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Microbial Production of Astaxanthin pigment using Marine shrimp

Bhavsar S. P., Singh K. G. & Sharma L. P.*
Department of Microbiology, Institute of Life Sciences, H.P.T Arts and R.Y.K College of Science,
Nashik-422005, Maharashtra, India
*[E.Mail: prowlab@gmail.com]

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In this study, shrimp waste collected from local hotels of Nashik and Trimbakeshwar, was used as a source of Astaxanthin and *Lactobacillus* spp. Overall, 4 isolates of *Lactobacillus* from various shrimp body parts viz., head, stomach, tail etc. were obtained and identified using morpho-biochemical characteristics. Astaxanthin extracted from dried shrimp waste using hexane/acetone gave 4% recovery (~0.4g/10g of shrimp) and microbial fermentation using *Lactobacillus* isolates showed enhanced astaxanthin production. Detection of the extracted pigment was done using thin layer chromatography (TLC). The concentrate was subjected to TLC using silica gel G250 sheet and run with solvent system of hexane:acetone (2:3). Thin layer chromatographic separation of carotenoid extract from shrimp waste produced a distinct band at Rf ~0.3 corresponding to free Astaxanthin. It was further characterized using spectrophotometry with absorption maxima at 465 nm and using High Performance Liquid Chromatography (HPLC). Preliminary study was done on microbial fermentation for astaxanthin production so as to select the efficient microbial culture and significant fermentation factors. This investigation concludes that *Lactobacillus* spp. are efficient candidate for astaxanthin fermentation and that soyabean extract supports maximum astaxanthin yield as indicated by the FFD .

[Keywords: - Astaxanthin, Microbial fermentation, FFD, chitosan

Introduction

Astaxanthin, a caretenoid pigment, is characteristically produced by few birds, crustaceans and salmonid[1]. These natural pigments are in growing demand as advantageous over the chemical based colors. Astaxanthin has been explored for its several health benefits to human beings such as its anticancer role, its ability to enhance immune response and antioxidant [2,3]. *Haemotococcus pluvialis*, a fresh water alga, has been extensively studied for astaxanthin production [4, 5]. The production of astaxanthin by *H. pluvialis* been reported in varied growth conditions and the maintenance of either the vegetative motile stage or the resting stage has been proved to be a crucial factor in the process [6, 7, 8].

Phaffia rhodozym, an astaxanthin producing yeast is well known for its protective nature against toxic oxygen radicals. Use of this yeast for astaxanthin fermentation has extended the time period for about 6 to 7 days long [9]. Likewise, a few microorganisms have been reported to produce astaxanthin but its high cost production limits its use [10]. Present study was aimed to enhance astaxanthin yield using fast growing lactic acid bacteria in low cost substrates. This was done by optimizing the area with the most significant factor in the Fractional Factorial Design (FFD). Furthermore, the extraction costs were scaled down by using bio-solvents over the synthetic organic solvents. The post fermentation waste was used as a biofertiliser for crop improvement studies as it is environmental friendly.

Materials and Methods

Shrimp waste processing and Inoculum development:

Lactobacillus acidophilus NCIM 2660 and *Lactobacillus plantarum* NCIM 2595 were obtained from National collection of Industrial Microorganisms (NCIM), NCL, Pune. Four isolates were obtained from fresh shrimp. All cultures were subcultured on deMan, Rogosa and Sharpe (MRS) agar media (Difco, USA) incubated at 37^{o}C for 48-72 h under anaerobic conditions [11].

The isolated cultures were identified according to their morphological, cultural and biochemical characteristics. Further for identification purpose, Gram character, catalase, cytochrome oxidase, growth at 15^{o}C at 45^{o}C in 1 week, acid &/or gas production from sugar such as glucose, arabinose, mannitol, raffinose, lactose, sorbitol, xylose and sucrose [12]. Lactic acid bacterial cultures were maintained using MRS broth, MRS butt and MRS agar plates and checked for viability every 8-12 days. Shrimp waste comprising of head and body carapace was collected from local hotels in Nashik. The waste was dried in hot air oven at 55˚C for 48 h. Dried waste was ground using laboratory grinder and was stored in bottles at room temperature [11].

A loopful of *Lactobacillus* culture was streaked on sterile MRS agar plates and incubated at room temperature for 48 h in anaerobic jar. A well-isolated colony was transferred to sterile MRS broth (5 ml), incubated at room temperature for 48-72 h in anaerobic jar and added to fermentative broth (100 ml) to obtain 10^4cfu/ml.

Experimental Design:

In the first stage, various crude wastes as substrates (soyabean extract, molasses, coconut milk and whey) were processed individually and screened for the ability to increase the yield of astaxanthin, using microbial fermentation. Soyabean water was extracted by soaking soyabean for three hours and filtered after boiling for half an hour in water bath. Left over coconut was ground to obtain coconut milk. Whey and molasses were obtained from diary and sugar industries respectively [4]. In the second stage, based on the sugar and protein content, crude wastes were further diluted viz., molasses (1%, 3%, 5%), coconut milk (10%, 20%, 30%), soyabean (20%, 40%, 60%) and whey (1%, 2%, and 3%) [13].Using various substrate in different proportions, the microbial fermentation was carried out. In the third stage, depending on the yield of astaxanthin obtained, the best supporting substrate was selected and optimization of production medium was done by Fractional Factorial Design (FFD). The experimental design involved two factors; shrimp waste (3%, 7%) and soyabean extract (15%, 25%). Altogether four various combinations of the two factors were selected based on the preliminary fermentation study, each experiment was performed in triplicates viz., total 12 experimental runs were performed to asses only one response i.e., astaxanthin yield. Finally, the analysis of variance (ANOVA) was performed using the statistical software (Design Expert Version 7, Inc. USA).

Crude astaxanthin produced in the fermentation process using shrimp waste and soyabean mixture was extracted using different vegetable oils viz., mustard oil, soya bean oil, sesame oil and blend oil (mixture of corn oil, canola oil and hydrogenated soyabean oil) from waste. These oils were collected in a *throw-away* status from local Lord *Shani* temples and restaurants in Nasik, after their regular usage [14]. Homogenized fermented shrimp waste was mixed with oil in 1:2 ratios and heated in a water bath at 70^0C for 2 h; further filtered using a muslin cloth and the filtrate was centrifuged at 3000 rpm for 10 min. The pigmented oil layer from the supernatant was passed through a separating funnel. The volume of pigmented oil recovered was recorded and the astaxanthin content in the diluted pigmented oil was measured spectrophotometrically by obtaining λ max in each type of oil against the particular oil as blank spectrophotometer (Chemito UV 2100). The carotenoid yield was calculated and presented as total carotenoid, as astaxanthin [14].

Analytical Procedures:

The concentrate was subjected to Thin layer chromatography (TLC) using silica gel G250 sheet and eluted with two different solvent systems in proper proportions: (i) hexane : acetone (2:3; 3:1) and (ii) benzene : ethyl acetate (1:1) [15]. Astaxanthin is a red-orange colored pigment. Visual color assessments represent crude measures to characterize and classify the pigment. Concentrated astaxanthin pigment was dissolved in solvent (acetone or IPA:Hexane) and/or oil to make concentration of 1mM. Absorbance maxima at 400-1100 nm was determined using spectrophotometer (Chemito UV 2100). The extracted astaxanthin was analyzed by reverse phase HPLC (WATERS 2489). Mobile phase used was acetonitrile: methanol: water (47:47:16 v/v) and the sample was eluted through a 5µm spherical C18 column (Sun fire, 4.6×250mm) at a flow rate of 1.5 ml/min. HPLC injection was performed manually with manual injector. Absorbance was monitored at 465 nm on UV VIS detector (WATERS 2489) and the peak was obtained using the EMPOWER software.

Residual fermented waste was filtered through filter paper. Recovered solids were washed thoroughly several times using distilled water and de-proteinised by boiling in 3%aqueous sodium hydroxide (Hi Media, Mumbai) for 15 min. After draining the alkali, the process was repeated for the removal of residual protein from the waste and washed with tap water. Proteinised shell waste was demineralized by 1N HCl (Qualigens, India) at room temperature for 1 h. Acid was drained off and washed thoroughly with tap water followed by distilled water. Chitin was dried at ambient temperature (30± 2°C) and pulverized into powder using a dry grinder. Chitosan was prepared by deacetylation of chitin by treating with aqueous sodium hydroxide (1:1; w/ w) at 90 to 95^0Cfor 2 h. After deacetylation the alkali was drained off and washed with tap water followed by distilled water. Finally, the chitosan was dried at 30 ± 2°C. Chitosan was assayed using bromocresol purple-calorimetric method [16].

The chitosan powder obtained from the post-fermentation waste was utilized in an eco-friendly by applying to 5 seeds of each corn and fenugreek in triplicates at 3 different time periods (30 min, 60 min and 120 min) to evaluate time intervals for pre-treatment so as to establish

whether time exposure to the chitosan powder would have any effect on the biological response of the seeds. Seeds were pretreated by first shaking the seeds and chitosan powder vigorously for one minute to ensure the even distribution of the chitosan powder on the seeds. Treated seeds and the untreated group (control) were then sown in labeled small plastic pots containing top soil and placed outside in sunlight. Plant growth was evaluated for 15 days for each treatment in triplicate [17].

Results

Depending on the morphological, cultural and biochemical characteristics lactose fermenting bacteria from fresh shrimp body parts were isolated and identified as

1. *Lactobacillus fermentum*, 2.and 4.*Lactobacillus brevis*, 4.*Lactobacillus buchneri* (Table 1).

Table 1. Morpho-Biochemical characteristics of the isolates

Isolate No	Gram's reaction	Morphology	Catalase Test	Oxidase Test	Growth at 15°c in 1 week	Growth at 45° c in 1 week	Sugar Fermentation: Acid & Gas from Glucose	Arabinose	Mannitol	Raffinose	Lactose	Sorbitol	Xylose	Sucrose
1	+		-	-	+	+	+	+	+	+	+	+	+	+
2	+		-	-	+	+	+	+	-	-	+	-	+	+
3	+	SR	-	-	+	-	A	-	+	-	+	+	+	+
4	+	SR	-	-	+	-	A	-	+	-	+	+	+	+

+ : Positive Results; - : Negative Results; SR: Short Rods; AG: Acids & Gas Productions
A : Only Acid Production

On the basis of the primary fermentation study, out of all the isolates, *L. brevis* exhibited maximum production of astaxanthin (~0.35 g/10g) Fig. 1. Combination of 3% shrimp waste and 25% of soyabean extract gave highest yield of astaxanthin as shown in Table 2a, 2b, 2c.

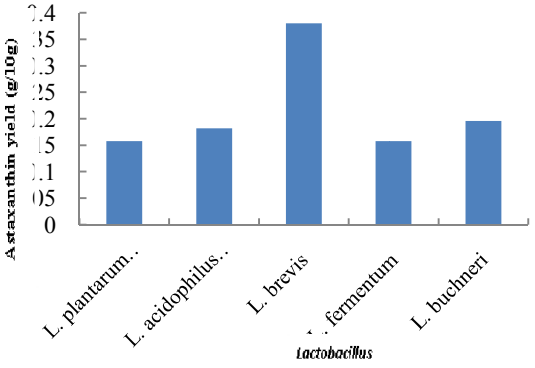

Fig 1.- Astaxanthin yield using microbial fermentation

Table2a:Optimization of Astaxanthin fermentation using FFD

Run	Block	Factor 1 A: Shrimp g/100ml	Factor 2 B: Soyabean extract ml/100ml	Response 1 Astaxanthin yield by microbial fermentation g/10g	g/L
1	1	7	15	0.13	1.3
2	1	3	25	0.28	2.8
3	1	3	25	0.32	3.2
4	1	7	15	0.14	1.4
5	1	3	15	0.11	1.1
6	1	7	25	0.21	2.1
7	1	3	15	0.12	1.2
8	1	7	25	0.24	2.4
9	1	3	25	0.30	3.0
10	1	7	15	0.11	1.1
11	1	3	15	0.14	1.4
12	1	7	25	0.19	1.9

Model being significant at degrees of freedom (Df) 3 at 99 % probability with R square value 85%. The result table below shows analysis of variants (ANOVA) for astaxanthin yield, the contours plot/ response curve (Fig. 2.).

Table2b:Optimization of Astaxanthin fermentation using FFD

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	0.04069	3	0.01356	36.1703	< 0.0001
A	0.00140	1	0.00140	3.75555	0.0886
B	0.03740	1	0.03740	99.7555	< 0.0001
AB	0.00187	1	0.00187	5	0.0558

Result: Significant

Table2c:Optimization of Astaxanthin fermentation using FFD

Std. Dev.	0.019365	R-Squared	0.931337
Mean	0.180833	Adj R-Squared	0.905588
C.V.	10.70871	Pred R-Squared	0.845508
PRESS	0.00675	Adeq Precision	12.22384

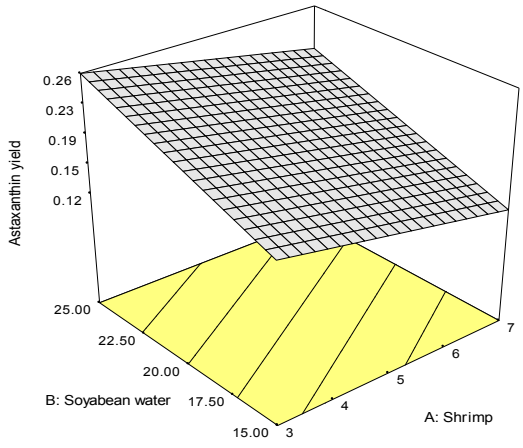

Fig 2.-Regular 2-way FFD analysis for fermentation optimization of Soyabean water (ml/100ml) and Shrimp waste (gm/100ml)

Final Equation in Terms of Coded Factors:
Astaxanthin yield =+0.18-0.011* A+0.056 * B-0.013 * A* B
Final Equation in Terms of Actual Factors:
Astaxanthin yield =
-0.14042+0.019583 * Shrimp+0.017417 *Soyabean extract-1.25000E-003 * Shrimp * Soyabean extract

The absorbance maxima of all oil extracted pigment were 465 nm. Highest astaxanthin yield of 103 µg /10g waste was obtained by extraction using soyabean oil and lowest (72.24 µg /10 g waste) using blend oil from local eatery (Fig.3.)

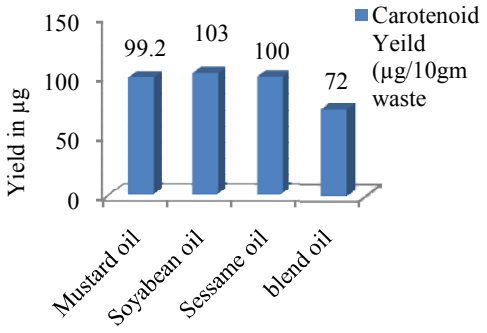

Fig. 3- Astaxanthin extracted using different oils

Characterization of astaxanthin

Red-orange color was obtained after chemical and oil extraction of pigment, which is characteristic of astaxanthin pigment (Fig. 4). Absorbance maxima were determined using spectrophotometer. The λmax obtained was 465 nm, which is similar to standard astaxanthin.

Fig. 4- Red-orange colored Astaxanthin Pigment

Thin layer chromatographic separation of carotenoid extract from shrimp waste yield a distinct band at Rf = 0.3 corresponded to astaxanthin.

HPLC analysis

A single sharp peak of astaxanthin was obtained at 465 nm which indicates that the astaxanthin was present in a pure form (Fig. 5).

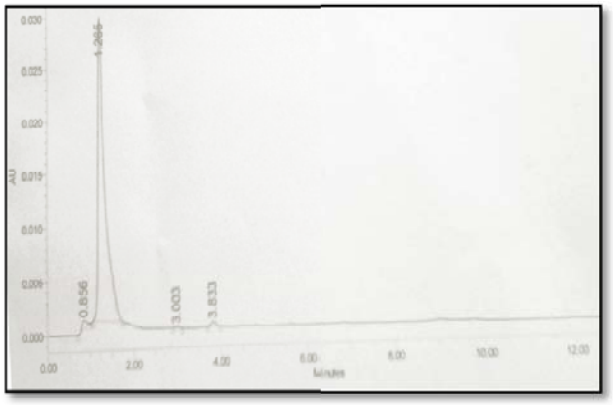

Fig. 5- HPLC chromatogram of Astaxanthin

Reutilization of Chitosan from residual shrimp waste as a plant growth enhancer

On pre-treatment of the corn seeds and fenugreek seeds, they were planted in suitable conditions. The root and shoot length was measured. It was observed that the pre-treatment step of 2 h enhanced the root and shoot development of the plant seeds (Table 3, Table 4).

Table 3. Effect of chitosan on pre-treated Corn seeds

Length in cm (SD)	Untreated (Control)	* Time of soaking in chitosan		
		30min	60min	120min
Shoot	17.1±0.32	18.3±0.25	25.23±1.36	28.3±1.18
Root	10.6±0.57	16.1±0.25	19.5±0.4	24±0.95

*Corn seeds were soaked in chitosan powder for different time intervals

Table 4. Effect of chitosan on pre-treated Fenugreek seeds

Length in cm (SD)	Untreated (Control)	* Time of soaking in chitosan		
		30min	60min	120min
Shoot	3.5 ±0.35	5.8±0.4	6.26±0.25	7.43 ±0.4
Root	10.8 ±0.26	8.5±0.3	9.93±0.2	14.9±0.25

*Fenugreek seeds were soaked in chitosan powder for different time intervals

Discussion

The seafood processing industry is one of the major food processing industries in India. During 2003-04, 129,785 ton of shrimp waste were produced. Processing of shrimp invariably generates solid waste in the form of head and body carapace. As the waste generation from processing of Indian shrimps ranges from 48 % to 56 % of the total weight depending on the species [14], it can be estimated that the solid waste generation shrimp processing industries in India, would be around 125,000 to 150,000 ton per annum. At present, a small quantity of this waste is used in the dry form as an ingredient in animal feed and the production of chitin. However, large quantities of this byproduct are being wasted, resulting not only in the loss of valuable components, but also in environmental pollution.

Shrimp waste is one of the important natural sources of carotenoid [18]. The carotenoid content in the waste from Indian shrimp was

found to vary from 35 to153 µg/g depending on the species, the major pigment being astaxanthin and its esters [14]. The recovery of these valuable components from the waste would improve the economics of the shrimp processing plant. Shrimp waste is one of the cheapest raw materials for recovery of carotenoids and extracted carotenoid would be a cheaper alternative than synthetic carotenoids.

The traditional chemical method for disposing the shrimp waste creates a problem due to large amounts of toxic waste which without further treatment would pollute the environment. Similarly to obtain Astaxanthin from algae and yeast cells is a tedious, costly and time consuming process. Yield of Astaxanthin by *Haematococcus pluvialis* and *Xanthophyllomyces dendrorhous*is reported to be 30mg/g of biomass and 4mg/g [19] respectively whereas from shrimp waste using *Lactobacillus* spp about 23.12mg/g of Astaxanthin was recovered [19,11]. Thus simple method using bacteria was carried out to accomplish both purposes; disposal of shrimp waste as well as enhanced production of Astaxanthin. In the present investigation, use of *Lactobacillus* species was aimed to decrease the pH of the medium. This decrease in pH is required to obtain the isoprenoid form of carotenoid which is a stable form of the pigment favored by acidic environment and also proteins present in the shrimp waste are hydrolyzed due to the action of proteolytic activity of proteases produced by lactic acid bacteria. This deproteinization also acts as a source of chitin which otherwise is obtained using chemicals such as HCl and acetic acid [20]. In the same manner, increase in *Lactobacillus* Probiotic therapy encouraged us to use the organism for fermentation. Lactic fermentation is a simple and environmentally friendly method to extract highly unstable carotenoid pigments [21]. The marine shrimp, *Panaeusse misulcatus* accumulates astaxanthin esters as major carotenoids and microbial method of extraction of astaxanthin is more effective than chemical method. In the present work, soyabean extract was used as a cost effective substrate for the microbial fermentation as it is promising source for higher yield of astaxanthin and proved to be efficient as observed in the Fractional Factorial Design.

For the steady recovery of these carotenoids from shrimp waste, use of organic solvent has been limited to analytical purposes only [22]. Solvents such as acetone, acid and alcohol have been used for extraction of carotenoids from crustaceans waste [5, 14]. A method has been patented for extraction of carotenoids from shrimp waste using a solvent mixture [11]. In the present work, use of mixture of solvents i.e. IPA:Hexane (1:1) gave more yield of astaxanthin as compared to only acetone. Acetone or IPA:Hexane helps in removal of pigment at 50^{o}C whereas in DMSO pigment is soluble. Aiming to reduce the production cost and with an attempt to make it synthetic chemical-free, the extractability of shrimp waste carotenoids was investigated using various waste vegetable oils. Several organic solvents such as acetone, benzyl alcohol, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone and ethanol have been permitted for use in food industries as carrier or extraction solvents, although levels of use depends on the type of foods in which they are used.

Carotenoids are a group of oil soluble pigments. The oil solubility characteristics of carotenoids have led to studies on the recovery of these pigments in oils. Anderson developed a process for extraction of carotenoids from shrimp processing waste where soyabean oil was added to the waste, mixed, heated, and the oil fraction recovered by centrifugation. In the present study it was observed that soyabean oil gave highest carotenoid yield whereas blend oil gave lowest carotenoid yield than any other vegetable oil studied. In aquaculture feed preparations, vegetable oil or fish oil is commonly used as a source of energy. The use of pigmented oil in feeds serves the dual purpose of pigment carrier as well a source of lipid energy. The pigmented product demonstrated R_f value of 0.3 and absorption maxima at 465 nm which confirmed the production of Astaxanthin [15]. The TLC spot showed fluorescence when viewed under UV light. It is important to select an HPLC method that meets the analysis objective. The pigment dissolved in organic solvent was read at 465 nm. Usually chromatogram contains many pigments and their degradation products therefore HPLC is used to discriminate the pigment. In the present work the chromatogram showed a single sharp peak which indicated that the extracted pigment was present in a pure form. HPLC separated pigment peak are identified by comparison of retention time (t_R) values with those of standards or by UV-VIS spectroscopy [23].

Thus using waste soyabean oil and using industrial effluent i.e. soyabean extract the yield of astaxanthin, an important pigment having potent anti-oxidant, anti-inflammatory and anti-cancerous property can be enhanced.

The chitosan extracted from the shrimp shell waste was utilized for pre-treating the fenugreek seeds and corn seeds before plantation.

The Sorption property of the chitosan effectively enhanced the absorption capacity of the seeds towards ions, salts, citrate, minerals etc. and this effect was reflected the root and the shoot development of these plantlets [24].

Conclusion: Data obtained in the present work strengthens the hypothesis that *Lactobacillus* spp. are efficient candidate for astaxanthin fermentation and that soyabean extract supports maximum astaxanthin yield as indicated by the FFD.

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