Int.J.Curr.Microbiol.App.Sci (2016) *5*(6): 370-376

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 6 (2016) pp. 370-376**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.506.042

Characterization and Cytotoxicity Assay of Pigment Producing Microbes

Kamla Malik[1*], Jayanti Tokas[2] and Ramesh Chand Anand[1]

[1]Department of Microbiology, College of Basic Science & Humanities, Chaudhary Charan Singh Haryana Agricultural University, Hisar 125004, Haryana, India
[2]Department of Biochemistry, College of Basic Science & Humanities, Chaudhary Charan Singh Haryana Agricultural University, Hisar 125004, Haryana, India
**Corresponding author*

Keywords

Pigment producing bacteria, fungi, extraction, cytotoxicity.

Article Info

Accepted:
15 May 2016
Available Online:
10 June 2016

A B S T R A C T

The pigment producing bacteria and fungi were isolated from soil, damping site, compost, industrial effluent, spoiled fruits and vegetables. A total of fifteen bacterial and nine fungal pigment-producing isolates were obtained and screened on nutrient agar and potato dextrose medium, respectively. The pigments from selected isolates were extracted by solvent extraction (acetone and methanol). The antimicrobial activity of selected isolates was determined by disc agar diffusion technique against standard isolates of *Staphylococcus aureus, Klebsiella pneumonia*, *Escherichia coli* and *Fusarium oxysporum.* The cytotoxicity was studied by using yeast toxicity test (YTT). Bacterial isolates BP-8 exhibited the inhibitory effect against *Saccharomyces cerevisiae* and *E.coli.* The R_f value of pigment extract from different bacterial isolates varied from 0.86 to 0.91 whereas it was 0.92 by fungal isolate FP-6 as determined by thin layer chromatography (TLC).). The bacterial isolates BP-2 and BP-6 were Gram+ve and BP-8 was Gram-ve. Out of these BP-6 and BP-8 were non spore former. These bacterial isolates belonged to the genus *Bacillus, Micrococcus* and *Serratia.* The fungal isolate was identified as *Aspergillus sp.*

Introduction

Now a day, there is an increasing demand for natural colors/pigments in various fields *viz.* food production, textile industries, paper production, cosmetic, pharmaceutical, printing and dye industries over synthetic colors by virtue of its carcinogenic and teratogenic nature (Unagul *et al*., 2005). Natural pigments not only have the capacity to increase the marketability of products but they also display advantageous biological activities as antioxidants and anticancer agents (Malik *et al*., 2012). These are easy to produce as compared to synthetic pigments and are economical as well (Tibor, 2007). Natural pigments have some limitations including solubility, sensitivity and short stability upon exposure to light, pH and high temperature. In this regard, a lot of attention is now being paid for synthesis of biocolors, also known as microbial pigments, by using the

microorganisms (Cho *et al*., 2002). Thus, there is an ever growing interest in microbial pigments due to several reasons like their natural character, safe, fast growth, production being independent of seasons and geographical conditions. Moreover, the predictable yield and cost effectiveness can be further enhanced by growing on low-cost substrates. Various types of microbial sources like bacteria, yeasts, mold and algae are used for production of microbial pigments. Microbial pigments have numerous beneficial properties like anticancer, antiproliferative, immuno-suppressive, antibiotic, biodegradability etc. Many microorganisms, including bacteria, fungi, yeast and mould etc. are employed for the industrial production of various pigments by using fermentation technology (Kumar *et al*., 2015).

Microbial pigments are of industrial interest because they are often more stable and soluble than those from plant or animal sources and can grow rapidly which can lead to high productivity and produce a product throughout the year (Jiang *et al*., 2005). Microorganisms are known to produce a variety of pigments like carotenoids, melanins, flavones, quinones, and more specifically monascins, violacein or indigo (Dufosse, 2007). In this way the pigments from microbial sources are a good alternative, therefore they are promising source of food colorants (Aberoumand, 2011; Ahmad *et al*., 2012). Food grade pigments such as β-carotene, arpink red, riboflavin lycopene and monascus pigments are used in food industries (Joshi *et al*., 2013). In pharmaceutical industry pigments like anthocyanin, prodigiosin and violacein are widely used to treat diseases. Several microbial pigments are also being used in textile industries (Kumar *et al*., 2015). In the present work, efforts have been made for screening, extraction, purification of pigments and identification of selected pigment producing bacterial and fungal isolates.

Material and Methods

Isolation and Screening of Pigment Producing Bacteria and Fungi

Samples were collected from soil, damping sites, compost, industrial effluent, spoiled fruits and vegetables. For isolation of pigment producing bacteria and fungi by using enrichment culture technique was used. The pigmented colonies were purified by streaking. The purified colonies of bacteria were then transferred on nutrient agar (NA) and fungal isolates on potato dextrose medium (PDA) slants. These cultures were maintained at 4°C.

Extraction of Pigments

The pigment from the selected bacterial and fungal isolates was extracted using different solvents *viz.* acetone, ethyl acetate, and methanol. The bacterial isolates were grown for 24- 48h in nutrient broth followed by centrifugation at 8000 rpm for 20 min. Both the supernatant and bacterial cell pellets were extracted using 95% (v/v) methanol and 99% (v/v) acetone in the ratio of 1: 5 until the pellet was colorless, i.e., complete pigment extraction was achieved. The fungal isolates were grown in potato dextrose broth. The culture broth was extracted using ethyl acetate and centrifuged at 10,000 rpm for 10 min. The extract was scanned in the range of 400 to 600 nm to find out the maximum absorption spectra by UV-visible spectrophotometer. Methanol was used as a blank.

TLC Analysis of Pigments

Qualitative analysis of pigments was carried out by Thin Layer Chromatography (TLC).

TLC plates were prepared by applying silica Gel G slurry which was dried at 100°C for one hour. Thereafter, 20µl of samples were spotted on the baseline of the TLC plates at 1.0cm interval and then allowed to dry at room temperature. The sample applied on TLC plates was placed in a presaturated TLC chamber containing mobile phase (benzene/ acetone in the ratio of 2:1 v/v). Then the plate was taken out and dried for few min. After 45 min., the TLC sheet was carefully removed and the Retention factor *(Rf)* value was calculated according to the following equation from the chromatogram.

R*f*= Distance travelled by the compound/ Distance travelled by the solvent front

Cytotoxicity Assay

Pigment producing bacteria and fungi were grown in nutrient and potato dextrose broth. Pigments were extracted in different solvents (methanol and acetone). The antimicrobial activity of pigments was tested by agar well diffusion technique against clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Fusarium oxysporum*. Methanol was used as control. The plates were incubated at 30^0C for 24h after which activity was measured by the presence of zone of inhibition surrounding the well. The experiment was done in triplicates. The antibacterial activity was expressed as the mean diameter of zone inhibition (mm).The yeast toxicity test (YTT) was also done using *Saccharomyces cerevisiae*.

Identification of Selected Isolates

The selected bacterial isolates were further identified on the basis of morphological and biochemical characteristics, as outlined in Bergey's manual of systematic bacteriology (Brenner *et al*., 2005).

Result and Discussion

Screening and Selection of Pigment Producing Isolates

Twenty five bacterial and fifteen fungal isolates were obtained from different samples *viz.* soil, dumping sites, compost, industrial effluent, spoiled fruits and vegetables on nutrient agar and potato dextrose medium, respectively. Out of these 15 bacterial and nine fungal isolates were found to produce pigments of different colors such as red, yellow, pink, white and light blue. Table 1 presents the colony characteristics of selected pigment producing isolates. Preliminary morphological observations revealed that the colonies were circular, raised, smooth, yellow, pink and red in color.

Characterization of Pigment

Pigment producing bacterial and fungal isolates were grown in nutrient and potato dextrose broth, respectively and by centrifuged. Bacterial pellets were extracted using methanol and acetone in the ratio of 1:5 (v/v) and fungal isolates in ethyl acetate. The pigment extract was then analyzed by scanning the absorbance in the wavelength region of 400-600 nm using UV-Vis spectrophotometer. The maximum absorbance of red pigment (BP-8) was obtained at 532nm; orange (BP-1) at 450nm, yellow (BP-6) at 468 nm, pink (BP-5) at 540 nm and black (FP-6) at 300nm. Similar studies have been carried out in *Serratia marcescens* where the maximum absorbance of red pigment by it was found to be at 534.76 nm using UV–Vis analysis spectra (Ahmad *et al*., 2012; Song *et al*., 2012). The maximum extraction of an orange pigment from *Salinicoccus* sp. M KJ997975 was done by using acetone: methanol (5:5) (v/v) solvent. The extracted pigment showed

maximum absorbance at 450 nm (Bhat and Marar 2015).

The TLC analysis of extracted pigment from the different bacterial isolates showed different R_f values. The Rf value of pigment producing selected isolates r The R_f value for BP-1, BP-5, BP-6 and BP-8 were 0.91, 0.86, 0.88 and 0.87, respectively (Table 2). However, it was 0.92 for fungal isolate FP-6. The R_f value of the extracted red pigment from *Serratia marcescens* KH1R KM035849 has been reported to be in the range of 0.64-0.96 when determined by TLC method (Vora *et al*., 2014). The R_f value of prodigiosin (red) pigment was 0.73 which is near to our results (Mohammed *et al*., 2012).

TLC analysis of extracted orange pigment from *Salinicoccus* sp. M KJ997975 showed R_f value of 0.65 in hypophase which suggested the presence of xanthophylls.

Table.1 Colony characteristics of selected pigment producing isolates

Isolate No.	Colony characteristics
Bacterial Isolates	
BP- 1	Orange, raised, shiny and smooth
BP- 2	Off White, irregular, flat
BP -3	white, flat, rough and irregular
BP- 4	Light blue, irregular and raised
BP- 5	Pink, raised and irregular
BP -6	Yellow, raised and smooth
BP- 7	white, flat, irregular and rough
BP- 8	Red, raised, smooth and round
BP- 9	Light yellow, raised, round and smooth
BP 10	Pinkish white, flat, irregular and rough
BP-11	yellow, raised, gummy, and smooth
BP-12	Off White, raised, round and smooth
BP-13	Dark yellow, raised and shiny
BP-14	Colorless, flat, irregular and rough
BP-15	Pale yellow, raised and round
Fungal Isolates	
FP-1	Pinkish white, flat, irregular and rough
FP-2	White and cottony
FP-3	Blackish brown and powdery
FP-4	Pinkish white, cottony and fuzzy edges,
FP-5	Yellowish white
FP-6	Black, powdery
FP-7	Off White, puffy, thick
FP-8	White, cottony and fuzzy edges
FP-9	Brownish with White edges

Table.2 Rf value of pigment producing selected isolates

Isolate No.	R_f value
BP-1	0.91
BP-5	0.86
BP-6	0.88
BP-8	0.87
FP-6	0.92

Table.3 Morphological and biochemical characteristics of pigment producing bacterial isolates

Test	Bacterial Isolate		
Morphological characteristics			
	BP-2	BP-6	BP-8
Pigment	Off- white	Yellow	Red
Colony morphology	flat, Irregular	raised, circular	raised, circular
Cell shape	rods	cocci	rods
Gram reaction	+	+	-
Spore formation	+	-	-
Optimum temperature growth	30^0C	30^0C	35^0C
Biochemical characteristics			
Catalase test	+	+	-
Voges-proskauer	-	-	+
Urease	-	+	-
H_2S production	-	-	-
Glucose fermentation	AG	AG	AG
Genus	*Bacillus*	*Micrococcus*	*Serratia*

AG- Acid and gas production

Cytotoxicity Assay of Purified Pigment From Bacteria and Fungi

Red pigment extracted from bacterial isolate (BP-8) showed clear inhibition zone against *Staphylococcus aureus* (4 mm), *Escherichia coli* (5 mm) and *Saccharomyces cerevisiae (8 mm)*. The prodigiosin pigment showed antibacterial activity against *Staphylococcus aureus* (17.5 mm), *Bacillus cereus* (10.5 mm), *Escherichia coli* (5 mm) (Gulani *et al*., 2012).

The red pigment produced by BP-8 isolate showed the inhibitory effect against *E.coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae.* The red pigment extracted from *Serratia marcescens* KH1R KM035849 exhibited antibacterial activity against *B.cerus, S.aureus* and *E.coli* with inhibition zone 12 mm, 07 mm and 06 mm, respectively (Vora *et al*., 2014).

Identification of Selected Isolates

The pigment producing bacterial isolates were characterized on the basis of morphology and biochemical test. Microbiological and biochemical

characteristics of pigment producing bacterial isolates (Table 3). These bacterial isolates BP-2 and BP-6 were found to Gram+ve and BP-8 as Gram-ve. Out of Gram+ve bacterial isolates BP-2 was spore former whereas BP-6 was nonspore former. Moreover, BP-2 isolate was rod shaped and BP-6 as cocci. Both of them were catalase positive and negative for Voges-proskauer and hydrogen sulfide production. So for urease activity is concerned, it was absent in BP-2 but present in BP-6. The color of pigment of isolate BP-2 was off-white and yellow of BP-6. It may be said that BP-2 isolate belong to *Bacillus* and BP-6 to *Micrococcus*. Isolate BP-8 showed raised, circular red pigmented colonies. In addition to Gram-ve and nonspore formation, it showed positive for Voges-proskauer test. It probably may belong to *Serratia.* Two bacterial isolates (MJ-O and MJ-Y) obtained had been shown to produce pigments. One of the bacteria (MJ-O) produced orange coloured pigment and the other one (MJ-Y) produced yellow colored pigment. The morphological characteristics of these bacteria were Gram positive, cocci and non-motile. Hence MJ-O was identified as *M. nishinomiyaensis* and MJ-Y as *Micrococcus luteus,* respectively (Bhat *et al*., 2013).

In conclusion, different pigments were produced by bacterial and fungal isolates. These pigments were extracted in methanol and acetone in the ratio of 1:5 (v/v). The maximum absorbance of red pigment (BP-8) was obtained at 532nm; orange (BP-1) at 450nm, yellow (BP-6) at 468 nm, pink (BP-5) at 540 nm and black (FP-6) at 300nm.

The R_f values of pigments ranged from 0.86 to 0.92. Red pigment produced by BP-8 isolate showed the inhibitory effect against *E.coli, Staphylococcus aureus* and *Saccharomyces cerevisiae*.

The results indicated that the purified pigment contains antimicrobial substances, and it possesses the ability to inhibit the growth of human pathogens. On the basis of morphological and cultural characteristics, the bacterial isolates were found to resemble to the genus *Bacillus, Serratia* and *Micrococcus*.

Acknowledgments

Authors are thankful to Chaudhary Charan Singh Haryana Agricultural University, Hisar for providing facilities and financial support for the completion of this research work.

References

Aberoumand, A. 2011. A Review article on edible pigments properties and sources as natural biocolorants in foodstuff and food industry. *World J. Dairy and Food Sci.*, 6: 71-78.

Ahmad, A.S., Ahmad, W.Y.W., Zakaria, Z.K. 2012. Application of bacterial pigments as colorant: The Malaysian perspective. Springer Briefs in Molecular Science, New York Publishers, pp. 57-74.

Bhat, R., Marar, T. 2015. Media optimization, extraction and partial characterization of an orange pigment from *Salinicoccus* sp. MKJ 997975. *Int. J. Life Sci. Biotechnol. Pharma. Res.*, 4: 85-89.

Bhat, S.V., Khan, S.S., Amin, T. 2013. Isolation and characterization of pigment producing bacteria from various foods for their possible use as biocolours. *Int. J. Recent Scientific Res.*, 4(10): 1605-1609.

Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. 2005. Bergey's Manual of Systematic Bacteriology, Springer-Verlag, New York Publishers, pp. 408-420.

Cho, YJ., Park, JP., Hwang, HJ., *et al*. 2002. Production of red pigment by submerged culture of *Paecilomyces sinclairii*. *Lett. Appl. Microbiol.,* 3: 195– 202.

Dufosse, L. 2007. Microbial production of food grade pigments. *Food Technol. Biotechnol.*, 44: 313–321.

Gulani, C., Bhattacharya, S., Arijit, D. 2012. Assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potentials. *Malaysian J. Microbiol.*, 8: 116-122.

Jiang, Y., Chen, F., Hyde, K. 2005. Production potential of water-soluble *Monascus* red pigment by a newly isolated *Penicillium* sp. *J. Agri. Technol.*, 1: 113-126.

Joshi, V.K., Attri, D., Bala, A., Bhusan, S. 2013. Microbial pigments. *Indian J. Biotechnol.*, 2: 362-369.

Kumar, A., Vishwakarma, H., Singh, J. 2015. Microbial pigments: Production and their applications in various industries. *Int. J. Pharmacy, Chem. Biol. Sci.*, 5(1): 203-212.

Malik, K., Tokkas, J., Goyal, S. 2012. Microbial pigments: A review. *Int. J. Microbial Res. Technol.,* 1(4): 361-365.

Mohammed, H., Naseer, J., Aruna, K. 2012. Study on optimization of prodigiosin production by *Serratia marcescens* MSK1 Isolated from air. *Int. J. Adv. Biol. Res.*, 2: 671-680.

Song, C., Makoto, S., Osamu, J. *et al*. 2000. High production of prodigiosin by *Serratia marcescens* grown on ethanol. *Biotechnol. Lett.,* 22: 1761–1765.

Tibor, C. 2007. Liquid chromatography of natural pigments and synthetic dyes. *J. Chromatography Library*. 71: 11-19.

Unagul, P., Wongsa, P., Kittakoop, P., Intamas, S., Srikiti-Kulchai, P. 2005. Production of red pigments by the insects pathogenic fungus *Cordyceps unilateralis* BCC 1869. *J. Industrial Microbiol. Biotechnol.,* 32: 135-140.

Vora, J.U., Jain, N.K., Modi, H.A. 2014. Extraction, characterization and application studies of red pigment of halophile *Serratia marcescens* KH1R KM035849 isolated from Kharaghoda soil. *Int. J. Pure and Appl. Biosci.*, 2: 160-168.

How to cite this article:

Kamla Malik, Jayanti Tokas and Ramesh Chand Anand. 2016. Characterization and Cytotoxicity Assay of Pigment Producing Microbes. *Int.J.Curr.Microbiol.App.Sci.* 5(6): 370-376. doi: http://dx.doi.org/10.20546/ijcmas.2016.506.042