Nat. Volatiles & Essent. Oils, 2021; 8(5): 3714 - 3723

NVEO
Natural Volatiles &
Essential Oils

Analysis the antibacterial activity *Pseudomonas aeruginosa* pigment on *Escherichia coli*

Dr.V.Jeyanthi Kumari

Assistant Professor of Zoology,A.P.C.Mahalaxmi College for Women,Thoothukudi, Tamil Nadu, India.

Email: jeyanthikumari@apcmcollege.ac.in

ABSTRACT

Biodegradability, non-toxic nature and therapeutic nature of the natural pigments, colorants and dyes make an alternate and attractive source for human use. The current study aimed to screen and identify pigment producing *Pseudomonas aeruginosa* from garden soil using pseudomonas isolation agar and its species level was identified under the guidance of Bergey's manual. Different incubation times were provided to optimize its maximum pigment production. There was a slight colour change in the media observed, for the first three days during the incubation of *P.aeruginosa* in nutrient broth. The colour change was notable from the fifth day onwards. On the seventh day, it was dark green and continued on until the end of the incubation time. The validity of this experiment was demonstrated by colorimetric method. The pigment concentration was low (0.076±0.18) during the third day of colorimetric reading. The concentration of pigments was gradually increased at the time of the fifth and seventh day of colorimetric observation. The concentration of pigment production in optical density was increased at 9th day observation (1.873±0.16). The colorimetric reading in the following days was more or less the same. The density of the pigments remained intact.

Keywords: *Pseudomonas aeruginosa*, biodegradation, pigments, optical density

INTRODUCTION

Biodegradability, non-toxicity and non carcinogenicity of the natural dyes and colorants derived from natural flora and fauna make an alternate source for human use (Joshi *et al*., 2003). Plants and microorganisms are the natural colorant resources of nature and are great alternatives to synthetic dyes and pigments currently employed **(**Parekh *et al*., 2000). In today's living conditions, the impact of natural pigment is rapidly spreading and raising awareness among people. The side effects of chemical colouring agents cannot be listed and they are the important cause of diseases that affect the human species (Kamla *et al*., 2012).

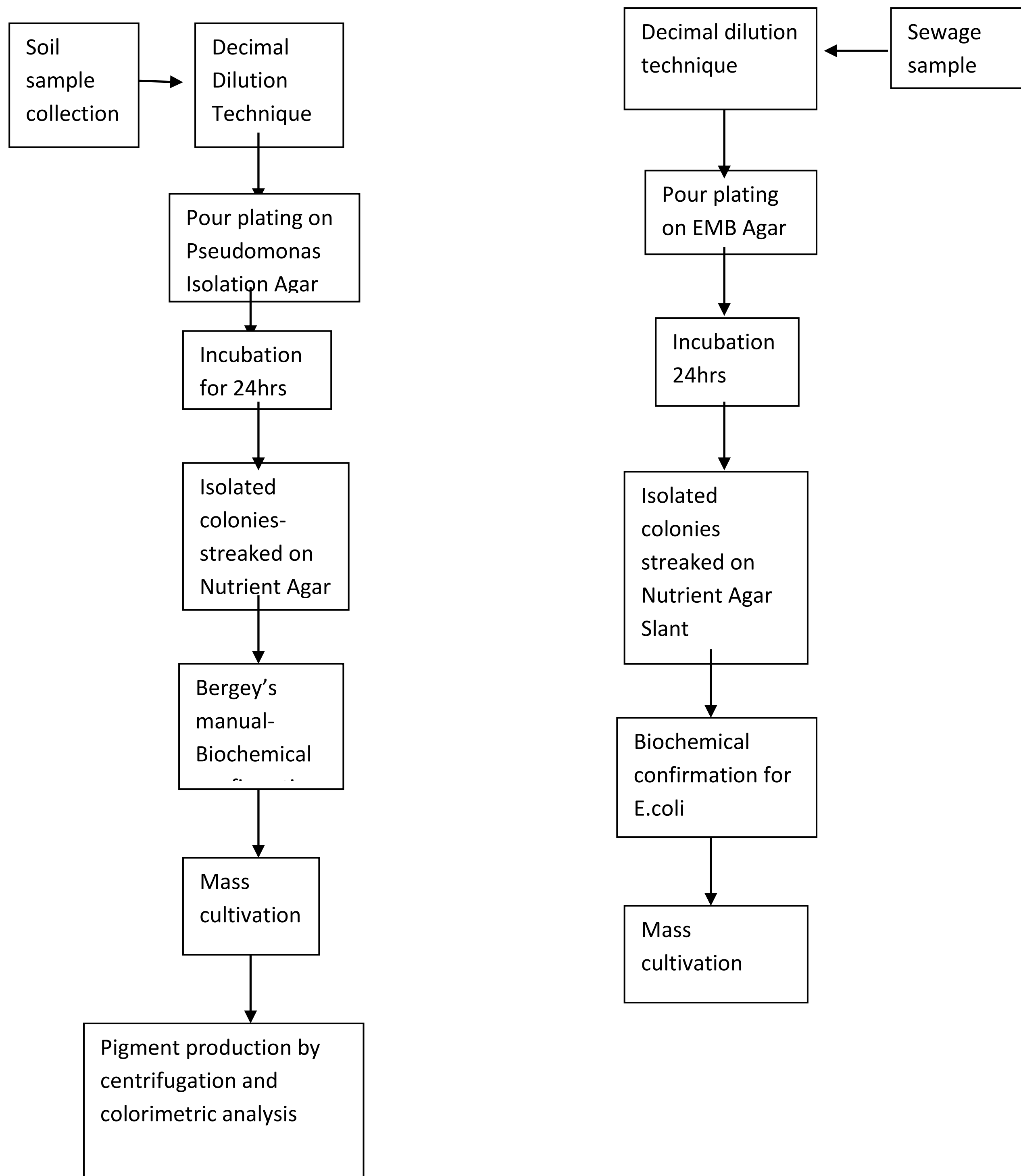
Among natural pigments, pigments from microbial sources are potentially good alternatives to synthetic pigments (Dufossel,2006). Pigments responsible for bright colours are synthesized almost exclusively by bacteria belonging to genera *Pseudomonas, Streptomyces, Nocardia, Sorangium, Brevibacterium, Burkholderia*and*Bacillus*. Among them *Pseudomonas* is most eligible pigment producing agent (Yuodim *et al*., 2002). Pigments posses some inhibitory effects towards many bacterial strains and microorganisms commonly present in medical and industrial process. It is nontoxic, safe and inorganic antibacterial agent.

Behind the discovery of many diseases, it has become important to bring our rare species of plants and antimicrobial agents for those dreadful diseases (Hawkey 2008; Jemal *et al*., 2011). The phytochemical analysis of many plants emphasized that the secondary metabolites are bioactive compounds with medicinal properties like antibacterial, antifungal, antioxidant, antidiabetic and anticancer (Gurib-Fakim 2006; Phillipson 2007).

Pharmaceutical industries turned researchers to find potential components in these pigments that have important therapeutic applications (Muhammed numan *et al*.,2018). The toxic effect of many drugs compel pharmacist to search new sources of drugs that are safer in use and antibiotic of broad spectrum potential. The current increase in the drug resistance in the pathogen might be due to the increased use of present antibiotics. Products of the microbesin particular isolated from novel ecosystem can be a potential alternative source for new drugs (Mellouli *et al*., 2003). Considering the pharmacological potential of bacterial pigments, and realizing the importance of natural pigments and its positive impact on society, the

work plan was designed to synthesize natural pigments from *Pseudomonas aeruginosa.*

Schematic Explanation



Materials and Methods

Glassware preparation

All glasswares used were cleaned with 6N HCl to remove residual iron and rinsed with distilled water. All

growth media and reagents were prepared in the same water.

Isolation of *Escherichia coli*

Sewage sample was collected in a sterile conical flask and was streaked on EMB agar plates using quadrant streak method. The incubation time of 24-48 hrs were provided at 37°C. *E. coli* were appeared as metallic sheen on EMB agar plates. Then the strains were maintained on nutrient agar slants at 4°C. Thus obtained colonies were stored in agar slants for further microbiological, biochemical and physiological characterization. Cultures obtained were made free of contamination at frequent intervals by streak plating and sub-culturing. The isolated bacterial cultures by using selective media were subjected to the following tests adopting the scheme recommended by Bergey's Manual, (1998). A loopful of *E. coli* bacterial strain was inoculated into 50ml of sterile nutrient broth was incubated on a rotary shaker for 24hours to activate the strain. Muller Hinton Agar medium was used as a bacterial culture medium in the antibacterial assays.

Isolation of *Pseudomonas aeruginosa*

The soil samples were collected from the botanical garden of Millerpuram, Thoothukudi, at a depth of 5cm from the substratum. It was collected in sterile containers aseptically and transported immediately to the lab and processed for bacteriological analysis. The collected sample was serially diluted from 10^{-2} to 10^{-9} and the diluted samples were plated on both nutrient and Pseudomonas Isolation Agar. The plates were incubated at 37^0C for 24hrs. After incubation, the total Bacterial Count from Nutrient Agar plate and the individual colony from Pseudomonas Isolation Agar (PSI) were screened for pigment production. The selected strain was streaked on nutrient agar slant and stored at 4^0C for further analysis. The bacterial cultures on PSI are identified based on the morphological and biochemical characteristics outlined by Kannen et al., (1978).

After biochemical confirmation, stored *Pseudomonas* culture was quadrant streaked on Nutrient agar plate to check its purity. Well isolated purified colonies were stored for experimental purposes. The mass cultivation has done by preparing 100 ml nutrient broth and was sterilized at 121 lbs for 15minutes followed by the cooling of the medium. The stored *P.aeruginosa* was inoculated aseptically into the sterile nutrient broth.

Pigment production

An incubation period of 10 days was provided. But once every two days, the sample was collected, centrifuged at 8000 rpm for 15 min and both the supernatant and bacterial cell pellets were collected. Bacterial pellets were then extracted using either 95% (v/v) methanol or 99% (v/v) acetone in the ratio of 1:5 until the pellet was colorless, i.e., complete pigment extraction was achieved and then the pellet was discarded. The pigment extract was then analyzed by scanning the absorbance in the wavelength region of 580nm using a colorimeter. This was performed for 14days and results were collected in triplicate (Kamla *et al*., 2012).

Antibacterial Activity

To test the antibacterial activity of pigment, agar well diffusion method (Kirby-Bauer method) was followed (Bauer *et al*., 1966). Cultured *E.coli* was used for this assay. A sterile cotton swab was dipped into the bacterial suspension and evenly streaked over the entire surface of sterile Muller Hinton agar plate to obtain uniform inoculum separately. Wells were punched on the plates using sterile borer (8mm). The plates were allowed to dry for 5 min. pigment extract (25, 75, 100µl) were dispensed into each well using sterile micropipette. Streptomycin (10µl) was used as positive control. The plates were incubated overnight at 37°C. Antibacterial activity was determined by measuring the diameter of zone of inhibition (mm).

Result and Discussion

Pigment Production

During the incubation of *P.aeruginosa* in nutrient broth, for the first four days, there was a slight colour change in the media. The colour change was notable from the fifth day onwards. On the ninth day, it was dark green and continued on until the end of the incubation time (Plate 2). The validity of this experiment was demonstrated by colorimetric observation also. The pigment concentration was low (0.076±0.18) during the third day of colorimetric reading. At the time of the fifth and seventh day of colorimetric observation, the concentration of pigments was gradually increased (Fig1). The concentration of pigment production was high during the ninth day of optical density(1.873±0.15).

Analysis of antibacterial activities

Using selective media, the bacterial cultures were isolated and they were subjected to various microbiological, biochemical and physiological analysis (Plate1 &3) to identify its species level and it was tabulated (Table 1). The antibacterial activity was expressed in terms of Zone of Inhibition and the amount of 25, 50, 75µl extracts were used for the assay (plate4 &5). The results of the antibacterial activities are presented in Table2. The antibacterial activity was increased with increasing volume of extract. The pigment extracts of 75µl volume showed significant antibacterial activity against *E.coli* isolates tested . The growth inhibition zone measured for *E.coli* ranged from 2to 7mm (Table 2). The colorimetric reading in the subsequent days was more or less the same. The density of the pigments remained intact.

Table1 Microbiological, biochemical and physiological analysis of isolates

Properties analysed	***E.coli***	***P.aeruginosa***
Microbiological Analysis		
Simple staining	Rod	Rod
Gram staining	-	-
Spore staining	-	-
Motility	+	+
Biochemical Analysis		
Catalase	+	+
Oxidase	-	+
Hydrolysis of Gelatin	-	+
Casein	-	-
Starch		-
Lipid	+	+
Fermentation of		
Lactose	-	-
Dextrose	+	-
Sucrose	(-)	-
Arabinose	-	+
Xylose	-	-
Manitol	-	+
H_2S production	-	(+)
NO_3production	+	+

indole	-	-
MR reaction	+	-
VP reaction	-	-
Citrate utility	-	+
Urease activity	-	-
Physiological Analysis		
Temperature		
4° C	-	-
15° C	-	-
30° C	+	-
41° C	+	-
50° C	-	-
65° C	-	-
Effect of P^H		
5.7	-	+
6.8	+	+
8.0	-	-
Nacl tolerance		
2.5%	-	+
5%	-	(+)
7%	-	-
Growth on MacConkey	-	+

+ positive results (90-100% positive)
- Negative results (0-10% positive)
(+) weak positive (76-89% positive)

Table 2 Antibacterial activities of pigment on *E.coli*

Fraction of the Extract (µl)	Zone of Clearance (mm) *E.coli*
25	2±0.19
50	4±0.13
75	7±0.09

Standard deviationd±mean

Plate1.Isolated colonies of *P.aeruginosa*

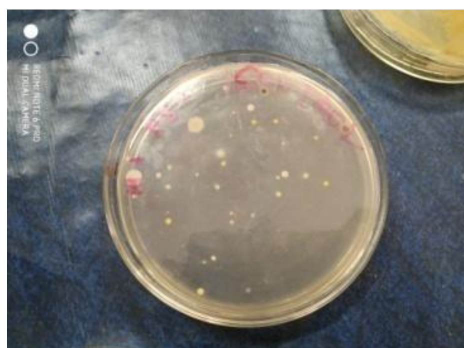


Plate 2 Piment production during 10th day of Incubation

Plate.3.Growth of *E.coli* on EMB Agar

Plate.4.Zone of Inhibition

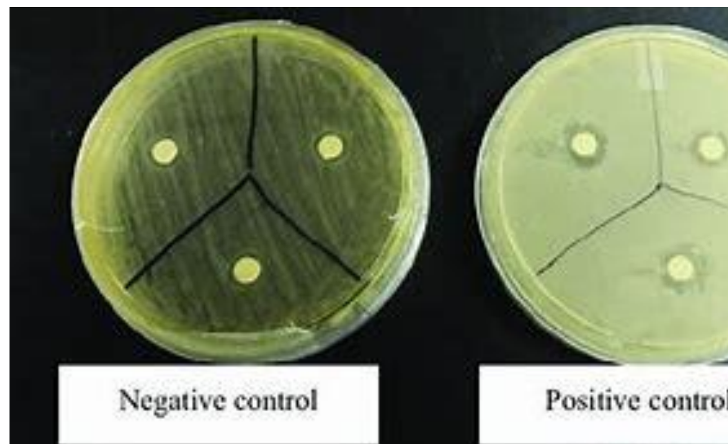
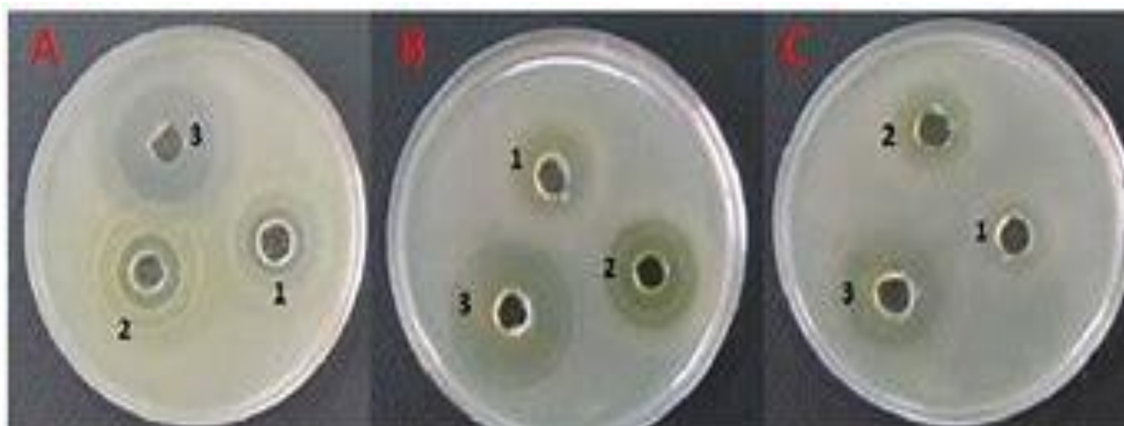


Plate.5.Antibacterial effect of the pigment on *E.coli*



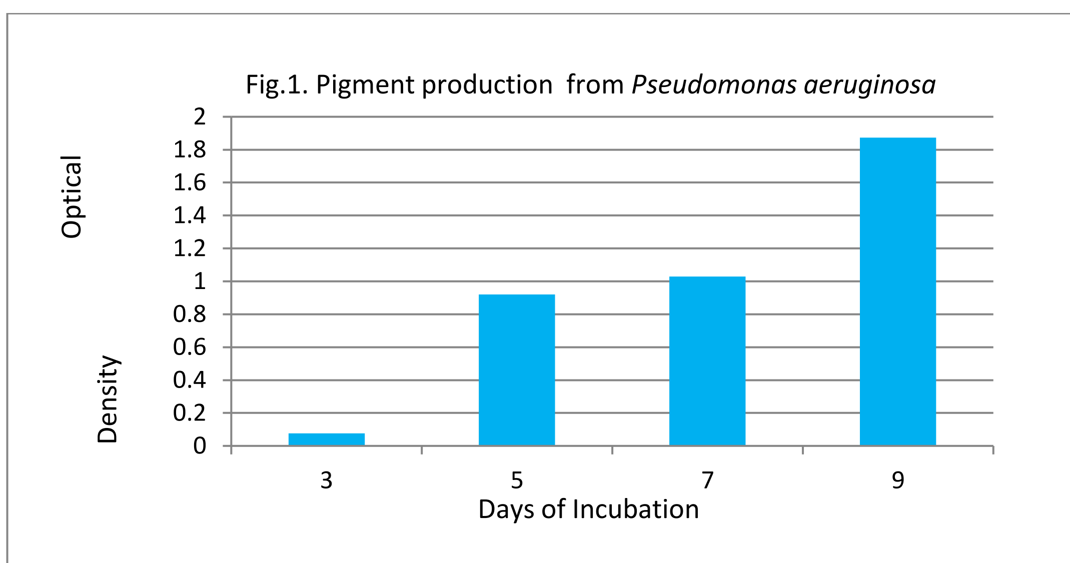


Fig.1. Pigment production from *Pseudomonas aeruginosa*

Microbial pigments are the characteristic feature of some bacteria which may be useful in identification. Bacterial pigments offer promising avenues for various applications due to their better biodegradability and higher compatibility with the environment (MeghaWaghela and Shabib Khan, 2018). The present experiment revealed that the microorganism *P.aeruginosa* isolated from the soil produces a large amount of pigments. The advantages of pigment production from microorganisms comprise easy and fast growth in cheap culture medium, independent from weather conditions and colors of different shades (Sinha *et al.*, 2017). According to the above assertion, the simple, easiest nutrient broth medium was used for the pigment producing *P.aeruginosa* respectively.

These compounds have remarkable anticancer, immunosuppressive, inflammatory, antimicrobial and antioxidant activities (Korkina 2007). Recently, a review has been published on the biosynthesis of these compounds from bacteria (Singh *et al*., 2017). With the passage of time the investigation of new, vital and bioactive compounds from bacterial sources increased as compared to other sources. For example, anthocyanin, a compound that have diverse biological activities and positively affect health and have the property to reduce the risk of cancer (Kong *et al*., 2003; Katsube *et al*., 2003; Lazzè *et al*., 2004; Martin *et al*., 2003; Kim *et al*., 2012). Anthocyanin is also involved in reducing the chances of inflammatory insults and have role in modulating the immune response (Youdim *et al*., 2002; Wang and Mazza 2002). As another example, violacein has the properties such as antiviral (Sánchez *et al*., 2006), anticancer (Ferreira *et al*., 2004; Kodach *et al.*, 2006), antiprotozoan (Matz *et al.*, 2004), antioxidant activities (*Konzen et al., 2006*) and antibacterial activities (Nakamura *et al.,* 2002).

There is growing interest in microbial pigments due to their natural character, safety to use, medicinal properties, nutrients like vitamins, production is independent of season and geographical

conditions, and controllable and predictable yield (Nakashima *et al*., 2005).Again bacterial pigments can be produced from waste material thus environmental pollution can be minimized (Joshi *et al*., 2003; Kamla*et al*., 2012). In the present work, the periodical colorimetric observation of pigment production revealed that the highest production or colour change occurred on the 9th day of incubation and very low pigment production on 3rd day of incubation. After 9th day, not much notable increase in the optical density was observed. This in turn indicated that, there was no more pigment production in the medium.

Sewage is commonly a cloudy aqueous solution containing minerals and organic matter. The sewage water without treatment was simply discharged into the nearest large body of water such as river, which leads to contamination of rivers and lakes chronically affecting the flora and fauna. Bacteria are considered as serious pathogens, which cause diseases and subsequent economic losses. Among the bacterial diseases, Gram-negative bacteria such as *E.coli* and *Enterobacter* predominantly affected not only human beings but also the fishes. The microorganisms present not alone in the sewage water but also in the wastewater from the distilleries which contains the microbial stains such as *Enterobacter* sp, *Corynebacterium* sp and *Micrococcus* in large amount (Vasanthy and Thamarai selvi, 2006). This is what led to the discovery of today's drugs and acted as a stimulus.

In today's fast-paced world, there is an urgent need to discover new antimicrobial and anti-tumour agents following the discovery of many infectious diseases. Because of safe and effective constituents of plant products, the interest in medicinal plants has been shown all over the world. Particularly the presence of active components and principles are acting as a good immunomodulators.

The reason may be, once the nutrient content in the media began to decrease, the bacteria began to produce pigments to save their lives (antagonism) so it is for this reason, that bacteria not producing pigments in its early days began producing pigments in later days when there is food shortage come. Chidambaram *et al*., (2013) clearly mentioned that, *Pseudomonas aeruginosa* produces a variety of extracellular pigments and they are biologically active metabolites that function in microbial competitiveness.

Acknowledgment: I would like to thank the Chairman, Director and Principal of APC Mahalaxmi College for women for providing facilities and their constant encouragement to carry out this research study.

Funding: None

Data availability: All datasets and statistical report analyses during this study are included in the manuscript.

Ethics Statement: This article does not contain any studies with human participants or animals.

References

1. Bauer,A.W, W.M.Kirby, J.C.Sheris and M.Truck. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Path*.45:149-158.
2. Bergey's Manual of Determinative Bacteriology.1998. John G.Holt-Noel.R.Krieg Peter H.A., Sheath James T.Stanley and Stanley T. Williams (Eds.) 9th Edn. Lipponcott Williams and wilkins Publications, Philadelphia, PA.19106.USA.
3. Chidambaram, K.V, A.Z.Zainul, and A.A.Wan. 2013. Bacterial pigments and their applications.*Process Biochemistry*.48:1065–107
4. Dharmadhikari, S.D., M.P.Shrivastava, and P.G.Dashputra. 1986. Study of analgesic property of *Nyctanthes arbor-tristis* (Prajakt). *Ind J Pharmacol*. 18(1): 19-60.
5. Dufossel,L.2006. Microbial Production of Food Grade Pigments.*Food Technol. Biotechnol. 44*: 313– 321.

6. Ferreira CV, Bos CL, Versteeg HH, Justo GZ, Durán N, Peppelenbosch MP. Molecular mechanism of violacein-mediated human leukemia cell death. *Blood.* 2004;104(5):1459–1464. doi: 10.1182/blood-2004-02-0594. [PubMed] [CrossRef] [Google Scholar]
7. Gurib-Fakin.A. 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Mol Aspects Me.* 27:1-93.
8. Hawkey,P.M. 2008. The growing burden of antimicrobial resistance.*J AntimicrobChemoth*.62.1-9.
9. Jemal A., F.Bray, M.M.Center, J.Ferlay, E.Ward and D. Forman.2011.Global Cancer Statistics. *Cancer J lin*.61:69-90.
10. Joshi,V.K., D.Attri, and A.Bala.2003. Microbial pigments. *Ind Journal of Biotechnolog*.2:362–369.
11. Kamla,M., T.Jayanti, and G.Sneh.2012. A review on microbial pigment. *Int J Microbial Res Technol*.1:361–365.
12. Kannen, D., N. N. Gerber, and R. Bartha. 1978. Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. *J. Bacteriol*. 134:690-92.
13. Katsube N, Iwashita K, Tsushida T, Yamaki K, Kobori M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J Agric Food Chem.* 2003;51(1):68–75.
14. Kim HW, Kim JB, Cho SM, Chung MN, Lee YM, Chu SM, Che JH, Kim SN, Kim SY, Cho YS. Anthocyanin changes in the Korean purple-fleshed sweet potato, Shinzami, as affected by steaming and baking. *Food Chem.* 2012;130(4):966–972.
15. Kodach LL, Bos CL, Durán N, Peppelenbosch MP, Ferreira CV, Hardwick JC. Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells. *Carcinogenesis.* 2006;27(3):508–516. doi: 10.1093/carcin/bgi307
16. Kong J-M, Chia L-S, Goh N-K, Chia T-F, Brouillard R. Analysis and biological activities of anthocyanins. *Phytochemistry.* 2003;64(5):923–933. doi: 10.1016/S0031-9422(03)00438-2.
17. Konzen M, De Marco D, Cordova CA, Vieira TO, Antônio RV, Creczynski-Pasa TB. Antioxidant properties of violacein: possible relation on its biological function. *Bioorg Med Chem.* 2006;14(24):8307–8313.
18. Korkina L. Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cell Mol Biol.* 2007;53(1):15–25
19. Lazzè MC, Savio M, Pizzala R, Cazzalini O, Perucca P, Scovassi AI, Stivala LA, Bianchi L. Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis.* 2004;25(8):1427–1433.
20. Martin S, Giannone G, Andriantsitohaina R, Carmen Martinez M. Delphinidin, an active compound of red wine, inhibits endothelial cell apoptosis via nitric oxide pathway and regulation of calcium homeostasis. *Br J Pharmacol.* 2003;139(6):1095–1102. doi: 10.1038/sj.bjp.0705347. [PMC free article] [PubMed] [CrossRef] [Google Scholar]
21. Matz C, Deines P, Boenigk J, Arndt H, Eberl L, Kjelleberg S, Jürgens K. Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. *Appl Environ Microbiol.* 2004;70(3):1593–1599.
22. Meghawagela., and Shabib khan.2018. Isolation, characterization of pigment producing bacteria from various food samples and testing of antimicrobial activity of bacterial pigments. *DAV International Journal of Science*.7:1-7.
23. Mellouli L, Ameur-Mehdi RB, Sioud S, Salem M, Bejar S. Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated Streptomyces sp. US24 strain. *Res Microbiol.* 2003;154(5):345–352.
24. Nakamura Y, Sawada T, Morita Y, Tamiya E. Isolation of a psychrotrophic bacterium from the organic residue of a water tank keeping rainbow trout and antibacterial effect of violet pigment produced from the strain. *Biochem Eng J.* 2002;12(1):79–86.
25. Nakashima T, M.Kurachi, and Y.Kato. 2005. Characterization of bacterium isolated from the sediment at coastal area of Omura bay in Japan and several biological activities of pigment produced by this isolate. *MicrobiolImmunol*. 4:407–415.
26. Parekh,S., V.A.Vinci, and R.J.Strobel. 2000. Improvement of microbial strains and fermentation processes.*ApplMicrobiolBiotechnol*. 54:287– 301.
27. Phillipson, J.D.2017.Phytochemistry and pharmacognosy. Review. Phytochem.68:2960-2972.

28.Sánchez C, Braña AF, Méndez C, Salas JA. Reevaluation of the violacein biosynthetic pathway and its relationship to indolocarbazole biosynthesis. *ChemBioChem.* 2006;7(8):1231–1240.

29.Singh M, Kumar A, Singh R, Pandey KD. Endophytic bacteria: a new source of bioactive compounds. 3. *Biotech.* 2017;7(5):315

30.Sinha, S., S.Choubey, A.Ajay Kumar, and P.Bhosale. 2017. Identification, Characterization of pigment producing bacteria from Soil and Water and Testing of Antimicrobial Activity of Bacterial Pigments, *Int. J. Pharm. Sci. Rev. Res.*42: 119-124.

31. Vasanthy, M ., and C.Thamaraiselvi. 2006. Bioremediation of distilary spent wash using indigenous microbial strains and Cashew nut hall carbon. *J. Ecotoxicol. Environ.Monit*.16 : 301-305.

32.Wang J, Mazza G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor α in LPS/IFN-γ-activated RAW 264.7 macrophages. *J Agric Food Chem.* 2002;50(15):4183–4189. doi: 10.1021/jf011613d. [PubMed] [CrossRef] [Google Scholar]

33.Youdim KA, McDonald J, Kalt W, Joseph JA. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *J Nutr Biochem.* 2002;13(5):282–288. doi: 10.1016/S0955-2863(01)00221-2. [PubMed] [CrossRef] [Google Scholar]

34.Yuodim, K.A., J. McDonald, and W.Kalt.2002.Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *J Nutr Biochem*.13:282– 288.