Nitrogen (N) deficiency is one of the most common factors for reduction in yield of legume crops particularly in Ethiopia. The utilization of biological nitrogen fixation (BNF) by legume-Rhizobium symbiosis is most prominent symbiosis found in nature; so for increasing legume production and soil fertility we should use organic fertilizer such as bio fertilizer. The aim of this study was to isolate, identify and characterize the fababean nodulating Rhizobium isolates. Fababean (Vicia faba L.) rhizobia were isolated from nodules collected from four fababean growing area of Arsi zone of Ethiopia. A total of twenty rhizobia isolates were collected and characterized based upon their morphological and biochemical characteristics. Out of total, twelve isolates were selected as representative rhizobia samples. The result of this study showed that all isolates were fast-growing and failed to absorb Congo red. Cell size ranged from 2.1 to 5.2 mm. The smallest and largest colony diameter (2.1 and 5.2 mm) was recorded in isolates BKF3 and MRFB10 respectively on YEMA medium. Isolates were capable of utilizing 50-100% of the tested carbohydrates. 75% of all isolates metabolized all the nitrogen sources and all isolates were utilized glutamine. All isolates were sensitive to neomycin and ampicillin at 20μg/ml concentration.

**Keywords:** Rhizobia, Fababean, Isolates, Nitrogen fixation Ethiopia.

**INTRODUCTION**

Leguminous plants (pulses) are important as human food and animal feed they provide not only high quality protein but also a variety of nutrients such as vitamins, minerals, and other nutrients. Most species of the Leguminosae form symbiotic associations with nitrogen-fixing root nodule forming bacteria known as rhizobia. They fulfill most of their N requirements through this process. Therefore, the legumes are an essential part of the terrestrial nitrogen cycle and used to sustain ecosystem functioning (Spret, 2001). They occupy 12-15% of the earth’s arable land and account for a third of human dietary protein needs and for up to 2/3 of subsistence livelihood (Graham and Vance, 2003). Legume plants (Fababean, field pea, chickpea, soybean, etc.) show a vast diversity in morphology, habitat and ecology. However, all legume species can be distinguished from non-leguminous plants as they produce pods as a common feature.

Fababean (Vicia faba L.) is one of the earliest domesticated cool season food legumes in Ethiopia. Ethiopia is the second largest fababean producer in the world next to China (Teklay et al., 2014). Fababean plays a great role in every aspect of Ethiopian life not only as food but also the straw and the seed as feed for animals, haulms as firewood, green manuring and for compost preparation (Comlanvi, 2011). It plays an important role in the restoration of soil fertility through atmospheric nitrogen fixation, which provides agricultural sustainability (Romner et al., 2013).

**Rhizobia** are a genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran and Hoben, 1994) and they are able to bring out nodule formation on legumes are called rhizobia. Rhizobia are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes until the point where nodulation becomes possible. Rhizobia are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile rod-shaped (approximately 0.5-0.9 μm in width and 1.2-3.0 μm in length) and heterotrophic (Somasegaran and Hoben, 1994).

Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7. Rhizobium growth normally occurs under aerobic conditions. When fixing nitrogen, low levels of oxygen are required to produce the enzyme nitrogenase...
and hence, *Rhizobium* is able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

*Rhizobia* are of great importance for nitrogen acquisition through symbiotic nitrogen fixation in a wide variety of leguminous plants. These bacteria differ from most of other soil microorganisms by taking dual forms, i.e., a free-living form in soils and a symbiotic form inside of host legumes. Therefore, they should have a versatile strategy for survival, whether inhabiting soils or root nodules formed through *rhizobia*-legume interactions. The objective of this study was to isolate, identify and biochemical characterization of root nodulating *rhizobium* isolates from fababean (*Vicia faba L*) nodules.

**MATERIAL AND METHODS**

The experiments were carried out at the Department of Microbiology, College of Natural Science, and Addis Ababa University, Ethiopia.

**Collection of samples**

Plant samples were collected from different locations of Arsi zone fababean growing area such, Bekoji, Meraro, Kersa and Munese. A total of 20 (five for each area) samples of fababean nodules were collected and big sized and pink colored nodule preferably on tap root were selected and transported to the laboratory for further investigation by following the method given by Vincent (1970).

**Isolation of Rhizobium from root nodules**

In the laboratory nodule samples were tagged properly and named as BKFB1-BKFB5 for Bekoji, MRFB6-MRFB10 for Meraro, KRFB11-KRFB15 for Kersa and MUFB16-MUFB20 for Munese. The nodules (collected from inoculated host fababean) was surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo chlorite for 2-minutes (Lupwayi and Haque, 1994). The surface sterilized nodules were then rinsed with sterile distilled water six times to completely remove the sterilizing chemicals. The nodules were crushed with sterile glass rods in1 drop of sterilized 0.85% NaCl. The crushed nodules were transferred to YEMA medium (DIFCO). They were then incubated at 28±2°C and periodically checked for colony formation.

**Autoclaved at 121°C for 15 minutes, pH adjusted to 7, Taken from –Lupwayi and Haque. (1994)**

**Purification and preservation**

Colonies of isolates was picked with sterile inoculating loop and streaked on sterile YEMA plates and incubated at 28±2°C. The purity and uniformity of the colony type was carefully examined through repeated re-streaking and a single well isolated colony picked to YEMA slant containing 0.3% (W/V) CaCO₃ in a culture tube and incubated at 28±2°C. After sufficient growth the culture slant were preserved at 4°C (Somasegaran and Hoben, 1994).

**Presumptive test**

1. **Gram staining test**
   
   Gram stain reaction was carried out to confirm that all isolates were gram negative and does not contain any gram positive bacteria or contaminants. Gram’s procedure was done as indicated in Lupwayi and Haque, (1994).

2. **Congo red absorption**
   
   Colonies was tested on Congo-Red (CR-YEMA) (Somasegaran and Hoben, 1994). Stock solution of Congo Red (CR) prepared by dissolving 0.25g of CR in 100ml sterile distilled water,10ml from this stock solution added to one liter of YEMA. The broth culture suspension inoculated into YEMA-CR medium, and the plates were wrapped with aluminum foil to provide a dark condition and incubated to at 28±2°C observe CR absorption or not.

3. **Acid and alkaline production on BTB**

   They were tested for production of acid or alkaline by incorporating bromothymol blue (BTB) as reaction indicator on yeast extract manniotl agar (YEMA) according to Somasegaran and Hoben (1994). After 48 hours of growth, a loop full of *Rhizobium* culture (10⁹cells/ml) streaked on YEMA-BTB plate, fast growers changed the BTB in to yellow color, but slow growers changed into blue color, and results were recorded after 3-5 days of incubation.

   YEMA:------------------------1 liter
   BTB-(0.5 % w/v in 95% ethanol)--------5ml
   pH-------------------------------6.8

4. **Growths on Peptone Glucose Agar (PGA) Medium**

   Isolates inoculated on PGA incorporated with bromoresol purple in order to check a change in pH of the medium associated with the presence of contaminants in the preserved culture further checked on PGA medium with 10 µ g/ml Bromoresol purple (BCP) dye.

---

**Table 1: YEMA Composition**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount (gm/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.2g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>D-manitol</td>
<td>10g</td>
</tr>
<tr>
<td>Agar</td>
<td>15g</td>
</tr>
<tr>
<td>Distil water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

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Composition of PGA medium
Glucose.................................5g
Peptone..................................10g
Agar......................................15g
BCP stock solution.....................10ml
PH.........................................6.7
Distilled H2O............................1liter
Autoclaved at 121°C for 15 min. Source: Somasegaran and Hoben (1994)

The BCP (Bromo cresol purple) prepared as stock solution by dissolving 1g/100ml of ethanol (Somasegaran and Hoben, 1994). The pH adjusted at 6.8 by 1N NaOH and HCl (Lupwayi and Haque, 1994). The bacterial culture suspension was inoculated on the medium and incubated at 28±2°C; finally presence/absence of bacterial colonies was checked.

Colony Morphology
The cultural characteristics of the isolates were performed according to (Lupwayi and Haque, 1994). Each bacterial isolates From YEMB were inoculated on the YEMA medium and incubated for 3-5 days. A single colony of each isolate was characterized by colony appearance, diameter, color, shape and extra cellular polysaccharide production.

Biochemical characteristics
Carbohydrate Utilization
Isolates were checked for their ability to utilize different carbohydrate sources. Different sources of carbohydrate can be checked up. This may include glucose, maltose, D-fructose, glyceral and Gluconate. The test was carried out according to Somasegaran and Hoben (1994). Ten percent distilled water solution of each carbohydrate (w/v but v/v for glyceral) was prepared and heat stable carbohydrates (glucose and D-fructose) were autoclaved together with the medium, but heat labile carbohydrates (gluconate, maltose and glyceral) were filter sterilized using disposable membrane filter of 0.22μm sizes and added to the basal medium (YEMA) after sterilization when the medium temperature was reduced to 50°C. Finally, a loop full of 72 hours old YEM broth culture of each rhizobia isolate was streaked on the plates of incorporated carbohydrates under test and incubated at 28±2°C for 3 to 5 days and growth was recorded as (+) for positive growth and (-) for no growth in relation to the positive control YEMA plates.

Amino Acid Utilization
The isolates were streaked on different amino acids including: Glutamine, Peptone, Glycine, alanine and L-lysine in order to determine the ability of the isolates to utilize the amino acids as a nitrogen source. They were added at concentration of 0.5g/l to a basal medium (Somasegaran and Hoben, 1994). Finally 48hr old rhizobia suspensions were inoculated in to these basal media and incubated at 28±2°C for 3-5 days.

Intrinsic antibiotic resistance (IAR)
The resistance of isolates to antibiotics was tested by streaking them on solid YEMA medium containing freshly prepared filter sterilized antibiotics using 0.22 μm sized membrane filters: Tetracycline, Erythromycin, Streptomycin, Penicillin and Neomycin and two concentrations (10 and 20 μg/ml). The stock of each antibiotic solution was first prepared by dissolving 2g of each antibiotic in 100ml of water as described in Lupwayi and Haque, (1994). Erythromycin was dissolved in ethanol and the other was dissolved in sterilized distilled water. The filter sterilized solutions of each antibiotic was added to sterile YEMA cooled to 50°C and mixed thoroughly. The isolates then tested by streaking the culture on each cooled plate and incubated at 28 + 2°C for 3-5 days. The results were recorded qualitatively either as +/− for growth and no growth, respectively.

RESULT AND DISCUSSION
Isolation and Morphological characteristics of rhizobia
A total of 20 root nodule samples collected from different fababean growing areas from Arsi zone. The data in Table 1 shown that among 20 samples tested, 12 samples were found positive for the presence of Rhizobium on the basis of white mucoid growth on YEMA medium when incubated for 24 h at 28±2°C. Pure colonies of rhizobia isolates were microscopically examined to determine cell shape and size, Gram stain reaction and motility in liquid culture. Colony morphology of rhizobia isolates was studied to determine the opacity and viscosity. The data in Table (2) show that all rhizobia isolates tested had fast growth on YEMA medium, non-capsulated, short rod, Gram negative and motile. Cell size ranged from 2.1 to 5.2 mm. The smallest and largest colony diameter (2.1 and 5.2 mm) was recorded in isolates BKF3 and MRFB10 respectively on YEMA medium (Table 2). According to Somasegaran and Hoben, 1994 classification those isolates were classified as fast growing root nodule bacteria. Similar results were reported by Mona H. A. et al., 2016 on rhizobia isolated from fababean colonies of the isolates were appeared a sticky natural, indicating the production of mucous substances which is one of the characteristics of Rhizobia (Singh et al., 2013).

All the isolates were failed to absorb Congo red pigment when grown on YEMA-CR medium. The isolates change the color of Bromocresol purple, when grown on peptone glucose agar medium and changed the YEMA-BTB medium. This result is similar to the results of Abere Minalku et al., 2009 and Zerihun Belay and Fassil Assefa (2011) on isolation and characterization of rhizobia from fababean. Among all the isolates MRFB6, MRFB7, MRFB10, KRFB12, KRFB13, MUFB16, MUFB18 and MUFB19, showed large mucoid growth on YEMA medium, isolates
BKFB2, BKFB3, KRFB14, and MUFB20, showed small mucoid growth on YEMA media (Table 2).

### Table-2: Morphological characteristics of rhizobia isolated from fababean nodule

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Colony size (mm)</th>
<th>Colony type</th>
<th>Colony shape</th>
<th>Colony texture</th>
<th>Colony color</th>
<th>Gram rxn test</th>
<th>YEMA-BTB</th>
<th>YEMA-CR</th>
<th>PGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKFB2</td>
<td>2.5</td>
<td>SM</td>
<td>rod</td>
<td>butty</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BKFB3</td>
<td>2.1</td>
<td>SM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRFB6</td>
<td>3.5</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRFB7</td>
<td>4.8</td>
<td>LM</td>
<td>rod</td>
<td>butty</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRFB10</td>
<td>5.2</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>Yellow</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KRFB12</td>
<td>4.2</td>
<td>LM</td>
<td>rod</td>
<td>butty</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KRFB13</td>
<td>4.2</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KRFB14</td>
<td>3.2</td>
<td>SM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MUFB16</td>
<td>3.5</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MUFB18</td>
<td>4.5</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MUFB19</td>
<td>4.8</td>
<td>LM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MUFB20</td>
<td>2.8</td>
<td>SM</td>
<td>rod</td>
<td>elastic</td>
<td>White</td>
<td>-</td>
<td>Yellow</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LM= large mucoid, SM= small mucoid

### Biochemical characteristics of rhizobia isolates

#### Carbohydrate utilization

The data on table 3 showed that isolates were capable of utilizing 50-100% of the tested carbohydrates. All isolates utilized glucose and most of the isolates (85.3%, 58.3% and 50%) were capable of utilizing glycerol, D-fructose, gluconate and maltose. Girmaye et al. (2014) result more than 50% the fababean isolates were able to utilize and grow well on most carbon sources. The result, in general showed that the majority of isolates were able to use a broad range of carbon sources.

#### Amino acid utilization

The results of utilization of nitrogen sources showed that more than 75% of all isolates metabolized all the nitrogen sources and all isolates were utilized glutamine (table 3). The result which revealed that similar with Getahun Negash (2015) and Girmaye et al. (2014) who were identified that 37.1% and 48% of the isolates were able to metabolize all the nitrogen sources respectively.

### Table-3: Carbohydrate and amino acid utilization of rhizobia isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Carbohydrate utilization</th>
<th>Amino acid utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Maltose</td>
</tr>
<tr>
<td>BKFB2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BKFB3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRFB6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRFB7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MRFB10</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KRFB12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KRFB13</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KRFB14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MUFB16</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MUFB18</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MUFB19</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MUFB20</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Utilization%</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

### Antibiotics resistance test

Results presented in (Table 4) indicated different antibiotic resistance patterns among the rhizobia isolates. All isolates (100%) were resistant to tetracycline and streptomycin at concentration of 10μg/ml and most of isolates were resistance to erythromycin (75%), penicillin (25%) and neomycin (41.6%) at the concentration of 10μg/ml. All isolates were sensitive to neomycin and ampicillin at 20μg/ml concentration. In a similar study, fababean rhizobia showed sensitivity to ampicillin and kanamycin than other types of antibiotics (Girmaye et al., 2014). Finally, antibiotic resistance test indicated that all the isolates showed variation in tolerance to 20μg/ml concentration of tested antibiotics (Table 4).
CONCLUSION
The results from this study concluded that isolation and biochemical characterization of rhizobia isolates from faba bean nodules were to select and screen superior isolates for preparation of effective strains. From the result of this study rhizobia isolates were fast growing type and did not absorb red color when cultured in YEMA containing congo red medium. The tested isolates were utilized different carbon and nitrogen sources and able to tolerate wide range of antibiotics. Therefore, the presence of diversity from the study areas showed that getting effective rhizobial strains for the production of faba bean. Hence, studies needed molecular characterization for obtaining superior rhizobia strains from different area for production of bio fertilizer.

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Conflict of interest
The authors do not declare any conflict of interest.

REFERENCE