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PHOTO EFFECT AND BIO-AUTOGRAPHIC STUDIES OF INTRACELLULAR ORANGE FLUORESCENT PIGMENT PRODUCED BY *Bacillus endophyticus*

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ABSTRACT

Present study has focused on the effect of chemical (solvents) and physical (photo) conditions on pigment production and its bioactivity of intracellular orange fluorescent pigment (IOFP) extracted from soil bacterium *Bacillus endophyticus*. Standardization of pigment and its colour stability was confirmed by using different solvents (70% & 100% ethanol, hexane, heptane, ethyl acetate, acetone, petroleum ether, chloroform, methanol and distilled water), photo conditions (Dark, U.V light and White light) on pigment production and its bio-activeness by antibacterial activity using agar cup plate method against gram-positive (*Staphylococcus aureus, streptococcus pyogenes* and *Listeria monocytogenes)* and gram-negative (*Salmonella typhi, Vibrio cholera, Shigella Flexneri and E.coli)* human pathogens and purification of pigment by TLC coupled with bio-autographic studies. Acetone is proved to be the best solvent for extraction and the pigment was stable in all solvents without changing its colour except heptane. When compared to control (dark incubation) antibacterial activity of IOFP produced in U.V and W. Light was effective against all tested pathogens with slight differences in their antibacterial activity. TLC bio-autographic studies reveal that the separated pure band shows clear zone of inhibition under red back ground of live cells stating that, the compound is active against human bacterial pathogens. Hence this study concludes that, the production and biological activity of the IOFP was independent of light incubation, and TLC guided bio-autographic approach offers a rapid detection technique that avoids the testing of purified fraction once again.

Keywords: Anti-bacterial activity, Bio-autography, colour, intracellular, fluorescent, pigment, stability, light incubation

INTRODUCTION

Bacteria are known for their importance in producing pharmaceutically important bioactive compounds. Pigments produced by bacteria are one of such compounds that have been reported to have multi-disciplinary applications in food, textile, pharmaceuticals, cosmetics, Neutraceuticals and medical fields (**Chidambaram *et al*., 2009**).Various pigments like monascins, melanins, flavins, quinones, prodigiosins, violacein or indigo specifically carotenoids have been reported previously (**Chaudhari *et al*., 2013**). Carotenoids have highest range of biological significance due to epidemiologically correlated with a lower risk for several diseases (**Dufosse *et al*., 2009**), anti-tumor activity (**EL-Banna *et al*., 2012**).Planar chromatographic analysis hyphenated with the biological detection method is termed as bio-autography (**Lee *et al*., 2004**). It is an effortless, quick and easy on the pocket method for the screening of complex bio extracts from chemical and biological origin, with sub sequent bioassay-guided isolation (**Muller *et al*., 2004**). The major applications of bio-autography is fast screening of a large number samples for bioactivities such as antibacterial, antifungal, antioxidant, enzyme inhibition, etc. and in the target-directed isolation of active compounds (**Hostettmann *et al*., 1997; Aerts *et al*., 2000; Trivedi *et al*., 2017; Michael *et al*., 2017**).Our current research has focused on production of pigment in various light incubations, stability indifferent solvents and evaluation of anti-bacterial activity using traditional well diffusion method followed by bio-autographic studies of an orange fluorescent pigment produced *Bacillus* sp.

MATERIALS AND METHODS

Pigment source

Orange fluorescent bacterium used in this study was isolated from chilli rhizospheric soil at Achempeta, Guntur district, Andhra Pradesh, India.

Pigment extraction and standardization

Pigment extraction was done as per the procedure described by **Mantri Sairam, (2017)** (**Prasanna *et al*., 2009**), standardization using different solvents (70%ethanol-EtOH, 100% ethanol-Et-OH, chloroform-Chlrfm, acetone, petroleumether-P.ET, 70% methanol-MeOH, and distilled water-D.H2O). After centrifugation at 10000 rpm for 5min, colourless pellet was discarded and the coloured supernatant was measured for its optical density at 450nm (**Munoz *et al*., 2004**).

Colour stability of Pigment in various Solvent

Colour stability of acetone extracted pigment was determined by using different polar solvents. Dried pigment was dissolved in polar solvents like hexane, heptane, ethyl acetate and acetone at the concentration of 1mg/ml and vortex until the pigment was dissolved, and kept for 15 min to observe colour stability (**Marar *et al*., 2015**).

Effect of photo conditions (light incubation) on pigment production

Effect of light during incubation on pigment production was evaluated by incubating the bacterial culture under three different light sources such as ultraviolet radiation obtained by the use of a metal halogenated-high-pressure lamp (solar simulator SOL2, Germany) (290nm), white light (520nm by fluorescent tube light) and in dark (without light) for 48 hours at 35^{o}C. Extraction was carried out from incubated both using the above extraction procedure and pellet was collected in three vials having 5ml of acetone. Pigment production was quantitatively measured by absorption spectrophotometer (Shimadzu UV-1800, Japan) with the scanning range of 200 to 800nm and anti-bacterial activity was measured by agar well diffusion method (**Ralf *et al*., 2004**).

Anti-bacterial activity

Test organisms

To evaluate the anti-bacterial activity of the light incubated pigment, test organisms such as *Salmonella typhi, Vibrio cholera, Shigella flexneri, E.coli, Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes* were used.

Agar well diffusion method

Agar well diffusion method was employed to determine the bio-activeness of different light incubated pigments. Sterilized Muller and Hinton agar plates were pre seeded with human bacterial pathogens (*Salmonella typhi, Vibrio cholera, Shigella flexneri, E.coli, Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes)*, and agar wells were made by using 6mm stainless steel borer and loaded with 50µl of acetone extract at 1mg/ml concentration. Plates were incubated at 35^{o}C for 24hours. Anti-bacterial activity was measured in terms of zone of inhibition around the loaded well (**Balwos *et al*., 1992**).

Purification by TLC coupled with bio-autograhy

Purification by TLC was performed based on the protocol by **Mantri Sairam, (2017)** (**Prasanna *et al*., 2009**). Plates were left air dried for 3 days at room temperature for complete evaporation of mobile phase and localization of separated compound after running the thin layer chromatography (TLC). Surface of the TLC plate was sprayed with sterile physiological saline (0.85% NaCl) and 5ml of Muller-Hinton agar medium inoculated with 0.1ml suspension of *Salmonella typhi, Vibrio cholerae, Shigella flexneri, Streptococcus pyogenes, Staphylococcus aureus, Listeria monocytogenes* and *E.coli* (10^6cells/ml^{-1}) separately poured on the surface previously evaporated, saline prayed TLC plates. Inoculated plates were incubated in humid conditions over night at 37±1°C for 24hr. After incubation, TLC plates were sprayed with 2% (*m/V*) sterile triphenyl-tetrazolium chloride (TTC) solution and allowed to re-incubate for 6hr. observe zone of inhibition around the separated compound/band on the TLC plates (**Mantri Sairam *et al*., 2017; Choma., 2005; Magyarory *et al*., 2002**).

RESULTS AND DISCUSSION

Source of pigment

Bacillus endophyticus (*AVP-9Kf527823*) is the source of pigment which has been molecular characterized by **Nokku P *et al*.,2014**. **Abu., 2013,** reported that, the isolate shows multiple plant growth promoting traits. Similarly *Bacillus endophyticus* exhibiting yellow fluorescence isolated from cotton rhizosphere extensively reviewed for its anti-biotic metabolites. Unlike the fluorescent compounds produced by pseudomonad's, they yellow fluorescent compound produced by the *Bacillus* sp did not diffuse in to the medium, even from old cells which clearly shows that the fluorescent compound was strictly intracellular and located in nodules of the cytoplasm and was not associated with the cellwall (**Magyarosy*et al*., 2002**).

Production, extraction and standardization of pigment

In present study, production was observed when nutrient medium is supplemented with 1% glucose. Out of seven different solvents (70 % ethanol-EtOH, 100 % ethanol- EtOH, chloroform-Chlrfm, acetone, petroleum ether-P.ET, 70 % methanol-MeOH, and distilled water-D.H2O) used for the extraction of intracellular orange pigment, acetone was effectively extracted the pigment. This is due to the efficiency of acetone to disrupt the cell wall by dissolving the glycogen content of the cell wall (Fig. 1). Similarly, **Mariya patel, (2006)** reported that, highest amount of pigment was extracted in methanol as a solvent at concentration of 95% v/v while no extraction was observed in ethanol as well as acetone (**Nokku *et al*., 2014**). Chemical composition of the pigment generally decides the choice of organic solvent and total extraction of pigment. Carotenoids are lipophilic and soluble in organic solvents, such as chloroform, hexane, acetone, petroleum ether (Mariya., 2010). Production of the fluorescent pigment appears to be regulated by the nutrient source in the medium and by the type of medium (solid or liquid). Cells fluoresced when they were grown on NA containing 20g of peptone/liter but did not fluoresce when they were grown on NA containing 5g of peptone/liter and extraction and purification of the fluorescent bacterial biomass resulted in a yellow water-insoluble powder that fluoresced orange under long-wave length UV light(**Magyarosy *et al*., 2002**).

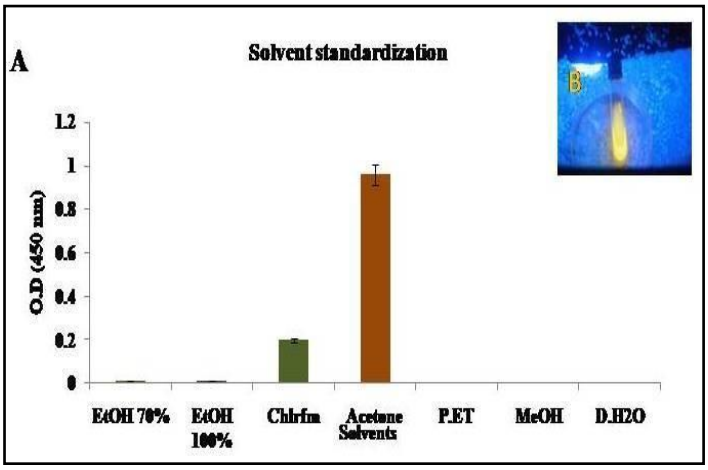

Figure 1(a) Extraction of fluorescent pigment using different solvents
(b) Extracted pigment under long wave length of U. V. light

Colour stability of pigment

Pigment has retained it's original orange colour on long standing in all solvents used except in heptane, where the pigment has changed to yellow and reverted to its original orange colour on addition of acetone indicating that pigment has poor stability in heptane due its lower polarity (Fig.2).

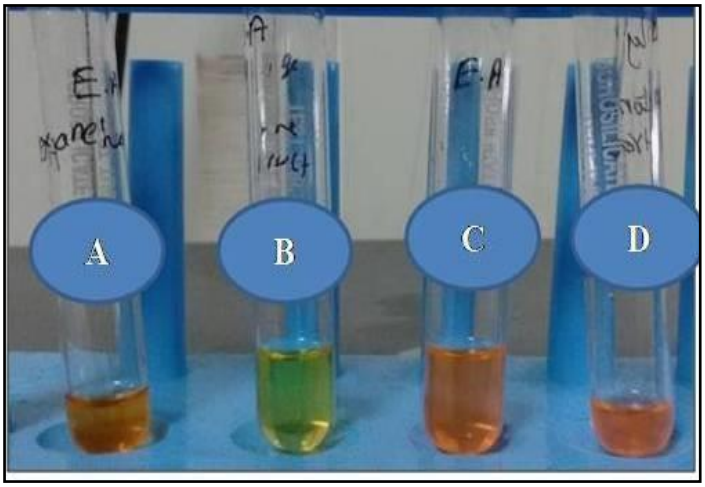

Figure 2 Colour reactions of crude pigment in different solvents
a) Pigment in Hexane; b) Pigment in Heptane; c) Pigment in Ethyl acetate; d) Pigment in Acetone).

Effect of various light incubation conditions on pigment production

Bacterial culture was incubated at three different light conditions and was extracted separately in acetone (Fig. 3). Absorbance spectra of all the three extracts shows maximum peak at 520nm (**Mantri Sairam., 2017**) with different absorbance values (Fig4). IOFP incubated in dark shows higher absorbance 1.10Au followed by white light 0.85Au whereas least absorbance was recorded IOFP incubated under U.V. light 0.61Au, indicating that, UV-A protection by acarotene-like pigment may be provides a UV-A screen there by shielding the sensitive components such as the DNA, and avoids death of the cell against radiation in this UV region, this results in lower production of pigment. The absorption spectrum of the three light incubated pigments were ranked as Dark incubation>White light incubation>U.V. light incubation respectively. Similar results were obtained then the absorption maxima for the extracted pigments were found to be 450-520nm which was the characteristic wavelength of carotenoid pigments (**Marar, 2015**). One reason for the shift in the absorption

maxima of the extract to shorter wavelengths compared to pure β-carotene in hexane could be the number of conjugated C=C bounds (**Ralf., 2004**).

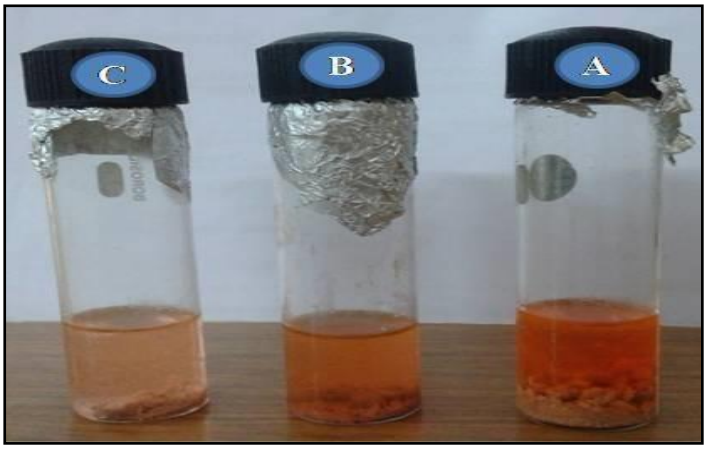

Figure 3 Acetone extract of orange pigment after different light incubations
A) Pigment extract from bacterium incubated in dark condition; B) Pigment extract from bacterium incubated in white light condition; C) Pigment extract from bacterium incubated in U.V. condition.

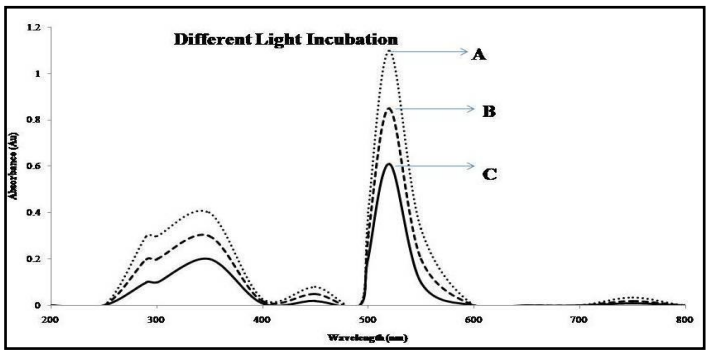

Figure 4 Wavelengths of three light incubated acetone extracts
A) Indicates the absorbance of IOFP under dark condition; B) Indicates the absorbance of IOFP incubated under white light condition; C) Indicates the absorbance of IOFP incubated under u. v. light condition.

Anti-bacterial activity

Anti-bacterial activity of three different light (Dark (control), U.V and W. Light) incubated IOFP has been evaluated by using 4 gram-positive and 3 gram-negative human bacterial pathogens. When compared to control (*Shigella flexneri-SF*, *Salmonella typhimurium-ST*, *E.coli-EC*, *Vibrio cholerae-VC*, *Streptococcus pyogenes-St.P*, *Staphylococcus aureus-SA* and *Listeria monocytogenes-LM*), IOFP incubated in u.v light condition, shows decreased zone of inhibition (percentages) in all tested organisms except *S. pyogenes* (*S. typhi* > 20%, *E.coli* > 18.37%, *S. flexneri* > 17.75%, *V. cholera* > 10.65% and ***S. Pyogenes* < 5.21%**). Wereas IOFP incubated in white light condition, all tested organism's shows decreased zone of inhibition except *S. aureus* and *L. monocytogenes* (*S. typhi* > 13%, *E.coli* > 6.12%, *S. Flexneri* > 12.91, *V. cholera* > 4.27%, *S. pyogenes* > 2.91%, ***S. aureus*** shows **< 3.09%**, and ***L. monocytogenes* < 3.41%**). In both cases, gram-positive bacteria did not affect much when compared to gram-negative bacteria this is due to the cell wall is composed mostly of a peptidoglycan layer, which maintains the osmotic pressure of the cytoplasm. Gram-positive bacteria have one cytoplasmic membrane with a multi layer of peptidoglycan polymer and hence, are highly susceptible to damage (**Małgorzata *et al.*, 2018**). In previous study, **Małgorzata Mizieli´nska, (2018)**, reported that, accelerated UV-A irradiation did not influence the activity of PLA films with nano-ZnO against *S. aureus* and *E.coli*, and this paper conforms the pervious findings. It indicates that, though IOFP was inhibiting all the test organisms, its performance was effect by different light conditions (**Małgorzata *et al.*, 2018**). The performance pattern of IOFP based on light conditions were ranked as Dark incubation>U.V. light incubation>White light incubation respectively Fig 5.

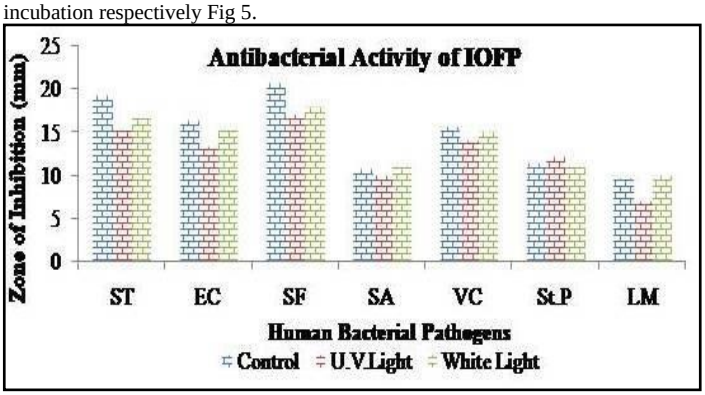

Figure 5 Anti-bacterial activity of IOFP incubated in three (Dark, white light and u.v. light) condition against human bacterial pathogens.

Purification and bio autography of IOFP

Single spot was separated from the crude acetone extract with R_f value 0.47 using chloroform:ethylacetate 3:1 ratio. Localized anti-bacterial activity of separated compound on the TLC plate was detected by agar overlay method and the activity of active spot was identified by bio-autography by spraying TTC solution as an indicator. Only living cells hydrolyse TTC to red colour formazan and forming the red colour background indicating the clear zone of inhibition against all inoculated pathogens. TLC bio-autography guided screening of anti-oxidant and antimicrobial activities of compounds in the crude extract is a quick approach and has been used by many researchers. Similarly, anti-bacterial activity of purified compound through TLC was reported by Abu., (2013), Fig 6.

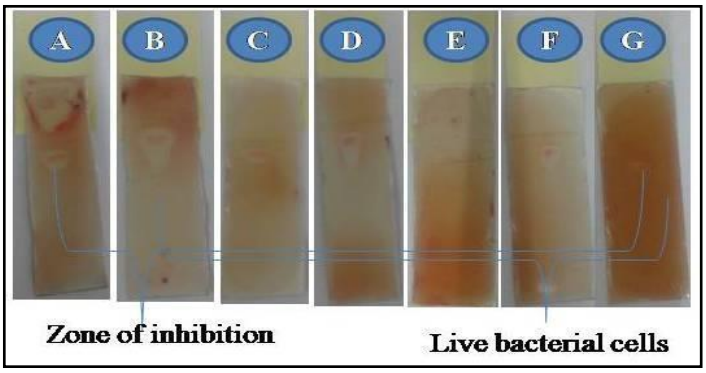

Figure 6 Bio autographic plates showing zone of inhibition around the separated pure compound against different human pathogenic bacteria (A. *Salmonella typhi; B. Vibrio cholera; C. Shigella flexneri; D. E. coli; E. Staphylococcus aurous; F. Streptococcus pyogenes*; G. *Listeria monocytogenes*).

CONCLUSION

By this study, we conclude that, the production of orange fluorescent pigment from *Bacillus endophyticus*, and the anti-bacterial activity was independent from light incubations, since all three light incubated pigment extracts shows the considerable anti-bacterial activity, but we noticed that, there is a minor effect in anti-biotic performance. The orange pigment was stable in all the tested solvents except heptane. And on other hand, TLC-guided bio-autography is method to screen and purify orange fluorescent pigment from *Bacillus endophyticus Kf527823*, is a rapid, coast affective method to detect the bio-activness of separated bands or compounds on the TLC plate itself. Overall result states that the production and bio activeness of pigment was stable in different light and solvents conditions.

Conflict of interest: The authors report no conflicts of interest

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