Wolkite University

College of Natural and Computational Science

Department of Biotechnology

Senior project on: Isolation, Morphological and biochemical Characterization of local Rhizobium from Root Nodules of Faba Bean, from Guraghe Zone

Senior Project research Submitted to Wolkite University College of Natural and Computational Science Department of Biotechnology for Partial Fulfillment of Bachelor of Science Degree in Biotechnology.

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APPROVAL SHEET

THIS RESEARCH PAPER ENTITLED WITH"ISOLATION, MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF LOCAL RHIZOBIUM FROMROOT NODULES OF FABA BEAN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF SCIENCE IN BIOTECHNOLOGY.

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Table of Contents

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List of table

List of figure

List of Abbreviations

BNF=Biological nitrogen fixation

N_2=Atmospheric nitrogen

Nacl= Sodium chloride

YMA=Yeast mannitol agar

YEMA= Yeast Extract Mannitol Agar

Sp=Species

H_2o_2=Hydrogen peroxide

H_2O=Water

Mg $(So_4)_2$= Magnesiumsulphate

KNO_3= Potassium Nitrate

K_2HP0_4= Dipotassium phosphate

$CaC0_3$= Calcium carbonate
NH_3= Ammonia

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Abstract

Symbiotic systems for biological nitrogen fixation (BNF) in agriculture are most promising. Accordingly, the present study will be conducted for the isolation, identification, and characterization of nitrogen-fixing bacterial strains Rhizobium from the legume plant bean which grows widely in almost districts area (Mola kalad, Buchu, Albazer and Koro) of Butajra Guraghe Zone. Nitrogen fixation is the reduction of N2 (atmospheric nitrogen) to NH_3 *(ammonia) which is made possible by the enzyme nitrogenase. A study was undertaken to investigate the occurrence of rhizobium from the root of beans. Strain of root nodulating bacteria was isolated from a root nodule of faba bean. The isolate were grown on yeast extract Mannitol agar medium and the morphological characterization was realized by gram stain. A total of four isolate were isolated on YEMA medium. The isolates were found circular, colony shape, rod shaped, whitish, entire colony margins and moist colony texture. Gram staining and caseinase test showed negative while carbohydrate utilization tests and catalase test registered positive. All four isolates were showed the characteristics of rhizobia. We recommend that the isolates re- identify, strain type confirmed through molecular characterization techniques and their efficiency should check.*

Key words; Rhizobium strain, carbohydrate utilization, fave bean

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Legumes are agronomically and ecologically important symbionts that lead to the development of new plant organ (legume nodule) in response to nitrogen fixing bacteria (Datta*et al.*, 2015). The present study reports the isolation of *Rhizobial*isolates from soybean plants and their characterization on the basis of morphological and biochemical characters. The importance of this information is that it will lead to the documentation of indigenous strains and as foundation for further study to determine whether the strains are high nitrogen fixers. Nutrient improvement of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries (Zsbrau, 1999). Large quantities of rhizobia in the soilhave been shown to be able to facilitate cultivation of legumes as it reliefs the use ofnitrogenous fertilizers in legumes (Deb *et al*., 2015). However, indigenous rhizobia canform barriers to the establishment of introduced, more efficient inoculants in nodules ofintended host plants. There is evidence of widespread suboptimal efficiency ofindigenous strains with legumes. But it is generally argued that indigenous populationsare greatly adapted to their local soil environments and may form additional effectivesymbioses than commercial inoculants isolated from a distant and unrelated soilenvironment (Gandee*et al*., 1999). Thus, selection of indigenous strains with highnitrogen fixing capacity, adapted to a range of environmental conditions at a specificsite, is a significant strategy to maximize legume production (Sajjad*et al.*, 2008).

1.2 Statement of the problem

The major portion of biological nitrogen fixation is carried out by the symbiotic nitrogen fixers such as rhizobium, this have agricultural as well as ecological importance. Much of nitrogen 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. Biological nitrogen fixation is the cheapest and environment friendly procedure in which rhizobia interacting with leguminous plants and fix aerobic nitrogen into soil. It has been proven that the presence of rhizobia increases plant productivity without any harm to human health and environments. So for maintaining soil fertility, cultivation of leguminous plants is important which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants.

In fact, in Guraghe Zone (Ethiopia), synthetic fertilizers gives short sustain high yield product, but it causes along term negative impact on the farm lands. In addition most farmers in guraghe zones are not aware about the use of native rhizobia inoculants as biofertilizers because the knowledge is not the first priority in agricultural production and even the rhizobia were not explored. Therefore a research project has been initiated with the objectives of isolation and characterization of Rhizobium from fava bean root nodules native to Guraghe farmers.

1.3 OBJECTIVES

Morphological and Biochemically characterization of Rhizobium bacteria isolated from Legume plant and screening isolates for salt and pH tolerance.

1.3.1 Specific objective

- ✓ Morphological characterization of Rhizobium bacteria collected from Legume plant
- ✓ Biochemically characterization of Rhizobium bacteria collected from Legume plant and soil of different parts of Ethiopia.
- ✓ Screening of Rhizobium isolates for salt and pH tolerance

1.4 Significance of the study

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). Consequently, there has recently been a growing level of interest in environment friendly sustainable agricultural practices and organic farming systems which include the use of bio-fertilizers as a substitute of chemical fertilizers. So, farmers benefited with its less cost and high productivity, and keep environment free of chemical.

CHAPTER 2: LITERATURE REVIEW

2.1 Rhizobium sp. and Nodulation

Eighty percent (80 %) of the atmosphere is nitrogen gas (N_2). Unfortunately N_2 is unusable by most living organisms. Plants, animals, and microorganisms can die of nitrogen deficiency, if surrounded by N2 they cannot use. All organisms use the ammonia (NH3) form of nitrogen to manufacture amino acids, proteins, nucleic acids, and other nitrogen-containing components necessary for life. Biological nitrogen fixation (BNF) process changes inert N2 to useful NH3. This process is mediated in nature only by bacteria and certain species of actinomycetes. In the free-living system, plants gain benefit when the bacteria die and release nitrogen to the environment, or when the bacteria are loosely associated with the roots of plants. In legumes and a few other plants, the bacteria live in small club-like growths on the roots called nodules. Within these nodules, N2 fixation occurs, and the NH3 produced is directly absorbed by the plant. Nitrogen fixation by legumes is a close/symbiotic relationship between a *Rhizobium* bacterium and a legume host plant (Adewusi*et al*., 2008).

Symbiotic N_2 fixation occurs through associations of plant roots with nitrogen-fixing bacteria. The symbioses are 1) between many leguminous species and *Rhizobiu*m or *Bradyrhizobium*, forming nodules on roots; 2) between a small number of non-leguminous genera and *Frankia*. The utilization often associative BNF technology in grass and cereal crops was found to be useful in the development of profitable agriculture technologies. The findings of several authors revealed existing associations of tropical grasses with nitrogen-fixing bacteria, that which under favorable conditions, may be contributing significantly to the N economy of these plants.

The bacteria belonging to the genera Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium and Mesorhizobium, which are collectively referred to as rhizobia.Rhizobial strains (Rhizobium trifolii, Rhizobium phaseoli, Rhizobium leguminosarum and Bradyrhizobiumjaponicum). Rhizobium bacteria able to form nodules on their host plants, and the fixation of N_2 by legumes play a key role in agricultural sustainability (Willams, 2006). Legumes such as beans, peas, nuts etc have been used in agriculture since ancient time and legume seeds or pulses were the first source of human food and their domestication. Legume plant posses a unique ability to establish symbiosis with nitrogen-fixing bacteria of the family Rhizobiaceae. The literature on the study of *Rhizobium* on field fava bean is inadequate.

Conversely, obtainable information concerning effect of *Rhizobium* on nodulation, plant growth, and nitrogen fixation has been reviewed in different reviews.

Rhizobium sp. is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. The Rhizobia are broadly classified as fast- or slow-growing based on their growth on laboratory media. Rhizobia are further classified according to their compatibility with particular legume species. *Rhizobium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into the soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers et al., 2003).

These bacteria at first infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. Sets of genes in the bacteria control different aspects of the nodulation process. One *Rhizobium* strain can infect certain species of legumes but not others. Specificity genes determine which *Rhizobium* strain infects which legume. Even if a strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. Such rhizobia are termed ineffective. Effective strains induce nitrogen-fixing nodules. Effectiveness is governed by a different set of genes in the bacteria from the specificity genes. Nod genes direct the various stages of nodulation.

Once inside the parenchyma cells of the root, rhizobia, and infected cells proliferate forming a nodule initial. Whether the nodule initial develops into a functional nodule is determined by the plant and a host of environmental factors. Within the successful nodule, rhizobia are surrounded by a peribacteroid membrane where they differentiate into bacteroids and begin fixing atmospheric nitrogen.One of the methods for sustainable agricultural includes the use of beneficial microorganisms for plants as they are able to promote plant growth by growing endophytically on plants, in symbiotic association with plants or as free-living cells in soil. The requirement for good agricultural practices is revitalizing the interest in biological nitrogen fixation and *Rhizobia*-legumes symbiosis, particularly those involving economically important legume crops in terms of food and forage.

Legumes are agronomically and ecologically important symbionts that lead to the development of new plant organ (legume nodule) in response to nitrogen fixing bacteria (Datta*et al.,* 2015). One of the ways of describing Rhizobia is according to their growth in solid media.

2.2 Rhizobium strain Characteristic

Morphological and biochemical characters; size, shape, colour, texture of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains or isolates. Typical morphological colony characteristics, when grown on standard yeast-mannitol medium, may include; form, elevation, colour and margin (Somasegaran and Hoben, 1985). *Rhizobium* strains secrete growth hormones like IAA which showspositive influence on plant growth and play an importantrole in formation and development of root nodules.

Biochemical characterization can be very helpful in confirming that isolates are rhizobium species. Among the important biochemical tests is the glucose assay which can be performed to determine the capability of microorganisms to utilize glucose as a sole carbon source for growth (Singh *et al*., 2008).Biochemical characterization plays an important role to differentiate between fast and slow growers of Rhizobium species (Singh *et al*., 2013

All the four strains were gram negative, rod shaped and mucous producing Phytohormone (Indole Acetic Acid).Bradyrhizobiumjaponicum was found to be negative to bromothymol blue test, while all other three strains were positive depicting the former to be slow growing and later to be fast.growing(Willams, 2006).Phytohormone (Indole Acetic Acid) *Bradyrhizobiumjaponicum* was found to be negative to bromothymol blue test, while all other three strains were positive depicting the former to be slow growing and later to be fast growing(AbhinavDatta eat al 2015) . All the strains were resistant to Penicillin and sensitive to Tetracycline. *Rhizobium phaseoli* produced starch hydrolysis while *Rhizobium trifolii* was positive in Caesinase test. *B. japonicum, R. trifolii* and *R. phaseoli* showed sufficient growth in Urease test and Lysine decarboxylase test.

All four strains hydrolyzed lipase and Catalase enzyme. *Rhizobium trifolii* and *Rhizobium phaseoli* utilized citrateand in utilization of carbon sources, fast growing strains were able to utilize carbon in comparison to slow growing one. Experiments for Indole Acetic Acid production under aerobic and anaerobic conditions showed maximum production in aerobic condition i.e., 0.4 µg ml-1 by *Rhizobiumtrifolii* and 0.6 µg ml-1 by *Rhizobium phaseoli*. *Rhizobium* was further applied as a biofertilizer for significant improvement in plant growth and yield(Deka*et al*., 2006).Further assessment of *rhizobium* genetic diversity is contributing both to the world wide knowledge of biodiversity of soil micro-organisms and to the usefulness of *rhizobium* collections, and it is developing long-term strategies to increase contributions of legume-fixed to agricultural productivity (Deshwal*et al.*, 2011).

2.3 Role of Rhizobium Bacteria

Rhizobium sp. is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. *Rhizobium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into the soil by interacting symbiotically with leguminous plants (Kiers et al., 2003). The bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium and Mesorhizobium*(Martinez Romero, 2003; Willems, 2006) are able to form nodules on their host plants inside of which they fix-nitrogen.*Rhizobia* are the gram negative bacteria which have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen *Rhizobium* inoculation increases nodule biomass, thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation (Adewusi*et al*., 2008).

Various researches demonstrated the ability of *rhizobium* to colonize roots of nonlegumes (Matiru*et al*., 2004) and act as phytohormone producer, phosphate solubilizer and to some extent, as nitrogen fixer (Afzal*et al*., 2008). *Rhizobium* was further applied as a biofertilizer for significant improvement in plant growth and yield. Bio-fertilizer promotes plant growth and productivity, which has internationally been accepted as an alternative source of chemical fertilizer. *Rhizobacteria*effectively colonize plant root and increases plant growth by production of various plant growth hormones, P-solubilizing activity, N2 fixation and biological control activity (Deshwal*et al.*, 2011).

CHAPTER 3: Materials and Methods

3.1. Description of the Study Area

This study was conducted in Gurage Zone Butajira Destrict,southern Nation Nationalities, and Peoples Region of Ethiopiafrom January to June, 2019 G.C. It is located around 158 k/ms away from Addis Ababa.The area has an elevation of 1910-1935meters above sea level with latitude and longitude of $8^0$12' 45''N and $37^0$48' 20''E.

3.2 Sample Collection Site

Representative soils amples were collected to isolate different Rhizobium bacteria from different location. Purposely four soil samples were collected from fourdifferent legume farming areas namelyMolakalad, Buchu, Albazer, and Koro using plastic bag. Samples were transported to Wolkite Universitylaboratory of Biotechnology department.

3.3 Planting*faba bean* on Sample Soil

Soils collected from different location were filled in to the plastic pots for faba bean seed planting. *Faba bean* seeds (Bulchavariety) were surface sterilized with 95% ethylalcohol and then with 3% sodium hypochlorite for (3 min). After being washed severaltimes with distilled sterilized water, sevenseeds were planted in each plastic pot with different soil samples in triplicate.

3.4Nodule Collection and Isolation of Rhizobia

Fresh and clean root nodules of faba bean (Bulchavariety) were collected from uprooted plants carefully after 45 days of growth. Then nodules were washed with sterile water to remove the adhered soil particles and placed separately on the blotting sheet to remove the excess moisture.Surface treatment with 95% alcohol for 10 sec was followed and washed with sterile water. Then these nodules were surface sterilized with 3% sodium hypochlorite for 3 min and rinsed five times with sterile distilled water.Noduleswere surface sterilized and macerated withdrops of sterile water in Petri dishes with the aidof glass rods. Then 0.1 ml of the suspensionwas streaked on plate containing YeastExtract Mannitol Agar (YEMA)containing ($MgSO_4$ .7H_2O 0.2g/L, NaCl 0.2g/L, K_2 H PO_4 0.5g/L, Yeast extract 0.5g/L, Mannitol 10 /L, Agar 10g, distilled water 1000ml) with the right calibration of pH (6.8-7). Plates were incubated at 28°C for 3-5 days according to Atlas, (2010).

3.5 Purification and Preservation of Isolates

The cultures were purified by picking a single isolated colony, transferred into 10 ml of sterilized yeast extract mannitol (YEM) agar media and sub-culturing on separate plates. Repeated sub-culturing was made by considering small/large and/or mucoid/dry colonies of a nodule as an individual culture until purity and uniformity was maintained. A single well isolated colony was transfer to YEMA slant containing 0.3% (w/v) $CaCO_3$. When sufficient growth was observed, the slant was stored at 4°C refrigerator for further characterization according to the method proposed by Howieson and Dilworth, 2016.

3.6 Morphological Characteristics of *Rhizobium* Isolates

The isolated native strains were characterized on the basis of morphological and biochemical characteristics according to Jordan, (1984). Isolated colonies were characterized on the basis of colony form, elevation, margin and color using the morphological keys (Aneja, 2003). After incubation for 2-3 days at 30°C microscopic observation of the isolates was done using Gram staining technique as described by Arora, (2003).

3.6.1 Gram Staining Method

Pure culture of bacterial strains were put for gram staining for more specific identification of the coloniesaccording to Zeenat*et al.*, (2017). The gram staining was done in laminar air flow hood. The slides were washed with ethanol, coloniesmarked with the help of inoculating loop/needle and finally heat fixed. Smears were stained in following steps; first crystal violet was applied on each slide and kept for 1 min then washed with distilled water. Iodine was applied on the slides as mordant for 1 min, washed with 95% alcohol for 30 sec and washed with distilled water. Finally Safranin was applied on the slides, washed with distilled water and air dried.

3.6.2 Salt, pH and Temperature Tolerance

The ability of the isolated *bacteria* strain to grow at different concentrations of salt were tested by streaking them on YEM medium containing 0.5%, 1.0%, 2.0%, 3%, 4%(w/v), NaCl.

Differences in pH tolerance were tested in YEM agar by adjusting the pH to 4.0, 5.0, 6.8, 7.0, 8.0 and 9.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates used as controls.

Differences in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 35°C, 40°C, 45^{o}C and 50°C. Control plates were incubated at 28°C for 48 hours.

3.7. Biochemical Characterization

3.7.1 Carbohydrate Utilization test

Carbon utilization of isolates was determined following Somasegaran and Hoben, (1994)method. Carbohydrates were prepared as 10% (W/V) solution in water. The basal medium was used with different carbohydrates (Glucose, Lactose, and Sucrose) substituted for mannitol. The medium was solidified with purified agar. Inoculawere prepared by diluting the 24 hoursyoung cultures with distilled sterilized waterto a density of 10^6 cells per ml theninoculated to the surface of carbohydratecontaining agar plates. Triplicate plates ofeach carbohydrate were incubated at 28°C for 7 days and scored for growth.Yeat extract agar medium without Mannitol and with Mannitol was used as a negative and positive control, respectively.

3.7.2 Catalase Activity

Catalase activity tests were performed to study the presence of catalysesenzyme in *rhizobium* spp. which hydrolyzes H_2O_2. Smear of strain was made on a clean and dry glass slide, and then a few drops of H_2O_2 were added to the slide. This test was performed as per the procedure of Ankur*et al*., (2017).

3.7.3Caesinase Test

Rhizobium isolates were inoculated on Skimmed Milk Agar medium containing (casein 0.5 g/L, yeast extract 0.25 g/L, glucose 0.1 g/L, and agar 1.5 g/L). Components were mixed with distilled water in a conical flask and sterilized. Inoculated plates were incubated at 34^{o}C for three days and color change was examined.

3.8 Salt and Acid Tolerance Test

Rhizobial isolates isolated from the four sites were tested for ability togrow in different salt and acid concentrations.The capability of the four *rhizobial*strains to grow in acidic media was tested by streaking on YEM agar plates of pH adjusted to 4, 5.0, 5, 6.8,7.0, 8.0 and 0.9 according to Jordan, (1984).The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM media containing0.5%, 1%, 2%, 3%, and 4% (w/v) NaCl according to El Sheikh and Wood, (1989).

CHAPTER 4. RESULTS

4.1 Morphological Characteristics of *Rhizobium* Isolates

Four rhizobial isolates, namely RS1, RS2, RS3 and RS4were isolated from faba bean plant nodules.The isolates were coded using a combination of the Rhizobium first letter and soil sample site name abbreviation.

Figure 1: Root Nodule observation from faba bean on plastic pots

Rhizobia can be described according to their growth in solid and liquid media. The colonies obtained in the study site were all circular in form, all convex in elevation, with entire or smooth margins with cream, white and transparent appearance on YEMA media after 48 days of incubation. All isolates were found gram negative.

A

B

Figure 2: Rhizobium isolation (A) and Sub-culturing(B) of Rhizobial bacterial colony on YEMA

Figure 3: Purification and preservaton of isolates

4.2 Rhizobial Growth At Different Salt, pH and Temperature Level

4.2.1 RhizobialGrowth at Different pH Level

Among the four isolates identified in this study isolate RB and isolate RKwere showed no growth at Ph 5 (fig 4). RBand RKisolateswerenot showed growth on pH<5and 9 (Fig.5,6 appendix and table 1).

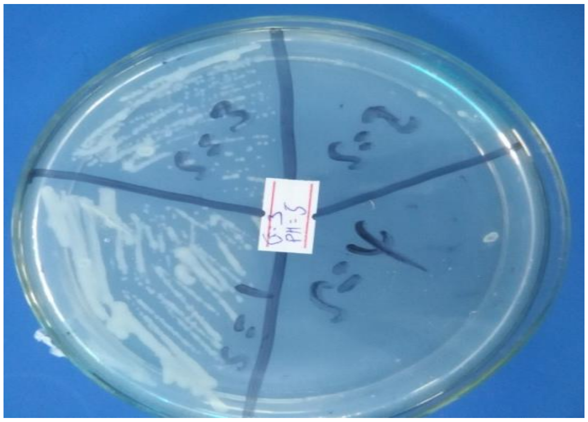


Figure 4: Growth of Rhizobium at ph=5

Table 1: Rhizobium isolates growth at different ph level

	P^H					
Name	pH4	pH 5	pH 6.8	pH 7	pH 8	pH 9
RM	++	++	++	++	++	+
RB	-	-	++	++	+	-
RA	++	++	++	++	++	+
RK	-	-	++	++	+	-

(++): High growth; (+): Moderate/low growth; (-): No growth

4.2.2 RhizobialGrowth at Different Temperature Level

All the four isolates were showed growth at a temperature of 35^0 C but, RB and RK isolates were registered less growth. No growth was maintained at the temperature of 50^0C by the two isolates. Paradoxically, RM and RA isolates were registered growth (fig 5).

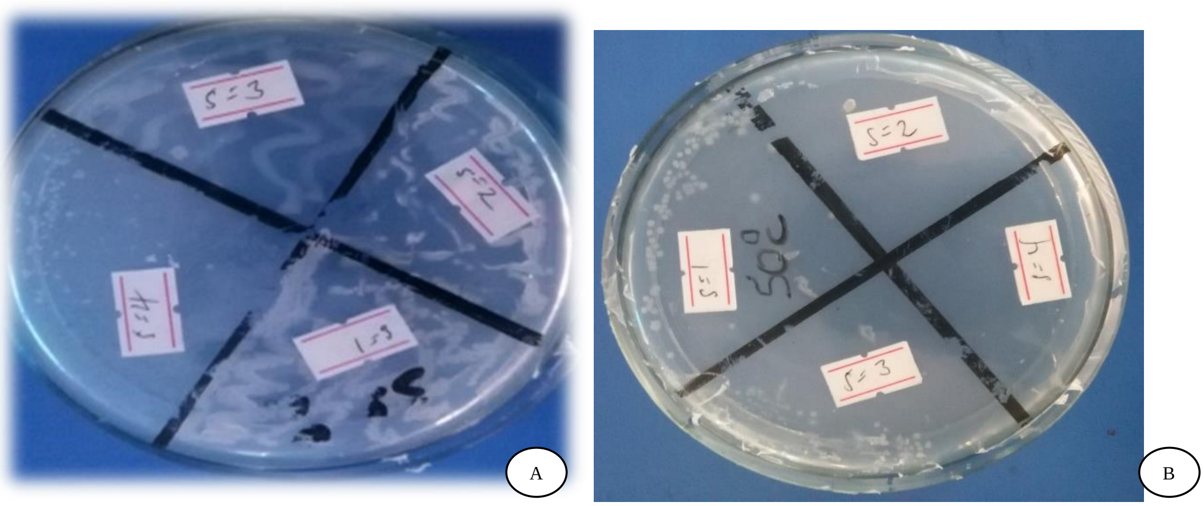


Figure 5: Growth of Rhizobia isolate at temperature 35(A) and 50(B) on 24 hours after inoculation

4.2.3RhizobialGrowth at Different Salt level

The growth of RS2 and RS4 Rhizobium isolates were showed less growth as the salt concentration increased from 2gm to 4 gm (fig.6).

Figure 6 : Rhizobium growth on 2gm NaCl(A), on 3gm NaCl(B) and on 4gm NaCl(C) containing YEMA

Table 4: Tolerance of Rhizobium strain to NaCl

	NaCl					
Name	0.2%	0.5%	1%	2%	3%	4%
RM	++	++	++	++	++	++
RB	++	++	++	++	++	++
RA	++	++	++	++	++	++
RK	++	++	++	++	++	++

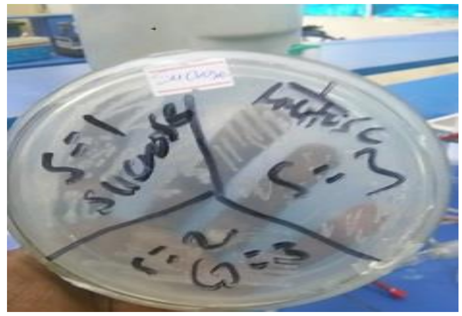
4.3 Biochemical Characterization of Rhizobial Isolates

Biochemical characterization of selected isolates was carried out on the basis of Carbohydrate Utilization test, Catalase Activity, Caesinase, Bromothymol Blue test and Urease test.

Table 2: Effects of various biochemical tests growth of Rhizobium isolates.

S.No	Carbohydrate Utilization			Catalase	Caesinase
	Glucose	Lactose	Sucrose		
RM	++	+	++	+	-
RB	++	+	++	+	-
RA	++	+	++	+	-
RK	++	+	++	+	-

Growth is signified by “+” and poor growth is signified by “-”.



Glucose

test (++)Sucrose test (++)

Lactose test

Catalase test (+)

Caesinase test (-)

Figure 7: Corbohydrate utilization test for Rhizobium isolates

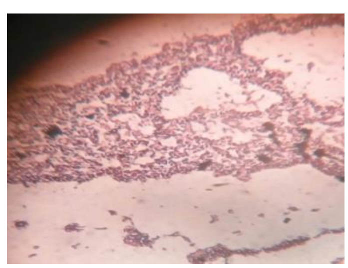


Figure 8: Gram stain of Rhizobial cells: Showing gram negative cells

Table 3: Morphological and cultural characteristics of Rhizobium isolate

Bacterial code	Isolation site	Colony shape	Color	Gram nature	Margin of colony
RM	Molakalad	Circular	Milkish White	negative	Entire
RB	Buchu	Circular	Milkish White	negative	Entire
RA	Albazer	Circular	Milkish White	negative	Entire
RK	Koro	Circular	Milkish White	negative	Entire

CHAPTER 5:DISCUSSION

5.1 Morphological characteristics of Rhizobia Isolates

One of the ways of describing Rhizobia is according to their growth in solid media. Morphological and biochemical characters; size, shape, colour, texture of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains or isolates. On the basis of morphological characters, the isolates were found circular in shape with entire margin, convex and milky to watery translucent appearance on YEMA medium. This study was different with the work of Gachande and Khansole (2011) that indicated *Rhizobium japonicum*and*Bradyrhizobiumjaponicum*colonies, which were circular in shape with whitish pink color. Our morphological result was in line with Rai*et al.*, (2013) and Gauri*et al.*, (2012). Datta*et al.*, (2015) also found that *Rhizobium* was Gram negative, motile, rod shaped and were fast growers as they showed convex elevation in Yeast Extract Mannitol medium. Table 3 represents the morphological and cultural characteristics of strains indicating *Rhizobium.*

5.2 Biochemical characteristics of Rhizobia

Biochemical characterization can be very helpful in confirming that isolates are rhizobium species. Among the important biochemical tests is the glucose assay which can be performed to determine the capability of microorganisms to utilize glucose as a sole carbon source for growth (Singh *et al*., 2008).Biochemical characterization plays an important role to differentiate between fast andslow growers of Rhizobium species (Singh *et al*., 2013).

5.3 Gram staining

Gram's staining of the isolates was confirmed by microscopic observations and the Rhizobium spp. was found to be gram negative. Gauri*et al.*, (2012) also reported that microscopic examination of *Rhizobium* revealed the isolates to be gram negative. The species of the genus *Rhizobium* have been separated into fast and slow growing types based on their rate of growth and their effect on the acidity of the YEM under laboratory conditions (Saeki et al., 2005 and Sharma *et al*., 2010). Freshly prepared YEM agar plates containing bromthymol blue at pH of 6.8 were found green incolor (figure not indicated). This result was in line with the work of Chen et al., 2001. The results of this study indicated gram-negative and rod-shaped cells as revealed by

Gram's staining technique (Fig. 8). This characteristic is typical of *Rhizobia*. The pure cultures of bacterial strains were put for gram staining for more specific identification of the colonies.

5.4 Rhizobium isolates Tolerance to pH

To observe pH tolerance of the Rhizobial isolates, they were grown in YEMA medium by adjusting pH from 4 to 9 and the plates were incubated at 28 0C for 36 hours. Growth of Rhizobia in soils is sensitive to pH; pH has been shown to limit survivaland persistence in soils.In the present study optimum growth for RM and RA isolate were found at pH 4 to pH 8.This isolates indicatedslight growth at pH 9, which was same as described by Yan *et al*., 2014 and Kaur*et al*., (2012).For the isolate coded as RB and RK; the optimum growth were at pH 6.8 and7 with no growth at pH 4, 5 and 9 (table 1). These isolates were shown a broad pH tolerance in this study. Based on the describtion given by Yan *et al*.,(2014) fast-growers are relatively more alkali tolerant and acid sensitive than slow growers. Our result was the same with that of Yan *et al*., (2014) intermesh of alkali tolerance characteristics but less sensitive to acidic for isolate RB and RK.Deora and Singhal, (2010) have proposed that slight variation in the pH of medium might have an enormous effect on the growth of *Rhizobium* which was same with what we observed in this study (Fig. 4 and table 1).Deora and Singhal, (2010) have proposed that slight variation in the pH of medium might have an enormous effect on the growth of *Rhizobium*. Rhizobial isolates were observed to be more sensitive to low pH than their host and this affects the establishment of the symbiosis, limiting the survival and persistence of the *Rhizobia* (Zahran, 1999).

5.5 Rhizobium isolates Tolerance to Temperature and Salt

Temperature is one of the major factors affecting rhizobial growth, survival in the soil and the symbiotic process itself (Niste*et al.*, 2013).The maximum temperature at which all the strains grew was 28-30 °C. To observe temperature tolerance of the Rhizobial isolates in this study, the YEMA medium containing Rhizobium was incubated at 35^{o}C, 40^{o}C, 45^{o}C and 50^{o}C for 24 hours. Maatallah*et al.*, (2002) reported maximum growth of *Mesorhizobia*between 20 to 40 ^{o}C. But, in this study RM and RA were found tolerating 50 ^{0}C and RB and RK not.

The ability of Rhizobium cultures to grow in different salt concentrations was tested by streaking them on YEMA medium containing 0.5%, 1%, 2%, 3%, and 4%(w/v) NaCl. In the present study, different isolates of *Rhizobia* were showed different growth rate at different concentration of NaCl. All isolates were shown growth at salt concentration of 4% (w/v). Our result was same with the report presented by AbhinavDatta (2015). The growth of RM and RK Rhizobium isolates were showed better growth at salt concentration ranging from 0.2% to 3% (table 4). Some bacteria heir capability of NaCl tolerance as some strains may grow at salt concentrations as high as 5% NaCl, others may not grow even at low NaCl concentration (100 mM) (Kucuk and Kivanc, 2008).

(++): High growth; (+): Moderate; (-): No growth

5.6 Starch Utility

Fast growing *Rhizobia* utilizes a wide range of carbon sources as compared to slow growing *Rhizobia*. In the present study, all the strains showed good growth on Glucose, Sucrose and Lactose (Table 5).Generally, fast growing rhizobia have been shown to have awider range of utilization of carbon sources than-slow growers. Slow-growers have been associated with utilization of mostly hexoses and pentoses but with limited utilization of disaccharides and sugar alcohols (Wagner *et al*., 1995).Based on the acid/alkaline reaction results on medium supplemented with glucose, allthe isolates were able to utilize glucose as a carbon source. Slow growers tend not topossess the catabolic enzymes for disaccharides. Fast growers on the other hand, canutilize disaccharides, cellobiose, sucrose and other such compounds not utilized by slowgrowers(Sawdosky*et al*., 1983). Previously it has been shown that both fast- and slowgrowersare able to utilize glucose as a carbon source, this corroborates with the findingsin this study.In the present study, all the strains showed good growth on Glucose, Sucrose and Starch.

5.7 Catalase and Caesinase

Bubble formation around bacterial colonies of all four isolates was showed positive Catalase test (table 2and fig.8). Thisfindings was same with AbhinavDatta (2015);Datta*et al., (*2015); Ankur et al., 2017 and Javed and Asghari*et al.,* (2008)and in contradiction to the results of Elsheikh, (1989).

In the present study, Caesinase test was found to be negative due to no clearing zone is observed around and/ or beneath the inoculums (figure 8). Datta*et al.*,*(* 2015), observed that caseinase test is negative in *Rhizobium leguminosarum*strain.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). This study confirmed that the root nodules of faba bean plants harbour the nitrogen-fixing bacterium- *Rhizobium.* The present investigation seems to be promising approach to consider a potential candidate Rhizobia isolate to be used in nitrogen fixation. Morphological characteristics varied among the 4 isolates obtained from therepresentative of foursites in the study area. The colors of colonies weretranslucent, cream and white. The elevation of all the colonies was convex. Biochemically, all Rhizobia were Gramnegative,mucus producing and acid 2 of them found alkali tolerant (>pH 9). Isolateswere fast growing with two slow growing, able to utilize glucose as a carbon source andunable to tolerate extremes of pH at 28°C.

6.2 Recommendation

- Further tests to confirm the isolates using genetic characterization and aninclusion of reference strains are being recommended.
- To confirm the isolates effects on plant growth and the ability of nitrogen fixations.

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Appendix

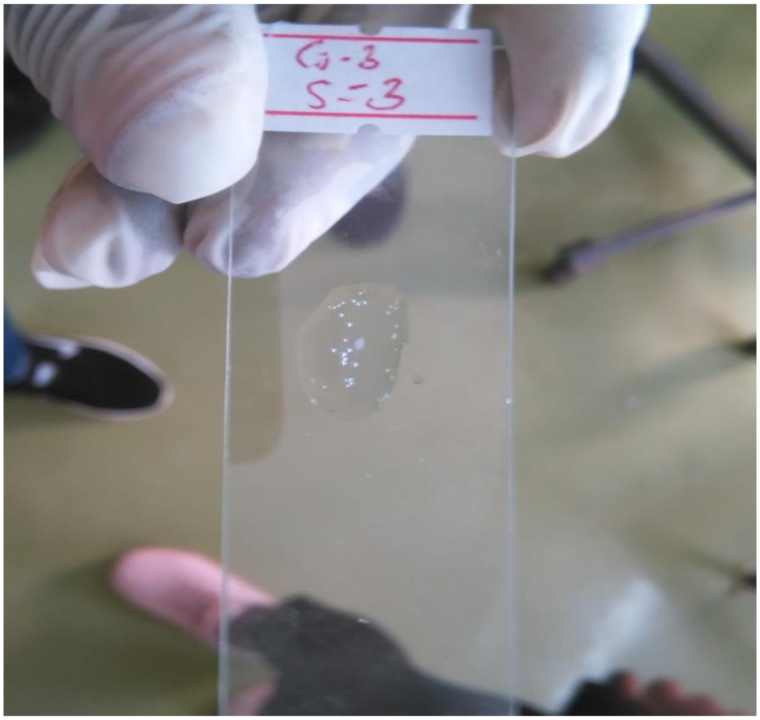


Fig.1:RS3 catalase test (positive)

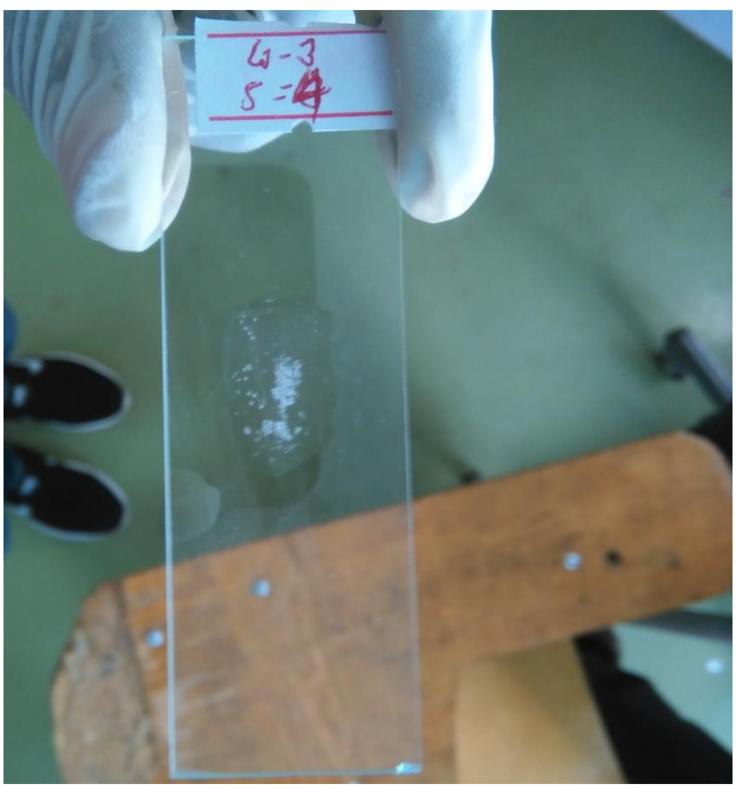


Fig .2.RS4 catalase test (positive)

Fig.3. Rhizobium isolate colony on YEMA

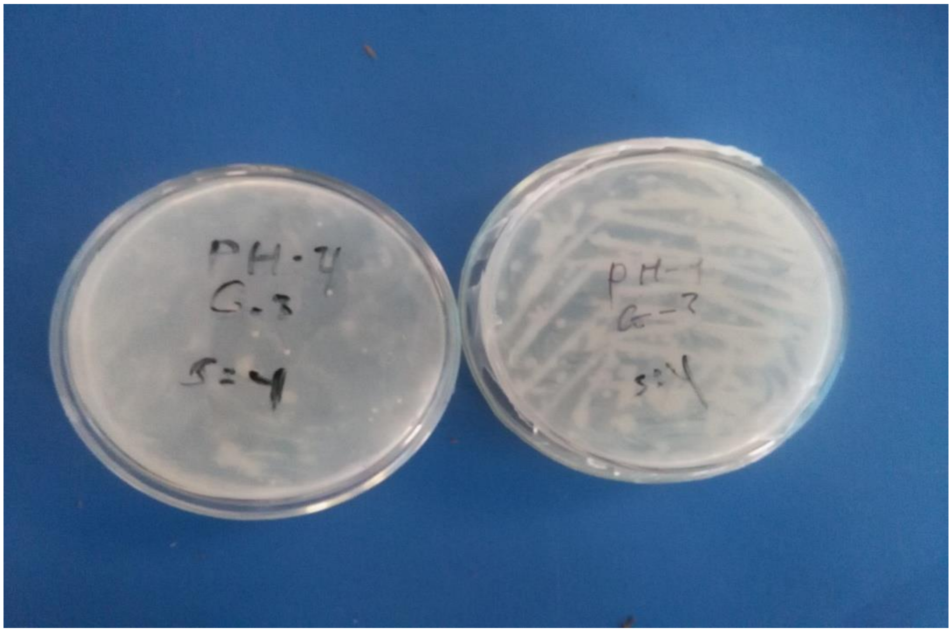


Fig.4. Growth of RS4 isolates at ph 4&9

Fig.5.growth of RS2 isolate at ph 9

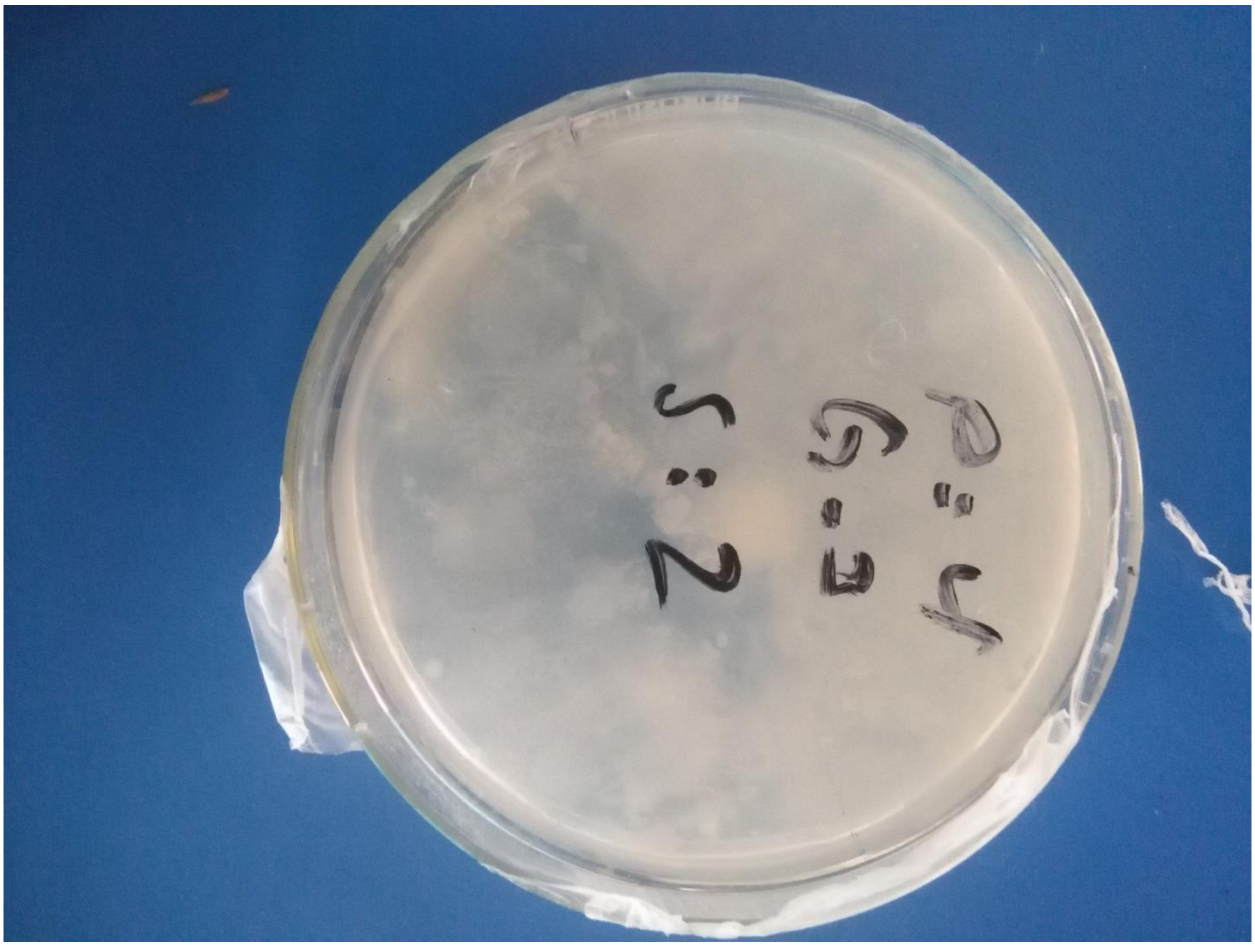
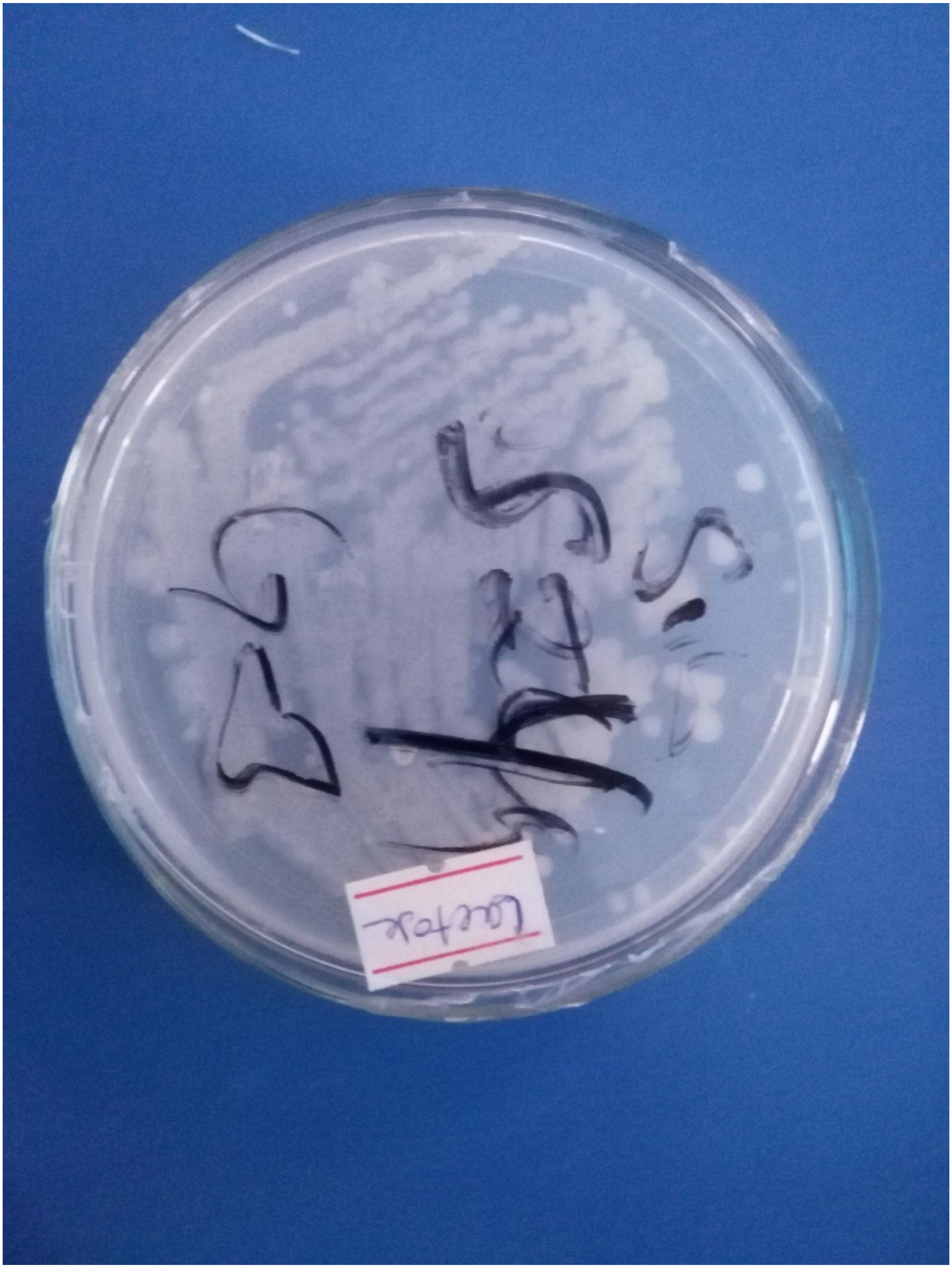


Fig .6. growth of RS2 isolate at ph 4


Lactose