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Isolation, identification and characterization of novel pigment producing bacteria from distillery spent wash

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ABSTRACT

An unidentified orange bacterial strain isolated from distillery spent wash when characterized for morphological, microscopic, biochemical and molecular features (16SrRNA sequencing) was identified as *Planococcus maritimus AHJ_2*. Small orange colonies measuring 2-3 mm in diameter on Luria Bertani agar medium was the striking feature noted in the organism. The bacterium could grow over a wide range of media, pH (3.0-11.0) and temperature (20-37°C) but optimal growth and pigmentation was observed in LB medium at pH 7.0 at 37°C containing 0.5 percent NaCl. Spectrophotometric, FTIR and HPLC analysis of pigments revealed the presence of carotenoid type of pigment.

KEYWORDS

Orange bacterium;
16S rRNA identification;
Growth;
Pigmentation;
pH;
Temperature.

INTRODUCTION

As against widespread use of synthetic dyes not known to be environment friendly, demand for natural pigments for coloring fabrics, foods/feeds, cosmetics and printing inks are increasing[1]. A number of natural pigments produced by plants contribute to enhanced immune system and reduced risk of degenerative diseases, such as cancer, cardiovascular diseases, macular degeneration, cataract and acting as anti-aging agents[2-5]. Of the various natural pigments, microbial pigments prodigiosin and violacein are types of red and violet bacterial pigments that have found application in medical areas due to their activities as immunosuppressive, anticancer, antibacterial and antifungal agents[6,7]. Carotenoids are currently produced for use as food colorants, nutritional supplements, cosmetics or health purposes[8]. In addition to their pigmenting abilities, carotenoids may function as antioxidants by quenching photosensitizers, interacting with singlet oxygen, and scavenging peroxy radicals[9]. The species of the various taxonomic groups' bacteria, fungi and yeasts are efficient natural producers of carotenoids. Facing the growing economic significance of carotenoids, much interest has been devoted to new supplies of this type of pigment[10,11]. The fermentation conditions, such as cultivation temperature, NaCl, pH[12], play important roles in the carotenoids forming activity of microorganisms as well as composition ratio of carotenoids.

In the view of the significance of biopigments, the present work in this paper deals with the characterization of the orange pigmented bacteria both for its iden-

tification and production of an intense orange carotenoid pigment.

EXPERIMENTAL SECTION

Materials

All chemicals and culture media used for the growth of pigment producing bacteria were purchased from Hi-Media laboratories (Bombay, India). Double glass distilled water was used for the experimentation. Solvents used for the extraction of pigments were of HPLC grade and purchased from Bombay, India.

Instruments

The instruments used in this study were UV/Visible spectrophotometer (UV Mini 1240 Shimadzu, Japan), commercial heavy duty shaker (REMI), cyclomixer and water bath (REMI).

Collection of distillery spent wash

Distillery spent wash from Shri Satpuda Tapi Sahakari sugar factory and distillery section, Purushottamnagar, Shahada was collected in sterile 500 mL Erlenmeyer flasks. Analysis of the sample was carried out in the research laboratory of Department of Microbiology, PSGVPM'S ASC College, Shahada.

METHODS

Enrichment of sample

1 mL of effluent sample was enriched in sterile 100 mL Nutrient Broth, Potato Dextrose Broth, and Luria Bertani broth each. All flasks were incubated for 48 hours on rotary shaker at 100 rpm (REMI, India Ltd.)

Screening and isolation

Enriched broth samples were diluted up to 10^{-5} and 0.1 ml of dilution was plated on sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate and sterile Luria Bertani agar plate. All the plates were incubated at 37^0 C up to 48 hours. Growth of organisms from each of the sterile nutrient agar plate, sterile starch casein agar plate, sterile potato dextrose agar plate, sterile Luria Bertani agar plates were observed and best pigment producing organisms were selected. These were further purified from mixed population. Six pigment producing bacteria were isolated from effluent samples. Out of six isolates, most prominent pigment producing bacteria grown on sterile nutrient agar as well as on sterile Luria Bertani agar were selected. A pure culture of the isolate was maintained on sterile LB agar (casein enzyme hydrolysate $10gL^{-1}$; yeast extract, $5gL^{-1}$; NaCl $10gL^{-1}$; pH 7) slants followed by storage at 4°C as master culture and working stocks. When needed, culture was inconsistently derived from a master culture by streaking on LB agar in order to maintain its genetic stability. The bacterial isolate when cultured on this medium at 37±0.5°C formed intense orange colored colonies after incubation period of 3-4 days and used for further studies.

Identification of isolates

Colony morphology and cell characterization

The bacterial isolate was plated on Luria Bertani agar, allowed to grow at 37±5°C for 3-5 days and then studied for different cultural and cell morphological parameters, such as colony size, elevations, margin and colony pigmentation. Motility (hanging drop method) and Gram's reaction of the bacterial cells were performed using standard methods.

Biochemical characterization

Identification of isolate was done on the basis of morphological, cultural, biochemical and physiological characteristics from MTCC-Chandigarh. The isolates were cross identified on the basis of biochemical characters conducted routinely according to the protocols of 'Bergey's Manual' on 48 hours grown bacterial cultures. The pre-sterilized Hi-carbohydrate biochemical kit (KB 002 and KB 009, Hi Media, Mumbai, India) was used for biochemical identification of the isolate.

Molecular identification of bacterial isolate based upon 16SrRNA sequence

Molecular identification of the isolate by 16S rRNA sequencing was done at 'National Center for Cell Science', Ganeshkhind, Pune. The determined sequence of this 16SrRNA fragment was submitted to GenBank for GenBank Accession (www.ncbi.nlm.nih.gov/ Blast). This sequence was blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/ Blast) for nucle-

otide homology. The maximum homology report (Taxonomy Blast Report) was identified.

Pigment production

Growth and pigment production was carried out in 500 mL Erlenmeyer flask containing 100 ml of Luria Bertani broth inoculated with 1 mL of 24 hours culture suspensions. Flasks were incubated on rotary shaker at 120 rpm for 72 hours.

Extraction and spectrophotometric analysis of pigment

For the extraction of the pigment a screening programs involving various methods were accomplished and the method found most suitable was used further for all the investigations. Culture samples from each flask were centrifuged at 10000 X g for 15 minutes. In the harvested cells 50 mL of HPLC grade methanol was added to the moist cell mass. Mixture was vortex for 5 minutes and kept for incubation up to 6 hours. All the pigments were extracted in methanol and colorless cell debris were removed by centrifugation. UV visible scanning spectra of the methanol extract containing pigment were recorded between 200 and 800 nm on UV visible spectrophotometer (UV Mini 1240 Shimadzu, Japan). The absorption maxima were thus determined.

Fourier transforms infrared analysis (FTIR)

FTIR analysis was carried out to detect the presence of functional groups in the extracted pigment. For pigment analysis, FTIR was considered as a complementary technique that could provide the molecular and structural information of organic and inorganic molecules present in the pigment.

Effect of medium pH on growth and pigmentation

In order to determine the optimum conditions for growth and pigment production, optimization of pH and salt concentrations were studied. In an experimental set up 500 ml Erlenmeyer flasks containing 100 ml of the growth medium LB broth was inoculated with a 100 µl of the inoculum. The pH of the growth medium was varied as 3, 5, 7, 9 and 11. The flasks were incubated on rotary shakers for 72 hours at 37°C. After incubation, the relative growth and extent of pigmentation was recorded spectrophotometrically. All the separate shake flask experiments were performed in triplicates and results were expressed as mean±SD.

Effect of incubation temperature

Effect of incubation temperature (20, 25, 30, 37, 45°C) on the bacterial cell growth and pigmentation was observed after growing the inoculated LB broth medium at different temperatures. After 72 hours of incubation period, the growth and pigmentation of the bacterial isolates was recorded spectrophotometrically.

RESULTS AND DISCUSSION

Colony morphology and cell characteristics

The colony morphology and cell characteristics of the bacterial isolates in Luria Bertani agar showed that within 3-5 days of incubation the bacterial isolate grew to form intense orange colonies (2-3 mm in diameter) having entire margin and smooth consistency (Photplate 1A). Gram staining of bacterial cells revealed the presence of Gram positive cocci.

Plate 1 : Growth and pigment production by the bacterial isolates in LB agar medium

Biochemical characteristics

The intense orange pigment forming isolate was identified on the basis of biochemical characters from Microbial Type Culture Collection, Chandigarh. The results of various biochemical tests are as depicted in TABLE 1. On the basis of biochemical characterizations, isolate was identified from MTCC, Chandigarh as a *Planococcus maritimus.*

Molecular identification of bacterial isolates based upon 16S rRNA sequence

16S rRNA sequences obtained from NCCS were

submitted to GenBank and are available as GenBank Accession Numbers JN873343.1 *Planococcus maritimus strain AHJ_2* (www.ncbi.nlm.nih.gov/Blast). The sequences were blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/Blast) for nucleotide homology. The maximum homology report (Taxonomy Blast Report) identified a high nucleotide homol-

Sr. No	Characteristics	Isolate 1
1	Configuration	Circular
2	Margin	Entire
3	Elevation	Raised
4	Surface	Moist
5	Pigment	Orange –Yellow
6	Opacity	Opaque
7	Gram's Reaction	+ve
8	Cell Shape	Coccus
9	Size mm	0.5-1
10	Spores	–
11	Endospores	–
12	Position	–
13	Shape	–
14	Sporangia	–
15	Motility	Motile
16	Anaerobic	–
17	Physiological tests	
18	Growth at temperature	
	4°C	–
	15°C	+
	25°C	+
	30°C	+
	37°C	+
	42°C	+
	55°C	–
19	Growth at pH	
	pH 5.0	+
	pH 7.0	+
	pH 8.0	+
	pH 9.0	+
	pH10.0	+
	pH 11.0	+
20	Growth on NaCl (%)	
	2.0	+
	4.0	+
	6.0	+
	8.0	+
	10.0	+
	11.0	(+)
	12.0	–
21	Biochemical Tests	
	Methyl Red Test	–
	Voges Poskauer test	-
	Casein hydrolysis	+
	Citrate	-
	Nitrate	-
	Arginine	-
	Ornithine	+
	Indole	-
	Gelatin Hydrolysis	-
	Starch hydrolysis	-
	Esculin hydrolysis	–
	Catalase test	+
	Oxidase test	–
	Growth on Mac-Conkeys	NLF
	Tween-20	-
	Tween-40	-
	Tween-60	-
	Tween-80	-
	Urea production	–
19	TSI Results	
	Butt	Yellow
	Slant	Yellow
	H_2S Production	-
20	Acid Production from	
	Trehalose	–
	Lactose	-
	Melibiose	-
	Adonitol	-
	Inositol	-
	Raffinose	+
	Cellobiose	+
	Maltose	+
	Xylose	-
	Sucrose	+
	Sorbitol	-
	Salicin	+
	Rhamnose	-
	Galactose	-
	Fructose	+

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ogy of the 16S rRNA (99% maximum identity in 100 % query coverage) with 16S rDNA/ 16S rRNA. From the analysis of the generated taxonomy report of the 16S rDNA gene sequence, this bacterial strain with highest score of (2628), and lowest E-value (0.0) was identified to be *Planococcus maritimus AHJ_2*. However, the bacterial strain under study showed a maximum of 99 per cent homology with the previously reported sequences. This established that the bacterial isolate identified as *Planococcus maritimus AHJ_2* is a novel strain that has not been reported earlier.

Separation and type determination of nature of the pigment

The methanol extract of the pigment was subjected to partition between immiscible solvents to separate the type of pigment produced by the organism. Extraction was achieved in the ether phase in the separatory funnel. Presence of the pigment in the diethyl ether phase exhibits the presence of carotenoids or carotenols.

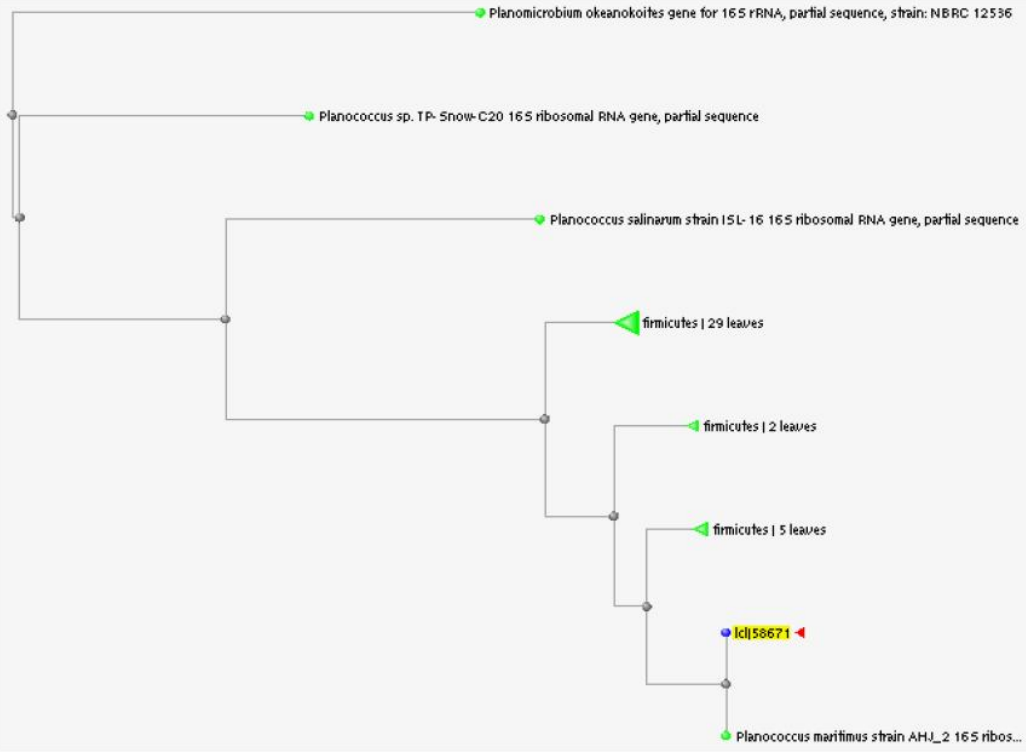

Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the phylum firmicutes

UV-visible spectra absorption spectra of the pigment

UV-visible absorption spectra of carotenoids pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids. The UV-visible absorption maxima were 466 nm in methanol. This absorption spectrum of the pigment was characteristic of carotenoids (Figure 2).

Identification of pigment by IR analysis

One of the main identification tests for pigment is IR spectrum. Pigment exhibited bright spectral absorption lines, 466.79 cm^{-1} peak related to the hydroxy group

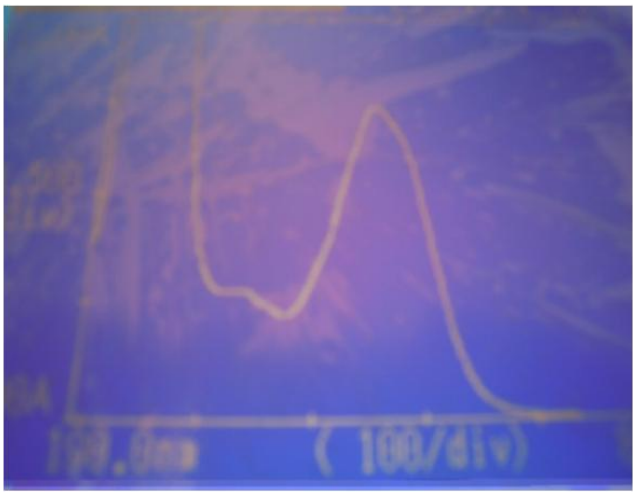

Figure 2 : Absorption spectrum of the pigment from *Planococcus maritimus AHJ_2*

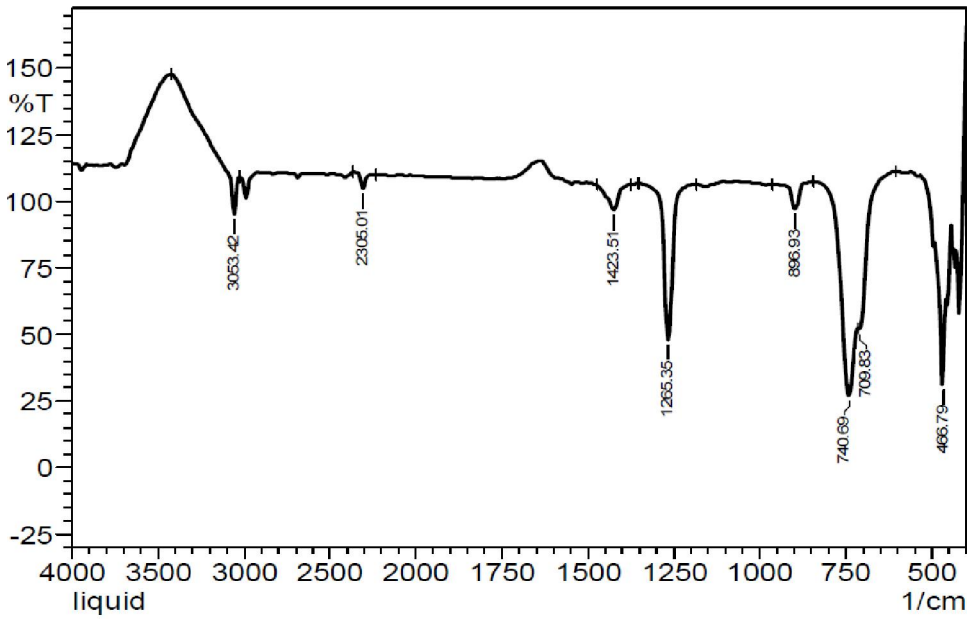

Figure 3 : FTIR analysis spectrum of the methanol extracted pigment

(OH), 709.83-740.69 cm^{-1} attribute to the O-H bend ing peaks, peak at 1265-1435 cm^{-1} related to the amino second group (NH), 2305-3053.42 cm^{-1} peaks related to amino group (NH) in pigment extracted from *Planococcus*. The IR spectral analysis of the methanol extracted pigment reveals the presence of carotenoids (Figure 3).

Effect of medium pH and incubation temperature on growth and pigmentation of the bacterial isolate

The effect of pH value of the growth medium on growth and pigment production of *Planococcus* wad studied. The isolate has shown remarkable ability to grow at pH values (3.0, 5.0, 7.0, 9.0 and 11.0) and produces orange and intense orange pigment over a wide range of medium pH. However, it showed its maximum growth and pigmentation efficiency at pH value of 7.0. This suggested that pigmentation was directly related with growth and that in spite of its capacity to grow over a wide pH range, the bacterial isolates was neutrophilic in its nature (Figure 4).

Temperature

In order to determine the optimum temperature, for the growth and pigmentation, the bacterial isolate was grown in LB broth and growth was observed at five different incubation temperatures (20, 25, 30, 37 and 40°C). All the incubation temperatures allowed the growth of

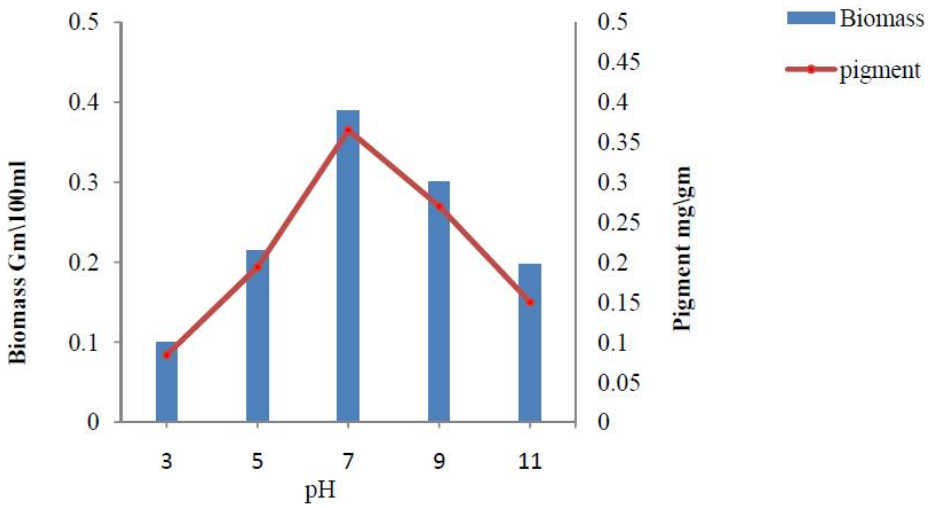

Figure 4 : Effect of pH on growth and pigment production

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the bacterial isolate indicating its temperature stability over a mesophilic range. However, the optimum growth of the bacterial isolate was observed in the cultures incubated at 37°C, followed by 30°C (Figure 5).

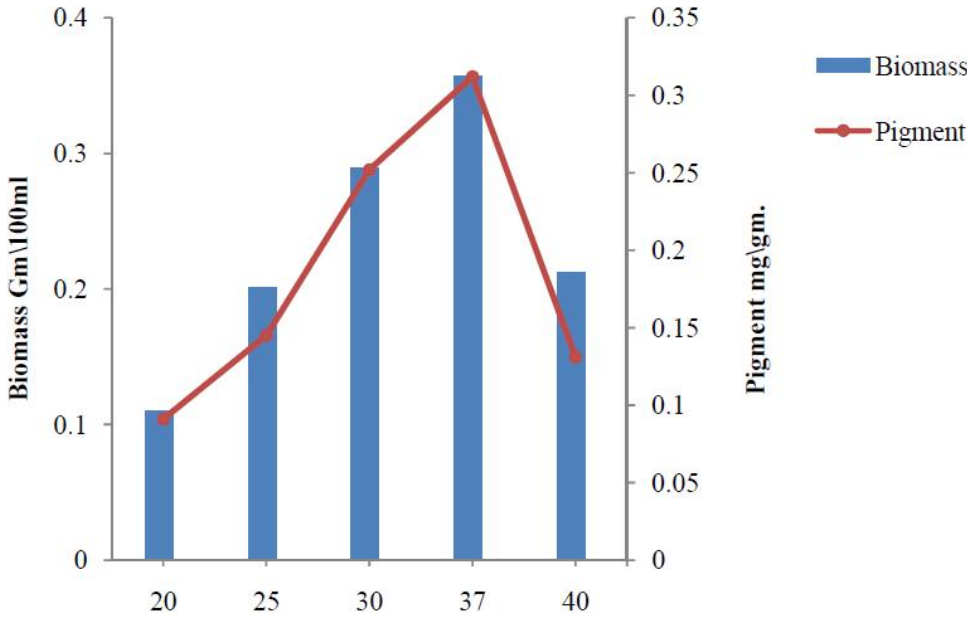

Figure 5 : Effect of temperature on growth and pigment production of *Planococccus*

CONCLUSIONS

Based upon various morphological, microscopic, biochemical and molecular parameters, an orange pigmented bacterial strain was isolated and identified as a novel strain of *Planococcus maritimus* AHJ_2. This strain could actively grow and was a potent producer of orange pigment in medium containing 0.5-1.0% NaCl and having initial pH of 6.0-7.0 and at an incubation temperature of 37°C. Preliminary investigations of the extracted pigment exhibited its close resemblance to carotenoids. Thereby studies related to its further analyses by sophisticated instrumentation and its applicability as food grade pigment are underway and are likely to yield encouraging results.

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