

Microbial properties of pigmented bacteria isolated from bioenhancer 'Amrit pani'

Neelima Garg*, RA Ram & Priti

ICAR-Central Institute for Subtropical Horticulture, Lucknow- 226 101, Uttar Pradesh
E-mails: neelimagargg@rediffmail.com, raram_cish@yahoo.co.in

Received 29 July 2016, revised 29 November 2016

Amrit Pani (Nectar water) contains consortia of beneficial microorganisms, out of which two pigmented bacteria, B₁ (yellow) and B₂ (orange) were isolated. Colonies of these microbes were smooth, 1-3 mm in diameter and identified as *Micrococcus luteus* (B₁) and *Micrococcus varians* (B₂). Both the bacteria were aerobic in nature, could be grown successfully on medium containing pH 7- 8 at 30 °C temperature. Isolated bacteria exhibited UV resistance and high pectinase activity 2.23 and 2.66 IU/mL, respectively. These bacteria have shown antimicrobial activity against *Colletotricum gloeosporioides* and *Fusarium solani* and extracted pigments shown DPPH activity (free radical scavenging activity) 86.11 and 59.02 %, respectively. Based on above study, *Amrit Pani* can successfully be used for the management of soil born diseases and fermentation of organic wastes.

Keywords: *Rishi Krishi*, *Amrit pani*, Honey, Pigmented bacteria, UV resistance

IPC Int. Cl.⁸: A01N 63/00, A61L, A61K, C12N, C12M, A01N, A23L 21/25, A01K 53/00

A number of organic farming systems based on traditional knowledge, viz. Biodynamic farming, Natural farming, Nateuco farming, *Panchagavya* farming, *Rishi Krishi*, *Jaivik Krishi*, Homa Organic farming, etc., have emerged in different parts of the country and use of organic liquid preparations for crop production is an age old practice in India. Based on traditional practices, *Rishi Krishi* was developed¹ and it was mentioned that 'Angara' rhizosphere soil of banyan tree (*Ficus benghalensis* L.); and *Amrit pani* are potent source to enhance soil biological properties. *Amrit pani* is a special bio-enhancer prepared by incubating cow dung along with cow ghee and honey. Twenty per cent solution is used for seed/seedling treatment and spray on field ready for sowing/transplanting. Seed or seedling treatment with *Amrit pani* can be done for better germination and management of soil born diseases. *Rishi Krishi* has been adopted by large number of farmers with satisfactory results in crops like maize, *jowar*, rice, wheat, pigeon pea, sugarcane, cotton, aonla, banana, mango, papaya, etc. In this study, pigmented microflora present in *Amrit pani* was characterized to give scientific explanation of its bio-enhancer properties.

Materials and methods

For preparation of *Amrit pani*, 250 g of cow's ghee is mixed thoroughly with 10 kg of fresh cow dung and 500 g honey or jaggery is incorporated and thoroughly mixed. The mixture is diluted with 200 L water and incubated for a week by stirring twice a day.

Isolation of pigmented bacteria

The experiment was conducted under aseptic conditions and *Amrit pani* sample was vortexed to form uniform suspension. One ml of suspension was transferred to 9 mL sterile distilled water and diluted the sample up to 10⁴ dilution by serial dilution methods and inoculated the sample on Nutrient Agar (Himedia M561) plates using pour plate technique. The inoculated plates were incubated at 37 °C for 24 h. Colonies producing pigment were isolated and sub-cultured. The purity of isolated colonies was confirmed by microscopic observation. Bacterial strains were inoculated on Nutrient Agar slants and stored in refrigerator (4 °C) for further study.

Microscopic identification

The bacterial isolates were gram stained and observed under a high power (100x) magnifying lens in light microscope.

Biochemical characterization

In order to determine the biochemical characterization of the bacterial isolates, a series of

*Corresponding author

biochemical tests were performed according to Bergey's manual of bacterial identification². The bacterial cultures were subjected to gram staining followed by different biochemical tests:

Catalase test

This test was performed by adding 1 drop of 3 % hydrogen peroxide and the effervescence was noted.

Motility test

Motility medium (HiMedia M260) was prepared and sterilized by autoclaving at 121 °C for 20 min. The tubes of motility medium were inoculated by stabbing into the top of the medium to a depth of about 5 mm and incubated in incubator shaker at 30 °C and the growth pattern of bacteria isolates was observed.

Urease test

Urea Broth (HiMedia M111) was prepared and filter sterilized and cooled to 55 °C. Aseptically sterile 40 % urea solution was added. Mixed well and distributed in 10 ml amounts into sterile tubes and inoculated with isolates (B₁ & B₂). The culture tubes were incubated for 48 h at 37 °C. After 48 h urea hydrolysis was determined by pink coloration of the broth. Broth colour changed from yellow to pink.

Gelatin test

Gelatin agar medium (HiMedia M920) plates were inoculated with bacterial culture and incubated for 5 days at 30 °C with an un-inoculated plate as a control. Incubated agar dishes were floated with mercuric chloride solution and allowed to settle the plate 5-10 min. Petri dishes were examined to see whether the medium is solid or floated around the line of bacterial growth.

Mannitol and glucose fermentation test

To determine the carbohydrate utilization, assays were performed with sucrose, dextrose, maltose, and mannitol broth with phenol red as an indicator. The ability of isolated bacteria to use glucose, lactose, and sucrose was observed by the production of gas.

Effect of physiological condition on growth and pigment of isolates

Effect of temperature

The investigation for standardization of optimum temperature for growth of the isolates were performed by inoculating the nutrient broth medium and

incubated at different temperature (20, 30, 40, 50 °C) for 48 h. After 48 hrs the growth was detected with OD₄₂₀ using UV-visible spectrophotometer.

Effect of pH

For the test of pH optimization, the pH of the nutrient broth medium was adjusted to 4, 5, 6, 7, 8, 9 using HCl or NaOH and inoculated by isolates. The inoculated medium was incubated at 30 °C for 48 h. Isolates growth was determined at 420 nm wave length.

Effect of aeration

Nutrient broth medium was inoculated with isolates (B₁ & B₂) in two different aliquots and incubated at 30 °C temperature with a pair of aliquot in aeration condition (on shaker at 100 rpm) and one pair in stationary condition. Growth of bacteria after 48 h was observed in terms of optical density at 420 nm wavelength using spectrophotometer

Effect of UV exposure on growth and pigment of isolated bacteria

B₁ & B₂ inoculum @ 1 x 10⁵ cells/mL (in log phase of growth) was added to sterile nutrient agar plates. The uncovered inoculated petri dishes were exposed to UV irradiation with a 15 W germicidal lamp (BUV-15, USSR) emitting 90 % of light at 254 nm for different time intervals (5, 10, 15, 20 min). Afterwards the plates were covered and incubated at 30 °C. The colony count appearing after 48 h of incubation was recorded.

Enzyme activity of isolated pigmented bacteria

The enzyme activity of isolates (B₁ & B₂) were examined by inoculation of cellulose and pectin containing broth with pH 8 for B₁ & pH 7 for B₂ and incubated at 30°C for 48 h. The cellulase and pectinase enzyme activities were assayed³⁻⁵. The change in colour was detected spectrophotomerically at 540 nm wavelength.

Antimicrobial activity of isolated bacteria (B₁ & B₂)

Antimicrobial activity of isolated bacteria was observed by plate assay method against *Colletotricum gloeosporioides* and *Fusarium solani*. Potato Dextrose Agar (PDA) was used to study the antagonistic activity since both types of microorganisms (fungi and bacteria) can grow on this medium. The bacterial strains were pour plated individually on sterile petri dishes. Approximately, a 5 mm mycelial plug was taken from the peripheral

edge of five days old cultures of fungal pathogens and each placed at the centre of the plates and the dual culture plates were incubated at 30 °C. Thereafter, the zone of inhibition (distance of the fungal mycelium measured in mm) was recorded after 3 days of growth⁷ and measured the colony size of fungi by Himedia colony size scale.

Extraction of pigment from isolates

The different solvents like chloroform, acetone, ethyl acetate and methanol were screened out for extraction of pigment but a modified procedure for the isolation of pigment was carried out; 48 h old inoculated nutrient culture broths which were incubated at 25 °C and 200 rpm used to harvest the cells by centrifugation at 5000 rpm at 4°C for 15 min. The resulting pellet was collected and mixed with 95 % (v/v) acetone in a sterile centrifuge tube and incubated at 60 °C in water bath for 30 min, sample was vortexed 2-3 times during incubation and centrifuged at 10000 rpm for 5 min, process was repeated until the pellet become colourless. Supernatant (coloured) was collected.

Characterization of extracted pigments

UV-VIS spectra absorption

Spectral analysis was done on extracted pigments dissolved in acetone using EC double beam UV- visible spectrophotometer. The extracted pigments were scanned in the range of 400 to 600 nm to find out the maximum absorption spectra. Acetone was used as a reference.

Determination of reducing activity of stable radical, 1-Diphenyl-Picrylhydrazyl (DPPH)

A 0.05 mM solution of DPPH in methanol was prepared. This solution was added to an equal volume of the solution of tested compound (extracted pigments). Methanol was used as a control solution. After incubation of 30 min at room temperature, absorbance was recorded at 517 nm. Reduction in absorbance of the reaction mixture indicated higher the free radical scavenging activity. The percentage of scavenging activity of pigments on DPPH radical was calculated as percentage inhibition of DPPH (I %) using the following formula:

$$I \% = \frac{(A_0 - A_s) \times 100}{A_0}$$

A₀= Absorption of control

A_s= Absorption of tested compound

Results and discussion

The isolated bacterium B₁ was yellow pigmented while B₂ orange pigmented. Both were gram positive *Cocci* with smooth colony and 1-3 mm in diameter in 48-72 h. Based on biochemical test results of pigmented bacterial isolates yellow pigmented bacteria (B₁) was identified as *Micrococcus luteus* and orange pigmented bacteria (B₂) as *Micrococcus varians* (Fig.1 & Table1). The growth and pigment production was affected by growth conditions, viz. pH, temperature and aeration as depicted in Fig 2 & Table 2. *Micrococcus luteus* (B₁) exhibited maximum growth and pigment formation at pH 8 where as *Micrococcus varians* (B₂) at pH 7, while 30 °C was the optimum temperature for both the bacteria. Aeration also affected the growth of both bacteria. In aerobic condition the isolates showed higher growth and pigment production compared to without aeration. Exposure of isolates B₁ & B₂ to UV irradiation upto 20 min resulted in only one log reduction in the bacterial population (Table 3). The pigment of both isolated bacterial colonies became more concentrated with increasing the UV exposure time. The petri plates which were exposed for 20 min under UV

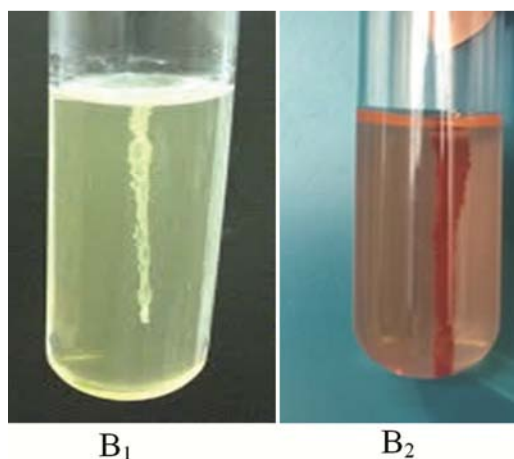


Fig. 1 — Motility test of B₁ & B₂

Table 1 — Biochemical characterization of pigmented bacterial isolates from *Amrit pani*

Biochemical test	B ₁	B ₂
Catalase test	Positive	Positive
Motility test	Non motile	Non motile
Mannitol fermentation test	Negative	Negative
Glucose fermentation test	Negative	Positive
Maltose fermentation test	Negative	Positive
Urease test	Positive	Negative
Gelatin test	Negative	Positive

Table 2 — Effect of growth condition on pigment production by isolates

pH	Temperature (°C)		Aeration		B ₁	B ₂
	B ₁	B ₂	B ₁	B ₂		
4	+	+	20	aerobic	++++	++++
5	+	++	30		++++	++++
6	++	++	40		+++	+++
7	+++	++++	50	anaerobic	++	+
8	++++	+++				
9	+++	+				

Table 3 — Effect of UV exposure on growth and pigment of isolated bacteria

SNo	UV exposure time(min)	B ₁ (Yellow pigmented bacteria)		B ₂ (Orange pigmented bacteria)	
		CFU/ml	Colony pigment	CFU/ml	Colony pigment
1	0	5.6*10 ⁴	++	6.2*10 ⁵	+
1	05	5.6*10 ⁴	++	6.2*10 ⁵	+
2	10	4.2*10 ⁴	+++	5.1*10 ⁵	++
3	15	3.8*10 ⁴	+++	3.7*10 ⁴	++++
4	20	3.5*10 ³	++++	2.9*10 ⁴	++++

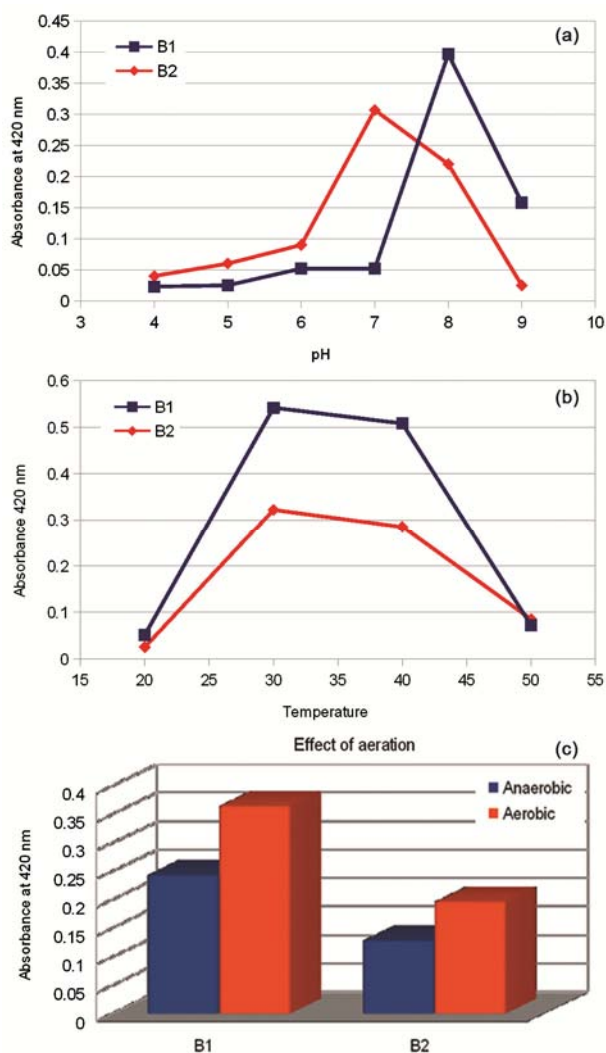


Fig. 2 — Effect of pH (A), Temperature °C (B), and aeration (C) on growth of bacterial isolates

Table 4 — Cellulase and pectinase activity of B₁ and B₂

Sample	Cellulase activity (IU/ml)	Pectinase activity (IU/ml)
B ₁ (yellow bacteria)	0.033	2.23
B ₂ (orange bacteria)	0.039	2.66

environment had highly pigmented colonies as compare the plates which are exposed for lesser period. This indicates the UV resistance property is related to pigment production. The pigmented bacteria were tested for cellulase and pectinase activity (Table 4). Both the bacteria were having high pectinase activity with 2.23 IU/mL for B₁ (yellow pigmented) and 2.66 IU/mL for B₂ (orange pigmented). These bacteria had antimicrobial activity against *Colletotricum gloeosporioides* and *Fusarium solani* as observed by plate assay method (Table 5). The extracted pigments (Fig. 3) were collected in plastic vials and further studied for UV-Vis. absorption and antioxidant properties. Maximum absorbance for yellow coloured pigment extracted from B₁ strain was observed at 460 nm and for orange coloured pigment extracted from B₂ strain at 440 nm (Fig. 4). DPPH free radical scavenging activity for yellow pigment extracted from B₁ showed very high activity with 86.11 %, whereas for orange pigment extracted from B₂ is 59.02 % scavenging activity. *Micrococci* were gram-positive, non-sporulating and non-motile bacteria with spherical cells, and often found in tetrads. The genus *Micrococcus* has several species, all described as strict aerobes and *M. luteus*, the type species of the genus *Micrococcus*, is an obligate aerobe. Netzer⁶ characterized the major carotenoids synthesized by

Table 5 — Antimicrobial activity of isolated bacteria (B₁ and B₂)

Fungus	Control colony size (mm)	Dual culture with B ₁ colony size (mm)	% reduction in colony	Dual culture with B ₂ colony size (mm)	% reduction in colony
<i>Colletotricum gloeosporioides</i>	18	5	72.22	6	66.67
<i>Fusarium solani</i>	16	6	62.5	8	50

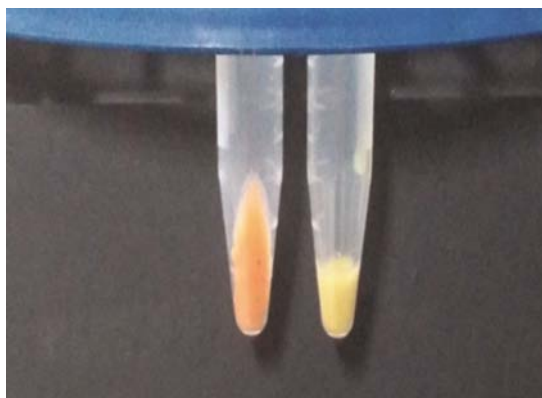
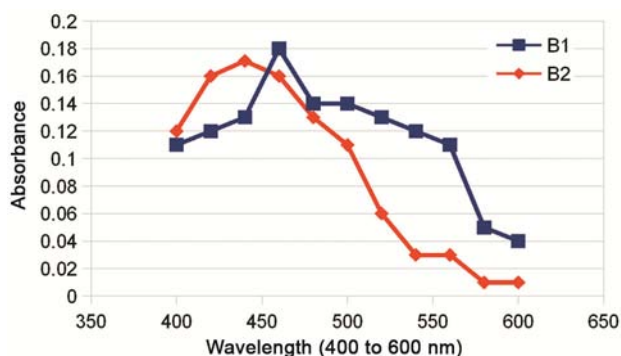
Fig. 3 — Pigment extracted from B₁ & B₂

Fig. 4 — UV-Vis. absorption properties of extracted pigment

the *M. luteus* strain NCTC 2665 as sarcinaxanthin. Kaliappan & Marimuthu⁷ have reported *M. luteus* to act against both gram positive and gram negative bacteria including *Coliforms*. There are reports that *Micrococcus luteus* bacterium produces a pigment that absorbs ultraviolet (UV) light at 350–475 nm, the same wavelength that causes mutations in DNA and triggers cancers such as melanomas^{8,9}. The pigment is also used in preparing sun screen lotions¹⁰.

The results indicated that these bacteria might be responsible for specific properties of bio-enhancer *Amrit pani* as seed dresser and for decomposition of organic wastes.

Conclusion

Isolated bacteria from *Amrit pani* had UV resistance property and high pectinase activity. They have also

shown antimicrobial activity against *Colletotricum gloeosporioides* and *Fusarium solani* and the pigment extracted from the microbes had shown free radical scavenging activity. It is concluded that *Amrit pani* can be successfully used for the management of soil born diseases through seed and seedling treatment and fermentation of organic wastes during composting.

Acknowledgement

Authors are thankful to the Director, ICAR-Central Institute for Subtropical Horticulture, Lucknow for providing all types of necessary facilities to complete the study.

References

- Desh Pandey, Organic Farming wrt. Cosmic energy, Non Violence Rishi- Krishi, Khede-Ajra, Kholapur, Maharashtra, 2003, 65.
- Holt JG, Gram-Positive cocci, In: *Bergey's Manual of Determinative Bacteriology*, edited by Holt JG, Krieg NR, Sneath PHA, Staley JT & Williams ST, 9th edn, Baltimore, 1994, 527-37.
- Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugars, *Anal Chem*, 31 (3) (1959) 426–428.
- Kumar D, Ashfaque M, Muthukumar M, Singh M & Garg N, Production and characterization of carboxymethyl cellulase from *Paenibacillus polymyxa* using mango peel as substrate, *J Environ Biol*, 33 (1) (2012) 81-84.
- Garg Neelima & Ashfaque M, Mango peel as substrate for production of extra cellular polygalacturonase from *Aspergillus fumigates*, *Indian J Hort*, 67 (1) (2010) 140-143.
- Netzer R, Stafsnes MH & Andreassen T, Biosynthetic pathway for γ -cyclic sarcinaxanthin in *Micrococcus luteus*: Heterologous expression and evidence for diverse and multiple catalytic functions of C50 carotenoid cyclases, *J Bacteriol*, 192 (2010) 5688-5699.
- Kaliappan U & Marimuthu K, Antibacterial activity of pigment produced from *Micrococcus luteus* KF532949, *Int J Chem Anal Sci*, 4 (3) (2013) 149–152.
- Mohana DC, Thppeswamy S & Abhisek RU, Antioxidant and ultraviolet protective properties of caretonoids isolated from *Micrococcus* spp, *Rad Prot Environ*, 36 (2013) 168-74.
- Surekha PY, Dhanya P & Sarath Josh MK, *Micrococcus luteus* Strain BAA2, A Novel Isolate Produces Carotenoid Pigment, *Electronic J Biol*, 12 (1) (2016) 83-89..
- www.medicaldaily.com/better-sunscreen-bacteria- scientists-find-pigment-absorbs-uv-1.