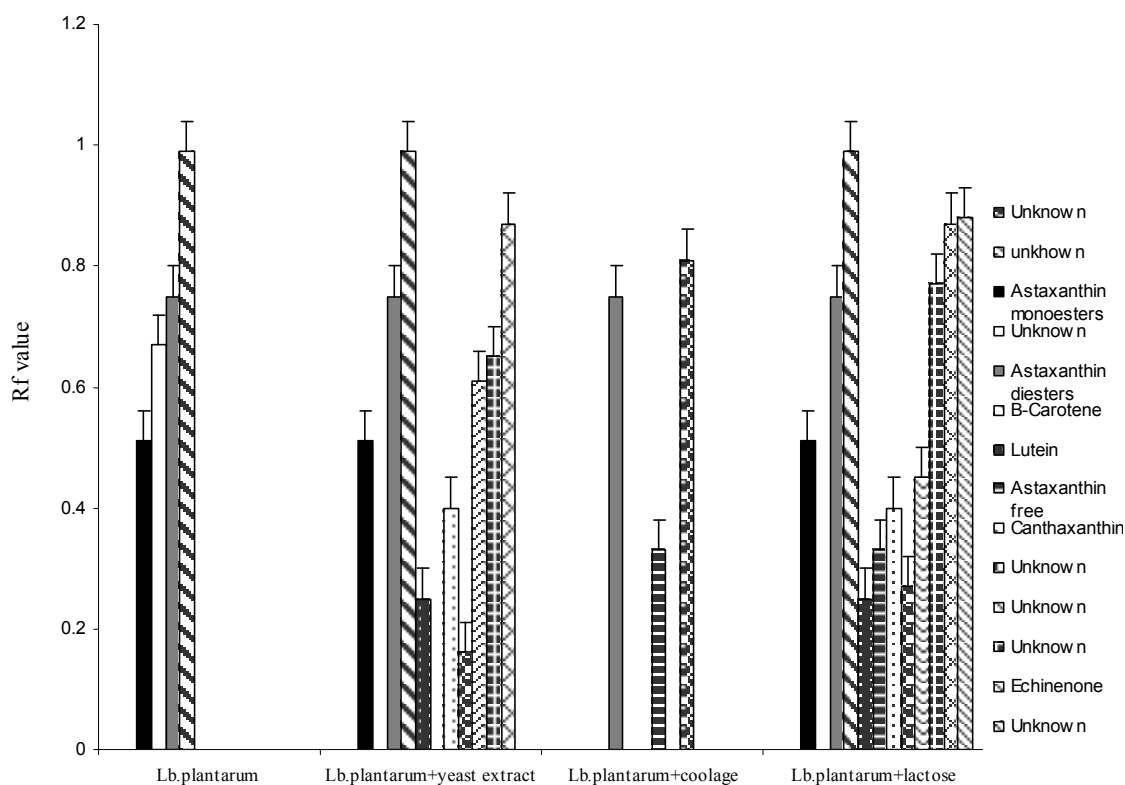
Carotenoid	Typical Rf value
β -carotene	0.99
Echinenone	0.87
Astaxanthin Di-esters	0.75
Astaxanthin Monoesters	0.50
Canthaxanthin	0.40
Astaxanthin Free	0.33
Lutein	0.25

Table 3: Microbial method of astaxanthin extraction

Bacteria	Rf value			
	Rf ₁	Rf ₂	Rf ₃	Rf ₄
<i>Lactobacillus plantarum</i> (PTTC 1058)	0.50	0.66	0.75	0.93
<i>Lactobacillus acidophilus</i> (PTTC 1643)	0.51	0.88	0.96	-

No significant difference was observed in carotenoid content between astaxanthin extraction by *Lactobacillus plantarum* (PTTC 1058) and *Lactobacillus acidophilus* (PTTC 1643) ($P < 0.05$)

Optimization of conditions for carotenoid extraction showed that, when lactose was added to fermentation medium, it gave a higher carotenoid yield than added yeast extract (Fig. 1). But the composition of both of them had a decreasing effect on the carotenoid yield. Results obtained from this study showed that the coolage at (-20°C) not only does not have an amplifying effect on the production of astaxanthin but also slightly reduces this effect (Fig. 1). IR spectrum result showed that, a characteristic absorbance at 1736 cm^{-1} was assigned to $\text{C}=\text{O}$ bond and the other peak in the spectrum belong to C-H, O-H and methyl groups were the same as in the molecule of pure astaxanthin. Results obtained from NMR analysis showed, a characteristic absorbance was assigned to methyl group in 2.07 ppm area, methyl-allyle groups at 1.21 ppm area and methyl-vinyl groups at 1.59 ppm area. Also O-H group and vinyl group were observed at 2.07 and 5.39 ppm area.

Fig. 1: Optimization of conditions for microbial carotenoid extraction by *Lactobacillus plantarum* (PTTC 1058) from shrimp waste.

DISCUSSION

Use of a mixture of polar and non-polar solvents for extraction of carotenoids from shrimp waste produces the highest yield. The extraction yield differed significantly ($p < 0.05$) between solvents. Solvent Group 2 gave significantly ($p < 0.05$) higher yield than Group 1 and 3. Britton *et al.*, (1985) recommended the use of water miscible polar organic solvents, usually acetone, methanol or ethanol, for extraction of carotenoids from tissues containing water. Delgado-Vargus *et al.*, (2000) discussed the advantages and disadvantages of various organic solvents for extraction of carotenoids and suggested that polar solvents are generally good extraction media for xanthophylls but not for carotenes. For wet tissues, use of non-polar solvents is not recommended as their penetration through the hydrophobic mass that surrounds the pigment is limited (Delgado-Vargus *et al.*, 2000). De Ritter and Purcell (1981) postulated that complete extraction of carotenoids from plant tissues could be achieved with samples of low moisture content by use of slightly polar plus non-polar solvents. Although the results obtained are for the waste from the species *Penaeus semisulcatus*, it would be applicable to waste from other species of shrimps. The residue available after carotenoid extraction may be used for the preparation of chitin/chitosan, thus having an integrated approach for efficient utilization of shrimp waste. Lactic fermentation is a simple and environmentally friendly method to extract highly unstable carotenoid pigments (Armenta-López, 2002). In this study, the pigment extracted from certain amount of shrimp powder 23.128 mg/g was calculated. Shahidi and Synowiecki (1991) reported that the carotenoid content in the shells of snow crab, *Chinocetes opilio*, was 14 mg/g. The carotenoid content in blue crab, *Callinectes sapidus*, was 4.63 mg/g (Felix-Valenzuela *et al.*, 2001). Thin-layer chromatographic separation of carotenoid extracts from *Penaeus semisulcatus* yielded eleven distinct bands at $R_f=0.33$ corresponded to astaxanthin, while yellow bands at $R_f=0.75$ and 0.99 corresponded to astaxanthin di-esters and β -carotene, respectively, as indicated by the TLC of standards. The orange bands at $R_f=0.51$, 0.40 , 0.87 and at 0.25 corresponded to

astaxanthin monoester, cantaxanthin, echinenone and Lutein respectively, as quoted in the literature (Lorenz Todd, 1998). The results indicated that astaxanthin, astaxanthin monoester and di-ester, and β -carotene are the major pigments in the *Penaeus semisulcatus*, while Lutein also could be separated from the shrimp waste extract using TLC. The NMR and IR methods of carotenoid extracts indicate that astaxanthin and its esters were the major carotenoids in the extract from *Penaeus semisulcatus*. The total content of astaxanthin and its esters was 23.128 mg/g. Astaxanthin and its esters have been found to be the major carotenoids in the marine crustaceans (Shahidi *et al.*, 1998). In the marine crab, accumulation of astaxanthin, β -carotene and zeaxanthin has been reported (Matsuno, Watanabe, and Nagata, 1974). Zeaxanthin and lutein were found to be major pigments in fresh water mullets (Matsuno, Nagata, and Chiba, 1975). Matsuno and Maoka (1988) reported that astaxanthin contributes 33–39 g/100 g of total carotenoids in meat and shell of marine crab *Paralithodes brevipes*, from Japanese waters. The present study indicates that the marine shrimp, *Penaeus semisulcatus*, accumulates astaxanthin esters, as major carotenoid and microbial method of extraction of astaxanthin is more effective than chemical method.

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