Role of Microalgae Pigments in Aquaculture

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Photosynthetic organisms are the primary producers containing organic pigments for harvesting light energy. There are three major classes of microalgal pigments, Chlorophylls, Carotenoids and Phycobilins. Chlorophylls are green pigments and Carotenoids are yellow or orange pigment, which is lipophilic and associated in chl-protein complexes while phycobilins are hydrophilic. In nature, carotenoids are produced principally by plants and their microscopic relatives, the microalgae. Animals, including humans, are not able to synthesize carotenoids thus ultimately they must obtain these pigments from the plants and microalgae. The basic structural elements of carotenoids are two hexacarbon rings joined by 18 carbon conjugated double bound chain. They are usually hydrocarbons (xanthophylls, e.g. lutein, violaxanthin, zeaxanthin, fancoxanthin, Peridinin). Some pigments in microalgae do not transfer excitation energy. These groups are called secondary careteniods e.g. Orange red coloured pigments (xanthophylls, astaxanthin and canthaxanthin) and they are over produced in some algal species when grown in unfavourable conditions. Secondary carotenoids accumulation is the main characteristic of many algae when growing under nitrogen limited conditions, which is often accompanied by a decrease in the chlorophyll content of the cell. Most carotenoids are polyunsaturated hydrocarbons, containing 40 carbon atoms, and comprising two terminal ring systems. Carotenoids that are composed entirely of carbon and hydrogen are known as carotenes, while those that contain oxygen are termed xanthophylls. The carotenoid fraction of the microalgae contains about 70 per cent monoesters of astaxanthin, 10 per cent detesters of astaxanthin, 5 per cent free astaxanthin, and the remaining 15 per cent consists of a mixture of β-carotene, canthaxanthin, lutein and other carotenoids that are also beneficial as antioxidants and provitamin - A activity.

Carotenoids have several roles in the microalgae photosynthesis apparatus functioning as,

i) Accessory light harvesting pigments transferring excitation to Chl a pigment.

ii) Structural entities within the light harvesting and reaction centre pigment protein complexes.

iii) Molecules required in the protection against excess irradiance, chlorophyll triplets and reactive oxygen species.

Within the various classes of natural pigments, the carotenoids are the most widespread and structurally diverse pigmenting agents. They are responsible, in combination with proteins for many of the brilliant yellow to red colours in plants and the wide range of blue, green, purple, brown and reddish colours of fish and crustaceans.

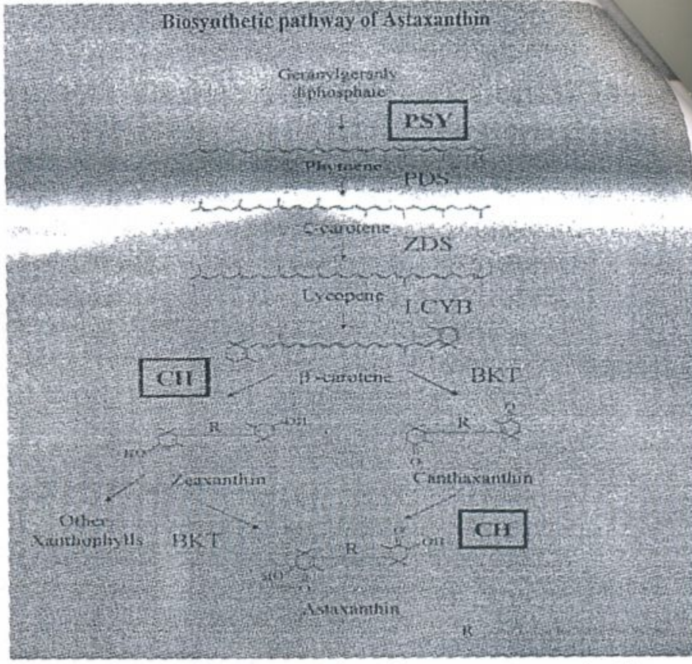
Astaxanthin (asta-ZAN-thin): Astaxanthin (3, 3-dihydroxy-a) is a group of natural pigments known as carotenoids. Carotenoids are a family of over 600 natural lipid-soluble pigments that are produced within microalgae, phytoplankton and higher plants. Astaxanthin is omnipresent in the nature, especially in the marine environment. Astaxanthin was first isolated and identified from lobsters in 1938. Since that time it has been found in a diverse array of animal such as birds, shrimp, crabs, crawfish, sea bream, plants and nearly all Salmon (Coho, Atlantic, pink, chum, Chinook and trout).

Astaxanthin is a symmetric molecule consisting of a short ring of polyene terminated by rings. The keto (O) and hydroxyl (OH) groups distinguish astaxanthin from other carotenoids such as β carotene. These pigments are found in the cytoplasm of microalgae or phytoplankton. In the natural aquatic environment, astaxanthin is biosynthesised in the food chain within microalgae or phytoplankton at the primary production level. Micro algae are consumed by zooplankton, insects or crustaceans, which accumulate astaxanthin, and in turn are ingested by fishes, which accumulate as astaxanthin in fish flesh. Astaxanthin is probably best known for elicit the pinkish-red colour in the flesh of salmon and trout, as well as shrimp, lobsters and crayfish etc.

Natural Astaxanthin from Microalgae: Natural sources of astaxanthin are numerous but nearly all are found in very low concentrations. Green alga *Haematococcus pluvialis* also referred as *Haematococcus lacustris* or *Sphaerella lacustris*, order of Volvocales, family Haematococcaceae is a rich source of astaxanthin, which accumulates as high as 10 to 30 g of astaxanthin per kg of dry biomass. *Haematococcus* is a motile alga about 20 - 30 um (micron) in size, which utilizes the available nitrate, phosphate and other nutrients to grow and reproduce. The alga occurs in nature world wide, where environmental conditions for its growth are favourable protective roles for astaxanthin. *Haematococcus* algae has a number of different forms during its life cycle and is normally found in ephemeral pools of fresh water where temperatures are cooler. Massive amounts of astaxanthin are produced when the cells undergo a dormant stage until the next influx of water and nutrients. Under these conditions Cells can remain viable in this encysted stage for decades. Japanese regulatory agencies have approved *Haematococcus* algae as a natural pigment for foods as well as in Aqua feeds. Advanced technology was developed to grow *Haematococcus* algae using pure culture conditions. The algae is cultivated employing a proprietary closed culture technology known as PhytoMax PCS (Pure Culture System) which automatically regulates cell culture conditions before transfer to open ponds for the final stage of astaxanthin production.

Other source of astaxanthin: Astaxanthin has been extracted from processed crustacean wastes of krill, shrimp, crab and crawfish. These extracts have been used in the diets of farm-raised salmon and trout, since salmonids cannot themselves produce the red carotenoid found in the muscle. However, crustacean waste products (oils and meals) generally contain less than 1000 ppm of astaxanthin. Furthermore, crustacean sources contain high amounts of moisture, ash, fluoride and chitin which limit the percentage of these products that can be included in salmonid feeds.

Biosynthetic pathway of Astaxanthin: The biosynthesis of astaxanthin is one of the most expensive molecules in metabolite terms which an algae or plant cell ever produces. Carotenoids participate as secondary light-harvesting pigments in the photosynthetic process of plants. Carotenoids are synthesized through the isoprenoid biosynthetic pathway, which is also responsible for such diverse compounds as prostaglandins, steroids, sterols, vitamins A, D, E and K. The pathway initiates at acetyl-CO-A and proceeds through phytoene, lycopene, β-carotene, and canthaxanthin before the last oxidative steps to astaxanthin. The astaxanthin molecule has two asymmetric carbons located at the 3 and 3" positions of the benzenoid rings on either end of the molecule. The long system of



double bonds between the ring structures is the conjugated polyene system, and plays important roles in the antioxidant features of the molecule. Astaxanthin, OH Carotenoids with these hydroxy-, keto-, methoxy-, epoxy-, or carboxyl- groups are collectively called "xanthophylls". Whereas unsubstituted carotenoids are called "carotenes", exemplified by beta-carotene. Fatty acids are estericed to the 3 or 3' hydroxyl groups, resulting in mono and diesters of astaxanthin. Esterified astaxanthin is more soluble in the cellular environment and inherently more stable to oxidation, thus it makes sense that the esterified form is the predominant type found in nature. Complexes of carotenoids and proteins generally called "carotenoproteins" are widely found in crustaceans. Astaxanthin is a red carotenoid pigment, but when complexed with various proteins, the light absorbance shifts and causes crustaceans to range in colour from green, yellow, and blue to brownish. The red colour of cooked crustaceans is actually produced when these proteins denature from the heat and release astaxanthin from their protein group.

Astaxanthin in fishes: Coloration plays a role in social structure as well as defence of fishes. Flesh colors of fish are predominantly dependent on the presence of special cells in the skin called chromatophores. These contain pigments or light scattering or light-reflecting organelles. In biology, any substance that can impart color to the tissues or cells of animals or plants can be called a pigment. There are four main groups of pigments that can be used to provide color in these cells in fishes: melanins, carotenoids, pteridines and purines.

i) Melanins are responsible for the dark coloration seen in fishes.

ii) Carotenoids, which are lipid soluble, dominate in giving the yellow to red colors. In fishes flesh (muscle), the carotenoids are the dominant pigment. Carotenoids are the major pigmenting compounds and cannot typically be synthesized by fish. The carotenoid (astaxanthin) found in wild salmon is used in aqua feeds to impart this natural, pink-red color to farmed salmon fillets. In contrast, most other pigmenting compounds can be made by the fish.

iii) **Pteridines** are water-soluble compounds and result in bright coloration like the carotenoids. Pteridines play a small role in coloration when compared to carotenoids.

iv) Purine compounds / guanine predominates are large amounts of guanine found in the silvery belly skin of most species of fishes. These basic compounds can be combined with other components like proteins, to produce the blue, violet, and green colour ranges seen in fishes.

Astaxanthin are used in aquaculture feeds to provide the colour associated with consumer products, such as the bright vibrant colours. Astaxanthin has biological functions related to growth, reproduction and tissue health in fishes and crustaceans, possibly due to the compound's strong antioxidant properties (Bell et al., 2000). Recent groundbreaking study in Norway by Christiansen and his colleagues demonstrated that Atlantic salmon fry have a growth and survival requirement for astaxanthin either in their diet or passed vertically from their mother. A recent study conducted by the US Food and Drug Administration assessed the astaxanthin concentration in a large variety of wild salmon species. This survey showed that the average astaxanthin concentrations ranged from 5-40 ppm of flesh.

Table 1: Astaxanthin ranges in fish muscle (Free esterified, 3 S-3' Isomer)

Species	Astaxanthin range	Astaxanthin average
Sockeye salmon	30 - 58 mg/kg	40.4 mg/kg
Coho salmon	9 - 28 mg/kg	13.8 mg/kg
Pink salmon	3 - 7 mg/kg	5.4 mg/kg
Chum salmon	1 - 8 mg/kg	5.6 mg/kg
Chinook King salmon	1 - 22 mg/kg	8.9 mg/kg
Atlantic salmon	5 - 7 mg/kg	5.3 mg/kg

(March, B E *et al.*, 1999)

Astaxanthin in marine crustaceans

Body colour of crustaceans is dependent on the qualitative and quantitative presence of carotenoids in the hypodermal chromophores and the pigmented layer of the epidermal exoskeleton. In lobsters and shrimps, astaxanthin is attached to a protein to produce the carotenoprotein and crustacyanin. This carotenoprotein imparts a blue colour in living Crustacea. In the presence of heat the carotenoprotein molecule is cleaved, which subsequently results in the characteristic astaxanthin red colour of cooked lobsters and shrimps. Most crustaceans contain a mixture of carotenoids in the carapace in addition to the blood, eyes, mid-gut gland, and ovary. The red carotenoid, Astaxanthin is the principal pigment in the carapace and internal organs of Penaeus shrimp species, accounting for 86 - 98 per cent of the total carotenoids. The majority of the astaxanthin within the epidermal tissue is in the mono-esterified form, meaning that one of the hydroxyl groups is esterified to a fatty acid, whereas, complexes of carotenoids and proteins called carotenoproteins and carotenolipoproteins dominate in the exoskeleton. Astaxanthin appears as a red pigment, but when complexed with various proteins, the light absorbance shifts and cause crustaceans to range in colour from green, yellow, blue to brown. Thus despite the fact that astaxanthin is the chromophore prosthetic group of the different carotenoproteins, many colours can be achieved. The red colour of cooked crustaceans is produced by the release of the individual carotenoid prosthetic group (astaxanthin) from the carotenoproteins.

Pigment deficiency in crustaceans

Blue Colour Syndrome

A nutritional deficiency of astaxanthin in the diet causes **Blue Colour Syndrome** in cultured *Penaeus monodon*. Studies of Blue Disease in farmed tiger shrimp *P. monodon* attribute the nutritional deficiency of carotenoids. Upon cooking, shrimp with Blue Disease assume a pale yellow colour rather than the bright red coloration characteristic of wild shrimp (Howell and Matthews, 1991). The effect of dietary carotenoids on pigmentation has been studied by feedig 100 ppm of various carotenoids. After four weeks of feeding a diet containing 50 ppm of astaxanthin, prawn with Blue Colour Syndrome resume their normal greenish brown pigmentation. Survival was higher in prawns fed with the astaxanthin diet. A positive correlation between survival and pigment concentration of tissues suggested that the carotenoids functioned as an intracellular oxygen reserve. Analysis of the tissues from the experimental groups verified that the astaxanthin-fed group increased in carotenoids 31.8 per cent, and had a normal appearance (Menasveta et al. 1993).

Pigment Deficiency Syndrome (PDS)

Degradation is associated with a loss of pigmentation and bleaching of the ovaries of mature females and larval egg yolks. This leads to low larval feeding rate in Z1, high levels of larval Z1 deformities and much reduced survival to larval stage zoea II. This condition has been termed "Pigment Deficiency Syndrome" (PDS). The use of carotenoids as pigments in aquaculture species is well documented and it appears their broader functions include a role as an antioxidant and provitamin-A activity as well as enhancing immune response, reproduction, growth, maturation and photoprotection.

Requirements of Astaxanthin

Astaxanthin is approximately 10 times stronger than other carotenoids including β-carotene, in terms of antioxidant activity and 100 times greater than vitamin E (alpha-tocopherol). Astaxanthin has strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen and has been proposed as the "super vitamin E". Additional benefits of this essential carotenoid include roles as an antioxidant and provitamin A activity, as well as enhancing immune response, reproduction, growth, maturation, photoprotection, and defense against hypoxic conditions common in pond cultures. Therefore, astaxanthin must be available in either their native habitat or manufactured diet to meet metabolic nutritional requirements. Astaxanthin is the optimal carotenoid for proper pigmentation of *Penaeus* shrimp. To significantly improve nauplii quality and Zoea survival, broodstock should be supplemented with 150 ppm of astaxanthin. In shrimp feed 75 - 150 ppm of astaxanthin (30 - 40 mg/kg) two months prior to harvest and 50 - 100 ppm astaxanthin during the entire culture period gives a good result for success culture.

Presently, combinations of krill oil / meal, crawfish oil, *Phaffia* yeast, and synthetic astaxanthin are used as carotenoids in shrimp feeds. Crawfish have low astaxanthin concentrations of 1500 ppm in the oils and *Phaffia* have 4000 ppm but *Haematococcus* algae have 10,000 - 30,000 mg/kg. An extensive body of data stresses the vital role of carotenoids in the physiology and overall health and concludes that carotenoids are essential nutrients that should be included in all aquatic diets at a minimum level of 5 - 10 ppm (Katayama T.,, 1971).

Quantification of microalgae pigments

Quantification of chls and carotenoids pigments are extracted in organic solvents (methanol, ethanol, acetone etc). The absorbance of the extract is determined spectrophotometrically and the pigment content is calculated. The separation and quantification of individual carotenoids can be achieved using high performance liquid chromatography equipped with an absorption or fluorescence detector.

Table 2. Quantification of Microalgae pigments

Pigments	Classes	Colour	Absorb value (nm)
Chlorophyll	Chl a, Chl b, Chl c	Blue or blue green	450-475 nm
		Red	630-675 nm
Carotenoids	Xanthophylls, Astaxanthin Canthaxanthin	Orange red colour	400-550 nm
Phycobilins	Phycoerythrobilin,		Blue green
	Phycocyanobilin	yellow or	
	Phycourobilin	orange light	500-650 nm

Conclusion

A different formulation of microalgae astaxanthin has already gained wide acceptance in the aquaculture markets as a pigmentation and vitamin source for salmon, trout, shrimp and ornamental fish. Researches also indicate additional benefits from dietary carotenoids beyond their sulting coloration and dramatically improve nauplii quality and zoea survival of shrimp. ***Haematococcus*** algae are now commercially available as a human supplementation form and provide the richest natural source of astaxanthin in the world. Research findings indicate that no adverse effects on health were observed from ***Haematococcus*** algae meal as the dietary source of astaxanthin. Synthetic astaxanthin is also produced by some companies through complex chemical reactions. However, it is not the same form as found in nature. It also has important metabolic functions in animals and man, such as conversion to vitamin A, enhancement of the immune response and protection against diseases, by scavenging of oxygen radicals. This shows a wide scope in the algal culture field and towards its further applications. ■

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**** More references may be had on request***