Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 3162-3168

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.707.369

Primary and Secondary Metabolite Profiling, Unravelling the Antibiotic Susceptibility from Culture - Lysed Symbiotic Colonies- of Diazotroph Bacteria (*Rhizobium leguminosarum*) Isolated from Root Nodules of *Dolichos Lab Lab.*

G.V. Pavan Kumar*, G. Pooja, G.V. Nagaraju and Y. Malyadri

Koringa College of Pharmacy, Korangi-533461, Kakinada, Andhra Pradesh, India

**Corresponding author*

Keywords

Rhizobium leguminosarum sp. LNRLS9, metabolites, Antimicrobial activity,

Article Info

Accepted:
24 June 2018
Available Online:
10 July 2018

A B S T R A C T

This is the first study demonstrating antibiotic potential of symbiotic bacteria and their metabolite profiling from cultured bacterial endosymbionts associated with root nodules of Leguminaceae plant *Dolichos lab lab*. Our study has significant scope and it has never been reported. Indian farming area especially semi arid areas host varieties of Legumes with novel secondary metabolites producing organisms. The natural metabolites extracted from root nodule-derived bacteria pave novel therapeutic remedy against the pathogens like *Bacillus subtilis*, *Escherichia coli* and fungal species *Aspergillus niger* when most of them are emerged as extreme drug resistant superbugs. In recent study the bacteria isolated from the root nodule were cultured by standard microbiology techniques. Most of the bacteria isolated were found to be Symbiotic nitrogen fixation of Gram-negative, motile, *Rhizobium leguminosarum*. The potential isolate LNRLS9 was mass cultured for extraction of primary and secondary metabolites. Preliminary screening for identification of Bacterial metabolites by chemical tests was performed using reagents. Ethyl acetate extract prepared from the lysed culture of the isolate was analyzed for antibiotic activity. The results of this study suggested that secondary metabolites produced by *Rhizobium leguminosarum* sp. (LNRLS9) could be used as a lead to control bacterial pathogens.

Introduction

Bacteria can populate diverse environments, from soil to water, even in the most extreme sites on the earth. These organisms can also be found on and inside other organisms, such as fungi, animals, and plants. The microbiome is a recently developed concept used to define the prokaryotic populations in close relationship with more-complex organisms (1, 2). Bacteria can flourish as endophytes inside plant roots, stems, leaves, and seeds (3) in crops (e.g., wheat, rice, maize, sorghum, and sugarcane), legumes (e.g. clover, common bean, and alfalfa), trees (e.g., *Populus*), grasses (e.g., switchgrass), and model plants (e.g., *Arabidopsis*) (4). Most common endophytic bacterial species have been

described as members of the phyla *Firmicutes* and *Proteobacteria* (5). Legumes are economically valuable plants in agriculture and establish symbiotic relationships with rhizobial bacteria (6). These bacteria contribute to the formation of plant root nodules, colonizing them and fixing atmospheric nitrogen. This phenomenon has been studied intensively (7). Rhizobial bacteria are commonly found in soil but also establish symbiotic relationships with legumes, inhabiting the root nodules, where they fix nitrogen. We hypothesize that given the long-term and intimate nature of legume-rhizobium relationships leads to nitrogen fixation with symbiotic growth and development of rhizobium.

The present communication deals with the isolation, Identification and characterization of the ethyl acetate crude extract for primary and secondary metabolites derived from Rhizobium microbial isolates from the root nodules of *Dolichos lab lab* plant and partial purification of the secondary metabolites and its evaluation for antimicrobial activity against the pathogens like *Bacillus subtilis, Escherichia coli* and fungal species *Aspergillus niger*.

Materials and Methods

Collection of nodulated roots of *Dolichos lab lab*

A total of ten nodulated plants were collected from organic farm garden of Acharya NG Ranga Agricultural University at Tadikonda village, Guntur district. Healthy beans plants were uprooted carefully and those plants possessing healthy nodules were selected and transported to the Department of Microbiology and Biotechnology Laboratory Complex of Koringa college of Pharmaceutical sciences in polythene bags for immediate processing.

Isolation of *Rhizobium* species from root noules

Isolation of *rhizobium* was done using yeast extract mannitol agar (YEMA) as described by Rajendran *et al.,* (8). In this, healthy, unbroken, firm and pink nodules were selected for the isolation. They were washed under tap water to remove adhering mud and soil particles, after which they were treated carefully with 5% hydrogen peroxide for surface sterilization. The nodules were repeatedly washed in sterile water for 3-4mins to get rid of the sterilant and then treated with 70% ethyl alcohol for about one minute and 0.1% $HgCl_2$ for two minutes. They were washed with sterile water (3 successive times) under aseptic conditions and crushed with sterile crucible. A suspension was made of the crushed nodules, plated on YEMA medium containing 1% Congo-red dye and incubated at 28 ± 1^0 C for 24 hours. Growth on YEMA plate was observed after the said incubation period.

Identification of *Rhizobium* species

The following tests were made to confirm identification of the isolated bacteria as *Rhizobium leguminosarum*.

Gram staining

Rhizobia are gram negative rod shaped bacteria. Tests were made to identify Rhizobia on the basis of their staining characteristics.

Staining procedure

The smear was fixed in a drop of normal saline & stained with crystal violet solution for 1 min. It was rinsed with water & the excess water was drained off. Iodine solution was added & allowed to act (iodine) for 1 min.

The iodine solution was drained off &

decolorized with iodinated alcohol (5 min). After washing with water, safranin was added & then rinsed with water. The slide was then observed under the microscope using oil immersion objective (9).

Mass scale production of Rhizobium culture

The effective strain (LNRLS9) was grown in 100ml of King's B media in 250 ml conical flask in orbital shaker at 28^0C, 120 rpm, for 24 hours. The selected medias (King's A, King's B, Nutrient broth and Nutrient broth with glucose) were prepared individually.

Each media was pored into fermenter, then the fermenter sterilized at 121^0C, 15 lbs pressure, for 20 min (10). After cooling the media was inoculated with pregrown inoculum of *Rhizobium leguminosarum* sp (LNRLS9). The fermentor was performed under fixed parameters, temp 28^0C, rpm-120, O_2-40%, pH-7.0 for 96 hours.

The turbidity of the culture was measured by estimating the optical density through spectrophotometer at hourly intervals. The culture was centrifuged at 10,000 rpm (Remi cooling centrifuge CTR 30 India) for 15min to get the cell free filtrate. These culture filtrates were used to study the efficacy against pathogens.

Extraction of crude metabolites from isolated culture with selected organic solvents

Crude metabolites were extracted from the effective growth medium by partitioning the filtrates with organic solvents Petroleum Ether and Ethyl acetate (11).

Isolation and partial purification of bioactive secondary metabolites

The filtrate was subjected for solvent extraction method to recover antibacterial metabolites in pure form (12). Most of the metabolites were expected to be in dissolved form with ethyl acetate fraction and so the Ethyl acetate fraction was further diluted with same solvent for enhancing the extraction of metabolites. Finally the entire volume was subjected to extraction under reduced pressure using rotary evaporator (Buchi R-210 Switzerland). The ethyl acetate phase contains antibiotic substances separated from aqueous phase. It was evaporated to dryness in water bath and was further dehydrated with Na_2SO_4. The residue obtained was used to identify the primary and secondary metabolites as well as the antibiotic activity.

Experimental investigations

Qualitative determination of primary metabolites

Primary metabolites directly involved in growth and development while secondary metabolites are not involved directly and they have been worked as biocatalysts. Primary metabolites are of prime importance and essentially required for growth of mushrooms. Many primary metabolites lie in their impact as precursors or pharmacologically active metabolites of pharmaceutical compounds such as antipsychotic drugs (13).

Chemical tests for determination of primary metabolites

Bio active Primary Metabolites are necessary for the actual growth or life of the organism. They

Will accumulate during growth of the organism and generally speaking do not degrade easily. Some of these metabolites are biologically active (14). Secondary metabolites are not involved directly and they have been worked as biocatalysts which are synthesized during secondary.

Metabolism and are potential source of drugs.

Test for soluble carbohydrates

Fehling's test

Five ml of Fehling's solution A and Fehling solution B was added to 0.5 mg of extract and boiled in a water bath. The formation of yellow or red precipitate indicates the presence of reducing sugars.

Test for protein & amino acids

Biuret test

To 0.5 mg of extract equal volume of 40% NaOH solution and two drops of 1% copper sulphate solution was added. The appearance of violet color indicates the presence of protein.

Ninhydrin test

About 0.5 mg of extract was taken and 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of pink or purple colour indicates the presence of proteins, peptides or amino acids.

Thin layer chromatography of crude extract containing metabolites

Thin layer chromatography (TLC) was carried out with the crude extract on silica gel (TLC silica gel. 60, 20 x 20, 0.5 mm, Merck and Co, Inc) with benzene: acetic acid : methanol (4:3:3) solvent system. The crude extract (30 µl) was spotted, and the solvent front was allowed to run for approximately 16 cm. The running lane was then dried thoroughly. After cut into portions (1 by 2.5 cm), these portions were scraped into micro centrifuge tubes and extracted with 100% acetone. The silica residue was removed by centrifugation and the supernatant was transferred to a second set of micro centrifuge tubes (15). The individual metabolites were again spotted on TLC plates for confirmation of metabolites.

Qualitative determination of secondary metabolites

Secondary metabolites are important mediators of ecological interactions between bacterial colonies and their environment.

HPLC-DAD analysis of the Ethyl Acetate extract

The obtained Ethyl Acetate extract was subjected to HPLC-DA detector screening.The chromatographic system consisted of an HP 1090M liquid chromatography equipped with a diode-array detector and HP Kayak XM 600 Chem Station (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230,260, 280, 310, 360, 435 and 500 nm. The UV visible spectrum was measured from 200 to 600nm. Sample (5 µl) was injected onto an HPLC column (125 X 4.6 mm, guard column 20 4.6 mm) filled with Nucleosil-100 C-18 (5 m). Separation was performed by a linear gradient using 0.1% orthophosphoric acid as solvent A and acetonitrile as solvent B. The gradient was from 0 to 100% solvent B in 15 min at a flow rate of 2 ml/min.The results were recorded.

Screening of *Rhizobium* strain for antibacterial and antifungal activity

Primary screening

A modified cross- streak method was used for antimicrobial activity. Single streak of *Rhizobium* strain was made on surface of the modified nutrient agar and incubated at 28°C. (16) After observing a good ribbon like growth of the *Rhizobium* on the plates, the overnight pathogenic bacterial strains, such as *Escherichia coli, Bacillus subtilis,* were

streaked at right angles to the original streak of *Rhizobium* and incubated at 28°C and the incubation distance was measured after 24-48 hrs. A control plate was also maintained without inoculating the *Rhizobium*, to assess the normal growth of bacteria.

Secondary screening

Determination of antimicrobial activity

The antimicrobial activity was determined by agar well method (17) purified extract obtained by the evaporation of the ethyl acetate extract. Then 50, 100µl of it were loaded into well bored and test organism (0.5 McFarland turbidity standards) was swabbed on Muller Hinton agar plates. A control bore and standard bore was also loaded in the Petri plate. Erythromycin 30mcg was loaded as positive control. The plates were incubated at 37°C for 18-24 hours and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter by using antibiotic zone scale (Himedia, Mumbai, India). The formation of inhibition zone around the pathogenic strains is due to the production of secondary metabolites by Rhizobium isolates.

Results and Discussion

Different chemical constituents have been found to possess wide range of medicinal properties, which may help in protection against various diseases. The quantitative estimation of primary and secondary metabolites reveals various chemical constituents present in the isolated extract. Secondary metabolites analysis is necessary for extraction, purification, separation, crystallization, identification of various compounds.

10 healthy root nodules of different sizes were isolated from the farm Garden of Acharya N G Ranga Agricultural University, Guntur. Isolation of Rhizobium species has always been faced with difficulties in comparison to their competitors like other bacteria and fungi from the soil adhered to the root nodules (18). This may be due to their long incubation period. Use of selective media was crucial for culturing the micro organisms. The cultural characterization of the *Rhizobium* isolates were Studied by using the selected Medias (King's A, King's B, Nutrient broth and Nutrient broth with glucose). Here the growth of *Rhizobium* is found to be abundant.

All the isolates were found to be *Rhizobium* and they produced highest biomass, when glucose was supplemented in the medium. Addition of other carbon sources such as starch, fructose, maltose, Mannitol, etc. to the medium also favoured the growth but the growth was less when compared with glucose. Both primary and secondary screening methods were used to screen *Rhizobium* for antimicrobial activity.The first screening was used to select the antimicrobial isolates and determine that *Rhizobium* was sensitive to the antibiotic. The secondary screening method was crucial to select the isolates for further studies.

The result of the screening revealed that the isolate was against bacterial culture as they showed broad spectrum activity with big zone of inhibition. Therefore the isolate were chosen for fermentation. The antibacterial metabolites from fermented broth were extracted in organic solvent (ethyl acetate) by solvent extraction method.

The crude extract were further analysed by thin layer chromatography on silica plates using with benzene: acetic acid: methanol (4:3:3) in the said ratios as solvent system. The extract produced two spots when the chromatogram was visualized under iodine vapour with R_f value of 1.2 for *compound* 1 and 0.53 for *Compound 2.*

Table.1 Primary metabolites in Ethyl acetate extract

S.no	Primary Metabolite	Test	Result
1.	Carbohydrates	Fehlings	++++
2	Proteins	Biurett	++++
3	Amino acids	Ninhydrin	++++

++++ Result Indicates presence of primary metabolites in extract

Table.2 Secondary metabolites in Ethyl acetate extract

S.no	Secondary Metabolite	Rt value in min
1.	N-acetyl phenyl alanine	6.7
2	3-methyl indole	7.0
3	amicoumacin antibiotic	7.3
4	3-methyl- indole	8.4
5	Indole compounds	8.9

Table.3 Antimicrobial activity of secondary metabolites in Ethyl acetate extract

Test organism	**Inhibition Zone (mm)***	**MIC µg/ml**	**Standard µg/ml**
Bacillus subtilis	16±0.4	2.5	2.33
Escherichia coli	24±0.6	1.5	2.33
Aspergillus niger	18±0.3	0.5	2.33

*Values are mean±SD of three replications

Chemical analysis is of paramount importance in identifying new source of therapeutically and industrially valuable compounds have been chemically investigated. In the present investigation primary and secondary metabolites were identified and analyzed using standard chemical test procedure. Qualitative analysis of primary metabolites were reported in Table 1, as proteins, amino acid and then carbohydrates.

HPLC-DAD analysis of the Ethyl acetate extract of *Rhizobium* sp. is shown in Figure 1. The peaks of the chromatogram were matched with the reference compound available in the database by UV-Visible spectrum. The peaks in the chromatogram having the same UV-Visible spectrum and retention time with that of the reference compound was identified and named. In the UV-Visible spectra various peaks observed corresponded to different compounds (Fig. 2). Peak at 6.7 min (1) represents N-Acetyl-phenylalanine, 7.0 min (2) 3-methyl-indole, 7.3 min (3) amicoumacin antibiotic, 8.4 min (4) 3-methylindole and Peak at 8.9 (5) corresponds to indole type compound (Table 2 and 3).

It is concluded that the present study was an attempt to identify and pick out versatile *Rhizobium* strains that display antimicrobial activity against a variety of microbial pathogens intrinsically. Our result indicates that we are able to identify the antagonistic activity of microbial growth. Further investigation is needed in order to determine the structure of active compounds and to scale up the production. The results are suggestive of primary and secondary bioactive compounds are commercially and pharmaceutically important.

Acknowledgements

The authors thank Management Koringa college of pharmacy and laboratory Techncians for their support to carry out the research work.

References

1. Aserse AA, Rasanen LA, Assef F, Hailemariam A, Lindstrom K. 2012. Phylogeny and genetic diversity of native rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia. Syst. Appl. Microbiol. 35:120–131.
2. Pflughoeft KJ, Versalovic J. 2012. Human microbiome in health and disease. Annu. Rev. Pathol. 7:99–122
3. Hardoim PR, van Overbeek LS, van Elsas JD. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16:463-471.
4. Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2012. Changes in the population of seed bacteria of transgenerationally Cd-exposed *Arabidopsis thaliana*. Plant Biol. (Stuttg.) 15:971–981
5. Mundt JO, Hinkle NF. 1976. Bacteria within ovules and seeds. Appl. Environ. Microbiol. 32:694–698.
6. Herridge DF, Peoples MB, Boddey RM. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18.
7. Guan SH, Gris C, Cruveiller S, Pouzet C, Tasse L, Leru A, Maillard A, Médigue C, Batut J, Masson-Boivin C, Capela D. 2013. Experimental evolution of nodule intracellular infection in legume symbionts. ISME J. 7:1367–1377.
8. G. Rajendran, F. Singh, A. J. Desai, and G. Archana, "Enhanced growth and nodulation of pigeon pea by co-inoculation of Bacillus strains along with Rhizobium sp.," Bioresource Technology, vol. 99, pp. 4544–4550, 2008.
9. *Rhizobium* identification was based on the Bergey's manual of determinative bacteriology, Vincent 1970.
10. Wilson, P.W. (1940). The biochemistry of symbiotic nitrogen fixation. University of Wisconsin press, Madison, Wisconsin.
11. Kokate C.K, Purohit A.P and Gokhale S.B., In: Pharmacognosy, 3rd edition. Niralin Prakashan, Pune. 1995.
12. Jo Handlesman A rapid method for isolation & identification of Rhizobium from root nodules. Journal of microbiological methods 9(1989) 29-33.
13. Jayaraman J. Laboratory Manual in Biochemistry. New Delhi: Wiley Eastern Limited, 1981.
14. Horbone, J.B., In: Phytochemical methods, 2nd edition. Chapman and Hall, NewYork,1984.
15. Rajan BM and Kannabiran K Extraction and Identification of Antibacterial Secondary Metabolites from Marine *Streptomyces* sp. VITBRK2 Int J Mol Cell Med Summer 2014; Vol 3 issue No 3, 131.
16. Balagurunathan R. (1992) Antagonistic Actinomycetes from Indian shallow sea sediments with reference to a alpha, beta unsaturated gamma lactose type of antibiotic from *Streptomyces griseobrunneus* Doctoral thesis, Annamalai Univ.India.
17. Perez M P& Bazerque P, antibiotic assay agar well diffusion method, Acta Biol Med Exp, 15 (1990)113.
18. Brooks G.H., Butel J.S. and Morse S.A. (2001) Antimicrobial chemotherapy. In: Medical microbiology (22nd edition) (eds.Jawetz, Melnick&Adelberg). International edition. Lange Medical books /McGraw Hill Publication.

How to cite this article:

Pavan Kumar, G.V., G. Pooja, G.V. Nagaraju and Malyadri, Y. 2018. Primary and Secondary Metabolite Profiling, Unravelling the Antibiotic Susceptibility from Culture - Lysed Symbiotic Colonies- of Diazotroph Bacteria (*Rhizobium leguminosarum*) Isolated from Root Nodules of *Dolichos Lab Lab.*. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 3162-3168.
doi: https://doi.org/10.20546/ijcmas.2018.707.369