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ORIGINAL ARTICLE **OPEN ACCESS**

Evaluation of Antagonistic and Antioxidant activity of Carotenoid pigments produced by *Planococcus maritimus* isolated from distillery spent wash

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ABSTRACT

In the present research work, an intense orange pigment producing bacterial strain was isolated from a distillery spent wash was characterized by morphological, physiological, biochemical, molecular features 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% methanol. The pigment revealed the presence of carotenoid type of the pigment. The striking applicability of the pigment was investigated and found that it exhibit strong antibacterial activity against four target bacterial pathogens of health significance like Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Proteus vulgaris, as well as antifungal activity against selected fungal pathogens Aspergillus and Penicillium species by in vitro techniques. Likewise the extracted pigment was evaluated for the total antioxidant potential by phosphomolybdenum and Ferric reducing antioxidant power assay and results represented in Ascorbic acid Equivalent (AAE).This studies revealed that newly isolated Planococcus maritimus strain from distillery spent wash as a non-pathogenic bacterium, can be a good source with antimicrobial and antioxidant activity for various applications in pharmaceutical, food and cosmetic products.

Keywords: *Carotenoids, Planococcus maritimus, antagonistic and antioxidant activity.*

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INTRODUCTION

Color is an important characteristic and vital constituent of food. Color is added to the food to make it attractive, appetizing, indicate its freshness, informing quality and safety. Biosynthesis of colorants for food, pharmaceutical and textile applications has attracted increased interests in recent years [1]. However, synthetic colorants are facing the challenges like dependence on non-renewable oil resources and sustainability of current operation, environmental toxicity, and human health concerns of some synthetic dyes [2]. 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 [3]. Natural dyes are non-toxic, nonpolluting, and less health hazardous [4] as well as their antioxidants and antimicrobial nature further adds to their positive effects [5]. 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 yields in pigments and lower residues compared to the use of plants and animals[6]. 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 [7, 8, 9]. Many studies have been made about investigation of antioxidant and vitamin effects of pigments. Another property of pigments is their antimicrobial effects that have very little research conducted about it [10].

Microbial pigments that can be used as natural colors as well as antimicrobial agents instead of antibiotics [11]. Thus investigations of the antimicrobial activity of natural bacterial pigments are a better source of antimicrobial compound than synthetic drugs therefore the investigation of the antimicrobial activity of natural products have created the new way for drug development in the control of antibiotic resistant pathogen [12]. Thus present research work is carried out to study the isolation and identification of pigment producing bacteria, and evaluation of antimicrobial and antioxidant activity of the pigment produced by isolate.

MATERIAL AND METHODS

Isolation of bacterial strain

Culture of pigment producing bacteria was isolated on Luria -Bertani agar (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 4^0C and sub cultured after every 30 days. The target bacterial pathogens for antibacterial assays were obtained from NCIM, Pune and maintained on nutrient agar plates at 37^0C for 24-48 h.

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). This sequence was 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) was identified [13].

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 until the broth attaining dark orange color. After incubation cells were harvested by centrifugation and washed thrice with distilled water by repeated centrifugation (13).

Pigment extraction

Extraction of pigment was carried out by centrifugation at 10,000 rpm for 10 min by the use of 80% HPLC grade methanol [14]. Pigment was completely extracted from biomass till cell pellet appeared as colourless. Estimation of extracted carotenoid pigment was carried out at 466 nm by spectrophotometer (UV, Mini Shimadzu).

Antibacterial activity of pigment extract

Pigment extracted in methanol was used for antimicrobial test against four target Gram positive and Gram negative pathogenic bacteria reported to affect health significantly [15, 16] using the agar disk diffusion technique in petridish. The pathogenic bacterial cultures - Gram positive bacteria like *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063*, and Gram negative pathogens like E. coli* NCIM 2065*, Proteus vulgaris* NCIM 4175, were used for the antibacterial activity by in vitro techniques using nutrient agar plates at 37^0C for 24-48 h. Antibiotic streptomycin (100µg/ml) was used as positive control and methanol was used as negative control. Paper disk of size 6 mm dipped in two different concentrations 100 µg/ml and 50 µg/ml were used for antibacterial activity. A clear zone of inhibition around the disc was the indicative of antimicrobial activity. Diameter of the zone of inhibition was measured in millimetre. The experiment was repeated in triplicate for each pathogenic bacterium. Similarly antifungal activity of pigment against fungus *Aspergillus niger* and *Penicillum* were tested

Antioxidant activity of pigment extract

Reducing power assay

The ferric reducing antioxidant power of the pigment sample was performed by the method previously reported method [17, 18, 19]. The methanol extract of test pigment sample was taken in various concentrations (0.2 - 1mg/ml). Ascorbic acid at various concentrations of 20-100ug/ml was used as standard. Test pigment sample was mixed with 2.5 ml of 50 µM phosphate buffer (pH 6.6), 2.5 ml 0.1% (w/v) potassium ferricyanide and incubated in a water bath at 50°C for 20 min. 2.5ml 1% (w/v) trichloroacetic acid solution was added to the mixture and centrifuged at 3000 rpm for 10 minutes. 2.5ml of the upper layer was carefully removed and combined with 0.5 ml of 5 mM ferric chloride and the absorbance of the reaction mixture was measured at 700 nm. A blank was run in parallel with all mixture without the pigment sample. The reducing power was expressed as microgram of ascorbate equivalent per mg dry weight of the test sample. Increased absorbance of the reaction mixture indicates increase in reducing power. The procedure was carried out in triplicates and the results were expressed as mean of triplicates.

Total antioxidant activity by Phophomolybdenum assay

Total antioxidant activity of methanolic extract of pigment was determined by the phosphomolybdenum assay. It is based on the reduction of Mo (VI) - Mo (V) by the antioxidant and subsequent formation of green phosphate/Mo (V) complex at acid pH. [20, 21]. 0.3 ml test pigment sample was added with a 3 ml of reagent solution containing 0.6 M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95^0C for 90 min. The absorbance of mixture was measured at 695 nm with

methanol blank. All the reaction was carried out in triplicate and results were expressed as mean of triplicate. Ascorbic acid in a concentration of 100mg/ml was utilized as reference standard. The antioxidant activity was expressed as (mean values) number of mg equivalent of ascorbic acid per 100 g of extract. The total antioxidant activity of the test sample was expressed as number of mg equivalent of ascorbic acid per gram of dry weight of the extracted pigment.

RESULTS

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*.** Molecular identification of isolate performed by 16S rRNA sequencing and submitted to GenBank and are available as **GenBank Accession Numbers JN873343.1**

Production and extraction of pigment

Intense orange colored pigment by isolate *planococcus* was found to be produced in Luria Bertani broth. Pigment was extracted by HPLC grade methanol.

Figure 1:- Biopigment production in shake flask level.

Antibacterial Assay

After incubation of plates at 37^0C for 24-48 h the highest zone of inhibition was observed against the pathogens *Staphylococcus aureus, E.coli* and *Bacillus spp.* which was 24.0 ± 0.21 mm, 14.0 ±0.42 mm and 19.0 ± 0.2 mm respectively at higher (100 µg/ml of) concentration of extract while minor zone of inhibitions 10.0 ± 02mm were also observed against the *Proteus* and *Bacillus* species. This data obtained revealed that the methanolic extract of the pigment showed effective antibacterial activity.

The literature survey reported that Carotenoid type of pigment produced by *Sporobolomyces* sp. was found to be highly inhibitory for *E coli* with the inhibition zone of 28 mm. The carotenoid pigment possessed good activity against *S. aureus* 26 mm. The pathogens like *S. faecalis* and *B. subtilis* were more effectively inhibited by the pigment extract of *Sporobolomyces* sp. producing a zone of 22 and 23 mm. [22].

Table 1:-Antibacterial activity of carotenoid pigment against human pathogens

Sl No	Sample	Concentration (µg/ml)	*E. coli*	*P. vulgaris*	*S. aureus*	*B. subtilis*
			Zone Diameter (mm)			
1	Pigment	100	14.0± 0.42	10.0± 0.2	24.0± 0.21	19.0± 0.2
		50	13.0± 0.32	07.0± 0.2	20.0± 0.2	08.0± 0.2

Values are average of triplicates with mean

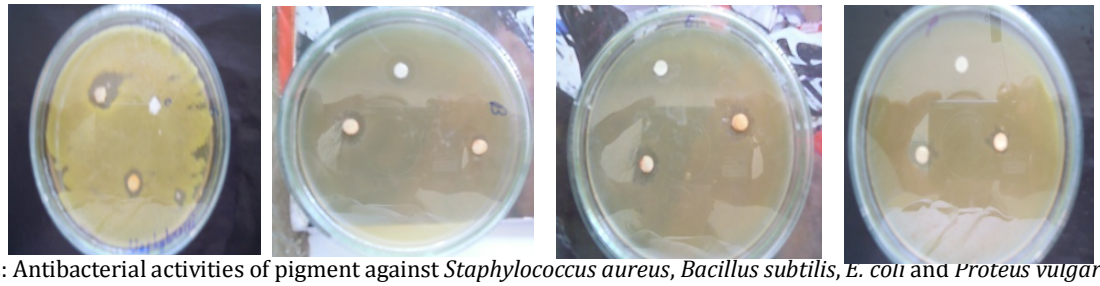

Figure2: Antibacterial activities of pigment against *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli* and *Proteus vulgaris*

The antifungal activity of pigment was determined against *Aspergillus* and *Penicillium* based on the inhibition of mycelial growth in fungus. The data revealed that very minor zone of inhibition was observed by inhibiting the growth of fungus Aspergillus. These results concludes that methanol extract of pigment have very less antifungal activity but shows strong antibacterial activity.

Antioxidant activity of pigment

Reducing power assay

The reducing activity of methanolic extract of pigment was analysed by reducing power assay and was found to be remarkable. The pigment exhibited strong antioxidant capacity. The reducing power was expressed in terms of microgram of ascorbate equivalent per mg dry weight of the test sample.

The experimental results revealed that reducing activity of pigment was found to be 50.2±0.31 µg of ascorbic acid equivalent per milligram of sample .

Total antioxidant activity by phosphomolybdenum assay

The total antioxidant potential of the orange pigment extract was evaluated using Phospomolybdenum assay. It results in direct estimation of reducing capacity of antioxidant. It quantitatively estimated total antioxidant activity of pigment, which is expressed as the number of equivalents of ascorbic acid (AA). Experimental results revealed that pigment exhibited stronger total antioxidant activity of 82.0±02 mg of ascorbic acid per gram of sample.

Discussion-

The isolated bacterial strain from distillery spent wash was identified as *Planococcus maritimus,* produces a prominent orange pigment on Luria Bertani medium. The pigment was extracted in methanol and identified as Carotenoids. The antibacterial activity of pigment against the human pathogens like *Escherichia coli, Proteus vulgaris, Staphylococcus aureus* and *Bacillus* were determined and found that it shows strong antibacterial activity and minor antifungal activity also. Antioxidant activity of the pigment was determined by reducing power assay and phosphomolybdenum assay and found that pigment revealed the prominent antioxidant activity which can be used in pharmaceutical and food industries.

CONCLUSION

In general natural products have immense important in human health care and prevention of diseases. Carotenoids are pigments which are commercially important in industries like cosmetics, food and medical as natural colorant. Antibacterial activity of the pigment proved the preservative characteristics of this natural food colorant and antioxidant property of this pigment can be used in the field of pharmaceutical, cosmetics and food industries.

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