

Eco-Friendly Extraction of Biopolymer Chitin and Carotenoids from Shrimp Waste

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Abstract: Astaxanthin a nutraceutical and chitin a natural biopolymer present in shrimp waste. In current chemical extraction methods HCl and NaOH are used for extraction and these chemicals are introduced into aquatic ecosystems are spoiling aquatic flora and fauna, pollute the environment and destroy astaxanthin. *Lactobacillus* species were isolated from gut of *Solenocera melanthera* and characterized phenotypically and genotypically. Initial screening experiments have shown to be an effective and identified as *Lactobacillus plantarum* based on morphological, biochemical characteristics and molecular analysis. Efficiency of fermentation has shown with good yield of astaxanthin and recovery of chitin. Hence this alternative microbial process is having advantage than existing hazardous, non- economical chemical process.

Keywords: Astaxanthin, Chitin, Shrimp waste, 16S rRNA gene sequence.

1. INTRODUCTION

Chitin is a major biomaterial of exoskeleton of shrimp. In the past few years shrimp production has increased tremendously from 30 % to 80% in catch and aqua culture respectively. Depending upon the species, size and shelling procedure as the production increases waste production increases from 40% to 50%. On the dry weight basis shrimp waste contain 14 to 30% chitin a natural biopolymer having many applications including in water treatment, textiles, used as conjugate in targeted drug delivery, food industries, cosmetics, antimicrobial and antioxidant activity [1]. Moreover, the waste also contains a nutraceutical, a xanthophylls carotenoid astaxanthin [2]. The production of nutraceutical astaxanthin from renewable sources is an economic interest due to high cost of synthetic production [3]. Astaxanthin is an excellent antioxidant, retina protector, food colorant and used in cosmetics with many applications [4-6].

The source for industrial production of chitin in shrimps and crabs by which successive chemical treatments with concentrated acids and alkalis leads to high energy consumption and generation of corrosive recovery products. The quality of chitin is diminished and astaxanthin is destroyed due to chemical treatment. Alternatively eco-friendly microbial process has been



used to reduce the use of chemical treatment [7]. Lactic acid bacteria (LAB) microbial fermentation process enhances astaxanthin recovery along with chitin without change in their quality. LAB are distributed in nature in several species like *L. plantarum*, *L. casei*, *L. acidophilus*, and *L. bulgaricus*, are commonly isolated from dairy products, fruits and vegetables, probiotics and ensilage of aquatic wastes [8]. With the initial work of Orla-Jensen (1919) the taxonomy of the *Lactobacilli* progressed slowly despite the economic and environmental importance of this group. Hence LAB is used as a starter culture. Using appropriate starter culture by Identifying and providing a practical means is always advantageous. This is due to their metabolites in preventing growth of unwanted microorganisms. They are strictly fermentative, producing either a mixture of lactic acid, carbon dioxide, acetic acid or ethanol (heterofermentative) or almost entirely lactic acid (homofermentative). [9-10]. Most of the LAB can grow between 10 to 50°C. The efficiency of LAB can be assay by demineralization and deproteinization. Hence, this paper focuses on isolation and phenotypic and genotypic characterization of lactic acid bacteria and the efficiency on extraction of biopolymer chitin and astaxanthin (nutraceutical) from shrimp waste.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Samples of deep sea shrimp (*Solenocera melanthero*) and waste were collected from Harbour of Visakhapatnam, Andhra Pradesh, India and processed. Samples were used for isolation of microorganisms. They were obtained in good condition to avoid any contamination. The samples were collected in sterile poly propylene bottles (Tarson, India) and transported to the lab under frozen conditions and stored in -20°C freezer.

2.2 Isolation of Lactic Acid Bacteria (LAB)

One gram samples for each were taken and homogenized in a mortar and pestle using sterile saline (0.85% NaCl) to make microbial suspension. All the samples were subjected to serial dilution using sterile saline of 10^{-2} to 10^{-7} by taking 1ml into additional dilution blanks by using micropipette. All the dilutions were spread onto the modified MRS agar plates (Triplicate for each dilution) and incubated in an incubator at 37°C for 24 – 48 h. Randomly picked colonies were transferred to MRS broth. The isolated bacteria were identified according based on morphological characteristics, Gram staining technique, motility test, endospore, milk coagulation activities, and growth at different temperatures, and pH. Different biochemical tests were conducted to identify lactic acid bacteria.

2.3 Initial screening experiments for selection of starter culture

For initial screening experiments 100g of waste was taken and homogenized in a warring blender (local made) and then added 100ml of hot water in order to destroy internal microorganisms present in the shrimp waste. Then the sample was drained and mixed with 100ml of distilled water, then added 5% inoculum, 15g glucose and 1.5% NaCl in 500ml conical flask. The flasks were flushed with nitrogen and sealed with parafilm (Hi-media). The

total contents were allowed to ferment for 72h. The pH and Total Titrable Acidity (TTA) were recorded at from 0hr to 72 hr with an interval of 24 hr. The TTA was estimated by determining ml of 0.1N NaOH required to increase the pH of one gm of fermented mass to 8.0 [8].

2.4 Genotypic characterization

Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation Kit. An isolated bacterial colony was picked and suspend in sterile water in a microfuge tube. Centrifuge it for 1 minute to remove the supernatant. Add 200 µl of Insta Gene matrix to the pellet and incubate at 56 °C for 15 minutes. Using 16S rRNA Universal primers (27F AGAGTTTGATCMTGGCTCAG, 1492R TACGGYTACCTTGTTACGACTT) gene fragment was amplified by using MJ Research Peltier Thermal Cycler. Unincorporated PCR primers and dNTPs from PCR products are removed by using Montage PCR Clean up kit (Millipore) and obtained fluorescent-labeled fragments were purified with an ethanol precipitation protocol. The sample was sequenced using ABI PRISM sequencing kit (Applied biosystems). Sequence data was aligned and analyzed for Identifying the Sample.

2.5 Construction of phylogenetic tree

For identification of closest relatives the sequences were subjected to nucleotide-nucleotide BLAST algorithm [9]. The CLUSTALX (1.81) programme was used to align the gene sequence generated with sequences of the selected members of the family *Lactobacillus* obtained from GenBank [10,11]. Phylogenetic analysis was performed by applying the ARB parsimony tool to different data sets by using MEGA 5 package. Number of bootstrap repetitions was 500.

2.6 Fermentation effect on recovery of chitin and astaxanthin (carotenoids)

To know the effect of fermentation on recovery of chitin and carotenoids the experiments were carried out in four different batches. Each batch comprising four numbers one for each at 0 to 72h. Each sample was filtered using cheese cloth to collect the fermentation liquor and the residue was washed three times with distilled water (1:100). The moisture on the washed residue was drained completely and weighed. Part of residue was used for analysis of carotenoids and remaining was dried at 55°C overnight and was considered as crude chitin. Deproteinization and demineralization efficiency were calculated [12].

3. RESULTS AND DISCUSSION

In the development of new starter cultures for shrimp waste fermentation the identification of the *Lactobacillus* species that dominate microbiota of fermented foods is a key step. Screening of bacteria from different environments led to isolation of a total 18 lactic acid bacteria. Cell and colony morphologies of all isolated strains appeared as short rounded cocci and colonies were greyish, white, smooth, circular, low convex with entire margins. Colonies of all strains on medium were 0.5 to 2mm in size. Moreover, all of them were gram positive and non motile.

3.1 Phenotypic characteristics of LAB

Optimum growth for all strains occurred at 7 % NaCl at 35 °C. The pH values above 6 was suitable, however growth at pH 6.5 was optimum. The biochemical tests results were of the highly reproducible and confirm that fermentation of sugars is a characteristic for identification of lactic acid bacteria. The classification according to the Nakagawa and Kitahara (1959), useful tests were catalase activity, fermentation of maltose, salt tolerance, pH sensitivity, growth, temperature and arginine hydrolysis [13]. The *Lactobacillus* species are Gram-positive, non motile, nonspore forming, cocci shaped cells, grow singly or in short chains, catalase negative and produce substantial amount of lactic acid. The *Lactobacillus* has shown growth at 15°C and 37°C, in 4% NaCl, cannot produce NH₃ from arginine, able to curdle milk and produce acid from all sugars except xylose, rhamnose and starch (Table1). The carbohydrate fermentation test results and other phenotypic characterization tests of the isolates indicated its relativity to *Lactobacillus plantarum*. Identification of lactobacillus species, based on phenotypic methods, such as fermentation patterns, may sometimes be difficult, due to an increasing number of lactic acid bacteria species which vary on less number of biochemical traits.

3.2 Genotypic characterization based on 16S rRNA sequence

The new isolate was similar from the facultative anaerobic lactic acid producing genera *Staphylococcus* and the *Pediococcus* which exhibit definite coccoidal morphology was reported [14-15]. These results are in accordance with *Pediococcus* bacteria as reported earlier [16]. The isolated *Lactobacillus* has shown the G+C values of 40-50 mol%. It is agreed that organisms exhibiting differences in G+C contents no greater than 2 % might be members of the same species [17-18]. Molecular identification based on 16S rRNA gene sequence analysis the LAB isolate showed 1475 bp. The above sequences were compared with the known sequences in the public databases in NCBI and the BLAST results are considered and given the results in a phylogenetic tree form [19]. The 16S rRNA gene sequences have been deposited into GenBank with the following accession numbers *Lactobacillus plantarum* KP_4 (Accession no. KX959989). The phylogenetic trees were generated based on sequence aligned data and have shown 99% similarity with *Lactobacillus plantarum* (Figure2).

3.3 Initial Screening experiments

The Initial screening experiments have shown that fermentation initial pH of shrimp waste with *Lactobacillus plantarum* was 8.30±0.02 and was found significantly lower than with other cultures. Similar inoculum level was reported in case of *Lactobacillus* 541. [12]. However, initial pH was adjusted to 6.5 by using acetic acid to lactic acid culture before fermentation. Reduction in pH was substantial in the present study in the first 24 h itself. The pH obtained after 72h of incubation was significantly lowers ($p \leq 0.05$) when compare to other cultures. Similar results were reported by Rao *et al.*, 2000. The lower the pH maintained, the more protein was left in the residue. Different acids (hydrochloric acid, citric acid and lactic acid) were used to maintain the pH 6.0. The pH reduction was observed in this study is similar with many other studies [20-21]. Combination of low pH and high salt concentration may be

advantageous during shrimp waste fermentation using salt resistant strains *L. plantarum* 541 and A6. It was also reported with *L. plantarum* 541, a high amounts of glucose (5-20%) were added to enhance acidification. Considering these possibilities of exploration, fermentation of shrimp waste with lactic acid bacteria was conducted at low pH without adjusting the initial pH by adding glacial acetic acid. Hence *lactobacillus plantarum* KP_4 was chosen for fermentation standardization conditions and for further studies.

3.4 Fermentation effect on chitin and carotenoids recovery

Under optimized conditions the fermentation effect on pH and TTA of shrimp waste was presented in (Figure 1). The Figure clearly indicates the significant ($p \leq 0.05$) reduction in pH and significant increase in TTA ($p \leq 0.05$). The reason is due to production of acids by lactic acid bacteria. The fermentation effect on demineralization (DM) and deproteinization (DP) of shrimp waste was presented in Table 2. At the end of 72 h of fermentation time the demineralization occurred by 70% and deproteinization by 92%. Demineralization was significantly different ($p \leq 0.05$), and deproteinization was also significantly different ($p \leq 0.05$) at different time intervals. The deproteinization is mainly due to proteolytic activity of LAB and also the *in situ* proteolytic enzymes present in shrimp waste [22].

Further, it is also reported that the fact of proteolysis is more effective at higher pH. Rao and Stevens (2000) used *L. plantarum* where they adjusted pH initially with glacial acetic acid since lowering of pH initially may reduce the spoilage of shrimp waste [12]. However, fermentation with *Lactobacillus plantarum* without adjusting the pH there was no spoilage of shrimp waste. It was shown that the recovery of carotenoids was more than 72% which was significant ($p \leq 0.05$) at 72h of fermentation time. It was noticed that the carotenoid content in residue was decreased than in liquor. Since residue undergoes many washing steps by which the carotenoids may be lost. Maximum carotenoid yield was observed by Chen and Meyers (1982) from crawfish waste using soy oil with 1:1 ratio of oil to waste, heating the waste with oil at a temperature of 80-90°C for a period of 30 min [23]. As carotenoids are degraded at higher temperatures, the lower temperatures for longer time are advisable for optimum yield of carotenoid from waste [24]. The carotenoid in pigmented oil can be further increased for extraction of pigments by reusing the pigmented oil from fresh waste. The advantage of oil carotenoid extraction process over solvent is that pigmented oil finds to use as carotenoid source in aquaculture feeds. Further the biopolymer from shrimp waste can be used in biomedical applications [25].

CONCLUSION

Natural biomaterial like chitin from renewable sources plays an important role in food industries, medical, tissue engineering and nanotechnology. Chemical extraction of chitin enhances environmental pollution with poor quality and degradation of astaxanthin. Eco-friendly microbial extraction of biopolymer is an alternative to hazardous chemical method. Furthermore, increase in number of significant studies in microbial extraction of biopolymer (chitin) enhances potential biomedical applications for human kind.

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1.2 Tables

Table 1. Biochemical characterization of isolated LAB strains

Characteristics	Group I	Group II	Group III	Group IV
Gram staining	+	+	+	+
Spore formation	–	–	–	–
Catalase activity	–	–	–	–
Amylase activity	–	–	–	–
NH ₃ from arginine	–	–	+	+
H ₂ S	–	–	+	–
Carbohydrate fermentation	+	+	+	+
Acid formation from				
Arabinose	+	–	+	+
Xylose	–	–	–	–
Glucose	+	+	+	+
Fructose	+	+	+	+
Maltose	+	–	–	+
Lactose	+	–	–	–
Sucrose	+	–	–	–
Ribose	+	+	+	+
Mannose	+	–	+	+
Trehalose	+	+	–	+
Melibiose	+	–	–	+
Raffinose	+	–	–	+
Rhamnose	–	–	–	–
Melezitose	+	–	–	–
Starch	–	–	–	–
Preliminary Identification	<i>Lactobacillus plantarum</i>	<i>Lactobacillus casei</i>	<i>Pediococcus acidilactici</i>	<i>Pediococcus pentoseus</i>

Sample	Weight of residue ^a (g)	Protein (g)	Ash (g)	DP (%)	DM (%)
0	12.5± 0.08 ^a	0.76 ± 0.02 ^a	2.42 ± 0.04 ^a	27.5±5.8 ^a	31.9± 2.4 ^a
24 h	10.4 ± 0.14 ^b	0.62 ± 0.04 ^b	1.89 ± 0.04 ^b	36.4±4.8 ^b	46.8± 0.9 ^b
48 h	8.25± 0.06 ^c	0.32 ± 0.02 ^c	1.12± 0.02 ^c	83.2±1.8 ^c	70.6± 1.2 ^c
72 h	7.84 ± 0.04 ^d	0.03 ± 0.02 ^d	1.05± 0.03 ^c	92.0 ± 0.4 ^d	70.0 ± 1.1 ^c
Fresh	18.5 ± 0.12 ^e	1.1 ± 0.04 ^e	3.45± 0.08 ^d	-	-

Table 2. Fermentation effect on the Deproteinization (DP) and Demineralization (DM) of shrimp waste

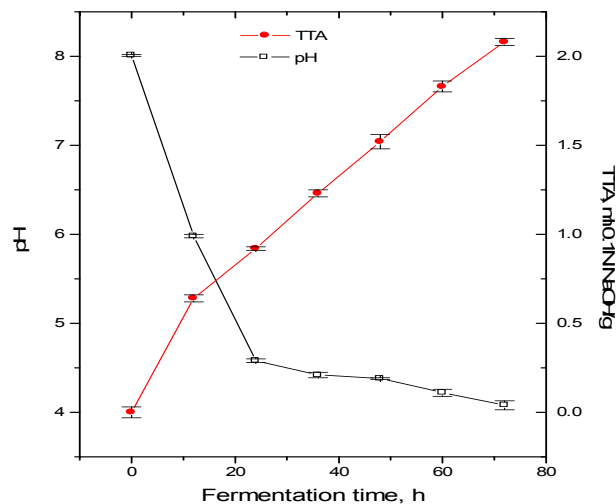


Figure 1. Changes in pH and TTA of shrimp waste during fermentation with *L. plantarum*

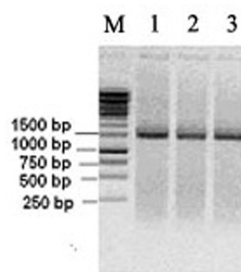
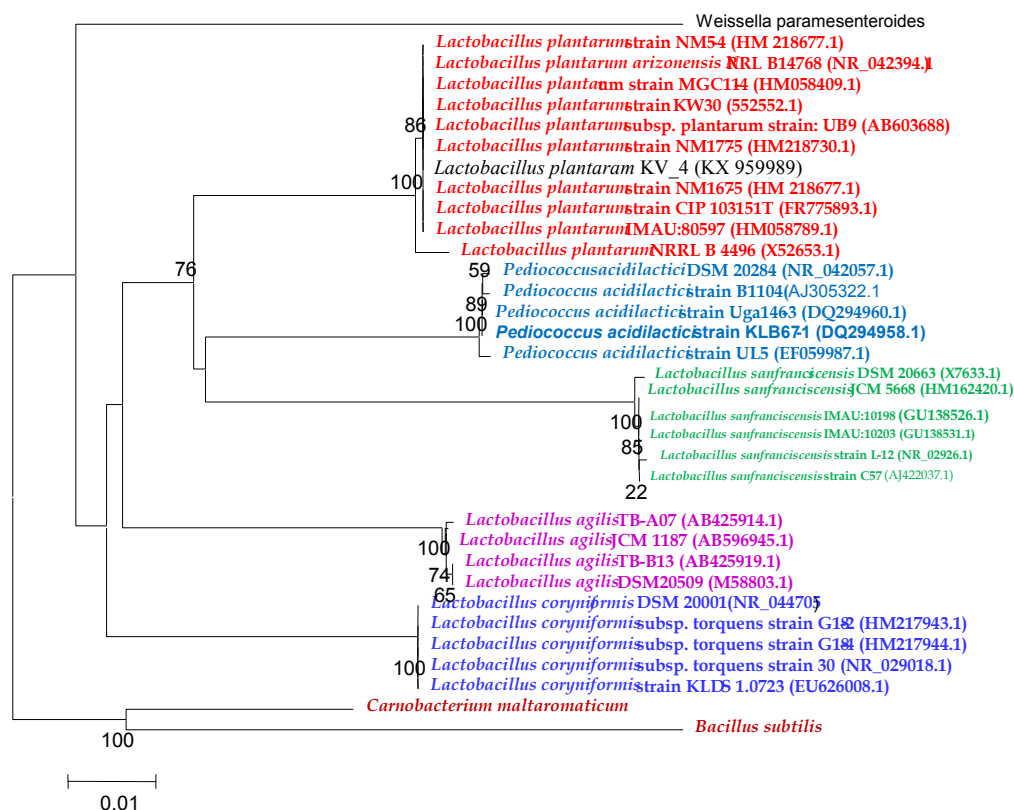


Figure 2. Amplified 16S rDNA of *Lactobacillus plantarum* (M: Marker , 1: (positive control) , 2 and 3 : *Lactobacillus plantarum* KP_4)

Figure 3. Phylogenetic dendrogram of 16S rRNA sequence of the isolated *Lactobacillus plantarum* strain KP_4 (Accession no. KX959989) obtained using Neighbour Joining (NJ) tree approach included in the MEGA.5 software.



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