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ISOLATION, BIOCHEMICAL CHARACTERIZATION AND METABOLIC FINGERPRINTING OF RHIZOBIUM FROM ROOT NODULES OF *CLITORIATERNATEA*

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ABSTRACT: One of the unique ability of leguminous plants is to establish symbiosis interaction with nitrogen fixing bacteria. The symbiotic association of such leguminous plants with rhizobia plays a major role in the agriculture crop production. The prime objective of this research paper was to isolate bacteria (Rhizobium) from root nodules of *Clitoriaternatea.* Therhizobial strains were further subjected to morphological, biochemical and metabolic characterization. Pure culture of rhizobial strains was isolated on selective YEMA medium. Antibiotic resistant pattern of all isolates was also evaluated against two antibiotics. In contrast, based on carbon utilization pattern a remarkable metabolic diversity was observed among therhizobial isolates recovered in the studies.

Key words: Root nodules, *Rhizobium*, *Clitoria*, Carbon source.

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INTRODUCTION

Plants have been a major source of medicine .Medicinal plants plays a crucial role in the lives of rural people, in remote parts of developing countries with limited facilities for health care. Medicinal plants have curative properties due to complex chemical substances of different composition, found as secondary plant metabolites in one or more parts of these plants. The plant metabolites are grouped as alkaloids, tannin, flavanoid, glycosides, corticosteroids, and essential oils etc. The importance of medicinal plants has been overlooked in the past, however, at present medicinal plants are looked not only as a source of affordable health care but also as a source of income. Many extracts from medicinal plant have been known to possess antimicrobial effects and used for the purpose of food preservation and medicinal purposes. Extraction and characterization of several active phyto-compounds from these green factories have given birth to some high activity profile drugs .The scope of genomics on genetic improvement of plants having medicinal value.

The Fabaceae (Papilionaceae) is the third largest land plant family, includes most of the important crop species and large number of legumes plant. Legumes are herbaceous woody plants that produce seeds in pods; for examples *Clitoria*, peas, beans, alfalfa etc. In legumes and few other plants roots, the bacteria live in small outgrowths on the roots called nodules. Within these nodules bacteria do nitrogen fixation and the plant absorbs the ammonia. Nodulation is also one of the best known models of symbiotic association. Leguminous plants in association with Rhizobium can fix significant amount of atmospheric nitrogen from air Rhizobium-legume symbiosis can increase yield with subsequent decrease in pollution (Hasan et al., 2015) leguminous plants proved to be useful tool for improving soil fertility through regenerative means.

The plant focus in our studies *Clitoriaternatea*, is one such legume plant that is yet to be characterized for its nodule flora.

Clitoriaternatea is an attractive perennial climber with conspicuous blue or white flowers. It belongs to the family Fabaceae and commonly known as "butterfly pea" and "shankhapuspi". (Manjula *et al.*, 2013) "Shankhapushpi" is an important drug of indigenous system of medicine. According to Ayurveda, Shankhpushpi is bitter, pungent, alexiteric, alternative, tonic, useful in bronchitis, epilepsy, leucoderma and teething troubles of infants etc. (Gaine *et al*., 2015) It is grown as an ornamental plant and as a revegetation species requiring little care when cultivated. Its roots fix nitrogen and therefore this plant is also used to improve soil quality (BhushanPahune *et al.*,2013) The plant is very useful as it has several therapeutic activities like antistress, anxiolytic, antidepressant, anticonvulsants, tranquilizing and sedative agent (ManjuLata Zingare *et al*.2008). In Southeast Asia the flowers are used to colour foods. In animal tests the methanolic extract of *clitoriaternatea* roots found to possess antidepressant, anticonvulsant and antistress activities (Shamsi *et al.,* 2014).

Legume plant posses a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. Bacteria of family Rhizobiaceae are symbiotic and effectively convert atmospheric nitrogen which is utilized by the host. Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil. This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (Gauri *et al*., 2011).This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops(Gauri *et al*., 2011).The rhizobia are a group of Gram-negative bacteria that form species-specific symbioses with legume plant (Bhatt *et al*., 2013).These bacteria infect the root nodules of leguminous plants, leading to the formation of lumps and nodules where the nitrogen fixation is takes place. The bacterium's enzyme system supplied a constant source reduce nitrogen to the host plant and plant furnishes nutrients and energy for the activities of the bacterium (Shetta *et al*., 2011). It provides a significant proportion of available nitrogen in the biosphere.

MATERIAL AND METHODS

Material used- *Clitoria* leaves, stem, and roots.

Collection of sample

Leguminous plant *Clitoriaternatea* (shankhpushpi) were selected for collection of root nodules from college garden.

(a)

(b)

Figure 1: Medicinal plant (a) Plant of *Clitoriaternatea* (b) Root nodules of*Clitoriaternatea*

Isolation of root nodulating bacteria rhizobium

Root nodules of *Clitoriaternatea* was used to isolate root nodulating bacterial strain .Plants were uprooted carefully and nodules were collected from the roots, washed thoroughly with sterile water and Surface treatment of nodules done with 95% alcohol for 30 sec. after that washed nodule immerged in 0.2% mercuric chloride for 2-3 minutes and immediately washed 5-6 times with sterile water as to remove traces of mercuricchloride. The nodules were transferred in sterile tube containing 0.5 ml water and crushed with a glass rod and milky bacterial suspension was obtained. Suspension was streaked on congo red yeast extract mannitol (CRYEMA) agar plate was incubated for 24hrs at 28^{o}c. After 24 hrs incubation colony shape, size, colour was noted.

Gram staining –

Gram-staining reaction was carried out by using a loopful of pure culture grown on YEM media (yeast extract mannitol agar media) and stained as per the standard gram's procedure. The isolates were classified as fast (medium turn yellow) and slow growers (medium turn blue) based on their reaction on the yeast extract mannitol agar supplemented with bromothymolblue.

Biochemical characterization of rhizobium

Thefollowing biochemical tests were carried out in growth medium at 28°C for 24 hours incubation.

Catalase activity-

Bacterial culture were flooded with hydrogen peroxide and observed for liberation of effervescence of oxygen around the bacterial colonies.

Oxidase test-

Bacterial culture flooded with kovac's reagent positive colonies turned lavender colour which became dark purple to black in 10 min.

Acid from glucose-

Mannitol in the YEM agar was replaced by equal amount of glucose and bromothymolblue was added to it, this media was inoculated with the bacterial strains and incubated. Change in color around the colonies was observed.

Starch hydrolysis test-

Starch agar medium was inoculated with rhizobium strain to analyze for starch utilization. Drops of iodine solution were spread on 24 hrs old cultures grown in petriplates. Formation of clear zone indicates utilization of starch.

Growth on glucose peptoneagar-

Glucose Peptone Agar (GPA) plates were streaked with rhizobium strains and incubated. Growth was observed after 48 hours.

Antibiotic resistance test-

The isolates were tested for antibiotic sensitivity by Kirby-Bauer disc diffusion method on YEM agar Cultures were inoculated by swabbing with bacterial suspension over the entire agar surface. The agar surface was allowed to dry for 3-5 minutes before applying the antibiotic discs. Chloramphenicol and tetracycline antibiotic discs were placed in Petriplate using sterile forcep. The plates were incubated at 28 °C for 24hrs. Resistance to an antibiotic was detected by the inhibition zone formed around the discs.

Metabolic fingerprinting of isolated bacteria based source utilization

Carbon source (C-source) utilization test was performed inscrew caps containing specific carbohydrates (Amarger *et al*. 1997) using M9 minimal medium. Five C- sources (maltose, sucrose, lactose, xylose and fructose) were used in the concentrations of 10, 20, 30, and 40% for each isolates. Culture was inoculated in the M9 medium (2 ml) containing 25 µg/ml bromothymol blue dye (pH 7.0) and incubated at 28°C with 150 rpm for 3-4 days. A control was maintained without sugar. The change in colour from green to blue or bluish green (alkali production) or yellow (acid production) indicated the utilization of the respective C-sources and no change in colour of the broth was taken as non- utilization.

RESULT AND DISCUSSION

Strains of root nodulating bacteria were isolated from the root nodules of leguminous plant i.e. *Clitoriaternatea* (shankhpushpi) from college garden. Nodules were agitated in a dilute sodium hypochlorite solution eliminated the need for a wetting agent and effectively sterilized nodules with highly convoluted surfaces such as those formed by *Rhizobium sp*. Surface contamination was checked by sterilizing 96 nodules in hypochlorite as described. Colonies of *Rhizobium* showed growth on YEM agar medium containing congo red after incubation at 28°C for two days. 5 bacterial colonies were obtained from root nodules. All isolates are white, mucilagenous, translucent, circular in shape, shiny, raised, entire and large or small colonies. (singh *et al*.2011,alemayehu 2009). All the strains showed growth in 48-72 hrs. and turned yeast extract mannitol broth containing bromothymol blue to yellow showing that fast growers and acid producers similarly.

The rhizobial isolates tested on YEMA plates containing bromothymol blue which indicated that fast-growing isolates (18) were found to produce yellow colonies due to acid production on the medium with high mucous after 2 days of incubation (shahzad *et al*. 2012, Sharma *et al*.2010). Microscopic examination revealed that the isolates were rod shaped and gram negative in nature (Keyser1982). All isolates were oxidase, catalase positive by the liberation of effervescence of oxygen around bacterial colonies and colour change respectively which complements the finding of lupwayi and hague (1994). Isolates showed a negative growth on glucose peptone agar (bhatt *et al.*). Positive results were obtained from the starch hydrolysis assay by applying iodine to inoculated plates, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas no growth areas appeared blue in colour. This implied that the isolates had the potential to hydrolyze starch present in the medium. De oliveria *et al*. (2007) also observed that *Rhizobium* strains obtained from different sources can utilize starch. Resistance patterns were observed of the *Rhizobium* strains to chloramphenicol and tetracycline antibiotics by the technique of antibiotic disc diffusion method. Occurrence of higher resistance to antibiotics like chloramphenicol and tetracycline was reported by Kahlon (1980).Similarly Kucuk and Kivnac, (2008) observed higher resistance of rhizobia against streptomycin, chloramphenicol and penicillin. This was further supported by occurrence of distinct rhizobia in tropical soils of India (Appunu *et al*. 2008). Thus, remarkable differences among the rhizobial isolates observed in the current study based on C-sources utilization may have ecological significance for their exploitation in future inoculation programmes.

Table 1: Morphological characters of root nodulating bacteria

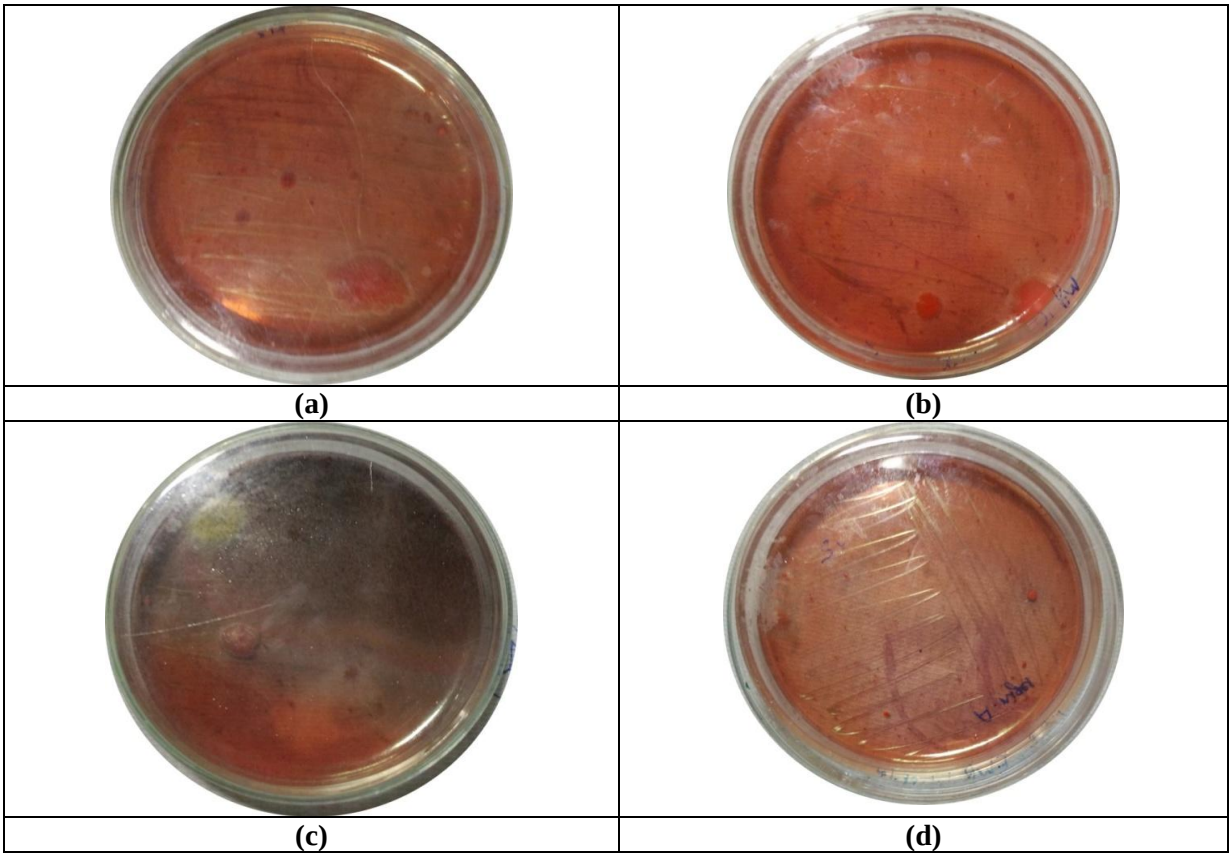
Characters	Result
Shape	Circular ,punctiform
Size	0.2- 0.8 cm
Margin	entire
Elevation	Convex, raised
Opacity	translucent
Colour	White, offwhite
Gram's nature	Gram -ve
Bacteria shape	Rod shape

Table 2: Biochemical test for *Rhizobium*

Culture	Antibiotic	Zone of inhibition
R_1L	Chloremphenicol	24.39
R_2L	Tetracycline	6.84
R_2S	Tetracycline	17.64
R_3S	Chloremphenicol	7.26

Table 3: Antibiotic sensitivity test

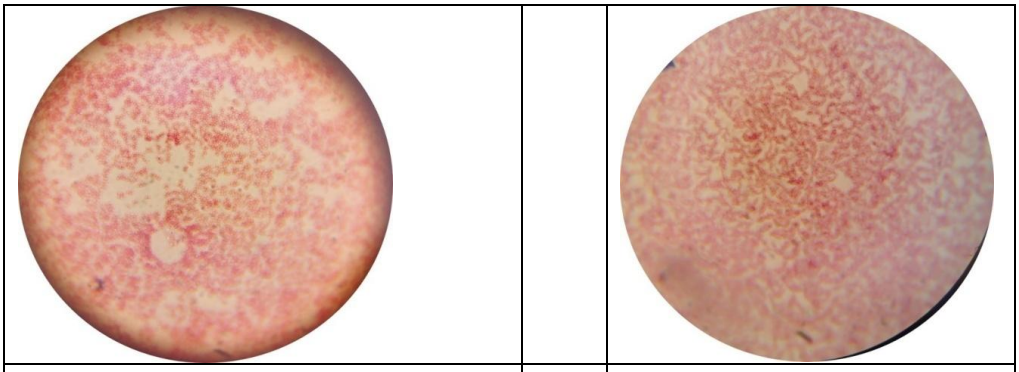
Strains	Catalase	Oxidase	Characterstics		
			Starch hydrolysis	Growth on GPA	Acid from glucose
R1S	+	-	+	-	+
R1L	+	-	+	-	+
R2S	+	-	+	-	+
R2L	+	-	+	-	+
R3S	+	-	+	-	+



(a) **(b)**

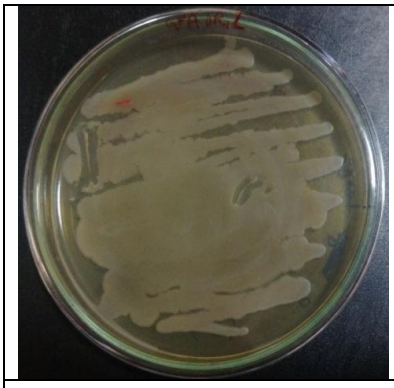
(c) **(d)**

Figure 2: Isolation (a, b, c, d) root nodulating bacteria



(a) **(b)**

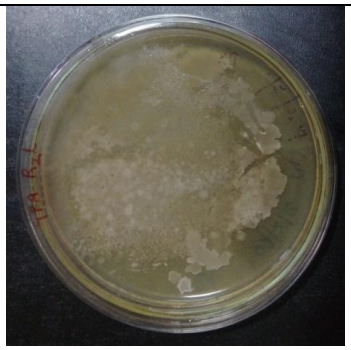
Figure-3: Gram staining (a) (b)



(a)

(b)

(c)



(d)

Figure 4: Pure culture of *Rhizobium* (a) R_1L (b)R_2S (c)R_3S (d)R_2L

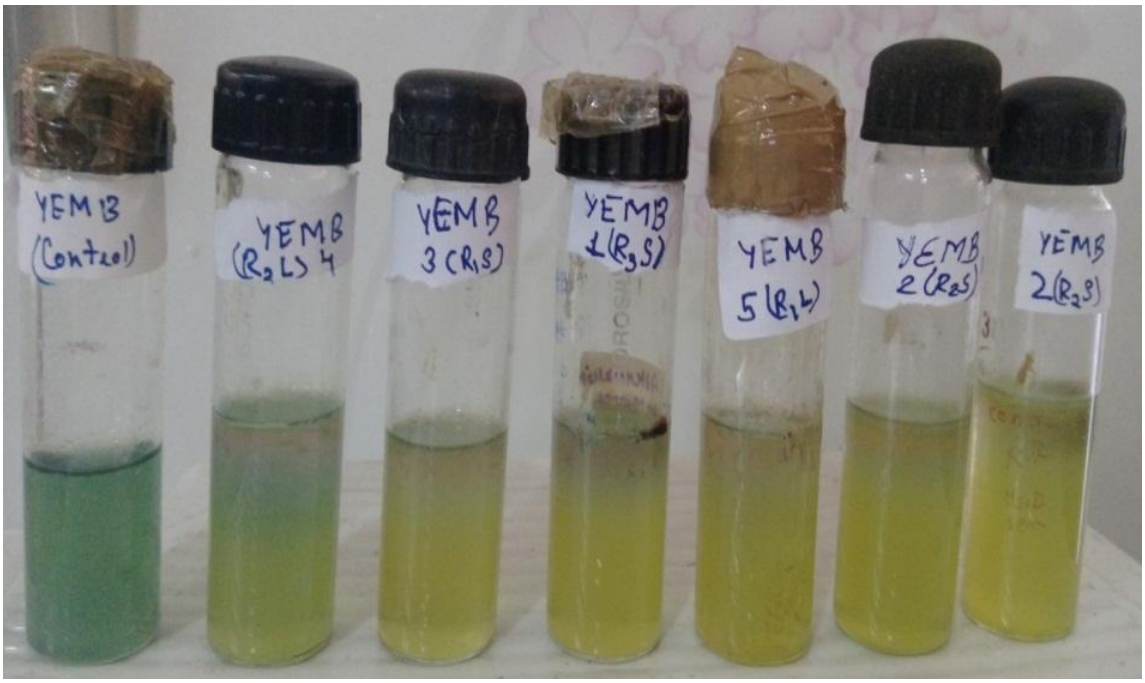
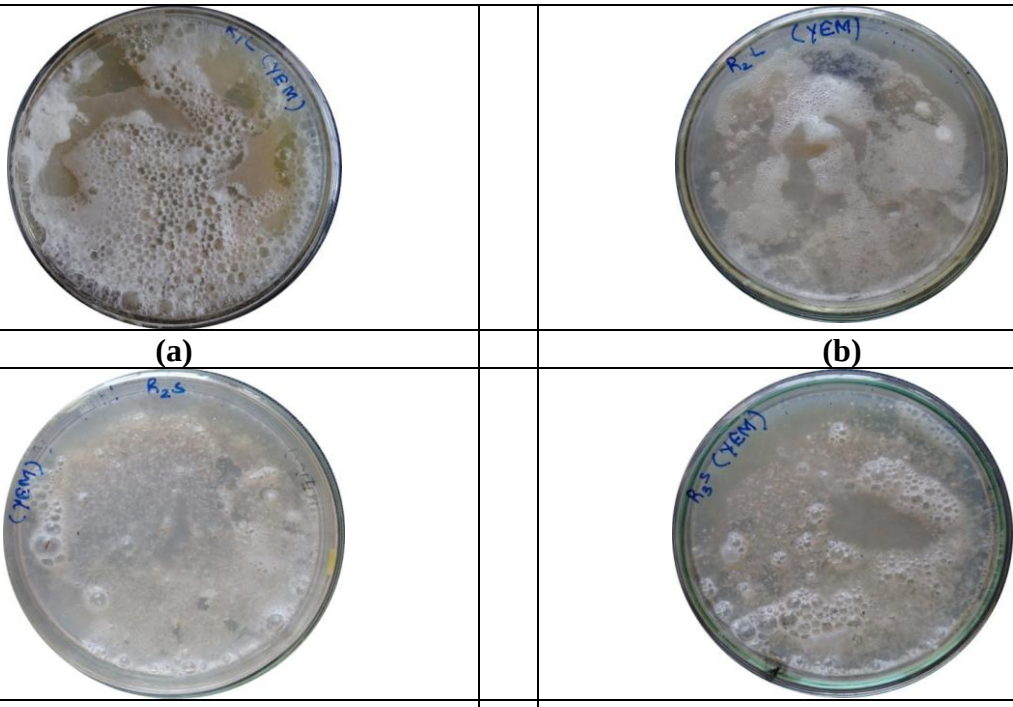


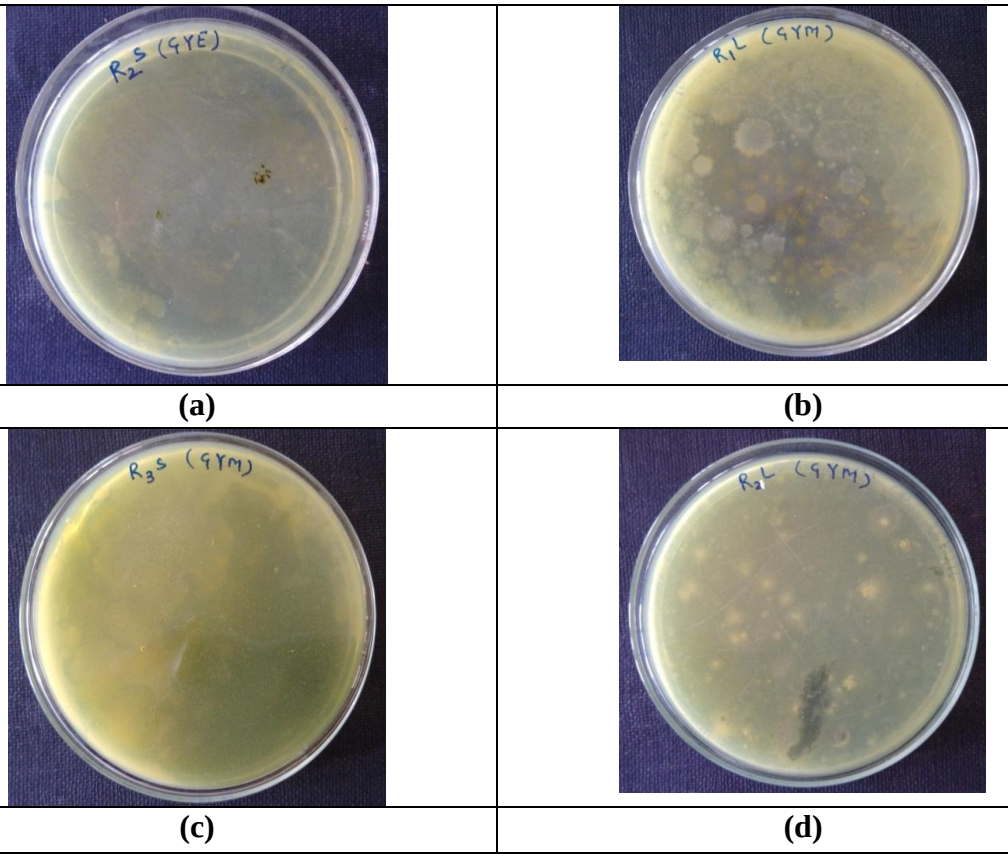
Figure 5: Biochemical test for fast and slow growers *Rhizobium*



(a) (b)

(c) (d)

Figure 6: Catalase test (a) R_1L (b) R_2L (c) R_2S (d) R_3S



(a) (b)

(c) (d)

Figure 7: Acid from glucose (a)R2S (b)R1L (c)R1S (d)R2L (e)R3S

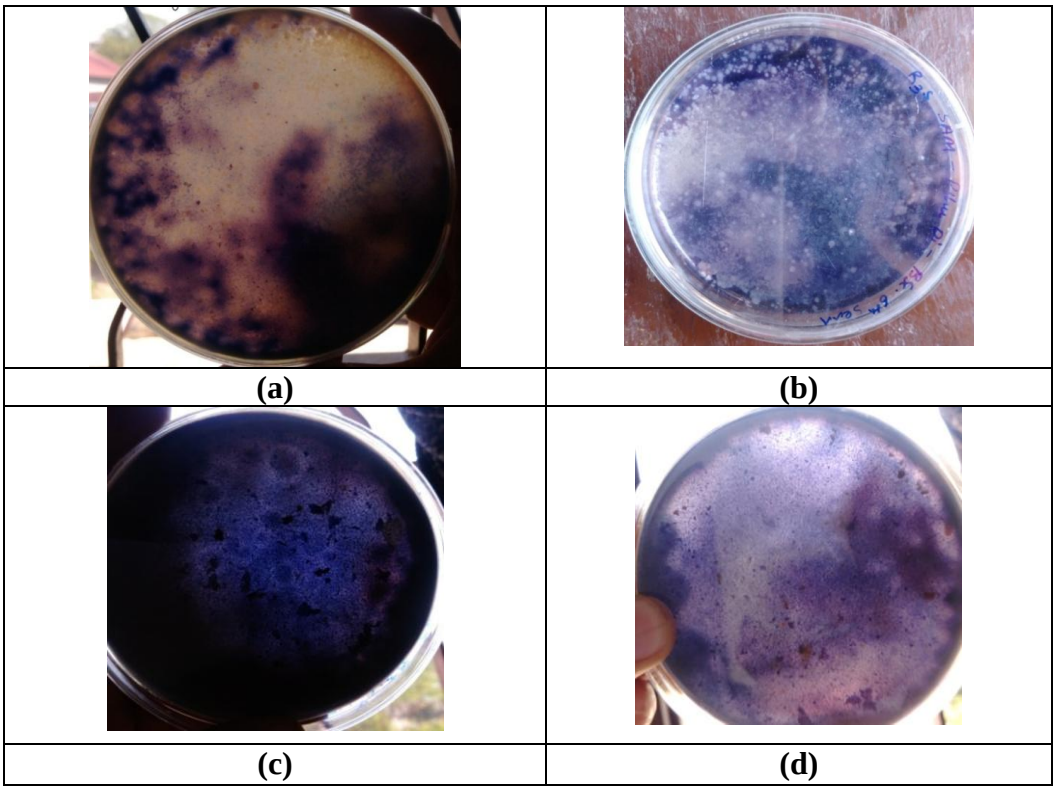


Figure-8: Starch hydrolysis test (a) R2L (b)R3S (c)R1L(d)R2S

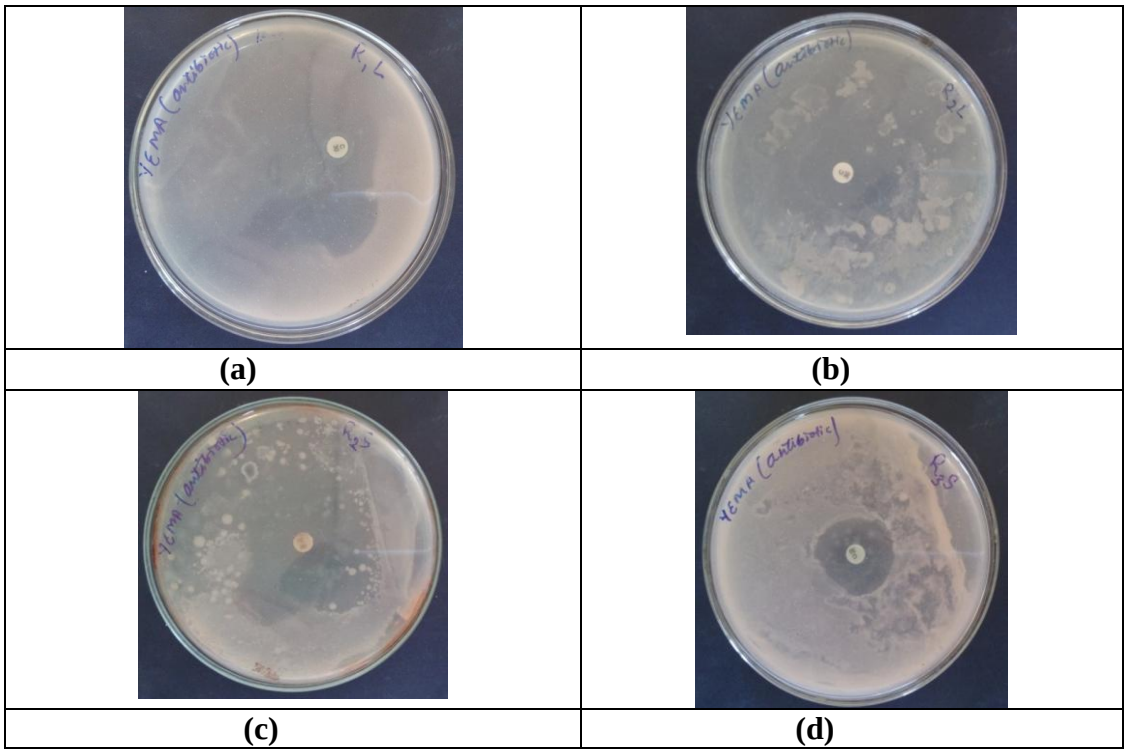
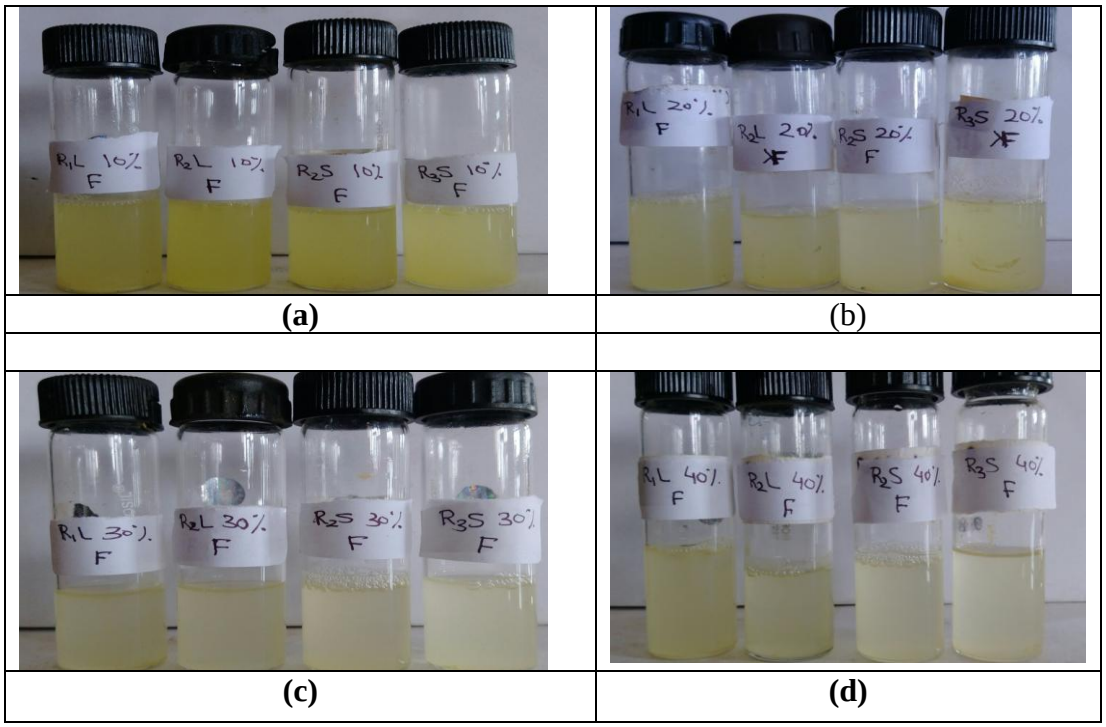


Figure 9: Antibiotic sensitivity test



(a) (b)

(c) **(d)**

Figure 10: Metabolic fingerprinting of *Rhizobium* based on carbon source (fructose) utilization (a)10% (b)20%(c)30%(d)40%

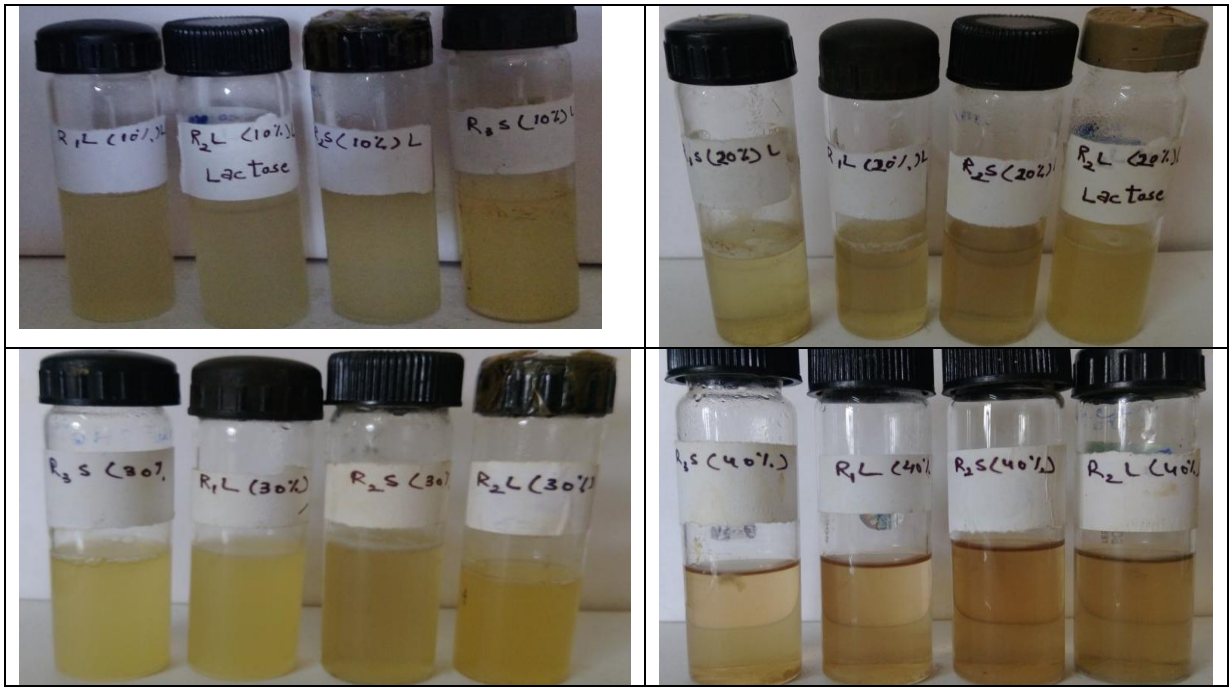


Figure 11: Metabolic fingerprinting of *Rhizobium* based on carbon source (lactose) utilization (a)10% (b)20%(c)30%(d)40%

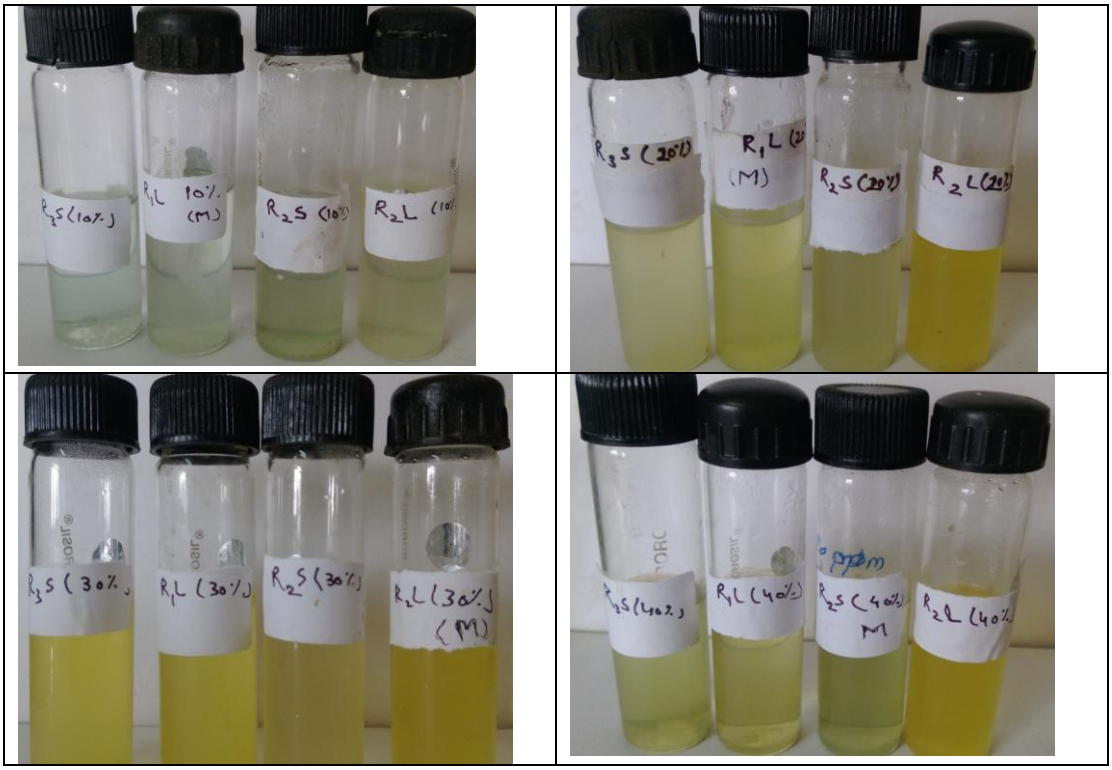


Figure 12: Metabolic fingerprinting of *Rhizobium* based on carbon source (maltose) utilization (a)10% (b)20%(c)30%(d)40%

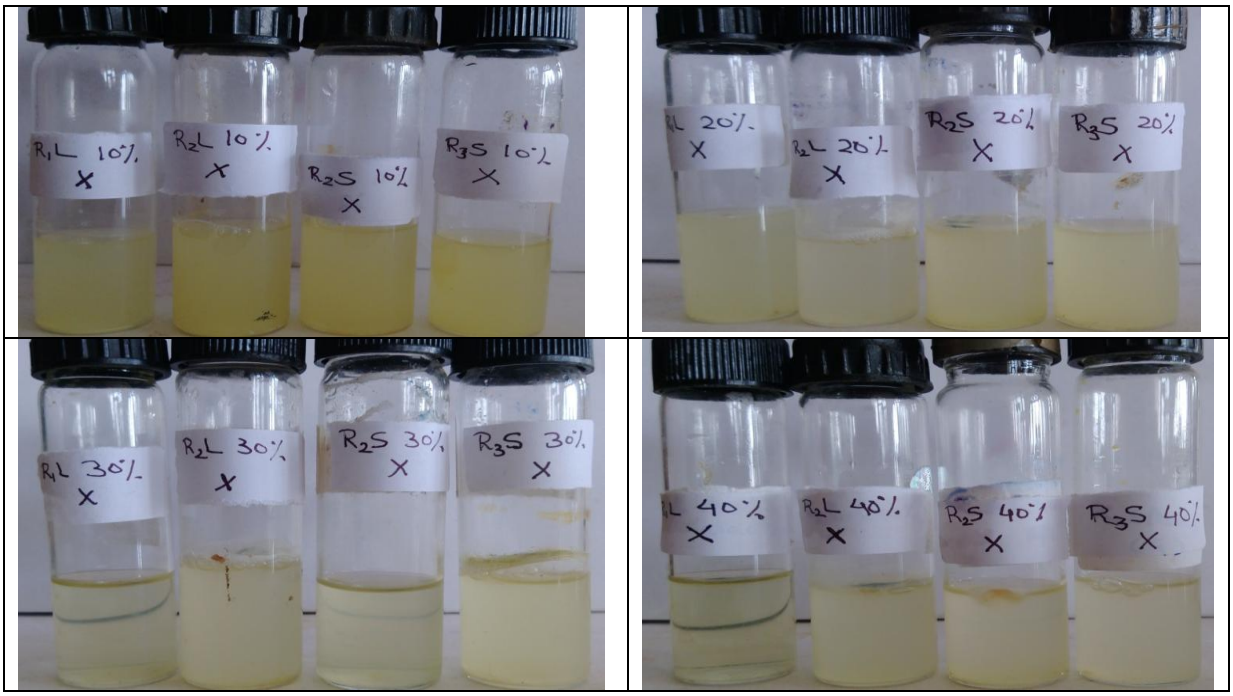


Figure 13: Metabolic fingerprinting of *Rhizobium* based on carbon source (xylose) utilization (a)10% (b)20%(c)30%(d)40%

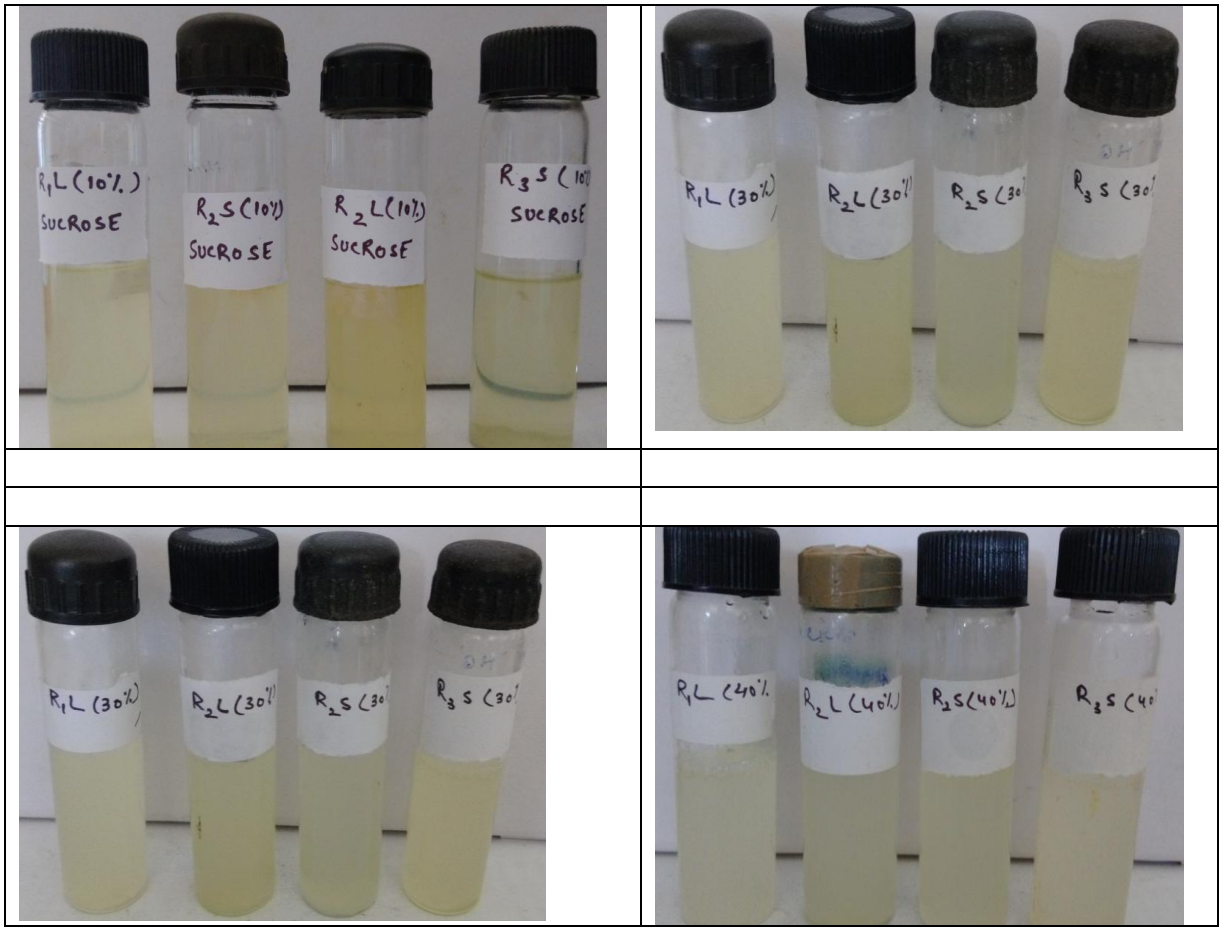


Figure 14: Metabolic fingerprinting of *Rhizobium* based on carbon source (sucrose) utilization (a)10% (b)20%(c)30%(d)40%

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