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Activity of novel yellow pigment produced by *Micrococcus yunnanensis* S-CSR-0010 against multidrug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

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ABSTRACT

This work was undertaken with an aim of isolating pigment producing bacteria with antibacterial property against multi drug resistant organisms. A novel yellow pigment producing bacterium isolated from local area was screened for antibacterial activity against pathogenic as well as multidrug resistant organisms. Pigment production was optimized under various parameters like pH, temperature and medium. Yellow pigment best extracted by using acetone as solvent, showed an absorption peak of 445 nm under UV-Vis spectrophotometer which is close to the absorption peak of beta carotene. This particular pigment showed inhibitory effect on *Pseudomonas aeruginosa* and multidrug resistant *Staphylococcus aureus* (MDRSA) with an inhibitory zone of 16 mm and 18 mm respectively. Bacterium was identified by morphological characteristics and 16S rRNA sequencing as *Micrococcus yunnanensis* S-CSR-0010, submitted to GenBank (accession number of KT443901). Optimum temperature and pH for pigment production was 30 °C and 7.0 respectively. The best medium for pigment production was found to be peanut broth. Present findings highlight the significance of yellow pigment isolated from soil. Further purification of pigment will be useful in discovering a promising drug.

Keywords: Absorption peak, antibacterial activity, beta carotene, *Micrococcus yunnanensis* S-CSR 0010, MDRSA, *Pseudomonas aeruginosa,* yellow pigment.

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INTRODUCTION

Pigments from natural sources have been obtained since long time ago and their interest increased due to toxicity problems with synthetic pigments. It may cause severe damage to vital organs or may be carcinogenic [1]. The waste of production process are also harmful. The natural pigments from plants also have instability against light, heat or adverse pH, low water solubility and are often not available throughout the year. Hence the microbial pigments are of great interest. Microbes can grow easily and fast in the cheap culture medium and independent from weather conditions. So, a work should be concerned on the bacterial pigments for finding cheap and suitable growth medium in order to reduce the cost and increase its applicability for industrial production.

Microorganisms can produce various pigments such as carotenoids, melanins, flavins, quinones, prodigiosins, monascins, violacein or indigo. Temperature of incubation is the main factor which depends on the type of microorganism. Carotenoids are yellow to orange red pigments present in varieties of plants, bacteria and fungi [2]. Recently, carotenoids are used commercially for nutraceuticals, cosmetic and pharmaceutical purposes [3]. Carotenoids can inhibit various types of cancer and can enhance immune response [4]. Also it can inhibit life style related diseases due to their provitamin action and higher antioxidant capacity [5]. In future, by genetic engineering of microbial pathway enzyme can produce high amount of carotenoids. Since man started suffering from various diseases started the search for antibiotics from microorganisms. Many bacterial infections caused by bacteria were resistant to antibiotics caused a major health care problem. Therefore, there is an urgent need to search antibiotics against multidrug resistant infections. In the present study, an attempt was made to isolate and characterize yellow pigment producing bacterial strains for carotenoid production and antibacterial activity.

MATERIALS AND METHODS

The media used in this study were obtained from HiMedia Laboratories, India and chemicals used were of analytical grade.

INSTRUMENTS USED FOR THE STUDY

Orbital shaking incubator (Remi Elektrotechnik Limited, India), UV-Vis spectrophotometer (Thermo Scientific Evolution 201), electronic balance (Shimadzu AY220).

ISOLATION OF YELLOW PIGMENT PRODUCING ORGANISM

Soil samples were collected from various locations near SIAS College and hostel premises, by scraping off the soil surface with a sterile spatula and about 10 g of soil was obtained from a depth of 2-5 cm. Bacteria present in the soil were isolated by serial dilution, spread plated on nutrient agar medium and incubated overnight at 30 °C. The bacteria were first inoculated onto nutrient agar plate and incubated at 30 °C for 24 h. The bacterial colonies formed were then transferred into Luria-Bertani –glycerol broth prior to storage at -20 °C.

IDENTIFICATION OF ORGANISM

Bacterial colonies were sub cultured on nutrient agar slants, identified by staining methods, and 16S rRNA sequencing, sequence comparison with the databases was performed using BLAST through NCBI database. The 1,485 bp 16S rRNA sequences of strain was submitted to GenBank database.

PRODUCTION OF PIGMENT

For maximum production of pigment, the medium used were nutrient agar (NA), nutrient broth (NB), peptone glycerol broth (peptone – 5 g, dipotassium hydrogen phosphate – 2 g, glycerol – 10 ml, distilled water – 1000 ml), peanut broth and Luria-Bertani LB broth. The cultures were inoculated by streak plate method to a suitable media on the petriplate or inoculated to a suitable broth. After incubating at a suitable temperature and time, pigment production was observed.

ISOLATION OF THE PIGMENT

Solvents used for the extraction were ethanol and acetone. Organism were cultured on suitable media on plates or broth and incubated at suitable temperature and time. Pigments were isolated from both plates and broth. Cells were scraped from the surface of the agar with scrapper, washed with distilled water and centrifuged to get cell pellet at 5,000 rpm for 10 min. Washed cells were treated twice with absolute ethanol and acetone for extraction of pigment. During each extraction, the suspension was agitated at room temperature, heated in a boiling water bath to extract the pigment completely by the ethanol and acetone from the cells. Again it was centrifuged at 6,000 rpm for 10 min, extraction with ethanol and acetone was done until the cells become colourless and supernatant was collected [6].

MAXIMUM WAVELENGTH ABSORPTION

The absorption spectrum of acetone extract of the cell pellet from the bacterial isolate was noted by using UV-Vis spectrophotometer.

EFFECT OF DIFFERENT TEMPERATURE ON PIGMENT PRODUCTION

Inoculum (5%) was added to 15 ml of nutrient broth in three 100 ml flask. Flasks were incubated at 24 ˚C, 26 ˚C, 28 ˚C, 30 ˚C, 32 ˚C, 34 ˚C and 36 ˚C. Nutrient broth without inoculum was used as control. Following 36 h of incubation, 1 ml was taken for pigment extraction. Pigment production was estimated as absorbance at 439 nm (OD) using UV-Vis spectrophotometer.

EFFECT OF DIFFERENT pH ON PIGMENT PRODUCTION

Nutrient broth was prepared in three 100 ml flask, pH of the medium were adjusted from 5.0, to 9.0. Nutrient broth without the inoculum was used as control. Following 36 h of incubation, 1 ml was taken for pigment extraction. Pigment production was estimated as absorbance at 439 nm (OD) using UV-Vis spectrophotometer.

EFFECT OF DIFFERENT MEDIA ON PIGMENT PRODUCTION

Nutrient broth, peanut broth, Luria-Bertani broth and peptone glycerol broth (15 ml) were prepared in 100 ml conical flask, pH of the medium prior to inoculate was uniformly adjusted to 7.5, fresh pigmented inoculum was added to each of the broth and incubated at 37 °C. Nutrient broth, peanut broth, Luria-Bertani broth and peptone glycerol broth without inoculum were used as control. Following 36 h of incubation, 1 ml was taken for pigment extraction. Pigment production was estimated as absorbance at 439 nm (OD) using UV-Vis spectrophotometer.

INOCULUM PREPARATION

An isolated colony of the multiple drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* were taken from nutrient agar and inoculated in to 5 ml nutrient broth in a test tube. This was incubated at 37 °C, 3-4 h to reach turbidity of 0.5 McFarland. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. This turbidity was considered approximately equal to 1.5 X 10^8 CFU/ml.

ANTIBACTERIAL ACTIVITY

Antibacterial activity was analyzed against multidrug resistant *Staphylococcus aureus* (MDRSA), strains of *Pseudomonas aeruginosa*. The cultures were spread in a solidified Muller Hinton agar medium using sterilized swab. Sterile paper discs (HiMedia) were immersed on pigment and placed on inoculated Petri plates. The plates were incubated at 37 °C for 24 h. The zone of inhibition was observed and antimicrobial activity was studied.

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RESULTS

ISOLATION AND CHARACTERIZATION OF THE SOIL ISOLATE

They were Gram positive cocci, yellow, smooth, mucoid or moist, opaque with entire margin arraged as clusters and are non motile, aerobic, produces yellow pigments (Fig.1). They grow at a temperature of 30 °C and pH 7.0. The 16S rRNA analysis showed that the isolated strain was *Micrococcus yunnanensis* S-CSR 0010. The individual yellow colour colonies obtained was sequenced, submitted to gene bank under the accession number. Phylogenetic analysis revealed, that the identified strain was closely related to *Micrococcus yunnanensis*. The crude pigment showed promising results against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

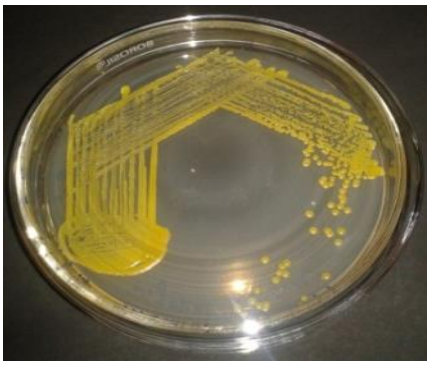

Fig. 1: Yellow pigmented soil isolate

EXTRACTION OF PIGMENT

Extraction for the yellow colored pigment was carried out using ethanol and acetone as a solvent. Acetone was found to be most suitable solvent for extraction. They are insoluble in water and soluble in organic solvents.

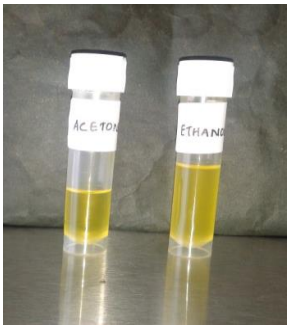

Fig. 2: Pigment extracted in acetone and ethanol as solvents

MAXIMUM WAVELENGTH ABSORPTION BY UV-VIS SPECTROPHOTOMETER

The absorption spectrum of acetone extract of the cell pellet from the bacterial isolates showed maximum absorption at 445 nm (Fig. 3) which is identical to the absorption spectrum of beta carotene reference sample.

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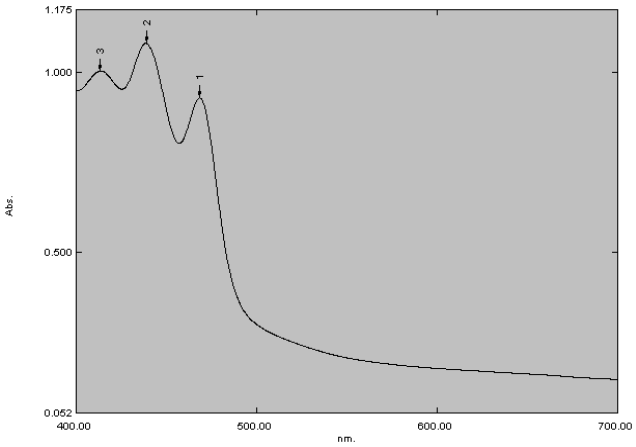

Fig. 3: Absorption spectrum of pigment

EFFECT OF TEMPERATURE ON GROWTH AND PIGMENT PRODUCTION

The maximum growth and pigment production was observed at 30 °C. There was a gradual and uniform decrease in growth and pigment production with the increase in temperature from 30 °C to 36 °C (Fig. 4).

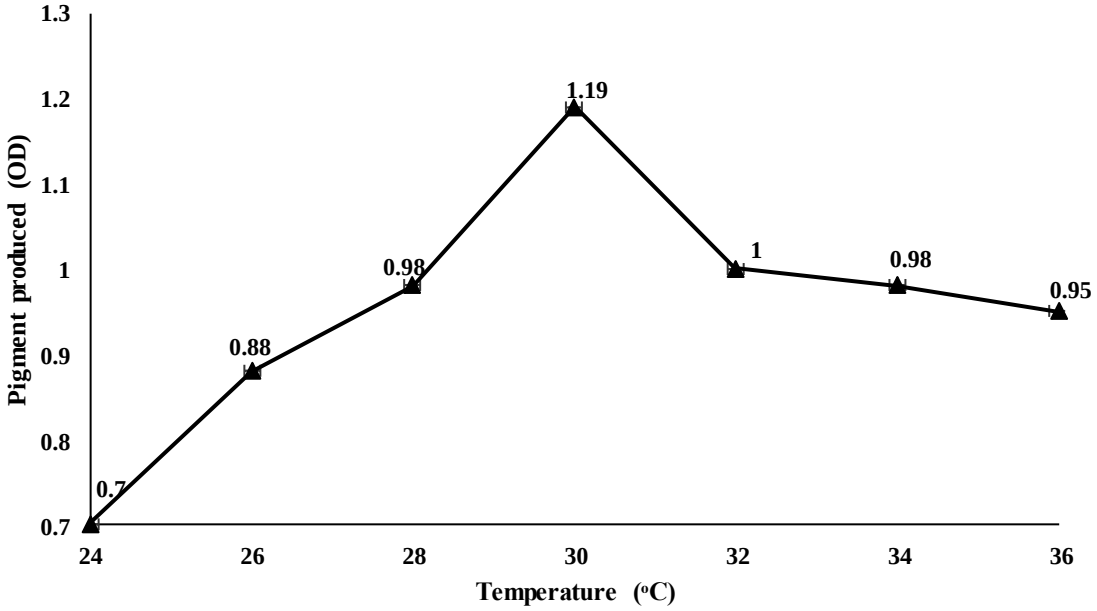

Fig. 4: Effect of temperature on pigment production, n=7

EFFECT OF pH ON GROWTH AND PIGMENT PRODUCTION

It was observed that the maximum growth and pigment production was obtained at pH 7.0. There was a gradual and uniform decrease in growth and pigment production with the increase in pH from 7.0 to 9.0 (Fig. 5).

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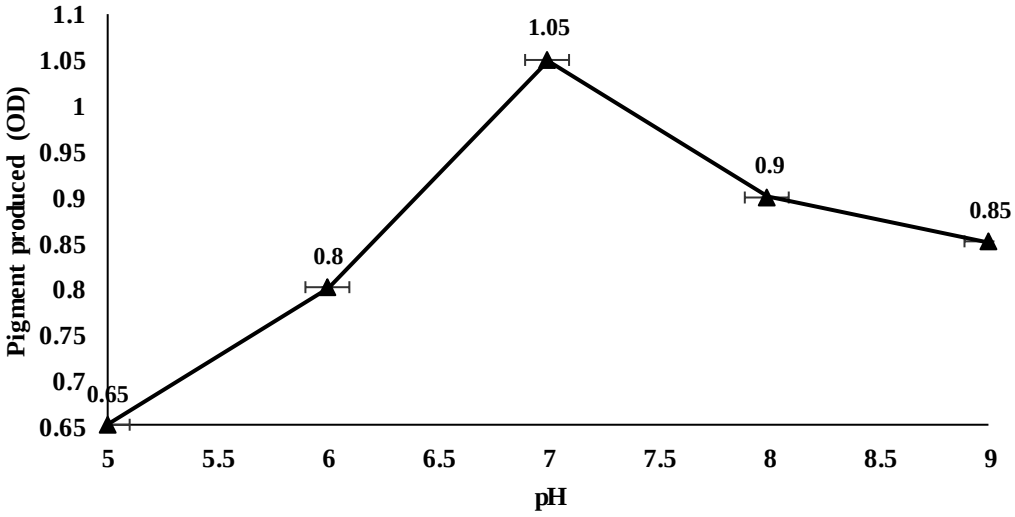

Fig. 5: Effect of pH on pigment production, n= 9

EFFECT OF DIFFERENT MEDIA ON GROWTH AND PIGMENT PRODUCTION

It was observed that the maximum production of pigment observed in peanut broth. It was observed that the powdered peanut increases pigment production (Fig. 6).

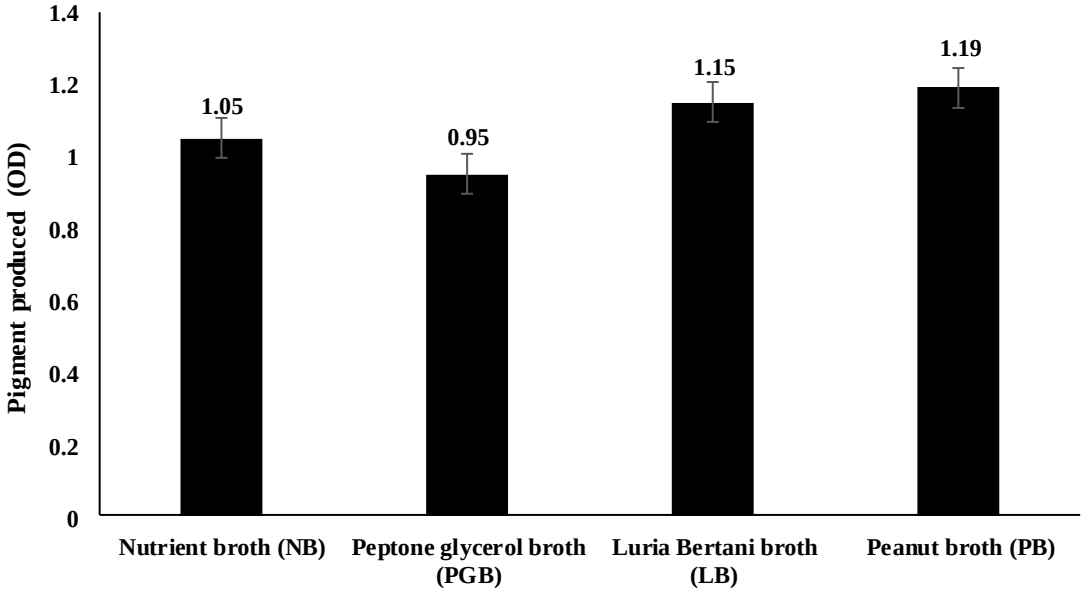

Fig. 6: Effect of media on pigment production, n=4

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ANTIBACTERIAL ACTIVITY EXHIBITED BY SOIL ISOLATE

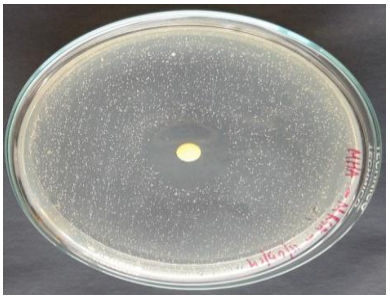

Fig. 7: The pigment gave zone of inhibition (18 mm) against *S. aureus*

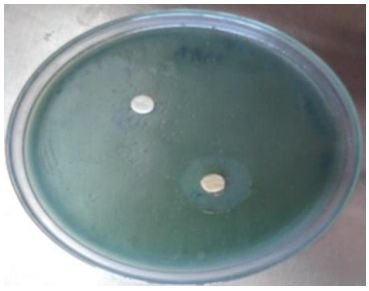

Fig. 8: The pigment gave zone of inhibition (16 mm) against *Pseudomonas aeruginosa*

The antimicrobial compound derived from the soil isolate will be useful in developing antibiotics against drug resistant bacteria.

DISCUSSION

Yellow pigment producing bacteria isolated from soil and the pigment was extracted by using ethanol and acetone as solvents. From the 16S rRNA sequencing, the organism was identified as *Micrococcus yunnanensis* S-CSR 0010 (accession no. KT443901). *Actinobacterium* sp. was first isolated from surface sterilized polyspora axillary roots [7]. Maximum absorbance was seen at 445 nm which was similar to the absorption spectrum of beta carotene (450 nm) [8, 9]. European Union committee considers that beta carotene produced by *Blakeslea trispora* is equivalent to chemically synthesized material used as food colorant [10]. Reports showed carotenoid production by *Myxococcus* sp. [11], *Streptomyces* sp. [12], *Sulfolobus* sp. [13], and *Mycobacterium* sp. [14]. To the best of our knowledge, this is the first report representing the carotenoid production from this particular organism. Bio-pigments synthesized by bacteria possess enormous efficiency as medically important products. Pigments from bacterial origin have no seasonal production problem and have high productivity. In order to increase the potentiality of the bacteria to synthesize large quantities of the pigment a comparative study of different media, role of temperature, growth of the organism in different media and pigment production must be studied. By analyzing the physiological characteristics it was found that, the maximum carotenoid production was seen at temperature of 30 °C and pH 7.0. A yellow pigment producing *Exiguobacterium* sp. showed a temperature and pH optima of 37 °C and 7.0 respectively [6].

The components of the different media should be analyzed and compared effectively, to deduce the most probable reason for the enhancement or the decline in pigment production. Keeping these objectives in mind, a media which could support the growth of the bacteria and at the same time prove efficient to trigger high levels of pigment formation was designed. The powdered peanut medium gave the highest yield at 30 °C. Peanut acts as a better source of substrate in enhancing pigment production in nutrient broth. Carotenoid production mainly depend on the environmental conditions, medium composition and the solvent used [15]. From antibacterial sensitivity test we can conclude that the pigment produced by *Micrococcus yunnanensis* showed antibacterial activity against multiple drug resistant *Staphylococcus aureus and Pseudomonas aeruginosa.* To the best of our knowledge, none of the bacterial pigments showed activity against multidrug resistant organisms. An *Actinomycete* sp. showed antimicrobial activity against multiple drug resistant *S. aureus* [16]. An *Actinomycete* 1B49 isolated from farming soil of Turkey showed activity against *S. aureus* with a zone of 16 mm [17]. *Chromobacterium* sp. produced violacein pigment, showed a zone of 15 mm against *Staphylococcus* sp. [18]. Violet pigment from psychrotrophic bacterium RT102 showed activity against *S. aureus* with minimum inhibitory concentration of 15 mg/L [19]. *Exiguobacterium* sp. from peninsular region showed activity against *S. aureus* with a zone of 12.5 mm [20]. Carotenoid pigment from *Sporobolomyces* sp. showed inhibitory activity against *S. aureus* and *P. aeruginosa* [21]. Pigment from *Pseudomonas aeruginosa* showed antibacterial activity against *S. aureus* with a zone of 17 mm [22]. The antimicrobial compound derived from the strain will be useful in developing antibiotics against drug resistant bacteria.

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CONCLUSION

Carotenoid pigment extracted from *Micrococcus yunnanensis* S-CSR 0010 showed high antibacterial activity against *Pseudomonas aeruginosa* and *MDRSA*. Since no one had attempted so far about the antibacterial activity of pigment against multidrug resistant organisms. A comparative study of different media, pH, and temperature were done in order to increase the potentiality of organism to synthesise pigment in very large quantities. Optimum temperature and pH for pigment production was 30 °C and 7.0 respectively. The best medium for pigment production was found to be peanut broth. Studies are in progress about the activity of this pigment against other multidrug resistant organisms.

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