



Purification and characterization of carotenoid pigment produced by *Planococcus maritimus* and its dyeing potentials

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ABSTRACT

The present research work revealed the production of an intense orange pigment from bacterial strain isolated from a distillery spent wash. From 06 different pigmented strains isolated, an intense orange pigment producing strain was characterized by morphological, physiological, biochemical, molecular features and FAME analysis and identified as *Planococcus maritimus*. This organism formed prominent orange colored colonies on Luria Bertani agar medium. The pigment was produced in shake flask condition and extracted in 80% HPLC grade methanol. The extracted pigment was separated in diethyl ether phase of separation by liquid-liquid extraction method and purified by TLC and HPLC. Thin Layer Chromatographic plate developed in methanol: toluene (6:5) mixture revealed the major yellow spot with migration rate of 0.94 and HPLC chromatogram of pigment extract exhibited a major peak with retention time 3.0833 which revealed the presence of carotenoids. Detailed characterization of pigment was done systematically by UV-Visible spectrophotometric, LC-MS and FT-IR method. The extracted pigment confirmed the presence of carotenoid pigment. Beside potential antimicrobial and antioxidant properties, pigment have effective dyeing potentials for textile and paper which finds broad applications in textile, paper, printing dye, cosmetic as well as food industries.

Keywords: Carotenoids, FAME analysis, HPLC, LC-MS, *Planococcus maritimus*, TLC.

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INTRODUCTION

Color additives are main ingredients of many products that make it look attractive and tasty [1]. Color determines the acceptance of a product and has paramount influence on human life. The colors may be synthetic dyes or organic pigments utilized in various food, textile or pharmaceutical industries [2, 3]. Many synthetic colors used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects like allergies, tumour, cancer and severe damages to the vital organs [4]. There has been much interest in the development of new natural colorants for use in the food industry due to strong consumer demand for more natural products [5]. Natural dyes are non-toxic, nonpolluting, and less health hazardous [6] as well as their antioxidants and antimicrobial nature further adds to their positive effects [7]. Nature produces many bio colorants from various resources including plants, animals, and microorganisms, which are possible alternatives to synthetic dyes and pigments currently employed in different applications. Natural dyes can be obtained from microbial sources are stable pigments such as anthroquinone, carotenoids, flavonoids, quinines, rubramines, canthaxanthine, riboflavin and the fermentation has higher yield in pigments and lower residues compared to the use of plants and animals [8]. Biocolors extracted from microorganisms proved to be safe and promising alternatives to other color additives, with no seasonal production problem and show high productivity. Thus there is an increasing demand for natural colors in the food, pharmaceutical, cosmetics, textiles and in printing dye industry [9]. Among pigments with natural origin, carotenoids represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity through the removal of oxygen radicals [10]. Carotenoids extracted from different sources have been used as a food supplement or nutritional replenishment for modifying the color of fats, oils, butter, margarine, cheese, drinks and juices [11, 12].

Thus present investigation is focused on the purification, characterization and identification of orange pigment produced by *Planococcus maritimus* and evaluation of dyeing properties of pigment applicable in textile, paper and printing industries.

MATERIAL AND METHODS

Isolation of bacteria

Prominent orange pigment producing bacteria was isolated on sterile Luria -Bertani agar medium (HI Media) from distillery spent wash of Shri Satpuda Tapi Sahakari sugar factory and distillery section, Shahada. Isolate was purified and maintained on slants of Luria- Bertani Agar (LB) medium at 4°C and sub cultured after every 30 days.

Morphological, biochemical and molecular identification of isolate

Morphological and biochemical identification isolate was carried out from Institute of Microbial Technology (IMTECH), Chandigarh, India. 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](http://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](http://www.ncbi.nlm.nih.gov/Blast)) for nucleotide homology. The maximum homology report (Taxonomy Blast Report) was identified.

Identification of isolate by Whole-cell Fatty Acid Methyl Ester [FAME] analysis

A whole cell fatty acid derivative of methyl esters (FAME) of the isolated strain was analysed using gas chromatography (GC). Chromatography was carried out using the Sherlock Microbial Identification System (MIDI, Inc. Newark, DE, USA). The bacterial culture was grown in triplicate on Trypticase Soy Broth Agar (TSBA) which consists of 30 g Trypticase Soy Broth and 15 g of agar for 24 hours at 28°C and fatty acids derivatives were separated from cell walls by saponification and extracted with hexane and methyl tertiary butyl ether. Extracts were analysed on a GC 689 (Agilent Technologies, USA) fitted with Ultra 2 phenyl methyl silicone fused silica capillary column 25 × 0.2 mm (Agilent Technologies, USA), using hydrogen as the carrier gas, nitrogen as the "make up" gas and air to support the flame. GC oven was programmed from 170 to 270°C at 5°C rise per minute with a 2-minutes holding at 300°C. Fatty acids were identified and quantified by comparison of retention time and peak area obtained and compared with the fatty acid standards. Qualitative and quantitative differences in fatty acid profiles were used to compute the distance for isolated strain relative to other strains in the Sherlock bacterial fatty acid ITSA1 aerobic reference library. This part of the work was accomplished with courtesy of Disha chemicals, Ahmadabad

Production of pigment

500ml Erlenmeyer flask containing 100 ml of sterile LB medium was inoculated with freshly prepared 100 microliter of the bacterial inoculum for growth and pigment production. Flask was placed in an incubating rotary shaker at 120 rpm for 48 -72 h.

Pigment extraction

Extraction of pigments was carried out by centrifugation at 10,000 rpm for 10 min by the use of HPLC grade methanol. Pigment was extracted from biomass by the method described earlier [13].

Purification of pigment

A three step purification protocol was applied for the purification and separation of pigment from crude methanol extract of pigment.

Liquid-Liquid extraction

In the first step of purification, in order to remove the traces of water and any impurities of cell extract present in the extracted pigment sample, phase separation was carried out by the modification method earlier described by [14,15].

The methanol extract of the pigment was subjected to partition between immiscible solvents to separate the type of pigment produced by the organism. 25 ml methanol extract of the pigment was added with 50 ml of petroleum ether in the separatory funnel. The distilled water was added slowly, along the walls of the separatory funnel to make an alcohol concentration of 90%. Two phases i.e petroleum ether and methanol phase were formed in the separatory funnel. Pigment sample concentrated in the methanol phase, was collected and made alkaline by adding 10 ml of 10% methanolic KOH solution, then diluted with water and shaken with 50 ml of diethyl ether. Again two phases were appeared, epiphase of diethyl ether and aqueous hypo phase. At this step pigment transferred to diethyl ether phase was collected.

In the second stage of partial purification, pigment sample collected from diethyl ether phase was subjected to TLC [16]. Commercially available silica coated TLC aluminum plate - silica gel G-60 F₂₅₄ (Merck Mumbai, India) 5 x 10 cm was used for TLC. For standardization of mobile system for TLC, various solvent systems comprising of methanol, petroleum ether, ethyl acetate, chloroform, acetone, diethyl ether, n-butanol, n-hexane, and toluene were used in different proportions for facilitating proper separation of pigment components, n-hexane: chloroform (4:6 5:5, 6:4), chloroform: methanol (6:4, 5:5, 7:3, 8:2, 2:8) and methanol: toluene (6:5) were used as a developing solvent with b-carotene as a standard. The final step of purification was achieved using HPLC equipped with photodiode array

detector [17]. Pigment extracted in methanol was evaporated to dryness at 30°C. Dried pigment was dissolved in 1 ml methanol and injected in a quantity of 50 µl into C₁₈ column with a 4.6 mm x 250 mm, 5 µm (Shimadzu LC8A, Japan). Separation was achieved by using reverse phase HPLC at a flow rate of 1.0 ml min⁻¹. Solvent and conditions for separation were methanol- water (70:30) for 20 minutes. Retention time was determined from the data obtained after analysis. This part of the work was accomplished with the courtesy of Shree industrial training and research Centre, Jalgaon.

Characterization of pigments

UV-Visible spectroscopy, LC-MS and FT-IR spectroscopic methods were applied for the characterization of pigment.

Ultraviolet-visible spectroscopic analysis

UV visible scanning spectra of the methanol extracted pigment was recorded between 200 and 800 nm wavelength on UV visible spectrophotometer (UV Mini 1240 Shimadzu, Japan). The maximum absorption of the sample was determined using methanol as a blank. B-carotene was used as standard.

Liquid Chromatography-Mass Spectroscopy (LC-MS) of pigments

Liquid chromatography-Mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography. LC-MS is useful for rapid detection of compound and its structural identification. It can able to scan more than 300 compounds present in the sample. For analysis of pigment by LC-MS, LCQ Fleet Ion Trap LC/MS model (Thermo Scientific, USA) equipped with automatic sample injector, Photodiode array detector (PDA), TSQ Quantum Access with Surveyor plus HPLC system APCI Mass selectivity detector was used. The mass spectrum was recorded in the positive ion mode in the mass range from m/z 50 to 1500. Purified pigment sample was submitted in SICART-Anand, Gujarat, for LC-MS analysis. This part of work was accomplished at SICART.

Fourier Transforms Infrared Analysis (FTIR)

Fourier transformed infrared spectra of purified pigment was analyzed on a FT-IR spectrophotometer. Sample dissolved in dichloromethane and deposited as a film on KBr plates and was scanned between 400 and 4000 wave number (cm⁻¹) on spectrum GX FTIR, Perkin Elmer. This FT-IR analysis part was carried out in the Department of Chemical Technology, North Maharashtra University, Jalgaon.

Applications of pigment in Dyeing of textile material

A variety of textile materials like cotton, nylon polyester, pure polyester, chiffon which were commercially available were selected for the present investigation [18]. Each cloth material was cutted into equal size of 2cm x 2cm pieces. Bacterial pigment in methanol 100 µg/ ml was applied to the cloth material in a warm surface. The cloth material was allowed to dry at room temperature for about 1-2 hours. All the dyed textile material was washed with tap water and allowed to dry at room temperature (30°C) in a shade. For all the experiments white cloth material were used as a control.

Applications of pigment as colorant in paper industry

Various paper materials like simple paper, filter paper, Whatman filter paper, A4 paper, art paper which were commercially available in the market were selected for the present study. Each paper material was cut into equal size of 2 cm x 2 cm [19, 20]. Bacterial pigment in methanol in varying concentrations of 100, 200, 300 µl was applied to the paper material on a warm surface. Then the paper material was allowed to dry at room temperature for about 15 minutes. For all the experiments white paper material was taken as a control.

RESULT

Isolation and identification of bacteria

The bacterial culture isolated from distillery effluent on the Luria-Bertani agar plate was identified on the basis of morphological, cultural, biochemical and physiological characteristics from Microbial Type Culture Collection-Chandigarh as *Planococcus maritimus* and available as GenBank Accession Numbers JN873343.1

Identification of isolate on the basis of whole cell fatty acid methyl ester profile.

The fatty acid composition of bacterial cell is important multiple character phenotypic test employed to reveal identity of isolate associated with rhizosphere [21].MIDI had identified more than 300 fatty acids and related compounds in bacteria and used fatty acids of 9-20 carbon in length for methyl esterification and profiling. Whole cell fatty acid profile of isolate (**Fig.1**) indicated the presence of straight-chain fatty acids at C_{10:0}, C_{12:0}, C_{13:0}, C_{14:0}, C_{15:0}, C_{16:0}, C_{17:0}, C_{18:0}, C_{19:0}, branched saturated fatty acids at isoC_{12:0}, isoC_{14:0}, isoC_{16:0}, anteisoC_{17:0}, monounsaturated fatty acids C_{15:1}w8c, C_{16:1}w5c, C_{17:1}w8c, C_{20:2}w6.9c, Hydroxy fatty acids - C_{10:0} 3-OH, C_{11:0} iso 3-OH, C_{12:0} 2-OH, C_{12:1} 3-OH, C_{12:0} 3-OH, C_{13:0} 2-OH, C_{14:0} 2-OH, C_{15:0} 2-OH, C_{15:0} 3-OH, C_{16:0} 3-OH, [22]. Fatty acid methyl ester analysis revealed that strain of effluent isolate contained typical fatty acids in the cell wall of which did not find any identity of available data in the library.

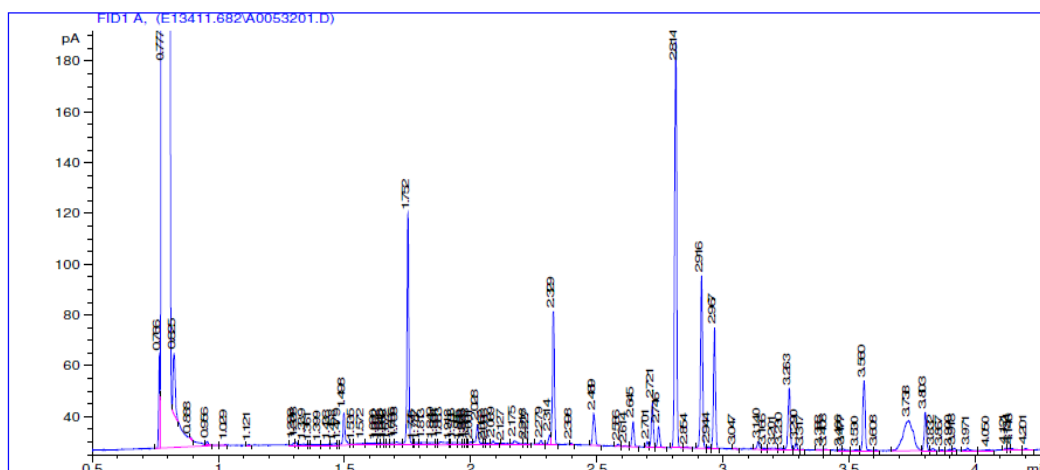


Figure 1: Whole cell fatty acid methyl ester analysis of isolate *Planococcus*

Production and extraction of pigment

Intense orange colored pigment production by isolate *planococcus* was found in Luria Bertani broth. Pigment was extracted by HPLC grade methanol at 80°C for 10 min until all visible pigment was extracted.

Purification of Pigment

Purification of pigment was carried out by phase separation method previously reported by Rodriguez-Amaya, 2001. 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 diethyl ether phase in the separatory funnel (Fig.2). Presence of the pigment in the diethyl ether phase revealed the presence of xanthophilic type of carotenoids [14].



Figure 2: Phase separation of pigment with diethyl ether

Thin Layer Chromatography (TLC)

Thin layer chromatography is the basic tool for the identification of pigment (Taylor & Davies, 1983). Thin Layer Chromatographic plate developed in methanol: toluene (6:5) mixture obtained after optimizing various solvents, showed the separation of pigment into two yellow spots with the same mobility and migration rate, R_f 0.94 and 0.92 respectively. From the literature studies, it revealed that obtained R_f values showed close similarity with the values of standard carotenoids [24].

HPLC Analysis of the pigments

HPLC is one of the most widely applied analytical separation technique. From the large complex mixtures HPLC separates the component present in the mixture. HPLC chromatogram of pigment extract exhibited (Fig.3) a major peak with retention time 3.0833m and minor peak with retention time 4.18 m which revealed the presence of carotenoids.

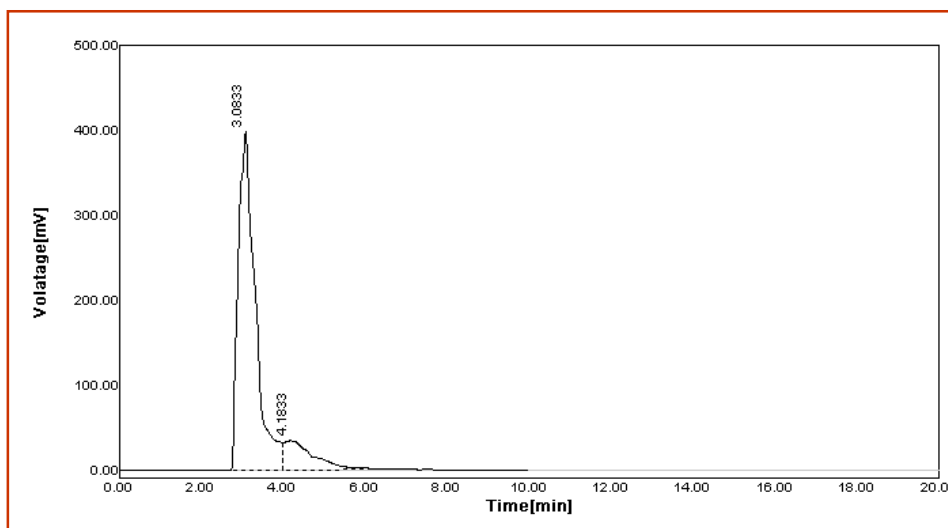


Figure 3: HPLC Chromatogram of pigment extracted from *Planococcus*

Characterization of pigment

Spectrophotometric analysis of pigment

UV visible scanning spectra of the pigment extracted in methanol scanned in the range of 200-800 nm, revealed the presence of major peak (Fig.4) with absorption maxima at 466 nm (UV Mini 1240 Shimadzu, Japan). The corresponding absorption was recorded as a 1.421.

The maximum absorption of the pigment between wavelengths of 300–600 nm indicates the presence of carotenoids [25]. Moreover, the earlier research reported that [26] most of the carotenoid pigments have maximum absorption range between 375 -505 nm.

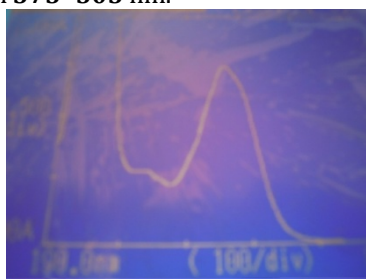


FIGURE 4: ABSORPTION SPECTRUM OF THE PIGMENT FROM *PLANOCOCCUS MARITIMUS*

Liquid Chromatography-Mass Spectroscopy (LC-MS)

The Liquid Chromatography-Mass Spectrum of the pigment (Fig.5) sample extracted from *Planococcus* illustrated the major peak with their relative molecular mass (m/z) (basepeak, M^+) 537 Da with and fragmentation pattern through 523.58, 152.17, 192.25, 299.42, 327.42, 509.67, 551.42, 565.33, 831.33, 1036.58, 1131.33, 1393.75 mass.

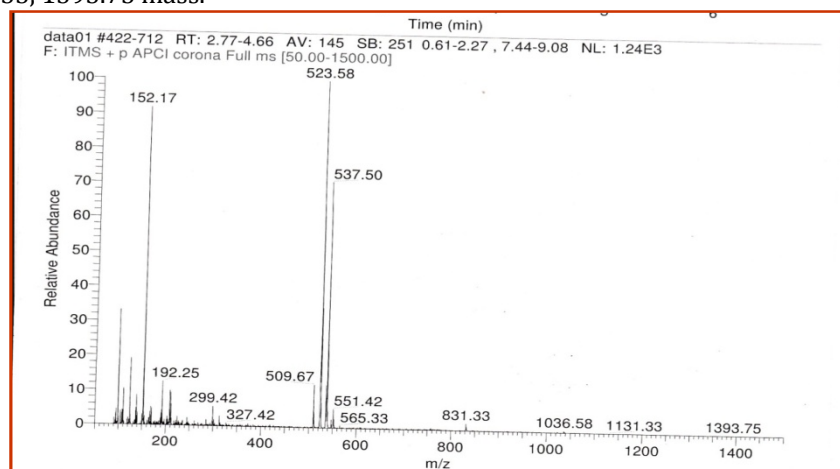


Figure 5: LC-MS analysis of pigment extracted from *Planococcus*

Identification of pigment by IR analysis

One of the main identification tests for pigment is IR spectrum. Fourier transform infrared spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. FTIR spectra are considered for the chemical profiles of pigment samples and it can be used to predict the functional groups present in the sample. The FT-IR absorption spectra of the pigment sample extracted from *Planococcus* exhibited the presence of following characteristic groups. CH stretching vibration absorption peak of alkenes (=C-H) at 3053.42 cm⁻¹, C=N stretching vibration peak at 2305.01 cm⁻¹, CH bending vibrations of alkanes (-CH₂) at 1423.51 cm⁻¹, primary or secondary alcohol (O-H) in plane bend at 1265.35 cm⁻¹, (-CH=CH) cis at 740.69 cm⁻¹, (C=CH₂) at 896.93 cm⁻¹, Poly sulphide (S-S) stretch at 466 cm⁻¹, (C≡C-H), alkynes of 709.83 cm⁻¹ [27] [Useful FTIR Info & Links]. The IR spectral analysis of the methanol extracted pigment reveals the presence of carotenoids (**Fig. 6**)

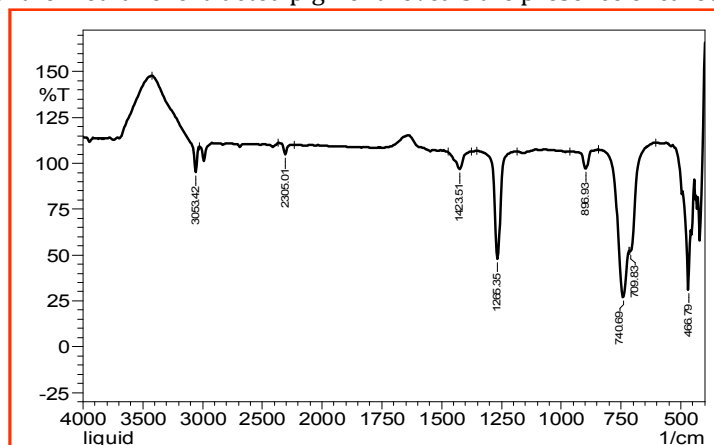


Figure 6: FTIR analysis spectrum of the methanol extracted pigment

On the basis of this spectroscopic characterization and literature survey, it may be concluded that the pigment purified from isolated species revealed the presence of carotenoid type. Literature survey suggested that Methyl glucosyl-3, 4-dehydro-apo-8'-lycopenoate, a novel antioxidative glyco-C-30-carotenoic acid produced by a marine bacterium *Planococcus maritimus* [28].

Applications of pigments as a colorant in textile industry

When pigment sample at 100 µg/ ml was applied to variety of textile materials like cotton, nylon polyester, pure polyester chiffon. Results obtained showed that the pigment produced from *Planococcus* can be effectively used as a colorant to color all the textile materials (**Fig. 7**) studied. Dyeing ability was found to be more on cotton cloth as compared to other cloths. It was reported that the pigment produced by *Pseudomonas fluorescens* VMRP2124 can be effectively used to dye all the textile materials like cotton, spun polyester, swiss cotton, propylene cotton and pure polyester [29].

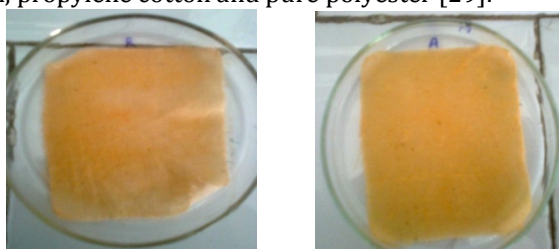
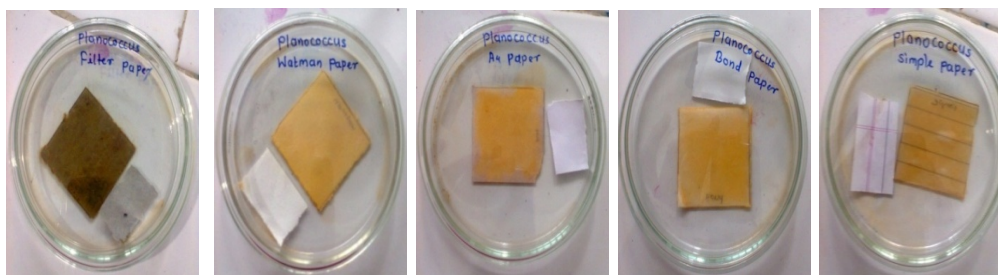


Figure 7: Application of pigment on textile materials

Application of pigments as a colorant in paper industry

Methanol extracted pigment at varying concentrations of 100, 200, 300 µl when applied on various papers with white paper material as a control, Results presented in the (**Fig.8**) revealed that pigment produced by *Planococcus* can be effectively used as a biocolorant for different grades of paper. The pigment in concentrations of 100 to 300 µl seemed to have a binding property with maximum absorbing intensity of orange coloration was observed at 300 µl concentrations with filter paper and Whattman paper.



Filter Paper Whatman Paper A4 Paper Bond paper Simple paper

Figure 8. Application of pigment to various types of papers

Discussion-

The bacterial strain isolated from distillery spent wash as a *Planococcus* produced a great amount of intense orange pigment in Luria Bertani broth. Isolate was identified on the basis of morphological, biochemical, molecular and FAME analysis, which belong to the genus *Planococcus maritimus*. Methanol extract of the pigment was separated in diethyl ether phase by liquid-liquid extraction method and purified by TLC and HPLC. Development of yellow spot with migration rate of 0.94 and retention time of 3.0833 in HPLC chromatogram confirmed the presence of carotenoid type of pigment. Purified pigment was further characterized by UV-Visible Spectrophotometric, LC-MS and FT-IR method and identified as a glucosylated carotenoid. Pigment have strong antibacterial and antioxidant activity (data not shown here) as well as it have effective dyeing potentials for textile and paper which finds broad applications in textile, paper and cosmetic industries.

CONCLUSION

Isolated organism *Planococcus maritimus* from distillery spent wash produces carotenoid pigments. It is desirable that synthetic pigments should be replaced by natural colorants. Carotenoids are one the most important natural colorant having antioxidant and antibacterial activity. Pigment produced by isolated strain was characterized by UV-Visible spectroscopy, LC-MS and FT-IR and found to be contained functional groups of carotenoid pigment. Purified pigment has strong antioxidant and antibacterial activity as well as it can be used as a colorant for textile, paper and printing dye industries.

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CONFLICT OF INTEREST

Author declares that there is no any conflict of interest.

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