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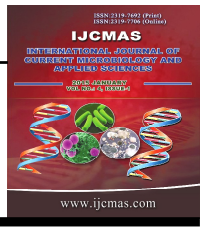

Original Research Article

Production Characterization and Aqua Feed Supplementation of Astaxanthin from *Halobacterium salinarium*

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Keywords

Astaxanthin, *Cyprinus carpio, Halobacterium salinarium,* Live stock

A B S T R A C T

Halobacterium salinarium is an obligate halophilic archaeon highly adapted to environments of extremely high salinity. *Halobacteria* produce large quantities of red-orange carotenoids. Carotenoids are a class of natural fat soluble pigments which produced orange-red colour. Astaxanthin is the main carotenoid extracted from *Halobacterium salinarium*, which cannot be synthesized by animals and must be provided in the diet. It is important to produce protein rich food in an aquatic system to improve the growth and bioenergetics of an aquatic species. In this study, astaxanthin was used as a supplement for aquafeed and tested against bioenergetics of the ornamental fish *Cyprinus carpio.* The weight, length of the *Cyprinus carpio* fed with astaxanthin is increasing with increasing content of halobacterial astaxanthin supplementation.

Introduction

Halophiles are salt loving organisms that grow best at the highest salinities (3–4.5 mol L^{-1} Nacl) forming dense blooms and resulting in the red colour of many brines. Common species of halobacteria are rod, cocci, or disc shaped, although triangular and even square shaped species exist. Halobacteria are classified as archea and belongs to the family halobacteriaceae. Ten genera have been reported, *Halobacterium, Haloarcula, Halococcus, Haloferax, Halorubrum, Halobaculum, Natronobacterium, Natranococcus, Natrialba* and *Natromonas* and an eleventh genus *Haloterrigena* has recently been proposed (Dym *et al.,* 1995).

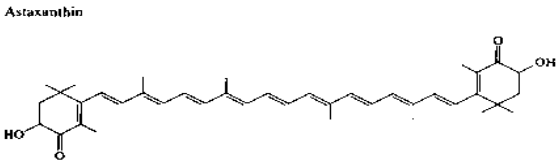
The first microbiological analysis was conducted on several closely related *Halobacterium* strains (originally designated as *H. salinarium, H. halobium, H. cutirubrum)* isolated in the mid-twentieth century from salted fish and meat from North America.

Halobacterium salinarium is an obligate halophilic archaeon highly adapted to environments of extremely high salinity. *Halobacterium salinarium* species primarily

inhabit thalassohaline salt lakes and solar salterns, thereby exhibiting optimal growth in the presence of NaCl concentrations between 2M and saturation. *Halobacterium salinarum* is an aerobe chemoorganotroph growing on degradation products of other organisms (Ng *et al.*, 2000).

Halobacteria produce large quantities of red-orange carotenoids. 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 colour in plant and animal products. In this group of pigments astaxanthin has important applications in human and animal food industries specifically pharmaceuticals and cosmetic industries. The Food and Drug Administration of the United States has permitted it for use in the aquaculture industry (Golkhoo *et al.*, 2006).



Molecular formula: $C_{40}H_{52}O_4$

Common name: 3,3'-dihydroxy-β-carotene-4, 4'- dione

Astaxanthin is the main carotenoid found in aquatic animals (Miki, 1991). This red orange pigment is closely related to other well known carotenoids such as β-Carotene, Lutein.

In many of the aquatic animals in which it is found, astaxanthin has a number of essential biological functions, ranging from protection against UV light, immune response, pigmentation and communication to reproductive behavior and improved reproduction (Meyer *et al.*, 1993).

Astaxanthin has antioxidant potency 1,000 times greater than Vitamin E and 10 times greater than any known carotenoid. It also stimulates and improves the immune response by enhancing immunoglobulin production in human blood cells.

Astaxanthin cannot be synthesized by animals and must be provided in the diet. Mammals lack the ability to synthesize astaxanthin or convert dietary astaxanthin into vitamin A; unlike β-carotene, astaxanthin has no provitamin A activity in these animals (Jyonouchi *et al.*, 1995).

Aquaculture is broadly defined as the farming of aquatic organisms such as prawns, mollusks, seaweeds, algae in addition to various fishes. In India Mumbai, Mysore, Bangalore and Kolkata aquarium are made in which ornamental fishes are cultured (Shamini and Bhatnagar, 2002).

Aquaculture is currently one of the fastest growing food production systems in the world. This is emerged as an industry and now possible to supply protein rich food throughout the world

In this present investigation the astaxanthin produced by marine bacterium *Halobacterium salinarium* was used as a supplement for aqua feed and tested against bioenergetics of the ornamental fish *Cyprinus carpio.*

Materials and Methods

Isolation of marine soil sample

The marine *Halobacterium salinarium* was previously isolated by primary selection process from the marine soil sample collected from saltpan region of Mukkani, Tuticorin District by serial dilution and pour plate technique.

Identification of the *Halobacterium salinarium*

The pure culture of *Halobacterium salinarium* was identified based on their morphology and colony characteristics. The organisms were maintained on Halophilic agar medium or Zobell marine agar medium and stored at 4°C (May, 2000).

Pure cultures of *Halobacterium salinarium* was selected and screened for astaxanthin pigment production in *Halobacterium salinarium* selective medium

Optimization of culture conditions

An attempt was also made to determine the optimum culture conditions for *Halobacterium salinarium* such as pH, temperature requirements for their maximum pigment production. Five different cultivation medium with different pH (5, 6, 7, 8 and 9) different temperature (30°C, 35°C, 40°C, 45°C and 50°C) and NaCl concentrations (5%, 7%, 10%, 15%, and 20%) were used and extracellular pigment production of the selected strain was recorded.

Extraction of astaxanthin pigment produced by *Halobacterium salinarium*

Pigment was extracted from the selected strain of *Halobacterium salinarium* by centrifugation at 8000rpm for 10mins. The precipitate was then resuspended in distilled water. After a short period, cellular lysis was occurred.

Later, the direct extraction of pigment with acetone was done (Calo, 1995). The pigment obtained was used for the further spectrophotometric and chromatographic analysis.

Spectrophotometric assay of astaxanthin

A separating funnel containing extracted pigment astaxanthin with acetone solution and 0.5 times of petroleum ether as well as two milliliters of a solution of saturated NaCl solution. The petroleum ether phase was collected after removal of the acetone layer, which was reextracted with acetone and the absorbance was read at 471nm. Astaxanthin composition was calculated by the following formula, using 1% extinction coefficient = 2100 (Weber, 1990).

$$\text{Extracted astaxanthin } (\mu g/g \text{ of bacterial cell}) = \frac{\text{ml. of petroleum ether} \times A_{471} \times 100}{21 \times \text{bacterial dry weight}}$$

Thin-layer chromatography

The broth culture medium was filtered and centrifuged at 3000rpm for 5 minutes. Three groups of solvents; Group1 Petroleum ether: Acetone: Diethylamine (10:4:1) Group 2 Hexane: Acetone (3:1) Group 3 Benzene: Ethyl acetate (1:1) were added to the extracted astaxanthin. Concentrated astaxanthin extract was spotted on a TLC plates and eluted with a mobile phase of hexane: acetone (3:1) The plate was then allowed to run approximately three inches above the sample line and then it was removed (Lorenz Todd, 1998). The R_f value can be calculated using the following formula

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

HPLC, NMR and FTIR analysis

The spot obtained from thin layer chromatography was redissolved in acetone and was subjected for HPLC, NMR and FTIR analysis then the results were recorded.

Astaxanthin feed supplementation and preparation

For the present study, the control and experimental feed were formulated by the following method described by New (1987). The experimental feed was prepared by using astaxanthin as a source of protein with 8 different concentrations such as 0.4g, 0.8g, 1.2g, 1.6g, 2.0g, 5.0g, 10.0g and control feed (devoid of astaxanthin). During the culture period of 40 days, feeding was done once in daily.

Biochemical analysis of feed

Protein is estimated by Biuret method (Lowry *et al.,* 1951) and carbohydrate is estimated by Phenol-Sulphuric acid method (DuBois *et al.* (1956). Bio energetics also estimated by the following formulae:

$$\text{Assimilation efficiency (AE)} = \frac{\text{Assimilation (A)}}{\text{Food consumption (C)}} \times 100$$

Gross growth efficiency (KI) = P/CX100

Net conversion (or)
net growth efficiency (K2) = P/AX100

$$\text{Assimilation efficiency (AE)} = \frac{\text{In. Final weight} - \text{In. Initial weight}}{\text{Experimental period (days)}} \times 100$$

$$\text{Food conservation ratio (FCR)} = \frac{\text{Dry food consumed}}{\text{Wet weight gain}}$$

Results and Discussion

The marine soil sample was collected from saltpan region of Mukkani, Tuiticorin District, the marine *Halobacterium* was isolated. The isolated culture was identified as *Halobacterium salinarium* due to its capability to produce the red orange coloured colony in the *Halobacterium salinarium* selective medium. The red orange colour colony produced on the halophilic medium indicates that the organism has ability to produce the astaxanthin. Among the five different media used in the present study, the maximum astaxanthin production was obtained in *Halobacterium medium-1*. The optimum condition for the production of astaxanthin was 35°C and at the pH 7.

An orange colour spot with R_f value of 0.50 observed through thin layer chromatography. The UV- spectral analysis showed that the maximum absorption at 471nm which indicates the astaxanthin pigment (Fig. 1). The HPLC analysis showed, the retention time at 3.685, it confirmed the astaxanthin pigment (Fig. 2). The NMR analysis revealed that the data for protons present in ($C_{40}H_{52}O_4$) astaxanthin (Fig. 3). The FTIR analysis revealed that the data for the functional groups of the astaxanthin pigment (Fig. 4).

Among the eight formulated diets, the protein and carbohydrate content was found maximum in the astaxanthin supplementary feed (T_7). The protein and carbohydrate content was found maximum in the *Cyprinus carpio* fed with astaxanthin supplementary feed (T_7) when compared with control. The fish growth characteristics such as length, weight, assimilation, metabolism, specific growth rate, gross growth efficiency, net growth efficiency and consumption efficiency in *Cyprinus carpio* were found better in the ten gram of astaxanthin supplementary diet fed to the animal than in control (Table 1). In the present study, it was found that the single source of astaxanthin enriched diet is found better FCR (1.79) could be used as alternative feed supplement.

Table.1 Bioenergetics of Fish Cyprinus carpio after treatment with Experimental Feed

Diets	Initial weight	Final weight	Production	Mid body weight $W=W_2-W_1/2$	Food consumed (C)	Faecal output (mg) F	Assimilation A= C-F	Metabolism R=A-P	Specific growth rate (%) $SGR=W_2-W_1/20\times 100$	Assimilation efficiency (%) AE=A/C× 100	Gross growth efficiency (%) $K_1=P/C\times 100$	Net growth efficiency (%) $K_2=P/A\times 100$	Consumption efficiency (%)= C/W/10× 100	FCR=Dry food consumed/Wet weight gain
T_1	5.15	5.45	0.30	0.150	0.36	0.050	0.310	0.010	1.50	66.66	83.33	103.33	24.00	2.18
T_2	5.19	5.47	0.28	0.140	0.40	0.090	0.310	0.030	1.40	68.50	70.00	90.32	28.00	2.17
T_3	5.23	5.50	0.27	0.135	0.96	0.129	0.831	0.561	1.35	86.56	28.12	32.49	38.50	2.06
T_4	5.38	5.93	0.55	0.275	1.06	0.206	0.854	0.304	2.75	80.56	51.88	64.40	43.65	2.09
T_5	5.60	6.30	0.70	0.350	1.64	0.236	1.404	0.704	3.50	85.60	42.68	49.85	46.82	2.00
T_6	5.67	6.40	0.73	0.365	2.36	0.243	2.117	1.387	3.65	89.70	30.93	34.48	65.56	1.86
T_7	6.02	7.60	1.58	0.790	3.45	0.296	3.154	1.574	7.90	92.76	45.79	50.09	48.65	1.79
T_8	5.24	6.23	0.99	0.495	1.16	0.120	1.040	0.050	0.19	89.65	85.34	95.19	23.04	1.65

T1-Experimental feed + 0.4g of astaxanthin, T2-Experimental feed + 0.8g of astaxanthin, T3-Experimental feed + 1.2g of astaxanthin, T4-Experimental feed + 1.6g of astaxanthin, T5-Experimental feed + 2g of astaxanthin, T6-Experimental feed + 5g of astaxanthin, T7-Experimental feed + 10g of astaxanthin, T8-Control feed devoid of astaxanthin (Trio).

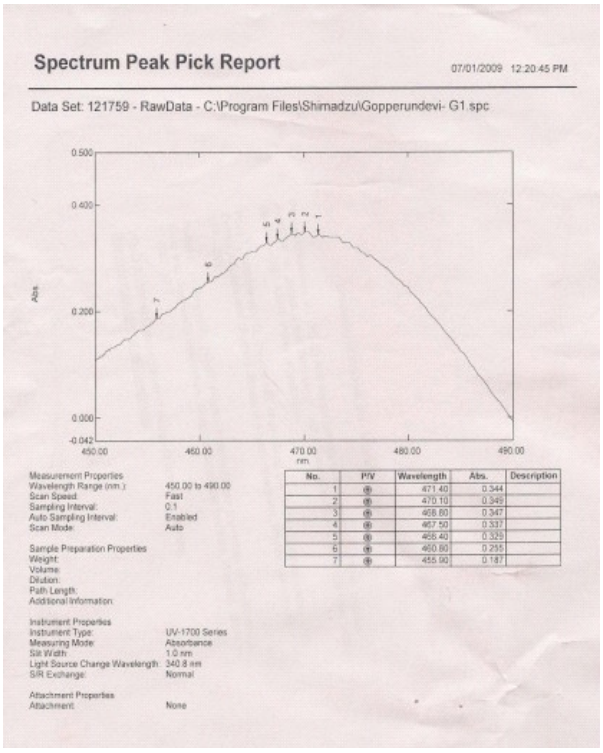


Fig 1 : UV Spectral Analysis of Astaxathin

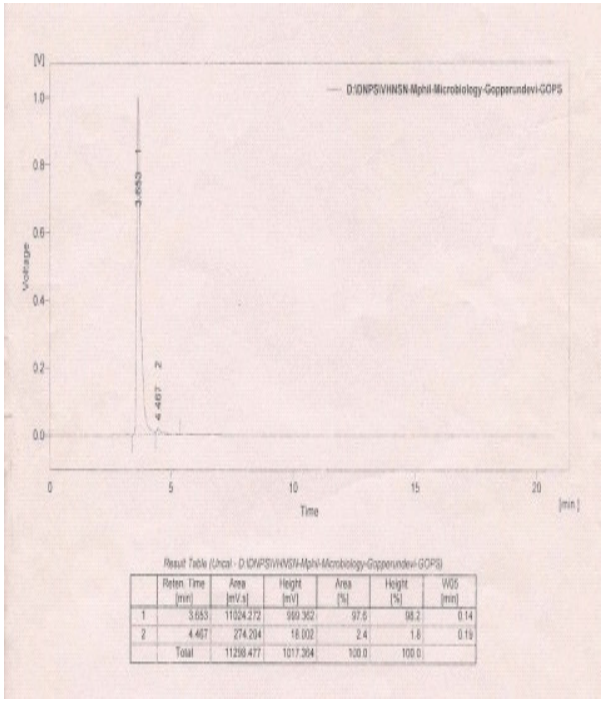


Fig 2 : HPLC Analysis of Astaxathin

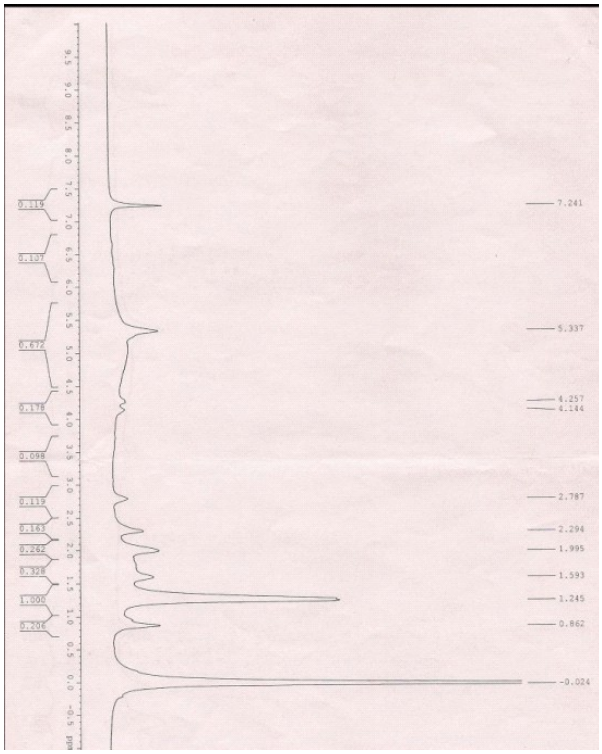


Fig 3 : H - NMR Spectral Analysis of Astaxathin

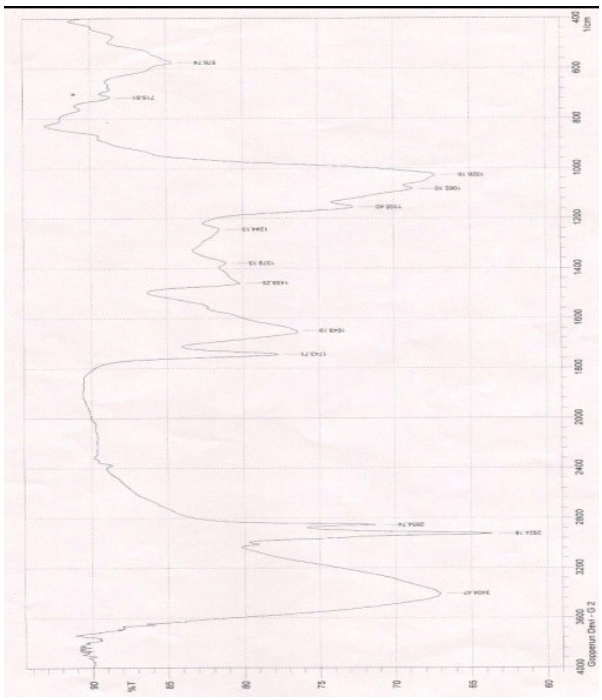


Fig 4: FTIR Analysis of Astaxathin

The present investigation reveals that the culture condition of *Halobacterium salinarium* significantly enhances high production of astaxanthin in high salt concentration. The feed supplemented with 10gms of astaxanthin increases the growth and bioenergetics of the test organism *Cyprinus carpio.* The obtained results which are similar to the previous study of astaxanthin production by an extremely halophilic archea bacteria isolated from high salt fermented Thai foods (May and Paukatong, 2000).

The astaxanthin produced by *Halobacterium salinarium* was subjected for characterization by number of analytical methods such as UV, HPLC, NMR, and FTIR. Hentschel *et al.* (2006) reported that the dry mass showed maximum absorption between 460–477 nm. The present study showed that the maximum absorption was obtained at 471nm. The results revealed that the organism produces the respective pigment astaxanthin.

The study on effect of formulated diets supplemented with astaxanthin at various concentrations of 0.4, 0.8, 1.2, 1.6, 2, 5, 10g per 100g of ingredients. According to the results, the weight and length of the *Cyprinus carpio* fed with astaxanthin is increasing with increasing content of astaxanthin supplementation. It is recommended from the present study that, astaxanthin could be used for the growth of the of the *Cyprinus carpio*. Wallat (2002) reported that, commercial feeds are continually modified, as they are subject to least-cost formulations, new research, and updates on nutrient requirements of fish. The present study suggests that the astaxanthin can be used as supplementary feed for aquaculture and improves their production. Feed supplement gained wide acceptance in livestock production and they are very applicable in aquaculture production systems. Fish fed with astaxanthin diets outperformed all other treatments with both superior growth rates and FCR values. Thus it can be used for aquaculture feed.

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