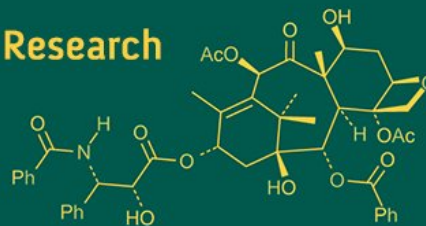
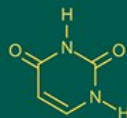
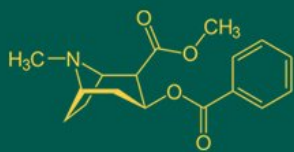


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(9): 1361-1367
www.biochemjournal.com
Received: 14-07-2026
Accepted: 17-08-2026

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Characterization of different *Rhizobium* isolates of lentil (*Lens Culinaris*) collected from different locations of Kolhapur district

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9Sq.10008>

Abstract

Lentil (*Lens culinaris* L.), commonly known as Masur, is an important cool-season pulse crop valued for its high protein content and its ability to fix atmospheric nitrogen in symbiosis with *Rhizobium* bacteria. The present investigation was carried out to isolate, purify and characterize native *Rhizobium* isolates from the root nodules of lentil plants collected from different locations of Kolhapur district. Eight root nodule samples were collected from lentil fields located in Gadhinglaj, Chandgad, Ajara and Bhudargad tehsils, from which four distinct *Rhizobium* isolates, designated RLGB₁ (Bhadgoun), RLCH₃ (Halkarni), RLAU₅ (Uttur) and RLKB₇ (Kadgaon), were successfully obtained using the Congo Red Yeast Extract Mannitol Agar (CR-YEMA) medium and serial dilution technique. Morphological analysis revealed that all four isolates were Gram-negative, rod-shaped, pink-staining, motile and gave a positive 3% KOH test. Cultural characterization on four different solid media (Nutrient Agar, Potato Dextrose Agar, CR-YEMA and Jensen's medium) revealed variation in colony colour, shape, margin, texture and elevation among isolates, with CR-YEMA medium supporting the maximum colony growth (12.70 to 16.20 mm), followed by Nutrient Agar (9.40 to 12.53 mm), Potato Dextrose Agar (9.47 to 12.40 mm) and Jensen's medium (8.40 to 11.60 mm). Among the isolates, RLGB₁ recorded the highest mean colony diameter (13.73 mm). Biochemical characterization revealed that all four isolates tested positive for catalase, oxidase, starch hydrolysis, methyl red and urease tests, while all were negative for indole formation, confirming their identity as *Rhizobium* species. Based on the overall morphological, cultural and biochemical profile, the isolates RLGB₁ which is followed by RLKB₇ showed the most maximum growth and were identified as promising isolates for further evaluation as potential biofertilizer strains for lentil cultivation.

Keywords: *Rhizobium*, lentil, root nodule, isolation, characterization, Kolhapur

Introduction

Lens culinaris L., commonly known as lentil or Masur, is an important cool-season legume of the family Leguminosae, widely valued for its protein-rich seeds, which serve as a staple dietary component, particularly as dal and as an affordable alternative to meat (Aykroyd *et al.*, 1982) [1]. India ranks as the second-largest producer of lentil in the world after Canada, and leads the world in area under lentil cultivation. In India, the crop is grown extensively in Madhya Pradesh, Uttar Pradesh, Rajasthan, Maharashtra and Bihar. Nutritionally, lentils are rich in protein, complex carbohydrates, minerals and vitamins, along with bioactive compounds such as antioxidants and phytoestrogens (Dhull *et al.*, 2022; Siva *et al.*, 2017) [10, 22]. As a legume, lentil is capable of fixing substantial amounts of atmospheric nitrogen through its symbiotic association with *Rhizobium* bacteria, with reported nitrogen fixation ranging from 10 to 129 kg N/ha depending on environmental conditions (ICARDA, 1989). Under favourable conditions, *Rhizobium* can fix about 57-100 kg N/ha, thereby enhancing soil nitrogen availability for plant uptake (Wagner, 2012) [25]. However, effective native *Rhizobium* strains are not present in all soils, which limits nodulation and nitrogen fixation efficiency. In such situations, isolation and inoculation of superior indigenous strains is essential to ensure efficient nodulation and improved productivity. Increasing concerns over the environmental and health hazards associated with excessive use of chemical fertilizers have intensified the search for eco-friendly alternatives such as biofertilizers. Since the efficiency of *Rhizobium* strains varies with soil type, host genotype and agro-climatic

conditions, it becomes essential to isolate and characterize native strains from the region of interest before their use as inoculants. Keeping this in view, the present study was undertaken to isolate, purify and characterize different *Rhizobium* isolates from root nodules of lentil collected from different locations of Kolhapur district, based on their morphological, cultural and biochemical characteristics.

Materials and Methods

The present investigation was carried out at the Department of Plant Pathology and Agricultural Microbiology, Govt. College of Agriculture, Pune, during 2025-26.

Collection of root nodule samples

Eight field-grown lentil plants having root nodules were randomly sampled from eight different sites located in the Gadhinglaj, Chandgad, Ajara and Bhudargad tehsils of Kolhapur district, with two villages selected from each tehsil. Plants were carefully uprooted and the loosely attached soil was removed by gentle shaking. Roots bearing mature, healthy (pink) nodules were washed thoroughly under running tap water and the nodules were collected in sterile polythene bags, properly labelled, transported to the laboratory, and stored at 4 °C until further use.

Isolation, purification and maintenance of *Rhizobium* isolates

Isolation of *Rhizobium* was carried out following the methods described by Somasegaran and Hoben (1994) [23]. Healthy pink nodules were surface sterilized in 0.1% mercuric chloride (HgCl₂) for 3 minutes, followed by 75% ethanol for 3 minutes, and rinsed thrice with sterile distilled water. Sterilized nodules were aseptically crushed with a sterile glass rod in 1 ml sterile water, and serial dilutions were prepared. Aliquots (0.1 ml) from 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions were streaked onto Congo Red Yeast Extract Mannitol Agar (CR-YEMA) medium and incubated at 28 ± 2 °C for 3 days. Translucent, white, elevated colonies typical of *Rhizobium* were selected and purified by repeated streaking on Yeast Extract Mannitol Agar (YEMA) medium. Pure cultures were maintained on YEMA slants at 4-5 °C. Isolates were designated using abbreviations derived from the host legume, tehsil and village of collection.

Morphological characterization

Gram staining was performed to determine cell shape, arrangement and Gram reaction, following standard procedure. Colony morphology (shape, size, elevation and colour) was recorded after 2-4 days of incubation (Nivaas *et*

al., 2019) [16]. Motility was assessed using stab inoculation into motility medium and incubation at 28 °C for 24 hours. A 3% potassium hydroxide (KOH) test was performed to confirm the Gram reaction, wherein the formation of a viscous, thread-like strand upon mixing bacterial growth with KOH solution indicated a Gram-negative reaction (Suslow *et al.*, 1982) [24].

Cultural characterization

The cultural characteristics of the isolates were studied by growing them on four different solid media viz., Nutrient Agar (NA), Potato Dextrose Agar (PDA), Congo Red Yeast Extract Mannitol Agar (CR-YEMA) and Jensen's Agar medium. Sterilized media were aseptically dispensed into Petri plates, inoculated with the respective isolates, and incubated at room temperature (27 ± 2 °C) for eight days. Colony colour, shape, texture, margin and elevation were recorded on the seventh day of incubation, and colony diameter was measured and analysed statistically using a Completely Randomized Design (CRD).

Biochemical characterization

Biochemical characterization of the isolates was carried out using standard assays, namely the catalase test (Blazevic and Ederer, 1975) [7], methyl red test, starch hydrolysis test (Eckford, 1927) [11], oxidase test (Collins and Lyne, 1970) [8], and indole formation test (Gordon and Weber, 1951) [13]. Each test was performed test along with appropriate controls, and the reaction of each isolate was recorded as positive (+) or negative (-) based on the visible response.

Statistical analysis

The colony diameter data were analysed using a Completely Randomized Design (CRD), and treatment differences were tested through analysis of variance (ANOVA), following the standard statistical methods described by Panse and Sukhatme (1985) [18].

Results and Discussion

Isolation and purification of *Rhizobium* isolates

Root nodule samples were collected from lentil fields located across eight villages in the Chandgad, Gadhinglaj, Ajara and Bhudargad tehsils of Kolhapur district. Isolation of bacteria from the root nodules was carried out using CR-YEMA medium and the serial dilution technique. Out of the eight samples processed, four *Rhizobium* isolates were successfully obtained, originating from Bhadgaon, Halkarni, Uttur and Kadgaon, and were designated as RLGB₁, RLCH₃, RLAU₅ and RLBK₇, respectively (Table 1).

Table 1: Root nodule samples of lentil collected from different locations of Kolhapur district

Sr. No.	Isolate	Tehsil	Village	Isolates obtained	Location (Lat./Long.)
1	RLGB ₁	Gadhinglaj	Bhadgoun	Yes	16°10'39.4"N, 74°21'54.6"E
2	RLGM ₂		Mahagaon	No	16°08'17.0"N, 74°21'55.2"E
3	RLCH ₃	Chandgad	Halkarni	Yes	16°09'54.2"N, 74°28'32.4"E
4	RLCA ₄		Adkur	No	16°00'51.5"N, 74°15'17.1"E
5	RLAU ₅	Ajara	Uttur	Yes	16°15'00.1"N, 74°16'21.8"E
6	RLAB ₆		Bahirwadi	No	16°16'25.0"N, 74°18'21.3"E
7	RLBK ₇	Bhudargad	Kadgaon	Yes	16°12'52.0"N, 74°03'52.8"E
8	RLBG ₈		Gargoti	No	16°19'10.1"N, 74°08'27.1"E

Morphological characterization of *Rhizobium* isolates

All four isolates consistently exhibited a Gram-negative reaction, indicated by pink coloration of the cells after Gram staining. Microscopic examination revealed rod-shaped cells

occurring singly or in pairs, reflecting typical bacillary and diplobacilli arrangements commonly seen in *Rhizobium* species. All isolates were motile and gave a positive 3% KOH test, confirming their Gram-negative nature (Table 2).

Table 2: Morphological characters of *Rhizobium* isolate of lentil

Sr. No.	Isolate	Gram staining	Stain colour	Cell shape	Cell arrangement	Motility	KOH test
1	RLGB ₁	-ve	Pink	Rod	Singly, paired	+ve	+ve
2	RLCH ₃	-ve	Pink	Rod	Singly, paired	+ve	+ve
3	RLAU ₅	-ve	Pink	Rod	Singly, paired	+ve	+ve
4	RLBK ₇	-ve	Pink	Rod	Singly	+ve	+ve

This result is in agreement with Nushair *et al.*, (2017) ^[17], who reported that *Rhizobia* strains isolated from lentil root nodules formed circular, pink and convex colonies, and were Gram-negative, rod-shaped and motile. Similar morphological features were also reported by Wani and Khan (2013) ^[26] and Zafar *et al.*, (2012) ^[27], who observed that *Rhizobium* isolates from the lentil rhizosphere were consistently Gram-negative and rod-shaped, features typical of beneficial soil bacteria involved in nitrogen fixation.

Cultural characterization of *Rhizobium* isolates

The colony characteristics of the four isolates differed

depending on the culture medium used (Table 3). On Nutrient Agar, RLGB₁ formed white, irregular, slimy colonies with lobate margins and raised elevation, while RLCH₃, RLAU₅ and RLBK₇ exhibited variation in colour and margin. On CR-YEMA medium, RLGB₁ produced pink-creamy, circular, smooth colonies with entire margins and convex elevation, while the remaining isolates formed creamy to white-creamy, circular, smooth colonies. On Jensen's medium, colonies were largely creamy and mucoid with either raised or convex elevation, while on PDA medium, colonies were creamy, mucoid to slimy, with smooth margins.

Table 3: Cultural characteristics of *Rhizobium* isolates on different solid media

Medium	Isolates	Colour	Shape	Texture	Margin	Elevation
1.NA	RLGB ₁	White	Irregular	Slimy	Lobate	Raised
	RLCH ₃	Dull white	Irregular	Dry	Undulate	Raised
	RLAU ₅	White creamy	Circular	Dry	Entire	Convex
	RLBK ₇	Whitish creamy	Irregular	Dry	Undulate	Raised
2.CR-YEMA	RLGB ₁	Pink creamy	Circular	Smooth	Entire	Convex
	RLCH ₃	Creamy	Circular	Smooth	Entire	Convex
	RLAU ₅	White creamy	Circular	Smooth	Entire	Convex
	RLBK ₇	White creamy	Circular	Smooth	Entire	Convex
3.Jensen's	RLGB ₁	Creamy	Irregular	Mucoid	Undulate	Raised
	RLCH ₃	Creamy red	Circular	Mucoid	Entire	Convex
	RLAU ₅	Creamy	Irregular	Mucoid	Undulate	Raised
	RLBK ₇	Creamy	Irregular	Mucoid	Entire	Raised
4.PDA	RLGB ₁	White creamy	Circular	Mucoid	Smooth	Convex
	RLCH ₃	Creamy	Irregular	Slimy	Smooth	Raised
	RLAU ₅	Creamy	Circular	Slimy	Smooth	Convex
	RLBK ₇	White creamy	Irregular	Mucoid	Smooth	Raised

The mean colony diameter of the isolates also varied among the different media (Table 4). CR-YEMA medium recorded the highest colony diameter, ranging from 12.70 to 16.20 mm, followed by NA (9.40 to 12.53 mm) and PDA (9.47 to 12.40 mm), while Jensen's medium showed comparatively lower colony diameter (8.40 to 11.60 mm). Among the

isolates, RLGB₁ recorded the highest mean colony diameter (13.73 mm), followed by RLBK₇ (12.28 mm), RLCH₃ (11.06 mm) and RLAU₅ (10.32 mm), indicating that CR-YEMA was the most suitable medium for colony growth of *Rhizobium*.

Table 4: Colony diameter (mm) of *Rhizobium* isolates on different solid culture media

Isolate	NA (mm)	PDA (mm)	CR-YEMA (mm)	Jensen's (mm)	Mean (mm)
RLGB ₁	12.53	12.40	16.20	11.60	13.73
RLCH ₃	11.50	10.57	12.70	9.47	11.06
RLAU ₅	9.40	9.47	14.00	8.40	10.32
RLBK ₇	12.33	11.90	14.63	9.27	12.28
Control	0.00	0.00	0.00	0.00	0.00
SE(m)±	0.48	0.33	0.51	0.31	0.60
CD at 1%	1.56	1.07	1.67	1.01	1.84

These findings are in agreement with Deka and Azad (2006) ^[9], who characterized fifteen *Rhizobium* isolates on enriched CR-YEMA medium and reported considerable variation among isolates with respect to colony configuration, margin, elevation and colour. Similarly, Satyadev *et al.*, (2018) ^[21] isolated nine *Rhizobium meliloti* strains from fenugreek root nodules on CR-YEMA medium and reported whitish, translucent, sticky, circular colonies measuring 3.2 to 5.2

mm in diameter, with variation observed mainly in colony size.

Biochemical characterization of *Rhizobium* isolates

Biochemical characterization, including catalase, oxidase, indole formation, starch hydrolysis, methyl red and urease tests, was carried out for all four isolates (Table 5). All the isolates tested positive for catalase, oxidase, starch hydrolysis, methyl red and urease tests, while all four

isolates tested negative for indole formation. This uniform biochemical profile supports the identification of RLGB₁,

RLCH₃, RLAU₅ and RLBK₇ as *Rhizobium* isolates.

Table 5: Biochemical characterization of *Rhizobium* isolates of lentil

Isolate	Catalase test	Oxidase test	Indole formation	Starch hydrolysis	Methyl red test	Urease test
RLGB ₁	+	+	-	+	+	+
RLCH ₃	+	+	-	+	+	+
RLAU ₅	+	+	-	+	+	+
RLBK ₇	+	+	-	+	+	+

The results are in agreement with Panwar *et al.*, (2012) ^[19], who characterized *Rhizobium* isolates from fenugreek root nodules based on catalase activity, oxidase activity, nitrate reduction and methyl red tests. Similarly, Rasool *et al.*, (2015) ^[20] isolated *Rhizobium* spp. from root nodules of

Vicia sativa and reported that the strains were positive for catalase, urease and oxidase activities, consistent with the findings of the present study.

Plates and Figures

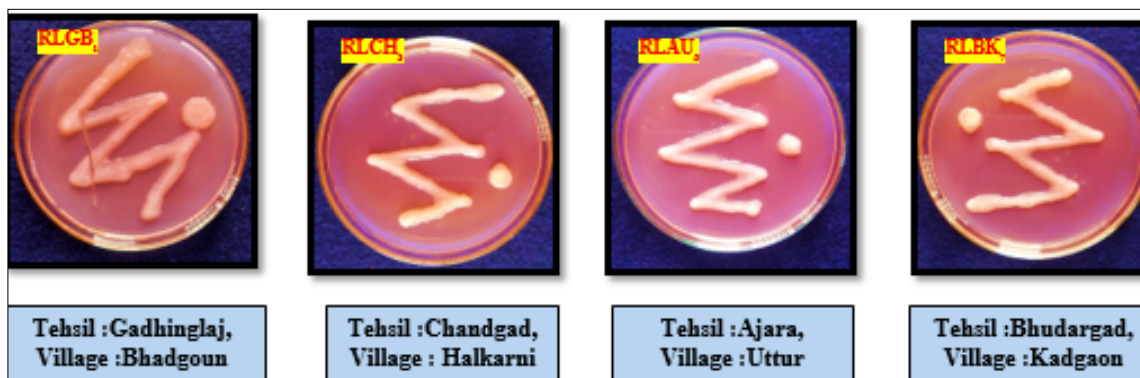
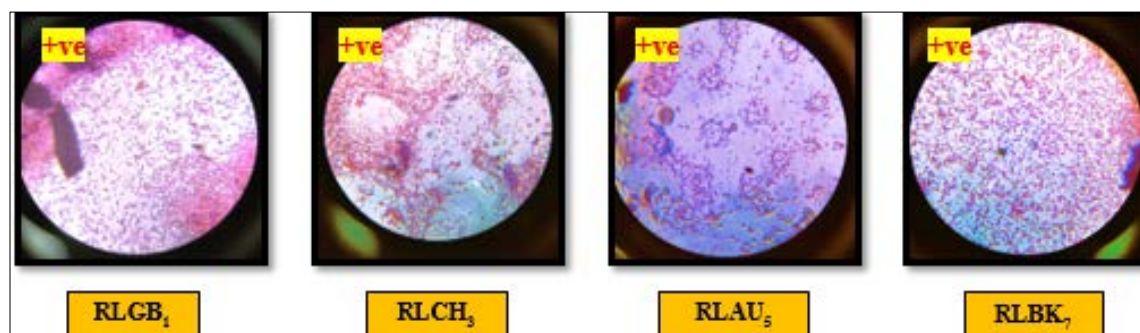


Plate 4.1: Pure culture of *Rhizobium* isolates of lentil.



1. Gram staining



2. 3%KOH test

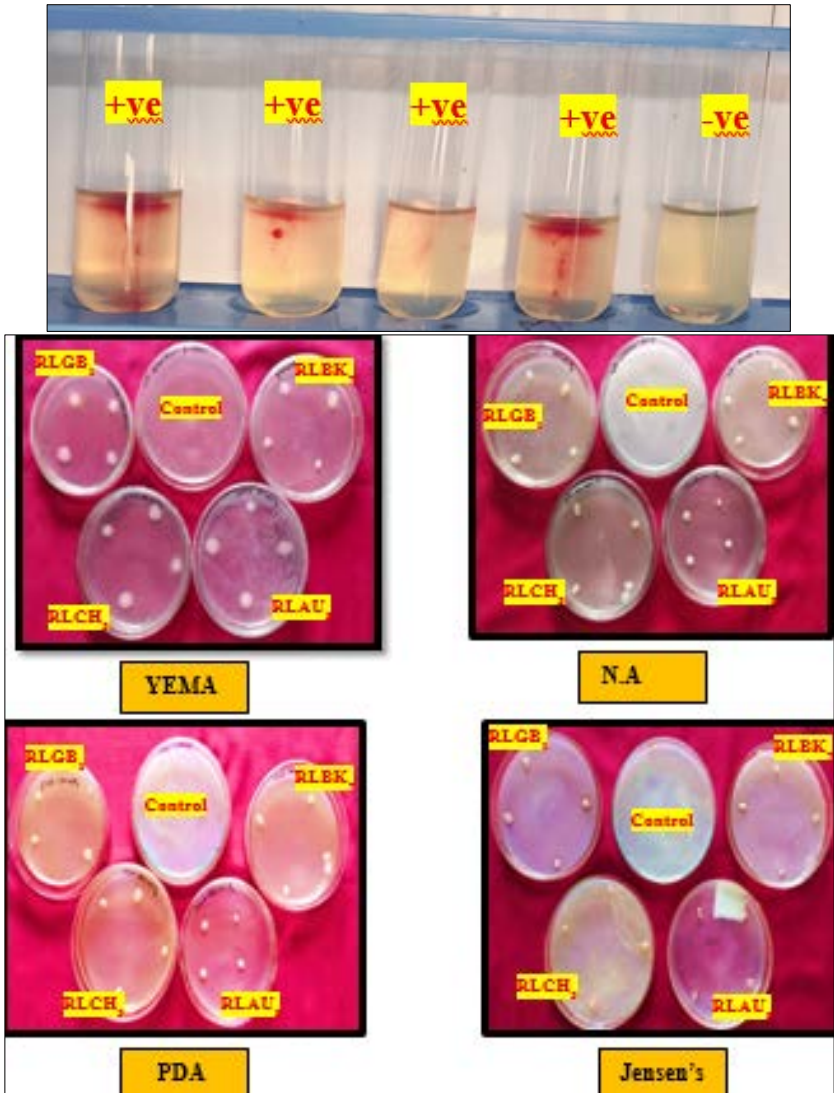
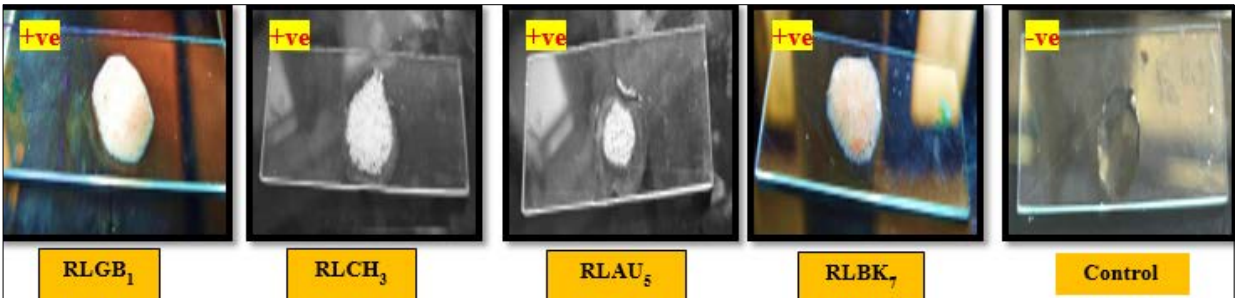
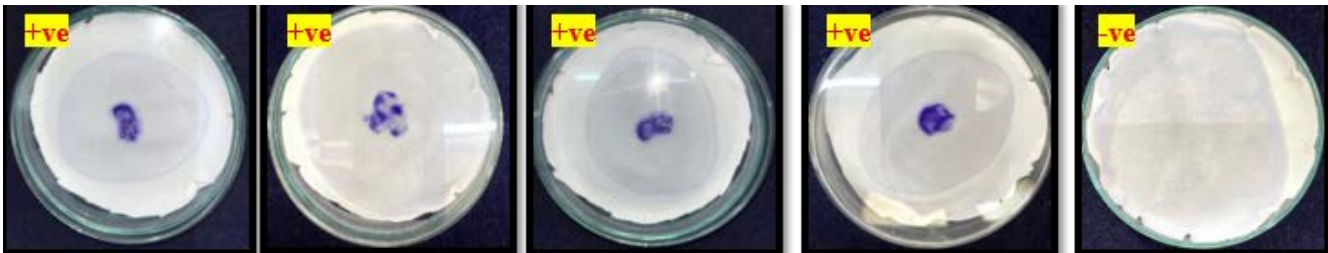


Plate 4.3: Cultural characterization of *Rhizobium* isolates on different solid media



1. Catalase test



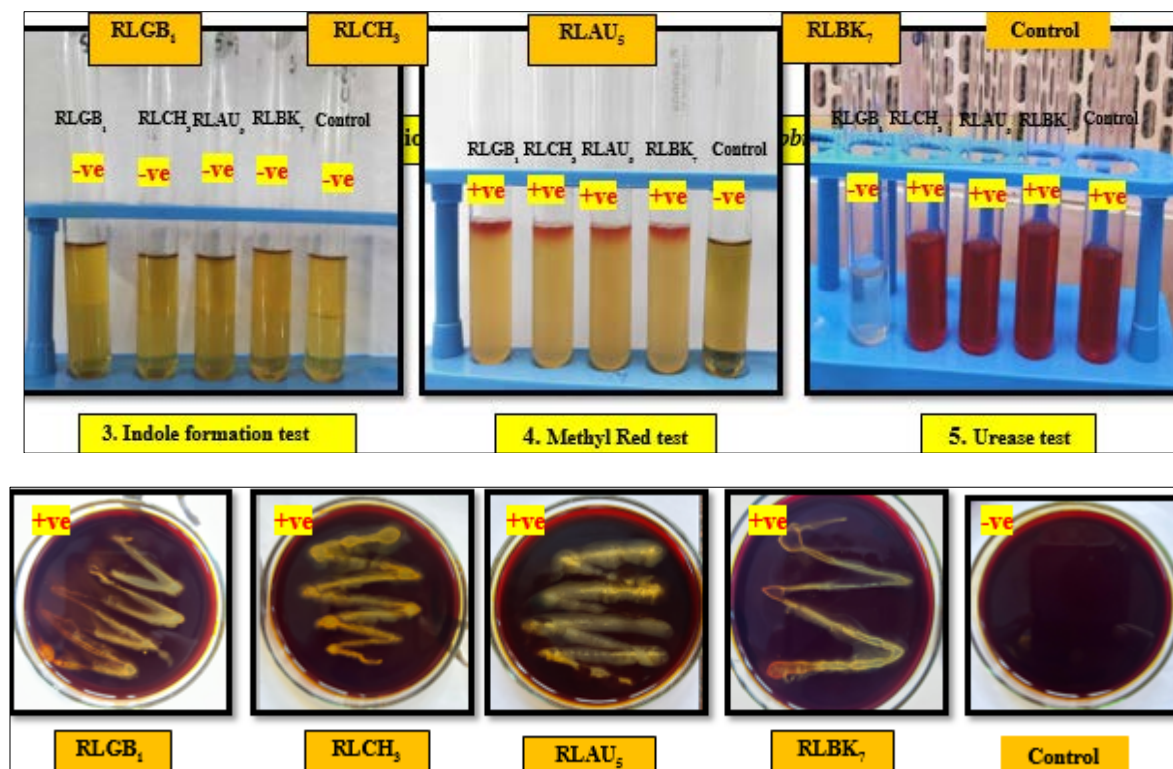


Plate 4.4: Biochemical characterization of *Rhizobium* isolates.

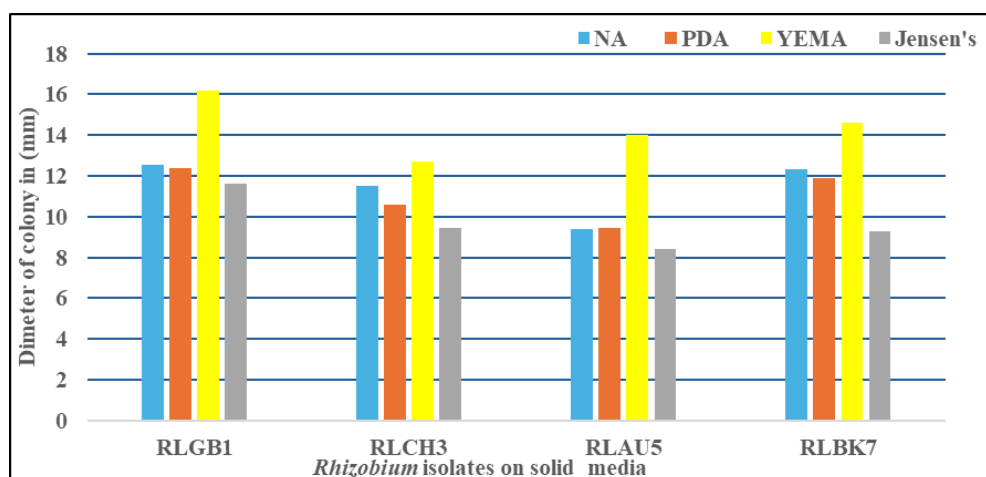


Fig 1: Growth of efficient *Rhizobium* isolates on different solid culture media of colony diameter (mm) of different isolates

Conclusion

Out of eight root nodule samples collected from different locations of Kolhapur district, four *Rhizobium* isolates, namely RLGB₁ (Bhadgoun), RLCH₃ (Halkarni), RLAU₅ (Uttur) and RLBK₇ (Kadgaon), were successfully isolated and purified using CR-YEMA medium. All four isolates were Gram-negative, pink-staining, rod-shaped, motile and gave a positive KOH test. CR-YEMA medium supported the maximum colony growth among the four tested media, and RLGB₁ (13.73 MM) recorded the maximum mean colony diameter. All isolates were positive for catalase, oxidase, starch hydrolysis, methyl red and urease tests, and negative for indole formation, confirming their identity as *Rhizobium* species. Based on their morphological, cultural and biochemical characteristics, isolates RLGB₁ and RLBK₇ showed the most maximum growth and merit further evaluation for their nitrogen-fixing efficiency and plant growth-promoting potential in lentil.

Acknowledgement

The author is thankful to the Department of Plant Pathology and Agricultural Microbiology, Govt. College of Agriculture, Pune, and BNF Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, for providing the necessary laboratory facilities to carry out this study.

References

1. Aykroyd WR, Doughty J, Walker A. Legumes in human nutrition. FAO Food and Nutrition Paper 20. Rome: Food and Agriculture Organization of the United Nations; 1982.
2. Al-Judy NJ, Majeed RE. Morphological, biochemical and molecular characterization of ten *Rhizobium* bacteria isolates. Iraqi Journal of Science. 2013;54(2):280-287.
3. Bantu N, Kumar GP, Devamani M. Isolation and purification of *Rhizobium* from French bean (*Phaseolus vulgaris* L.) root nodules. International Journal of Microbiology and Allied Sciences. 2016;3(1):12-16.

4. Beijerinck MW. Die Bacteroide der Papilionaceen-Knöllchen. Botanische Zeitung. 1888;46:725-735.
5. Berraho EL, Maâtallah J, Muñoz S, Sanjuan J, Lluch C. Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum* L.) growing in Moroccan soils. Agronomie. 2002;22(3):321-329.
6. Bhattacharya S, Chandra S, Gachhui R. Isolation and characterization of Rhizobium sp. from root of legume plant *Pisum sativum* and its antibacterial activity against different bacterial strains. International Journal of Research in Pure and Applied Microbiology. 2013;3(4):108-114.
7. Blazevic DJ, Ederer GM. Principles of biochemical tests in diagnostic microbiology. New York: Wiley and Company; 1975. p. 13-45.
8. Collins CH, Lyne PM. Microbiological methods. 3rd ed. London: Butterworths; 1970.
9. Deka AK, Azad P. Isolation of Rhizobium strains: cultural and biochemical characteristics. Legume Research. 2006;29(3):209-212.
10. Dhull SB, Kinabo J, Uebersax MA. Nutrient profile and effect of processing methods on the composition and functional properties of lentils (*Lens culinaris* Medik): a review. Legume Science. 2022;5(1):156.
11. Eckford M. The starch-digesting power of certain groups of bacteria. American Journal of Hygiene. 1927;7(2):211-220.
12. Fathy DM, Eldomiaty AS, El-Fattah HIA, Mahgoub ESI, Hassanin AA. Morphological, biochemical and molecular characterization of Rhizobia of faba bean plants grown in North Nile Delta Egypt. Pakistan Journal of Biological Sciences. 2021;24(6):672-679.
13. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiology. 1951;26(1):192-195.
14. International Center for Agricultural Research in the Dry Areas (ICARDA). Nitrogen fixation in lentil. Aleppo: ICARDA; 1989.
15. Nagalingam S, Ranganathan S, Arumugam K. Identification and morphological evaluation of indigenous Rhizobium strains from *Cajanus cajan* root nodules. Journal of Plant Nutrition and Soil Science. 2020;44(2):201-208.
16. Nivaas A, Rani P, Kumar S. Colony characterization of rhizobial isolates from leguminous crops. Journal of Microbiological Research. 2019;9(2):45-50.
17. Nushair SA, Al-Thani RF, Mahasneh AM. Isolation, phenotypic characterization, and screening of native root-nodulating bacteria associated with lentil crops. Jordan Journal of Biological Sciences. 2017;10(3):185-192.
18. Panse VG, Sukhatme PV. Statistical methods for agricultural workers. New Delhi: ICAR; 1985.
19. Panwar S, Chaudhary S, Sharma M, Sharma YK, Malhotra S, Mehta R. Morphological and biochemical characterization of Rhizobium isolates from fenugreek (*Trigonella foenum-graecum* L.). National Seed Research Centre. 2012;40(2):196-200.
20. Rasool S, Shawl AS, Ahmad M. Isolation, biochemical characterization of Rhizobium sp. from the root nodules of *Vicia sativa* L. and its growth promotion potential. Journal of Bioprocess Technology. 2015;103:45-52.
21. Satyadev, Prajapati, Dake MS, Surekha S, Godika S, Krishna Prasanna. Isolation and characterization of *Rhizobium meliloti* isolated from rhizosphere soil and roots of fenugreek from different locations. Indian Journal of Current Microbiology and Applied Sciences. 2018;7(6):1460-1467.
22. Siva N, Thavarajah P, Kumar S, Thavarajah D. Variability in prebiotic carbohydrates in different market classes of lentil, common bean, and chickpea. Frontiers in Plant Science. 2017;8:2116.
23. Somasegaran P, Hoben HJ. Handbook for rhizobia. New York: Springer Verlag; 1994.
24. Suslow TV, Schroth MN, Isaka M. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology. 1982;72(7):917-918.
25. Wagner SC. Biological nitrogen fixation. Nature Education Knowledge. 2012;3(10):15.
26. Wani PA, Khan MS. Nickel detoxification and plant growth promotion by multi metal resistant plant growth promoting Rhizobium species. Bulletin of Environmental Contamination and Toxicology. 2013;91(1):117-124.
27. Zafar M, Abbasi MK, Rahim N, Khaliq A, Shaheen A, Jamil M, Shahid M. Influence of plant growth-promoting Rhizobacteria on growth, nodulation and nutrient accumulation of lentil under controlled conditions. Pedosphere. 2012;22(1):60-67.