Isolation and Identification of Endophytic Bacteria from *Rumex nepalensis* based on the Protein Profile and assay their Growth Conditions

Hermon Kebede Zinabu

Advisor: Seid Mohammad (PhD)

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Sciences

Presented in partial fulfillment of the requirement for the degree of masters in Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

May, 2024

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Declaration

I declare that this Master thesis entitled "I**solation and Identification of Endophytic Bacterial Isolates from *Rumex nepalensis* based on the Protein Profile and assay their Growth Conditions**" is my original work. Thus, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

_____ _____ _____

Name of student signature Date

RECOMMENDATION

I the advisor of this thesis hereby certify that, I have read the revised version of this thesis entitled "Isolation and Identification of Endophytic Bacterial Isolates from *R. Nepalensis* based on the protein profile and assay their Growth Conditions'' prepared under our guidance by Hermon Kebede. Therefore, I recommend the submission of the revised version of the thesis to the department following the applicable procedures.

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Seid Mohammed (PhD)	_____	2-06-2024
Major Advisor	Signature	Date
_____	_____	_____
Co-advisor	Signature	Date

Approval Page

I the advisor of the thesis entitled "**Isolation and Identification of Endophytic Bacterial Isolates from *Rumex nepalensis* based on the Protein Profile and assay their Growth Conditions**" and developed by Hermon Kebede here by certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated in to the final version of the thesis.

Seid Mohammed (PhD)	_____	**2-06-2024**
Major Advisor/Supervisor	Signature	Date
_____	_____	_____
Co-advisor/Co-supervisor	Signature	Date

We the undersigned members of the board of examiners of examiners of the thesis by Hermon Kebede have read and evaluated for the thesis entitled "Isolation and Identification of Endophytic Bacterial Isolates from Roots of *R. Nepalensis* based on the protein profile and assay their Growth Conditions" and examined during open defense. This is therefore, to certify that the thesis is accepted for the partial fulfillment of the requirement of the degree of masters of Science in biotechnology.

_____	_____	_____
Chairperson	Signature	Date
Teshome Geremew (Ph. D)	_____	19/07/2024
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Abstract

Rumex nepalensis Spreng (Amharic: Yewsha Tult) belongs to the Polygonaceae (buckwheat) family. It occurs as a weed of grassland, particularly in disturbed areas with high fertility and bushland. The current study aimed to isolate the endophytic bacteria from the root, stem and leaf parts of *Rumex nepalensis* plant. The *R. nepalensis* samples were collected and transported to the Applied Microbiology laboratory. Endophytic bacterial isolates were isolated from the root, stem, and leaf parts of *R. nepalensis*. Various biochemical tests, morphological characterization and enzymatic activities were performed. Phosphate and zinc solubilization were carried out for these Endophytic bacterial isolates. The secondary metabolites of isolates were extracted using ethyl acetate, and the antibacterial activities of the extracts were evaluated. The effect of various conditions like pH value, temperatures, salt concentration, and carbon and nitrogen sources were assessed against these endophytic bacterial isolates. Data analyses were conducted using multiple software. In this study, eight endophytic bacterial isolates were isolated from the plant's leaf, stem, and root parts. Nearly all of these isolates were Gram-positive. These isolates were also identified as *Cronobacter skazakii* EIRS-2, *Cronobacter skazakii* EIRS-3, *Cronobacter skazakii* EIRR-3, *Proteus vulgaris* EIRR-2, *Psedomonas stutzeri* EIRL-1*, Serratia liquefaciens* EIRL-2, and *Serratia liquefaciens* EIRS-1 based on growth condition from the root, stem, and leaf part of *R. nepalensis*. Some of these isolates can produce amylase, protease and cellulase enzymes. The recent isolate shows phosphates and zinc solubilization and is used for plant growth promotion. The ethyl acetate extract of the isolates also indicated inhibitory activity against test organisms. Considerable amounts of CDW (g/L) and OD600nm were obtained from these isolates. These growth conditions, such as pH values (7.5), temperatures (30&32^{o}C), salt concentrations (1&0.05M), carbons (Mannitol and glucose) and nitrogen sources (Peptone and Yeast extract) were found to affect the growth of these plant growth-promoting bacterial isolates. In conclusion, these potential endophytic bacterial isolates can be employed for plant growth promotion at suitable environmental conditions by producing certain cellulolytic enzymes which may act as phytopathogens inhibitors.

Keywords*:* Antibacterial activities, Endophytic, Isolates, Plants and secondary metabolites

1. Introduction

1.1 Background of the study

The genus *Rumex* consists of 250 species such as *R. acetosa*, *R.acetosella*, *R. abyssinicus, R.crispus, R. nepalensis* and *R. sanguineus*. *R. nepalensis* Spreng (Amharic: Yewsha Tult) belongs to the Polygonaceae (buckwheat) family. The family comprises over 1200 species in 52 genera that are usually plants which erect and long taproots. The plant is palatable to cattle and is high in fibre and nutrition. It has a self-supporting growth form and simple, broad leaves and achenes. Epidermis in roots is single layered and Polygonal shape. It is a perennial herb that grows up to 1m tall with green or pale brown stem. Endodermis is single layered. The cells of endodermis are elongated. Pericycle is single layered having spherical shape. Xylem is oval shaped, phloem and pith is spherical. Stone cells are absent (Wangchuk, 2015).

Rumex nepalensis a plant that grows in a wet or moisturized environment and commonly found at higher altitudes that grows in fertile areas. It occurs as a weed of grassland, particularly on disturbed areas with high fertility and bushland at 700-4000m altitude (Farooq *et al.*, 2013). The plant has broad ecological tolerance and is a common weed in grass land areas. It Regenerates from tap root and establishes quickly as seedlings and difficult to remove. Plants can develop in associations with their ecosystem to grow in their natural environments. Most Rumex species are reported to treat constipation, wound, bleeding, inflammation, ulcer, tumor, mild diabetes, diarrhea, edema, jaundice, and hypertension and have diuretic and analgesic effects (Tura *et al*., 2017). Natural product extracts contain quite different chemical constituents that have distinct pharmacological and biological properties. The secondary metabolites comprise carbon-based compounds such as sugars, fatty acids, terpenoids, sterols, flavonoids, tannic acids, saponins, anthraquinones, vitamin C, steroids, and phenolic acids, and nitrogen-based compounds such as alkaloids, amino acids, and protein-based compounds (Shakir *et al*., 2018). Isolated endophytes from the plant species such as Escherichia coli, Aspergillus niger, Chrysophanol, Physcion, Ochrobactrum, Paenibacillus, Clostridium and Endocrocin are used for their constipation, bleeding, tinea, pain, and purgative activities (Mishra *et al*., 2018). In Ethiopia, the plant is traditionally used for stomach ache, rheumatism, tonsillitis, ascariasis, uterine bleeding, abdominal bleeding, child diarrhea, toothache, acute febrile illness, amoebiasis, eye infection, and liver disease, gastrointestinal and oral ulcers, as an antidote, laxatives, and cabbage (Wubetu *et al*., 2017).

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1.2 Statement of the problem

Endophytic bacteria are known in our country. Most scientists done their research on medicinal use and traditional application of *R.nepalensis*. No one was done the research on screening of these endophytic bacteria from the plant *R. nepalensis*. So, there is a gap on this area. These endophytes solubilize inorganic compounds in to usable forms and increase growth rate of the plant.

1.3 Significance of the study

Rumex nepalensis was a plant that grows in a wet or moisturized environment. It can serve as a nutrition source for cattles in rural areas. Endophytic microbes live within the plant tissues without causing any visible damage to the host plant. Endophytic bacteria have different function for the plant to produce plant growth hormones, phosphate solubilization, nutrient composition and fixation of Nitrogen. Entry of Endophytic bacteria in the plant roots occurs through root hairs and spaces between epidermal cells. In order to be Nitrogen used by plants, it must have to be converted into nitrates. Bacteria plays a vital role in changing atmospheric Nitrogen into nitrites and then to nitrates. The beneficial effects of bacterial endophytes on their host plant gives for a plant growth-promoting rhizobacteria (PGPR). So, the isolates play a vital role enhancing the growth of the plant and solve food scarcity of cattles.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study was to isolate and identify the Endophytic Bacterial from *Rumex Nepalensis* based on the protein profiles and assays their growth conditions to increase food availability of cattles

1.3.2 Specific objectives

The specific objectives of this study were to:

- Isolate and screen endophytic bacterial isolates from *R. nepalensis*
- Identify these screened endophytic bacterial isolates using MALDI TOF MS techqnues
- Evaluate the optimum growth condition for certain endophytic bacterial isolates.
- Evaluate the zinc and Phosphates solubilization against endophytic bacterial isolates
- Investigate the antimicrobial activity of the crude extracts of the endophytic isolates

2. Literature review

2.1. History of R. nepalensis

R. nepalensis Spreng (Amharic: Yewsha Tult) (Figure 1) belongs to the Polygonaceae (buckwheat) family. The family comprises over 1200 species in 52 genera that are usually plants which erect and long taproots. The genus Rumex consists of 250 species. Some of the species have medicinal values such as *R. acetosa, R.acetosella, R.abyssinicus, R.crispus, R. nepalensis, R. sanguineus, R. tuberosus, R.thyrsiflorus,* and *R. vesicarius* and is widely distributed in the world (Tonny *et al*., 2017). *R. nepalensis* is one of the 250 plant species in the Rumex genera. It is a herbaceous, perennial plant producing erect, branched stems 50–180 cm tall from a large rootstock that grows in fertile areas that are rich in nitrogen. The plant is palatable to cattle and is high in fibre and nutrition (Wangchuk, 2015). It has a self-supporting growth form and simple, broad leaves and achenes. Epidermis in roots is single layered. Polygonal shape parenchyma is compactly packed. Endodermis is single layered. Pericycle is single layered having spherical shape. Xylem is oval shaped, phloem and pith is spherical. Stone cells are absent. The plant is sometimes harvested from the wild for local use as a food, medicine, and source of tannin (Informa *et al*., 2017). In Ethiopia, the plant is traditionally used for stomach ache, rheumatism, tonsillitis, ascariasis, uterine bleeding, abdominal bleeding, child diarrhea, toothache, acute febrile illness, amoebiasis and liver disease, gastrointestinal and oral ulcers, as an antidote, laxatives, and cabbage (Wubetu et *al*., 2017).

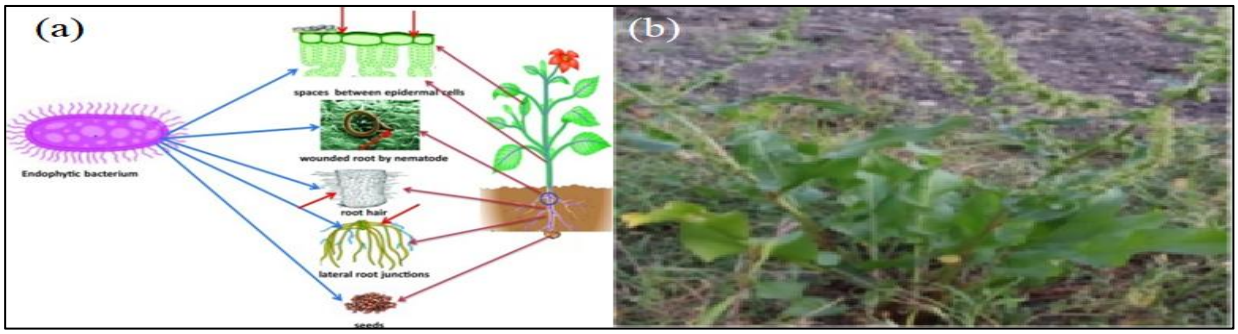


Figure 1 (a) Endophytic bacterial cells which recorded from the root part of *R. nepalensis.*

(b) Indicated that the leaf part of *R. nepalensis (Yilma,2021)*

2.2 Endophytic microorganisms

Endophytic microorganisms are microbes that live as close associations with plants. They spend most of their life cycle within the plant tissues without causing any visible damage to the host plant. Many endophytes secrete specialized metabolites or biologically active compounds (Liarzi *et al*., 2016). Endophytes are transmitted through the seeds or recruited from the soil rhizosphere. They enter the host plant through the cracks formed in the lateral root junction or wound caused by microbial or nematodes phytopathogens (*Mitter et al.,* 2017). Endophytic bacteria are the plant beneficial bacteria that thrive inside plants and can improve plant growth under normal and challenging conditions. They can benefit host plants directly by improving plant nutrient uptake and by modulating growth and stress related phytohormones. Endophytic bacteria are also having the potential due to their ability to produce plant growth hormones, phosphate solubilization, nutrient acquisition and fixation of Nitrogen. Act directly on the development and growth of plants by biological nitrogen fixation, acceleration of digestion, and phosphorus solubilization and production of phytohormones that confer resistant to biotic factors (Glick, 2012). Entry of Endophytic bacteria in the plant roots occurs through root hairs and spaces between epidermal cells (*Mitter et al.,* 2017). The root and leaves extracts of R. nepalensis have antimicrobial and antifungal activities (*Farooq et al*.,2013). *Escherichia coli* are used for antibacterial activity and *Aspergillus niger* for antifungal activity due to the presence of anthraquinone, steroid, saponins, reducing sugar, and tannin compounds (Surjeet. *et al*., 2011). Endophytic microorganisms are microbes that live as close associations with plants. Endophytic bacteria are also having the potential due to their ability to produce plant growth hormones, phosphate solubilization, nutrient acquisition and fixation of N2 (Glick, 2012). Endophytic bacteria have different roles for the plants. Phosphorus plays a key role in root development, root traits anatomy modifications and root hair density with a significant contribution in increasing yield of crops (Elhaissoufi *et al* .,2020). Zinc solubilizing microorganisms solubilize zinc through various mechanisms, one of which is acidification (*Kushwaha et al*., 2021). The biological nitrogen fixing bacteria and plants exchange nutrients because plants provide the carbon source required for microbial growth and in return, microorganisms provide the fixed nitrogen to plants and promote their growth (Santoyo *et al*., 2016).

2.3 Importance of Endophytic bacteria

Plant endophytes play a key role in the growth and development of plants. They can grow inside plants and improve plant growth under normal and challenging conditions and benefit host plants directly by improving plant nutrient uptake, modulating growth and stress related phytohormones. They can serve as a nutrient feeder for the plant. They share all the important traits consistent with the host plant growth promotion found in rhizobacteria. They can also, regulate secondary metabolites with significant medical properties and produce diverse biological effects. The Endophytic bacteria can not only promote growth of the host plant, but also help the host in tolerating stress conditions, and produce allelopathic effects against other competing plant species (Cipollini *et al*., 2012).

Endophytic bacteria differ from epiphytic bacteria by colonizing the interior of plants and from plant pathogens by not causing visible damage to the host plant. This colonization occurs at some stage of their development and considered as intermediate group between saprophytic pathogenic bacteria (*Mitter et al.,* 2017). The plants by themselves cannot fix atmospheric Nitrogen. So bacteria are the only microbes that have the ability to change atmospheric nitrogen first in to Nitrites and then in to Nitrates. As a result Nitrates can be used by plants as a natural fertilizer (Santoyo *et al*., 2016).

2.4 Mechanism of endophytic bacteria in plants

Bacteria established themselves on the roots surface; they start to penetrate into the root interior using specialized mechanisms. The process of penetration into the host can be active or passive. Passive penetration can occur at cracks present at root emergence areas, root tips or those deleterious organisms (Sheoran *et al.,* 2015). Active penetration by competent endophytic bacteria achieved by means of dedicated machinery of attachment and proliferation that involves presence of lipopolysaccharides, flagella, pili, twitching, motility and quarum sensing. After entry into the roots, the endophytic bacteria can spread systematically to colonize above the ground tissues. They can establish into the stem and leaves under natural conditions. The bacteria that migrate to the above ground plant tissue are then well adapted to particular endophytic niche. Bacterial movement inside plant is supported by bacterial flagella and plant transpiration stream (*Mitter et al.,* 2017). Migration along intercellular spaces requires the secretion of cell wall degrading enzymes such as cellulases and pectinases. Movement through xylem elements occurs through perfolated plates that allow movement of bacteria through large pores without requiring cell wall degrading enzymes (Sheoran *et al.,* 2015).

2.5 Role of endophytic bacterial cells for plant growth promotion

The biological nitrogen fixing bacteria and plants exchange nutrients because plants provide the carbon source required for microbial growth and in return, microorganisms provide the fixed nitrogen to plants and promote their growth (Santoyo *et al*., 2016).The atmospheric nitrogen is reduced to ammonia catalyzed by bacteria and archaea nitrogenase enzymes. It can be absorbed by plants from the soil as nitrate, ammonia, or in ammonium (NH4+) form. The microbes in plant has high nitrogen (N) fixation capacity due to the availability of various biological nitrogen fixing (BNF) microorganisms, such as free-living bacteria in soil and endophytic N-fixing in plants (Xing *et al*., 2016).Nitrate reductase (NR), glutamine synthetase (GS), and glutamine oxoglutarate aminotransferase have been used to assess the influence of endophytic bacteria on the uptake of N from the soil, its metabolism and use efficiency in plants (Bisht *et al*.,2020).

Phosphorus is an essential macronutrient directly involved in nucleic acids, cells division and growth of new tissues, which all regulate protein synthesis and energy transfer. This nutrient is needed for diverse cellular processes like photosynthesis, carbohydrate metabolism, energy production, redox-homeostasis, and signaling (Siedliska A, 2021).It plays a key role in root development, root traits anatomy modifications and root hair density with a significant contribution in increasing yield of crops (Elhaissoufi *et al* .,2020). Water-soluble phosphorus improves soil mineral fertility and increase P availability in soils; thereby plant P uptake will be enhanced leading to a higher plant productivity and yield (Meyer *et al*., 2018).

Phosphate solubilizing bacteria can be efficient in making Phosphorus more available to plants from both organic and inorganic sources by solubilizing and mineralizing insoluble Phosphate compounds (Chen, F *et al*., 2016).

Plants can uptake zinc as a cation but only a very minor portion of total zinc is present in soil solution as soluble form. Rest of the zinc is in the form of insoluble complexes and minerals Due to unavailability of zinc in soil, zinc deficiency occurs which is one of the most wide spread micronutrient deficiency. To reduce zinc deficiency, fertilizers have been applied in the form of zinc sulfate. Zinc solubilizing microorganisms solubilize zinc through various mechanisms, one of which is acidification. These microbes produce organic acids in soil which sequester the zinc cations and decrease the pH of the nearby soil. Zinc solubilizing bacteria are potential alternatives for zinc supplementation and convert applied inorganic zinc to available forms (Pandey et al., 2018).

2.5. Bioactive compounds of *Rumex nepalensis*

Phytochemical investigation of *Rumex nepalensis* has a large number of organic complex and biologically active compounds. *Rumex nepalensis* contains various constituents such as triterpenoids, stilbene glycosides, tannic acid, saponins, resveratrol, sterols, amino acids, quercetin, alkaloid, phenolic components, flavonoids, anthraquinones glycosides, anthraquinones and Vitamin C (Anusuya *et al*., 2012). The root and leaves extracts of R. nepalensis have also shown significant antimicrobial and antifungal activities. *R. Nepalensis* is a high value medicinal herb due to its high anthraquinone content. *R. nepalensis* has shown purgative, antioxidant, antifungal, antibacterial, antihistaminic, anticholinergic, antibradykinin ant prostaglandin, antipyretic, and anti-inflammatory, antialgal, insecticidal, analgesic and CNS depressant properties (Wahid *al*., 2013).The organic compounds will be isolated from root of *R.nepalensis* using common organic solvents such as ethyl acetate, per ether, n-butanol, ethanol and methanol systems (Anusuya *et al*., 2012).

2.6 Diversity of endophytic bacteria

There are different endophytic bacteria found in plant R. nepalensis such as *E. coli, Pseudomonas, Bacillus, Acinetobacter, Actinobacteria, Inquilinus, Pentoea* and *Sphingomonas*. These bacteria have different roles and uses. Some of the examples are E. coli for antibacterial activity and *Aspergillus niger* for its antifungal activity due to the presence of steroid, saponins, reducing sugar and tannin compounds (Surjeet *et al*., 2011). Pseudomonas, Bacillus, Inquilinus and Pentoea show positive plant growth properties.

2.7 The effect of soil and endophytic bacteria on growth of plants

Different plant species can coexist in a local area, and their presence may be determined by different soil microbes and nutrients (Carteron *et al*., 2020). Soil nutrients and soil microbes are two main factors that affect plant production, diversity and population development. Also Soil microbes and nutrients can influence plant photosynthetic performance, growth rates and yield (Castle *et al.,*2016). The microbes are common in soils of all plant communities and exert deep effects on plant performance directly through mutualistic or pathogenic interactions and indirectly by participating in nutrient cycling (Kutakova *et al*., 2020).

Soil nitrogen and phosphorus cycles are catalyzed mostly by the activities of soil extracellular enzymes, which are synthesized mainly by soil microbes. Soil nutrient conditions are governed primarily by soil microbes that may decompose complex organic matter and release mineral elements by secreting extracellular enzymes (Raiesi *et al*., 2018).

2.8 Identification of endophytic bacteria using MALDI-TOF MS

The isolated endophytic bacteria was investigated using the matrix assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) for identification based on growth conditions. The MALDI-TOF MS for isolate identification was performed following the methods of toubal (Toubal et al., 2018). The technique was used to analyze different types of organic molecules such as nucleic acids, organic molecule solutions, proteins, and completely microbial cells (Dekker and Branda.,2011). It was used for rapid identification of bacteria present in water, air and different surfaces. There were different approaches to identification at strain level using MALDI-TOF MS. The mass spectra was loaded for different *Bacillus* strains such as B.marisflavi, B.pumilus, B.infantis, B.subtilis, B, cereus and B. atrafaeus in a stack view (Alsayegh et al., 2021). MALDI-TOF MS relies on the detection of mass to charge ratio abbreviated as m/z of ribosomal proteins of the bacteria, which helps to provide a unique mass spectrum of the bacteria within a short period of time (Carbonnelle et al., 2011).

3. Material and methods

3.1. Description of Study area

The study was conducted at Adama Science and Technology University. Adama town is located at 8.54°N and 39.27°E, at an elevation of 1712 m, about 99 km southeast of Addis Ababa. It is located between the base of an escarpment to the west and the Great Rift Valley to the east. Adama is rapidly growing major city near Addis Ababa in central Ethiopia, with a total population of 456,868 in 2022 (Ethiopian statistics service web). The plant samples were collected from the Oromia region Arsi zone Diksis woreda.

3.2. Sample collection

Disease free and mature root of R. nepalensis was collected from Diksis. Fresh Plant leave, stem and root samples were transported to Applied Biology microbiology laboratory under aseptic conditions for isolation of Endophytic bacteria 24 h after collection.

3.3. Surface decontamination of collected root samples

Leave, root and stems were taken and washed thoroughly under plenty of running tap water to remove adhering soil particles and the microbes as show in in the figure 2 below. Root samples were cleaned with detergent sodium hypochlorite (2%) containing 0.1 tween 20 for 3 min. Finally, root samples were exposed to absolute alcohol for ˜10 sec and then washed three times with autoclaved distilled water (ADW). Surface decontaminated root was cut into pieces of about 1 cm^2 using sterile surgical blade. The root pieces were aseptically inoculated in Petri dishes containing Nutrient agar medium and then incubated at 30 °C for 18 to 20h in the dark. Plant samples were washed in sterile distilled water at every step of the surface sterilization process. For this surface sterilization, stem and leaf samples were placed on nutrient agar separately on three three Petridishes, incubated at 30 °C for five days as show in the Figure 2 below, and checked for feasible microbial growth.

Figure 2 Surface decontamination to isolate endophytic bacterial isolates.

3.4 Isolation, Incubation, growth initiation and preservation of endophytic bacterial isolates

Well grown colonies of cultivable bacterial putative-endophytes that are visible on the NA (on root legumes) would be randomly chosen for their further analysis. Pure culture of each putative endophyte was cultivated separately in universal bottles containing 10 ml LB medium.

The culture-cultivation was carried out at 30 °C, 160 rpm for 18h.The surface sterilized plant segments were cut into approximately 6 mm diameter disks under aseptic conditions and placed on nutrient agar plates, and they would be incubated for 72 h at 30 °C in an inverted position. After 72h, the plates were observed for bacterial growth surrounding the root, stem, and leaf sections. Endophytic bacteria emerged from the surface of plant segments were collected using an inoculation needle and further sub-cultured to obtain pure colonies. Single colonies acquired would be streaked on fresh nutrient agar plates for further purification. These pure colonies were preserved in refrigerator and used for further experimental procedures.

3.5 Characterization of Bacterial Isolates

All selected bacterial isolates were identified through morphological characteristics, such as colony color, margin, consistency, and texture, and microscopic characterization, such as gram staining, endospore staining, and motility. The biochemical and physiological tests like indole utilization, methyl red, and sugar utilization were performed according to standard procedures (Bullock & Aslanzadeh, 2013).

3.5.1 Microscopic characterization

3.5.1.1 Gram-staining

Gram staining was done on the endophytic bacteria to differentiate gram positive and gram species. Bacterial colonies were smeared on a drop of water on a clean glass slide and then left to air to dry. After that the slides were flooded with crystal violet dye for 45 sec rinsed under tap water. The slides were rinsed with 96% (v/v) ethanol for 20 sec and then rinsed under tap water. The slides were flooded with safranin for 1min and rinsed under tap water. Finally, the slides were allowed to dry and viewed under a microscope which is x100 oil immersion (Ebu et al., 2023).

3.5.1.2 Endospore staining

This activity was done on the endophytic bacteria to determine endospore producing ability. Bacterial colonies from pure culture were smeared on a drop of water on a clean slide and the smears were heated. Blotting papers were cut to fit the size of slides and placed on the slides. Then, the slides were flooded with 0.5% (w/v) of malachite green in a 100 ml of distilled water ordered to heat of a flame for 5min.Blotting papers were removed and the slides were rinsed with tap water .The slides were flooded with safranin (0.1%, w/v) for 1 min and then rinsed with tap water. Lastly, the slides allowed to dry and observed under a microscope of 100x oil immersion objective lens (*Hussey et al., 2011).*

3.5.1.3. Motility test

SIM medium (Beef extract 3 g/L; Na2S2O3 0.025 g/L; Peptic digest of animal tissue 30 g/L; Peptonized iron 0.2 g/L and Agar 3 g/L) was prepared and was poured into test tubes. After solidifying the test tubes were inoculated by stabbing in the center to half an inch. Then the test tubes were incubated for 24 hours at 37^{o}C. After 24 hours the test tubes were checked for the formation of a black precipitate (H2S production) and fuzzy area surrounding the stab line (motility) and then 2-3 drops of kovac‘s indole reagent was added to the tubes and the formation of red band on the upper and blackening from the center was checked (Vashist *et al.*, 2013).

3.5.2 Biochemical tests

Catalase test: Catalase test was conducted for obtained endophytic bacterial isolates. This test was applied to identify the presence of catalase which converts hydrogen peroxide to water and oxygen as follows. About 4 to 5 drops of 3% (v/v) H_2O_2 was added in a test tube and a small amount of isolated bacterial colony was added to the test tube and the formation of bubbles was examined and the result was recorded (Bullock & Aslanzadeh, 2013).

Citrate utilization test: Citrate test would be carried out to test the ability of the bacteria to use citrate as a carbon source. About 18-24hr colonies were streaked on Simmons citrate agar slants (g/l) (Bromothymol blue 0.08; $MgSO_4$ 0.2; $(NH_4)_2HPO_4$ 1 and Agar 15) and incubated at 30 C 2- 4 days (Bullock & Aslanzadeh, 2013).

Indole test: A sterilized tube containing 4ml of tryptophan broth would be inoculated with 18-24 h old culture of endophytic bacterial isolates and incubated at 30 ^{o}C for 24 h. Then, 0.5 ml of kovac’s reagent was added into test tube containing pure culture endophytic bacteria isolates, these efficiency for siderophores biosynthesis (Darkoh et al., 2015).

Methyl red test. This test determines if an organism metabolizes glucose and utilizes mixed acid fermentation pathway and produces strong acid end products (lactic, acetic, or formic) that are detected by the indicator methyl red. About 5 ml MR-VP broth tube was inoculated with the organism and would be incubated at 30 °C for 48-72hr. About 2.5 ml of the broth culture would be transferred to a fresh tube and inoculated with five drops of methyl red indicator (Bullock & Aslanzadeh, 2013).

Urease test*:* Urease test was used to determine the bacteria's ability to produce urease and convert urea into CO_2, H_2O, and NH_4. 18-24hr pure colony was streaked on urea agar slants prepared using 3 g of urea agar base (g/l) (Dextrose 1; Disodium phosphate 1.2; Monopotassium phosphate 0.8; NaCl 5; Peptone 1; Phenol red 0.012 and Agar 15) with addition of 40% (w/v) urea which will be added to the agar base after sterilization and cooling down. The cultures were incubated 30-32^{o}C for up to 5 days. The presence of urease was observed by color change from yellow to pink

Voges-proskaure test*:* The VP test detects the production of acetoin by organisms that utilize the butylene glycol fermentation pathway. In the presence of air and potassium hydroxide, acetoin is oxidized to diacetyl, which produces a red-colored complex. About 5 ml MR-VP broth tubes were inoculated with pure bacterial culture and incubated at 30 °C for 18–24rh. About 2.5 ml of the broth culture was transferred to a fresh tube and inoculated with six drops of α-naphthol followed by three drops of KOH. Then, The tubes were shaken and left for 10 min and color change was recorded (Bullock & Aslanzadeh, 2013).

3.6 Hydrolytic enzyme assay test

The presences of the following enzymes were analyzed. Amylase, Lipase, Cellulase, Protease and Xylanase. The pure cultures of the isolates were inoculated into solid diagnostic media by four isolated droplets. Enzyme index was used to compare the enzymatic production of different isolates (Carrim et al., 2006).

Amylase activity. Autoclaved distilled water was used for negative control and known producer strain for positive control. The strains were inoculated in to nutrient agar supplemented with 1%starch.After incubation for two days at 25^{0}C, Agar plate surfaces were treated with iodine solution which allow unstained zone around active amylase colonies (Luang et al., 2019).

Cellulase activity. The isolates were inoculated on to the medium containing $NaNO_3$ 1g, K_2HPO_4 1g, KCl 1g, $MgSO_4$ 0.5g, Yeast extract 0.5g, glucose 1g,Carboxymethylcellulose 5g and agar 15g. The plates were incubated at 25^{o}C for 5-8 days. At the end of the incubation Congo red solution (0.2%, w/v) was added to Petridishes and kept at ambient temperature for 20 min. Then the Petridishes were washed by adding 5 M of NaCl solution to remove excess dye and kept at room temperature for another 30 min. Colonies with the light-

yellow zone around the colony on a red background can be seen that shows Cellulase positive (Nxumalo *et al.*, 2020).

Protease test. Nutrient agar containing skimmed milk powder (1%, w/w) was used to prepare a protease substrate. Milk powder (10g/100ml) was sterilized at 110 ^{o}C for 5 min, cooled to 45 ^{o}C and added in to a basal medium in aseptic condition for two or three days at 25 ^{o}c A transparent zone formation was seen that shows a protease activity (*Rupali,* 2015).

3.7 Characterization of Plant growth promoting abilities of the isolated Endophytic bacteria

3.7.1. Phosphate solubilization test

The phosphate solubilizing ability of the isolated endophytic bacteria was tested by spot inoculating on Pikovskaya medium ($Ca_3(PO)_4$, 5 g/L; $FeSO_4.7H_2O$, 0.002 g/L; Glucose, 10 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 0.2 g/L; $(NH4)_2SO_4$, 0.5 g/L; KCl, 0.2 g/L; Yeast extract, 0.5 g/L; $MnSO_4.H_2O$, 0.002 g/L and Agar, 15 g/L at pH 7). The medium was dissolved by boiling and shaking gently, and then was sterilized by autoclaving at 121 ^{o}C for 15 min. The medium was poured into sterile Petri dishes and after solidifying was inoculated by fresh colonies of the isolated endophytic bacteria. The plates were incubated at 30 ^{o}C for 7 days and zone of phosphate solubilization was observed (Pande *et al.*, 2017).

3.7.2. Zinc solubilization test

The zinc solubilization ability of the endophytic bacteria was examined on minimal salts media. The minimal salts medium (Dextrose, 10 g/L; KCl, 0.2 g/L; K_2HPO_4, 0.1 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $(NH4)_2SO_4$, 1 g/L and Agar 15 g/L at pH 7-7.2) was prepared and insoluble zinc 1 g/L was added in the form of zinc oxide (ZnO). The medium was boiled to dissolve and was sterilized at 121 ^{o}C for 15 min. The sterilized medium was poured into sterile petri dishes and after solidifying the plates were spot inoculated with fresh colonies of the isolated bacterial colonies. The plates were then incubated at 30 ^{o}C for 14 days and examined for halo zones after incubation (Kamran *et al.*, 2017).

3.8 Evaluation of antimicrobial activities

The crude extract was prepared into two concentrations (1 g/ml and 0.5 g/ml) for the antibacterial activity analysis. Agar well diffusion method was used to evaluate the antibacterial activity of the extracted secondary metabolites from the isolates. Three bacterial strains *B. subtilis, E. coli,* and *Pseudomonas aeruginosa* were spread on Mueller-Hinton agar plates and 6mm wells were made using sterile cork borer. 50µl of the crude extracts and both the positive and negative controls were added in the wells. Ciprofloxacin 20 µg/ml was used as positive control and methanol was used as a negative control. The plates were incubated at 37oC for 24 hrs. After incubation the inhibition zones were measured using a ruler and recorded (Nxumalo *et al.*, 2020).

3.9 Data analysis

The data were analyzed using various software's. Mean values, standard errors, and standard deviation were calculated using Microsoft excel version 10. The effect of pH value, temperatures, and salt concentration, carbon and nitrogen sources against these endophytic bacterial isolates analyzed using Origin 2019b.

4 Result and discussion

4.1 Isolation of endophytic bacteria from R. Nepalensis

In this study, a total of eight endophytic bacterial isolates were obtained from the leaf, root and stem parts of *R. nepalensis* that were collected from the various areas. After *R. nepalensis* parts were sterilized and incubated for five to seven days, endophytic bacteria with various colors were detected which were emerged on the NA medium as indicated in the Figure 3 $_{a\text{-}f}$. As indicated in the Figure 3$_{a\text{-}f}$, the colonies of certain of these emerged endophytic bacterial isolates were found to be dark brown and white color. However, some of them characterized as white and white rhizoid colonies as illustrated in the Figure 3 $_{a\text{-}f}$ after 72 h of their growth.

In line with this study, it was reported that a total of 853 endophytic bacterial isolates were obtained from agronomic crops and prairie plants of which they were belonging to both Gram-positive and negatives (Ebu et al., 2023).The same authors identified these endophytic bacterial isolates as *Cellulomonas*, *Clavibacter*, *Curtobacterium*, and *Microbacterium* based on the 16S rRNA gene sequence, fatty acid analysis, and carbon source utilization efficiency. However, the recent of these endophytic bacterial isolates were belonged to *Proteus vulgaris, Psedomonas stutzeri, Cronobacter skazakii* and *Serratia Liquifaciens* based on the MALDI TOF MS characterization. In the previous study, endophytic bacterial genera such as *Acinetobacter, Brevundimonas, Frigoribacterium, Kocuria, Sphingomonas, Sporosarcina* and *Staphylococcus* were reported from the cultivar of the leaves of the common bean (*Phaseolus vulgaris*) using 16s RNA techniques (de Oliveira Costa et al., 2012).

As illustrated in the Figure 3$_{a\text{-}f,}$ these endophytic bacterial isolated were emerged on the NA medium after 5-7 days of incubation period at 30 ^{o}C These isolates with various colors were obtained from leaf, root, and stem parts of *R. Nepalensis.* These isolates were associated with *R. Nepalensis* and suggested that they could have promoted the growth of this plants by providing certain nutrients and minerals. In agreement with this study, it was reported that plant growth promoting (PGP) strains such as EU-A2SK1, EU-M4ARAct, and EU-E1RT3-1 which were identified as *Ewingella americana, Pseudomonas brenneri,* and *Pantoea agglomerans*, respectively were suggested for phosphorus (P) and potassium (K) solubilization (Rana *et al*., 2021). The same authors further stated that these endophytic bacterial isolates can assist for nitrogenase activity, production of indole acetic acids, and siderophores against maize (*Zea mays*)

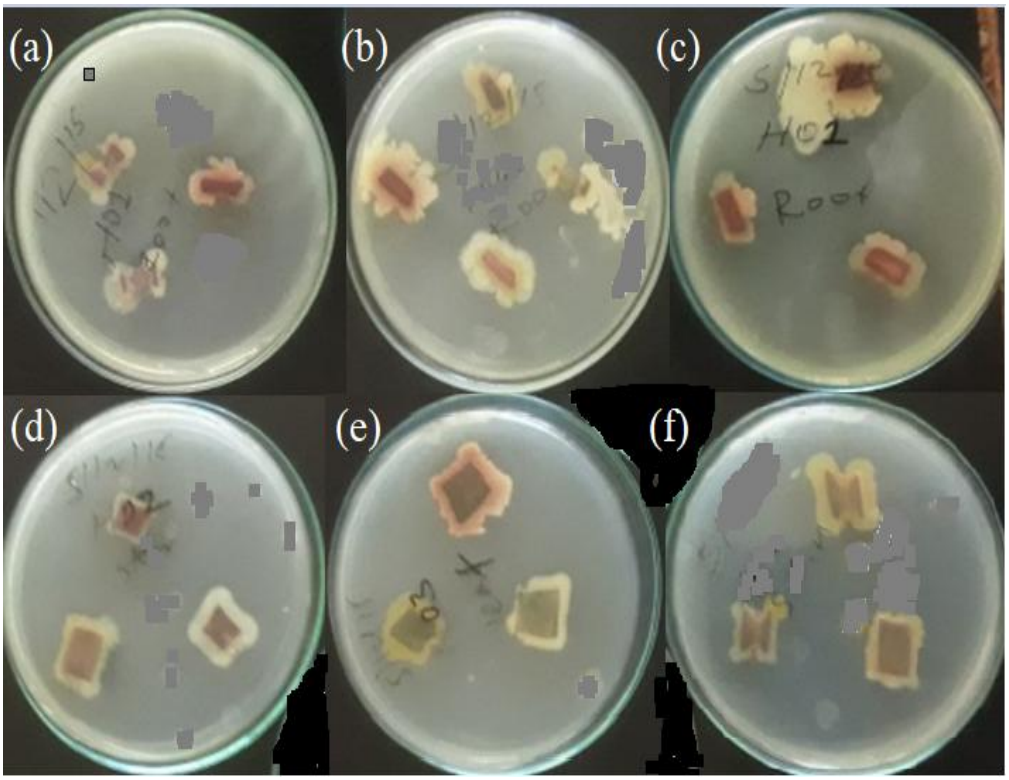


Figure 3, Bacterial isolation-a technique of separation of one species of bacteria from the mixed culture
(a), (b), and (c) Endophytic bacterial isolates which were obtained from the leaf part of *R. Nepalensis.* (d) and (e) These of endophytic bacterial isolates which were obtained from the root part of *R. Nepalensis* of which they were grown on the NA. (f) Indicated certain isolates of endophytic bacterial which were obtained from the stem part of *R. Nepalensis.*

4.2 Characterization of the endophytic bacteria

Gram staining: Gram staining was differentiated based on Gram reaction (Table 1). Most of these endophytic bacterial isolates were Gram-positive (Table 1) (Figure $4_{a\text{-}g}$). They are also rod-shaped in terms of morphology, as they were observed using a microscope. Gram negative bacteria are resistant to antimicrobial properties and gram positive bacteria are sensitive. In the previous study, specific Gram-positive endophytic bacterial isolates were also reported from various parts of *Artemisia annua, Moringa oleifera, Ocimum lamiifolium* and *Gloriosa superb* (Ebu *et al*., 2023; Kiros *et al*., 2023).

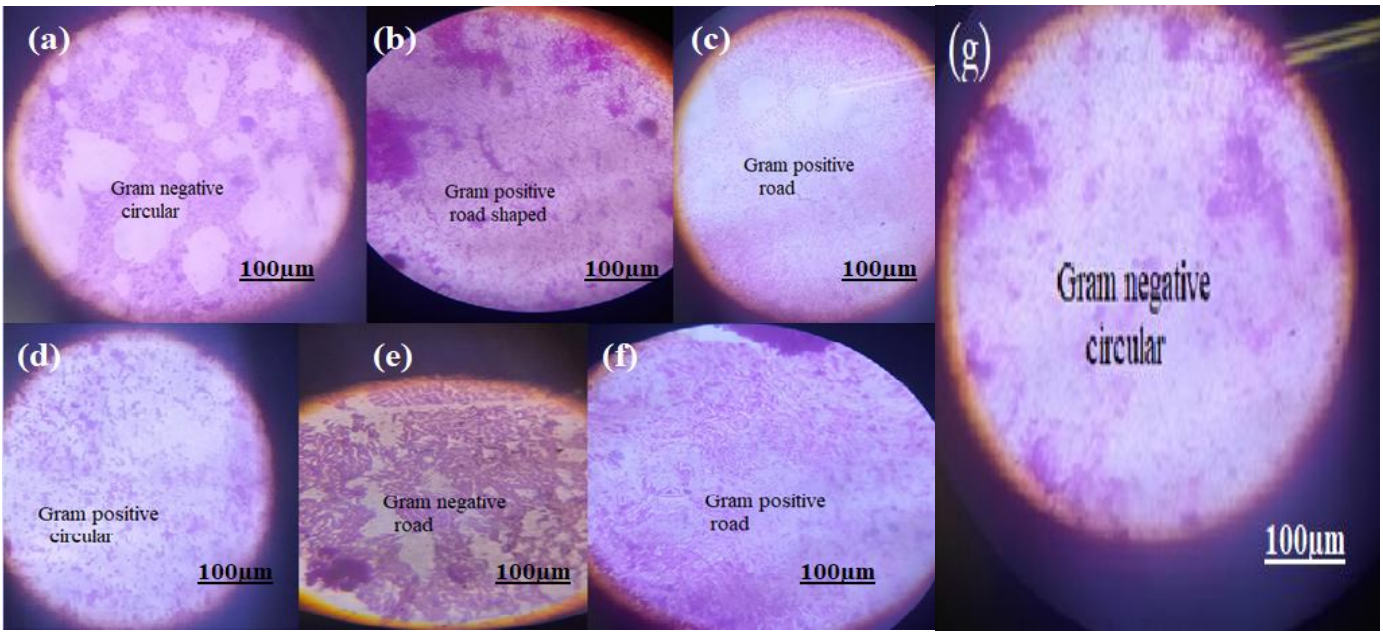


Figure 4: Gram staining: (a) Gram negative isolate (*Serratia liquefaciens* **EIRL-2), which was encountered from leaf part of** *R. nepalensis***. (b) and (c) were Gram-positive isolate (***C. scazaki* **EIRS-2)which were encountered from stem part of** *R.nepalensis.* **(d) Gram positive isolate (*Not identified* EIRR-1) was encountered from root part of** *R. nepalensis***. (e) Gram-negative isolate (*P. stutzeri* EIRL-1), which encountered from the leaf part of** *R. nepalensis* (f) **Gram positive isolate (***Cronobacter skazakii* **EIRR-3) was encountered from root part of** *R. nepalensis* **and (g) Gram-negative isolate (***Proteus vulgaris* **EIRL-1), which encountered from root part of** *R. nepalensis.*

Table 1: Endophytic bacterial isolates characterization and identification based on biochemical test and MALDI TOF MS techniques, respectively

S.N.	Type of Isolates	Sample sources	Gram-reactions	Catalase test	Indole test	Citrate test	Methyl red test	Urease test	Voges Proskauer test	Type of isolates based on protein profiles
1	EIRL-1	Leaf	-ve	-ve	-ve	+ve	+ve	+ve	-ve	*Psedomonas stutzeri* EIRL-1
2	EIRL-2	Leaf	-ve	NT	-ve	+ve	+ve	-ve	+ve	*Serratia liquefaciens* EIRL-2
3	EIRR-1	Root	+ve	-ve	+ve	+ve	+ve	+ve	-ve	NI
4	EIRR-2	Root	-ve	-ve	-ve	+ve	-ve	+ve	-ve	*Proteus vulgaris* EIRR-2
5	EIRR-3	Root	+ve	-ve	-ve	+ve	-ve	-ve	-ve	*Cronobacter skazakii* EIRR-3
6	EIRS-1	Stem	+ve	-ve	-ve	+ve	+ve	-ve	+ve	*Serratia liquefaciens* EIRS-1
7	EIRS-2	Stem	+ve	-ve	-ve	+ve	+ve	-ve	-ve	*Cronobacter skazakii* EIRS-2
8	EIRS-3	Stem	+ve	-ve	-ve	+ve	-ve	-ve	-ve	*Cronobacter skazakii* EIRS-3

Key: NT indicated not tested. NI indicated Not identified, -: Negative, +: Positive

EIRL indicated Endophytic bacterial isolates which obtained from the *R. nepalensis* leaf part. EIRR indicated Endophytic bacterial isolates which obtained from the *R. nepalensis* root part and EIRS indicated Endophytic bacterial isolates which obtained from the *R.nepalensis* stem part.

Motility test: The recent study showed that *S. liquefaciens* EIRS-1 was motile and negative for hydrogen sulphide and Indole tests, while *C. skazakii* EIRR-3 and EIRS-2 were non-motile, with no hydrogen sulphide, and the Indole test had negative results (Table 1). *P. vulgaris* EIRR-2 was motile, positive hydrogen sulphide and negative Indole test, while *C. Skazakii* EIRR-3, obtained from the root of R. *nepalensis* was non-motile, with no hydrogen sulphide and negative indole negative (Table 1). *S. liquefaciens* EIRL-2 and *P. stutzeri* EIRL-1, isolated from the leaf part of *R. nepalensis* were motile, positive for hydrogen sulphide and negative indole tests (Table 1, additional file: Figure-A_{1-2}). Motility media differentiated bacteria and other microbial isolates (Barrow & Feltham, 1993). Only motile bacteria can move away from the line of vaccination in the low concentration of agar; descendants of cells that have migrated throughout the medium show up, as evidenced by turbidity (cloudiness) in the medium. The higher concentration of agar makes the gel too firm to permit organisms to spread (Vashist *et al.*, 2013).

a) Shows indole test of the isolated endophytes from *R. nepalensis*. In this test Eight endophytic bacteria were characterized and from these strains the bacteria isolated from EIRR-1 (white rhizoid root) , *Proteus vulgaris* EIRR-2, *Cronobacter skazakii* EIRR-3 and *Serratia liquefaciens* EIRS-1were indole positive that change their color into red which shows the formation of a ring and they were able to produce tryptophan enzyme. These of endophytic bacterial isolates which were obtained from the root and stem part of *R. Nepalensis* (a) of which they were grown on the NA. (b) Shows that isolated endophytes R. nepalensis in that all were citrate positive since the green color was changed into blue that shows endophytes were utilize citrate as a source of carbon.

(c) In VP test from eight strains only *Serratia liquefaciens* (EIRS-1)has a positive result by forming red color in that the endophyte produces acetylmethylcarbinol from glucose-phosphate broth when alpha naphthol and KOH were added to Vp broth where as others were remain yellow that are negative result.

Methyl red test: EIRR-1, *Pseudomonas stutzeri* EIRL-1, and *S. liquefaciens* EIRL-2 were found to be positive against methyl red test. These isolates can utilize glucose and produce acid as end products by forming a red colour, while the others remain yellow and have a negative result, as shown in Additional file Figure A_2. Similarly, it was noticed that specific endophytic bacterial isolates, which screened from the Leaves, Roots, and Stems Parts of *Artemisia annua, Moringa oleifera*, and *Ocimum lamiifolium* Plants were reported as methyl red positive (Ebu *et al*., 2023) (Table S_1)

Urease test: In this study, eight strains of endophytic bacteria *Proteus vulgaris* EIRR-2 positive, *Serratia liquefaciens*- EIRL-2 and EIRR-1 exhibited negative results against urease tests (Table 1). Only *Proteus vulgaris* change the colour from yellow to pink to produce urease enzymes and convert urea into CO_2, H_2O, and NH_4. However, as indicated in Table 1, the rest of these endophytic bacterial isolates screened from various parts of *R. nepalensis* were negative for the urease test. This isolate cannot produce an entirely pink colour and cannot convert urea into CO_2, H_2O, and NH_4 (see figure 5). The positive control remains yellow because it has no endophytic bacteria.

Figure 5 Biochemical tests

4.3 Hydrolytic enzyme test

Amylase test: unstained zone around active amylase colonies was formed by endophytic bacteria which were isolated from stem EIRS-3(e), EIRS-1(c) and EIRS-2 (d), leaf part EIRL-2(g) and EIRL-1(a) (Additional file: Figure A3). But unstained zone around active amylase colonies was not formed by the endophytic bacteria isolated from root part (EIRR-2) (f), EIRR-3 (b) and EIRR-1.Thus,Amylase + bacteria can hydrolyze starch(amylose and amylopectin). They degrade amylose, amylopectin, cyclodextrins, glycogen and dextrin's, but they possess highest specificity toward starch (Luang et al., 2019).From our findings, the colonies showing clear zones of iodine solution were taken as positive starch-degrading bacterial colonies. All bacterial isolates show similar colony morphology and appeared as gram positive and rod shaped.

Cellulase test: In the present study, specific endophytic bacteria isolates obtained from various parts of *R. nepalensis* were found to be positive for cellulase enzyme (Table 2). Some of these isolates are including such as *P. vulgaris* EIRR-2, *C. Skazakii* EIRS-2, EIRS-3*, P. Stutzeri* EIRL-1 and *S. Liquefaciens* EIRS-1 of *R. nepalensis* were selected for their Cellulase producing ability. For this test, Carboxymethyl cellulose (CMC) agar was used. In this study, a total of 6 (75%) of the isolated bacteria were able to produce cellulose-degrading enzymes.2 (25%) isolates did not produce cellulose-degrading enzymes. Several studies on the isolation and characterization of cellulose-degrading bacteria from industrial wastes showed that only a small number of bacteria can produce large amounts of bioactive compounds capable of complete hydrolysis of crystalline cellulose in vitro (Nxumalo *et al.*,

2020).

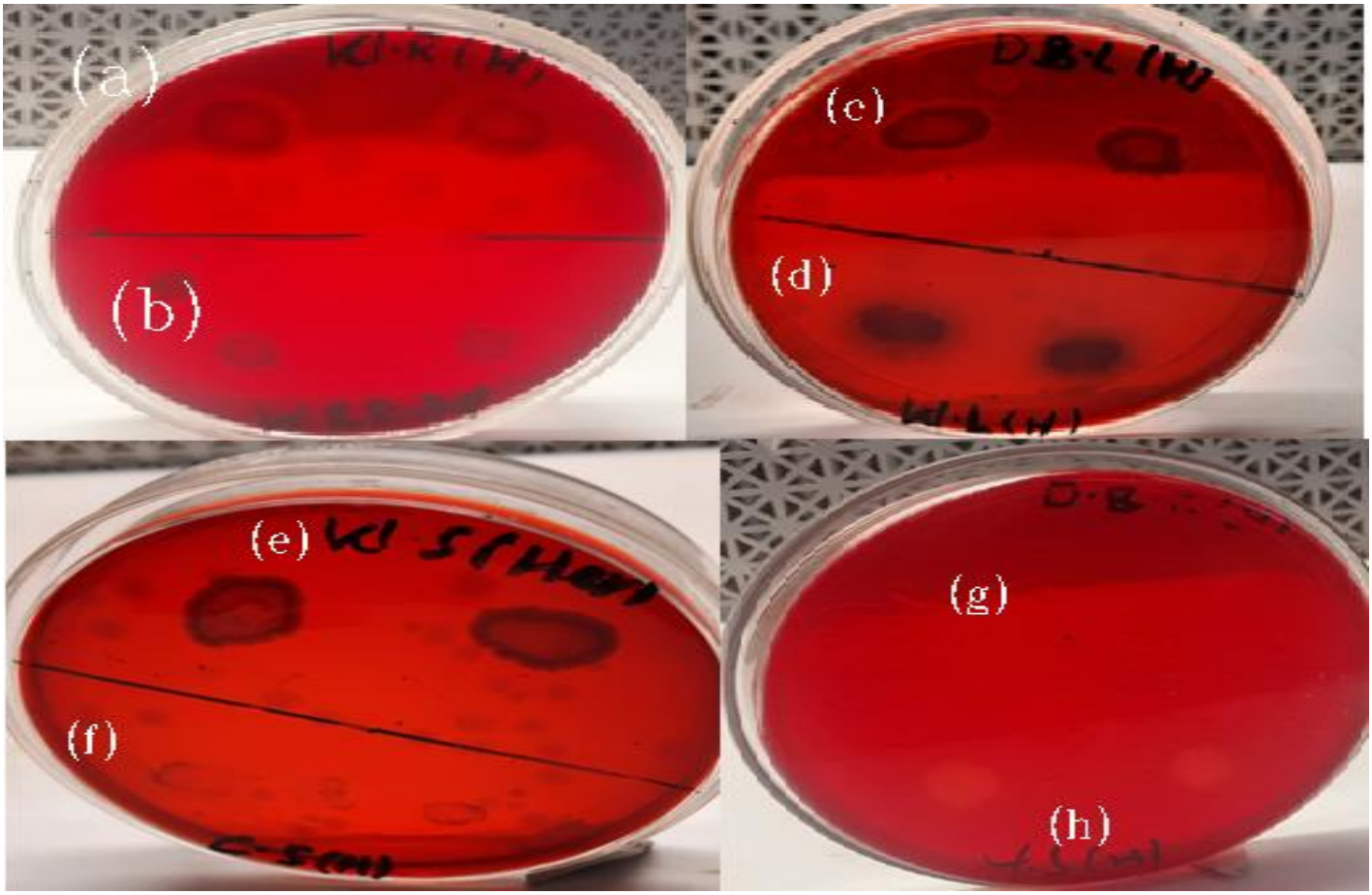


Figure 6.Cellulase test result (a) *C. Skazakii* EIRR-3, (b) NI EIRR-1 (C) *C. Skazakii* EIRR-3,(d) *S. Liquefaciens* EIRL-2 (e) *C. Skazakii* EIRS-2 (f) *C. Skazakii* EIRS-2 (g) *P. vulgaris* EIRR-2 and (h) *S. Liquefaciens* EIRs-1 which were isolated from root, stem and leaf of *R.nepalensis*.

Protease test: After the addition of skim milk powder to endophytic bacteria extracted from eight strains, a transparent zone formation was seen on three isolates, which were obtained from various parts of *R. nepalensis* against *C. skazakii* EIRS-2, *P. vulgaris* EIRR-2 *P. stutzeri* EIRL-1, *Cronobacter skazakii* EIRS-3, EIRR-1, *C. skazakii* EIRR-3, and *S. liquefaciens* EIRS-1 (Figure 7, Table 1& 2). However, the protease test did not detect against *S. liquefaciens* EIRL-2, which was obtained from the leaf part of *R. nepalensis*. (*Rupali,* 2015) reported that the amount of protease secretion by proteolytic isolates varied with the growth of bacteria and with the substrates used to detect proteolytic

activity.

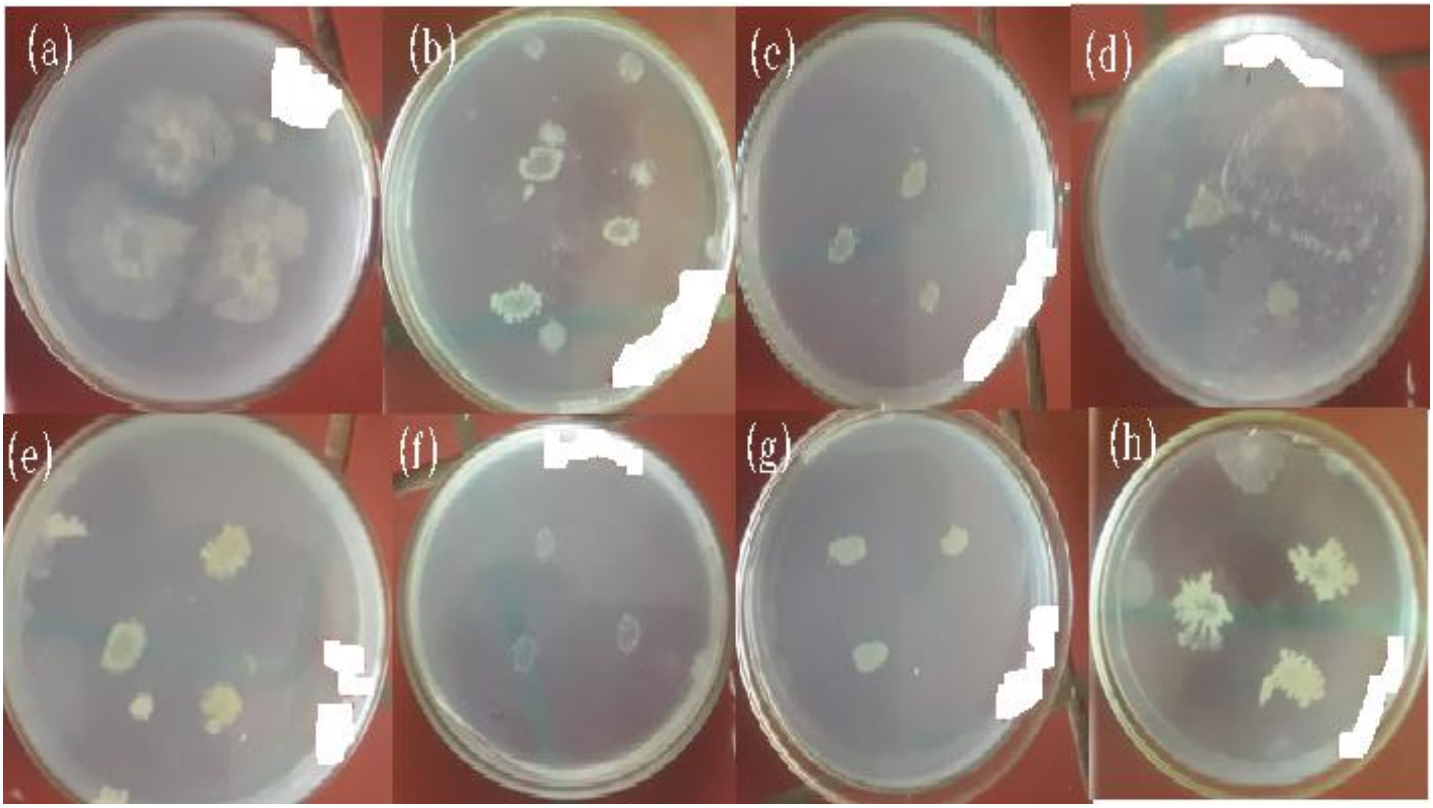


Figure 7. Protease test: As shown in the figure above (a-h), the bacteria (b, c and d) were isolated from root part, stem part (a,e and f) and leaf part (g and h) of *R.nepalensis* plant was tested for their protease enzyme utilization abilities.

4.4 Characterization of Plant growth promoting abilities of the isolated endophytic bacteria.

4.4.1 Phosphate solubilization test

The results obtained from this study showed that out of the eight endophytic bacterial isolates, 7 (87.5%) of them can solubilize the insoluble phosphate, which is added into Pikovskaya‘s agar in the form of tri-calcium phosphate. The isolate *EIRR-1 (NI)* showed the highest clearing zone (2.7cm) with a 1.93cm phosphate solubilizing index. Isolates *Cronobacter Scazaki* EIRS-2 showed the lowest clearing zone with 1.1mm and a solubilization index of 1.73cm (Figure 8). Further, others reported that endophytic bacterial cells, when used as bio inoculants, promote plant growth and yield, suppress specific pathogenic bacterial cells, can be used to remove certain chemical contaminants, as well as for phosphate and zinc solubilization, and contribute to nitrogen assimilation to the diversity of plants (Rosenblueth & Martínez-Romero, 2006). Complete data on the isolates with phosphate solubilizing ability is indicated in the appendices section.

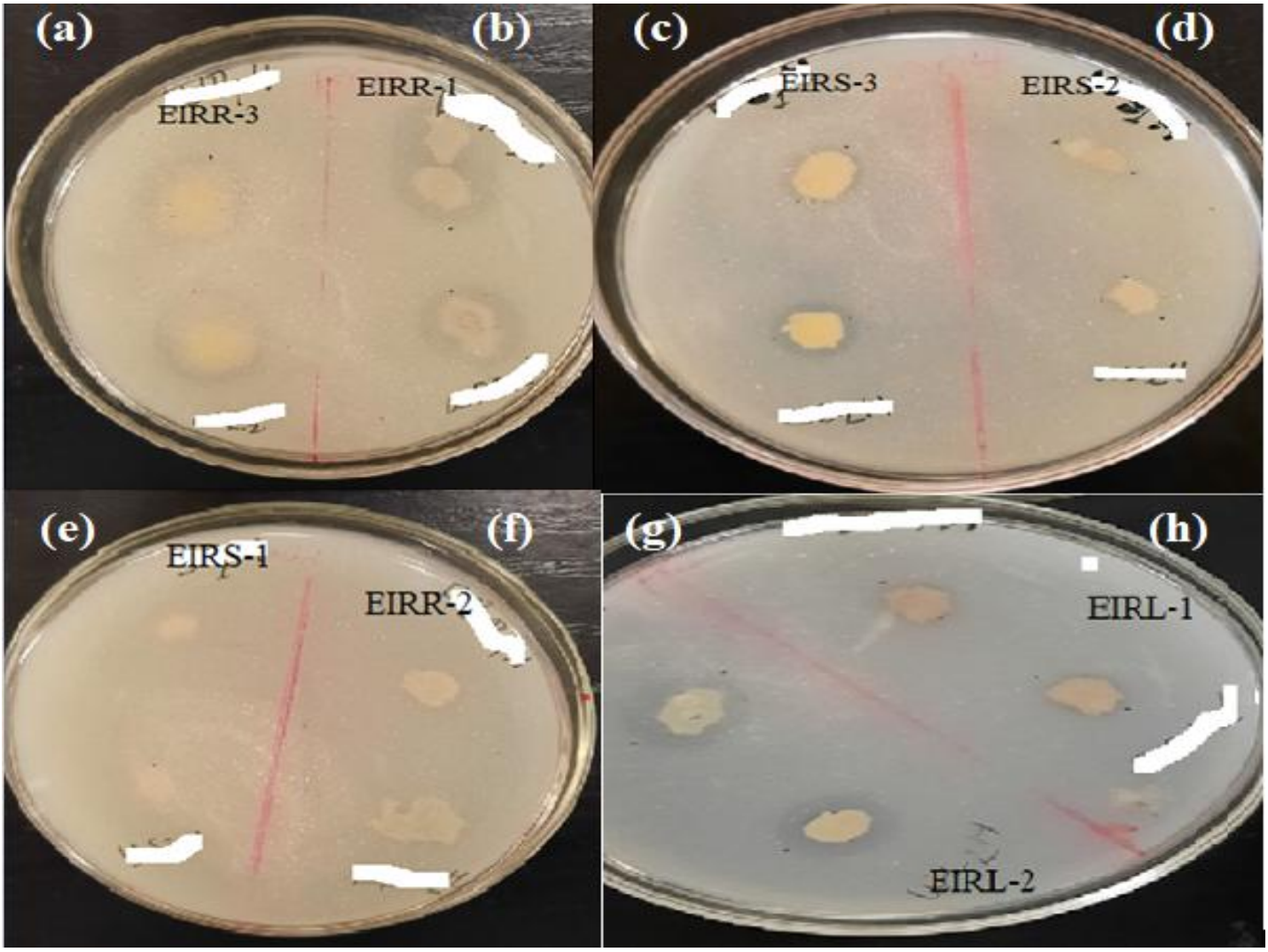


Figure 8: Certain types of phosphate solubilizing endophytic bacterial isolates which were encountered from different part of *R. nepalensis. (*a) Represents a bacteria isolated from white root(*Cronobacter skazakii*)(b)white rhizoid root colonies (c)cloudy stem (*Cronobacter skazakii*)(d) white stem colonies(*Cronobacter skazakii*) (e) yellow stem(*Serratia liquefaciens*) (f) darkbrown root colonies (*Proteus vulgaris*) (g) white leaf (*Serratia liquefaciens*) and (h) darkbrown leaf colonies (*Psedomonas stutzeri*).The bacteria labeled (a,b,c,d,g and h)shows high clear zone formation while those labeled (e and f)has a fewer clear zone formation as mentioned in the figure (8).

4.4.2. Zinc solubilization test

From eight of the isolated bacteria, as shown in the picture below, only *P. vulgaris* EIRR-2, *S. liquefaciens* EIRL-2, and *S. liquefaciens* EIRS-1 (Figure 9, Table 1) can solubilize the inorganic zinc-containing compounds for these isolates which obtained from various part of *R. nepalensis*. This bacterium was isolated from different Rumex parts but was the same bacterial species. It shows some precise zone formation around the colonies. Five isolated bacteria could not solubilize the inorganic zinc, as indicated in Table 2.

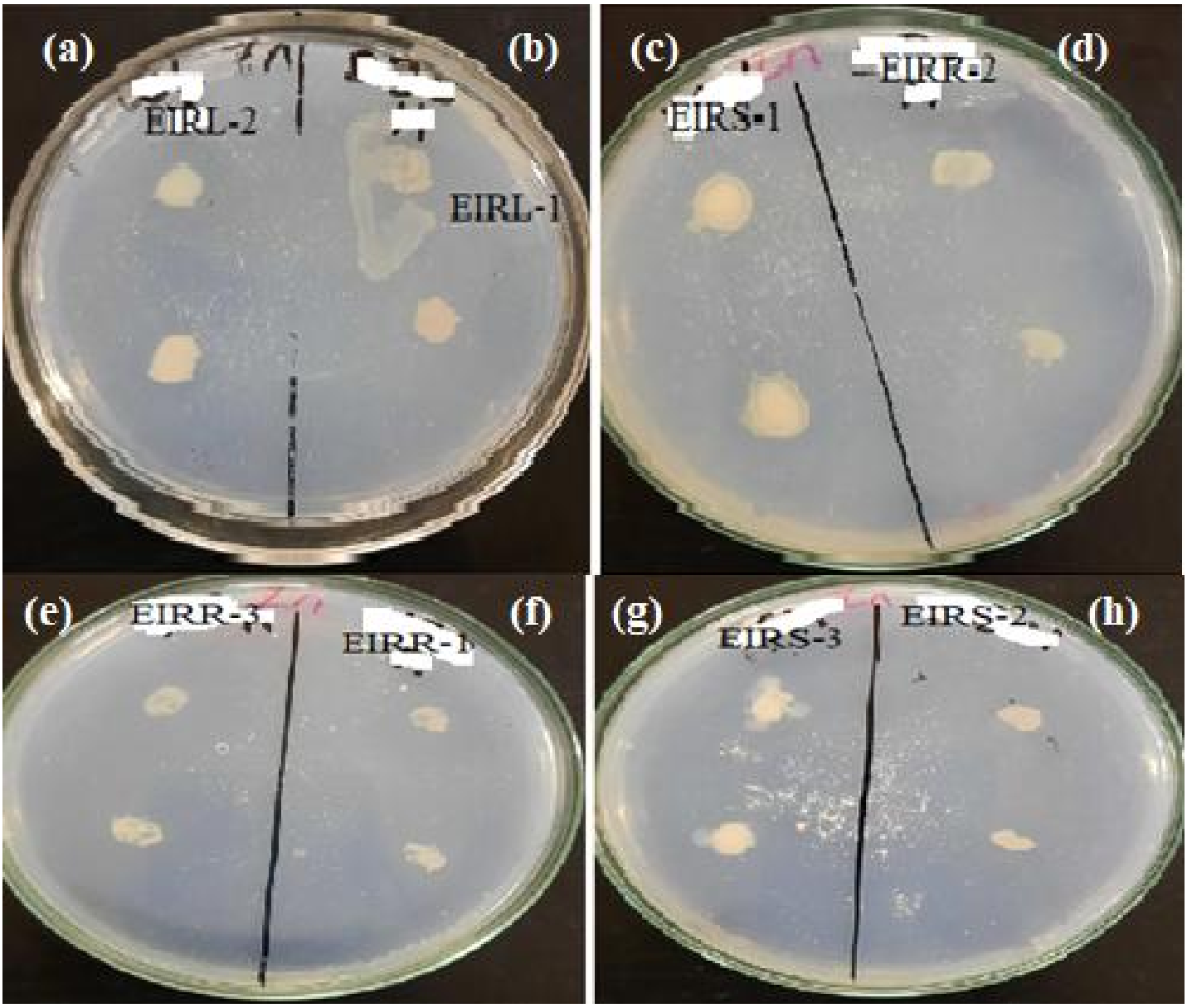


Figure 9: Zinc solubilizing ability of isolated endophytic bacteria. (a) Zn solubilizing ability of *S. Liquefaciens* strain isolate EIRL-2 isolated from leaf part of *R.nepalensis.* (b) Zn solubilizing ability of Isolate EIRL-1 *Pseudomonas stutzeri* strain isolated from leaf part of *R.nepalensis* (c) Zn solubilizing ability of Isolate EIRS-1 *S. liquefaciens* strain isolated from stem part of *R.nepalensis* (d) Zn solubilizing ability of Isolate EIRR-2 *P. vulgaris* strain isolated from root part of *R.nepalensis*.(e) Zn solubilizing ability of Isolate EIRR-3 *Cronobacter skazakii* and (f) Zn solubilizing ability of Isolate EIRR-1 NI strain isolated from root part of *R.nepalensis*. (g) Zn solubilizing ability of Isolate EIRS-3 *Cronobacter skazakii* and (h) Zn solubilizing ability of Isolate EIRS-2 *S. liquefaciens* isolated from stem part of *R.nepalensis*.

Table 2: Endophytic bacterial isolates and their efficiency of Phosphate, & Zinc solubilization, and various enzyme tests

S.N.	Type of Isolates	Amylase test	Protease test	Catalase test	Cellulase test	Phosphate solubilization	Zinc solubilization	Type of isolates based on protein profiles
						Solubilization test	Solubilization test	
1	EIRL-1	+ve	+ve	-ve	+ve	+ve	-ve	*Psedomonas stutzeri* EIRL-1
2	EIRL-2	+ve	-ve	-ve	+ve	+ve	+ve	*Serratia liquefaciens* EIRL-2
3	EIRR-1	NI	NI	NI	NI	NI	NI	NI
4	EIRR-2	+ve	+ve	-ve	-ve	+ve	+ve	*Proteus vulgaris* EIRR-2
5	EIRR-3	+ve	+ve	-ve	+ve	+ve	-ve	*Cronobacter skazakii* EIRR-3
6	EIRS-1	+ve	+ve	-ve	+ve	+ve	+ve	*Serratia liquefaciens* EIRS-1
7	EIRS-2	+ve	-ve	-ve	+ve	+ve	-ve	*Cronobacter skazakii* EIRS-2
8	EIRS-3	+ve	+ve	-ve	+ve	+ve	-ve	*Cronobacter skazakii* EIRS-3

Key: NI indicated not identified, -: Negative, +: Positive

These isolates are *C. skazakii* EIRS-2, EIRS-3, EIRR-3 and *P. stutzeri* EIRL-1(Table2). Santiago *et al.* (2011) have reported *Anabena*, *Calothrix*, *Providentia, Proteus, Serratia*, and *Trichoderma* sp can solubilize Zn and helped for the growth promotion various plants like wheat. Zinc is a micronutrient plant required to regulate the production of several proteins. It has both catalytic and structural roles in plants' metabolism in a deficient concentration. Zinc solubilizing isolates show clear zones around the colonies (Rana *et al*., 2012) (Figure 9).

4.5 Optimization of the isolated bacteria

The optimization test was done for those *Serratia liquefaciens* isolated from leaf part and *Proteus vulgaris* isolated from root part of *R.nepalensis* based on their performance of Indole test, urease, VP test, methyl red test, gram stain, and zinc and phosphate solubilization tests.

4.5.1. Optimization of PH

The study demonstrated that various pH value affect the CDW (g/L) and OD_{600nm} and hence the growth of bacterial species. For instance, variation in terms of CDW (g/L) ($p=0.028$, Figure 9b and Additional File: Table A_4) was detected between pH6 and pH7.5 against *P. vulgaris* EIRR-2 proving that pH is the other main growth factors for various microbial diversities. As indicated in Figure 9a&b, the highest CDW (g/L) was recorded for *S. liquefaciens* EIRL-2 (0.0255±0.0212 g/L) (Additional File: Table A_1) and *P. vulgaris* EIRR-2 (0.0084±0.0014 g/L) (Additional File: Table A_3), these isolated screened from the part of *R. nepalensis***.** Insignificant variations were detected among all pH values against *S. Liquefaciens* EIRL-2 in terms of CDW (g/L) (Figure 10a, Additional File: Table A_2)**.** From the selected two isolates, *S. liquefaciens* EIRL-2 show the higher (2.368± 0.0257) OD_{600nm} and CDW measurement at pH 7.5 respectively which was obtained from leaf part of *R. nepalensis.* There was slight difference in the measurements of their CDW and OD between EIRR-2 isolate that was extracted from root part of EIRL-2 isolate. Bokhari *et al.* (2019) previously reported optimum growth pH for *Citrobacter freundii* strain IG1 was 7.Also, Maalej *et al.* (2014) have reported that for EPS producing *Pseudomonas sp*. optimum pH for growth ranges from 7-8 with high cell density at pH 7.3.In addition with this, Wang *et al.* (2021) have reported that *Citrobacter freundii* has pH tolerance ranging from pH 5-10 and was strongly inhibited at pH 4 but did not survive at pH 3.As the PH value increases from lower to higher (6-8) the bacteria started to survive but beyond its optimum, the bacteria cannot survive (Figure 10).

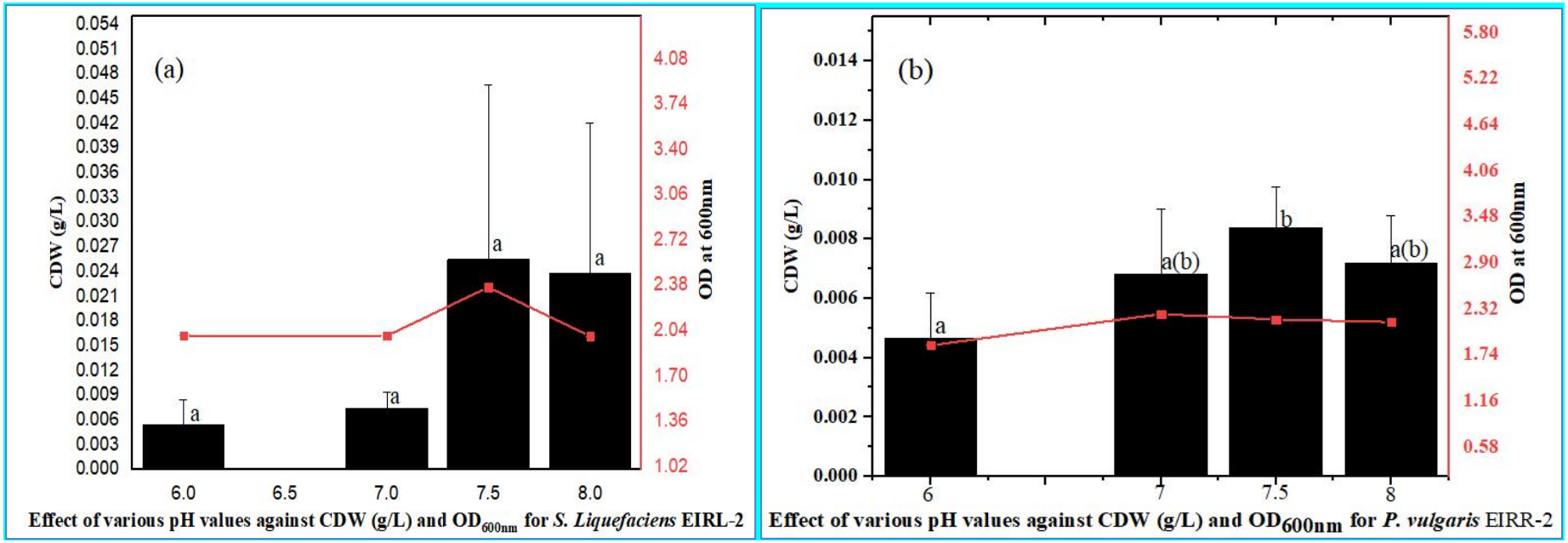


Figure 10: (a) Indicates various pH values against CDW (g/L) and OD_{600nm} for *S. liquefaciens* EIRL-2. (b) CDW (g/L) and OD_{600nm} against various pH values for *P. vulgaris* EIRR-2 which obtained from *R. nepalensis*, a medicinal plant. Data represented mean ± SD of three replicates (n = 3) of CDW (g/ml) for various pH values against *S. liquefaciens* EIRL-2 and *P. vulgaris* EIRR-2. According to One-Way ANOVA tool, there is no significant variation at 95% confidence interval using LSD test (P >0.05) with the same letters on the column bar for these isolates.

It was realized that both isolates (*S. liquefaciens* EIRL-2 and *P. vulgaris* EIRR-2) gave optimum CDW (g/L) at pH7.5 for these indicating that these isolates are neutrophilic bacteria (Figure 10a&b, Additional File: Table $A_{1\&3}$). These isolates were isolated from *R. nepalensis* suggesting that they could promote its growth. In line with this study, Fe (II)-oxidizing bacteria (FeOB) such as *Ferritrophicum radicicola, Sideroxydans lithotrophicus, S. paludicola,* and other new isolates were identified using 16SrRNA gene (Weiss et al., 2007). The same authors further confirmed that these bacterial strains grew on the Rhizosphere region of certain species of wetland plants (*Juncus effuses, Magnolia virginiana, Scirpus americanus*, and *Typha latifolia*) over 4.5.-7.0 pH range. It is the fact that these isolates gave the minimum CDW (g/L) (0.0054±0.003g/L for *S. liquefaciens* EIRL-2 and 0.004667±0.0015 g/L for *P. vulgaris* EIRR-2) (Figure 9a&b, Additional File: Table $A_{1\&3}$) at pH6.0 suggesting that they are not obliged to grow in acidic environment where other microbial diversities can grow and promote plant growth.

4.5.2 Optimization of growth Temperature

Various temperature ranges can affect the growth of endophytic bacterial species. The CDW (g/L) and OD600nm found to be determined by temperature ranges (Figure 11a). For instance the lowest (0.0018±.0012 g/L, 1.011±0.001 nm at 37^{o}C) and highest (0.031±0.0448 g/L at 32^{o}C recorded for *P. vulgaris* EIRR-2**,** 1.939±0.0040 nm at 30^{o}C) peaks of CDW and OD600nm were recorded against *S. liquefaciens* EIRL-2 (Figure 11, Additional File: Table A5&6). It was found that both of the selected isolates growth and CDW (g/l) production was at its peak at 30oC (Figure 11a). Based on the growth condition of the recent study, it was realized that *S. liquefaciens* EIRL-2, the entophytic bacterial isolate which extracted from the leaf part of *R. nepalensis* was mesophilic. In line with the recent study, it has been reported that various endophytic bacterial and other microbial species can be helpful to promote at a mesophilic temperatures ranges (*Goryluk-salmonowicz et al*., 2018; *War nongkhlaw* & Joshi, 2014). These of endophytic bacterial isolates were identified as *Bacillus* sp., 27 *Lysinibacillus*, *pantoea*, *Pseudomonas*, and *Serratia* sp., with potential to promote plant growth (War Nongkhlaw & Joshi, 2014). The same authors further illustrated that *B. siamensis* C53 and *B. subtilis cen* showed significant antagonistic activity against certain microbial pathogens. As it was observed from Figure 10a, significant variation predicted among 25 & 30 oC. (*p=0.006*), 25 & 32 oC (*p=0.015*),

and 30 & 37 oC (*p=0.009*). However, no variation detected among 25 & 35 oC (*p=0.*145), 25 & 37 oC (*p=0.*775), 30 & 32 oC (*p=0.*571), 30 & 35 oC (*p=0.*082), and 35 & 37 oC (*p=0.*226)

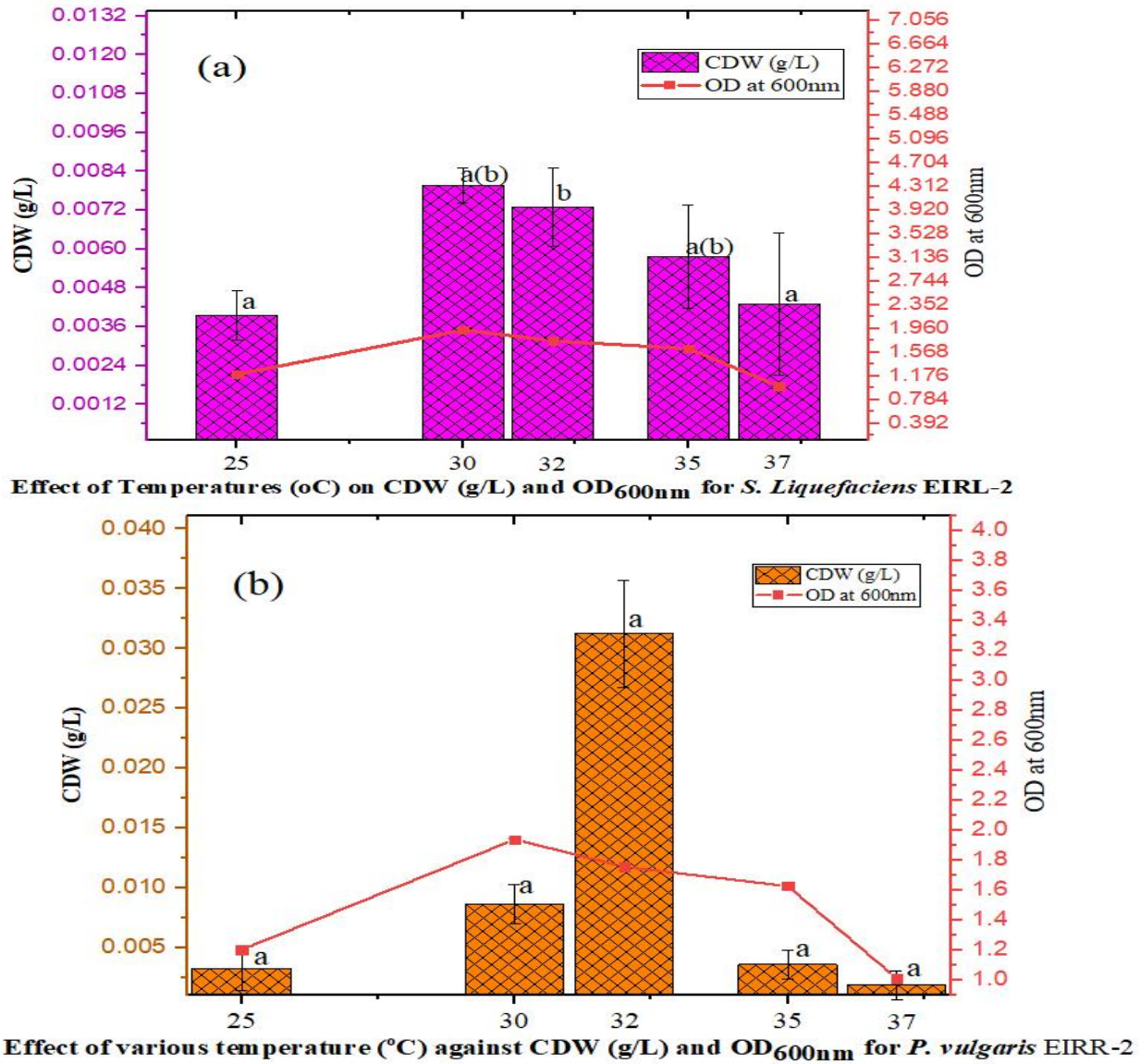


Figure 11: Effect of various temperatures (oC) on *S. liquefaciens* EIRL-2 and *P. vulgaris* EIRR-2 (a) Indicates various temperature range against CDW (g/L) and OD_{600nm} for *S. liquefaciens* EIRL-2. (b) CDW (g/L) and OD_{600nm} against various temperature range for *P. vulgaris* EIRR-2 which obtained from *R. nepalensis*, a medicinal plant. Data represented mean ± SD of three replicates (n = 3) of CDW (g/ml) for various temperature range against *S. liquefaciens* EIRL-2 and *P. vulgaris* EIRR-2. According to One-Way ANOVA tool, there is no significant variation at 95% confidence interval using LSD test (P >0.05) with the same letters on the column bar for these isolates**.**

P. vulgaris EIRR-2 which obtained from *R. nepalensis* gave various CDW (g/L) and OD_{600nm} against various temperature ranges (Figure 11b, Additional File: Table A7&8). The minimum (0.001833±.0012 g/L) and maximum (0.031267±0.0448 g/L) CDW (g/L) were detected at 37 and 32°C, respectively. Beshir (2022) found that *P. aeruginosa* grew at optimum growth temperature of 25 °C and the lowest cell Biomasses (CDW, 0.0021±6.33*10-5 g/mL) was obtained at 37°C. In was also reported that (Muktar, 2022) *Pseudomonas* grew at 30°C and minimum growth of OD_{600nm} & CDW (g/l) were obtained at 45°C. Sawai *et al.* (1977) stated that the optimal growth temperature of *Citrobacter freundii* to be between 30 and 37°C. Uddin *et al.* (2008) have reported that the bacterial fish pathogen *Citrobacter freundii* showed its peak growth at 36°C. Study conducted by (Chi, *et al.* 2018) demonstrated that *Enterobacter* LX3 isolate exhibited the ability to grow at 20-45°C but the maximum growth temperature against *E. cloacae* was reported to be 30°C, which is the same temperature recorded against *P. vulgaris* EIRR-2 and *S. liquefaciens* EIRL-2 (Figure 10a&b, Additional File: Table A5,6,7&8). However, in this experiment peak of OD_{600nm} and CDW (g/l) for *P. vulgaris* EIRR-2 was found to be larger at 30°C followed by 35 °C (0.0036±0.00117 g/L, 1.6287±0.0006 nm) (Figure 10a&b, Additional File: Table A7&8). Similarly, it has been reported that 0.55 g/l CDW have obtained from *B. pumilus* BNL 06 (Das et al., 2015), which isolated from *Brasssica nigra* L. This variation in terms of CDW (g/l) and OD_{600nm} happens due to the diversities of bacterial isolates, types of carbon & nitrogen sources and other growth factors as it was indicated in this study.

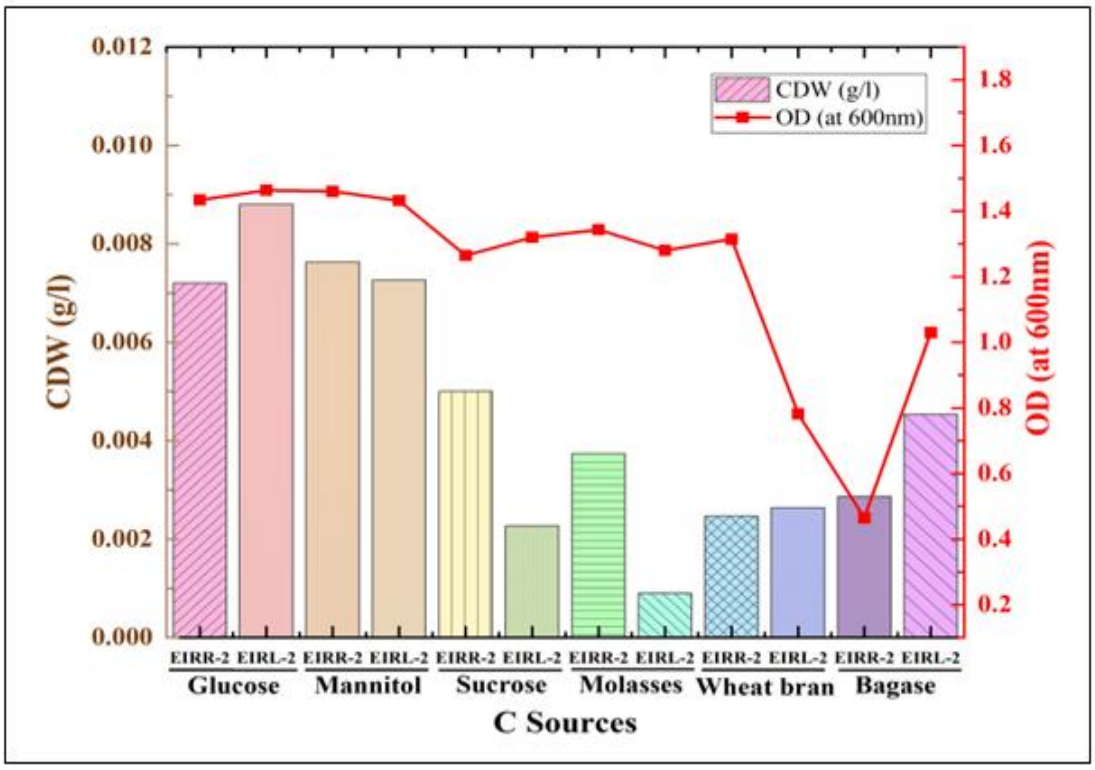
4.5.3 Carbon source and Nitrogen source optimization

These *P. vulgaris* EIRR-2 and *S. Liquefaciens* EIRL-2 exhibited maximum growth conditions against carbon sources. *P. vulgaris* EIRR-2 exhibited maximum growth in CDW (g/l) production and OD_{600nm} when Mannitol was used as the sole Carbon source (Figure 12a). The Highest OD_{600nm} and CDW (g/L) measurements for *P. vulgaris* EIRR-2 were 1.4596 and 0.00763, respectively, when grown on Mannitol as the sole carbon source. Wheat bran was the least preferred sole carbon source by Mannitol isolates, exhibiting the lowest OD_{600nm} and CDW (g/l) measurements, 0.4656 and 0.002466, respectively. In contrast, isolate EIRL-2 exhibited maximum growth in CDW (g/l) production and OD_{600nm} when glucose was used as the sole carbon source. The Highest OD_{600nm} and CDW measurements taken for isolate EIRL-2 were 1.4636 and 0.0088, respectively, when grown with glucose as the sole carbon source.

In contrast, in sucrose a reduction in growth OD_{600nm} was observed. Bacteria can grow on various carbon sources. Most bacterial isolates grow better on a medium containing glucose than these disaccharides and polysaccharides carbon sources. Thus, it may be because glucose, a monomer, can efficiently catalyze cellular functions such as growth and cell division. Disaccharides and polysaccharides cannot be efficiently catalyzed for cellular functions (Afroz, 2016).

P. vulgaris EIRR-2 exhibited maximum OD_{600nm} and CDW (g/l) measurement with yeast extract as the primary nitrogen source. The maximum OD_{600nm} and CDW (g/l) growth rate of the isolate under yeast extract was 1.94133 and 0.004966. In contrast, *S. liquefaciens* EIRL-2 shows maximum growth under peptone as the sole nitrogen source. The maximum OD_{600nm} and CDW (g/l) measurement was 2.16866 and 0.0095733 (Figure 12b). Singh *et al.* (2011) reported that when evaluating the usage of $(NH_4)_2SO_4$, peptone and yeast extract as nitrogen sources, the bacteria grew better when peptone was used as the sole nitrogen source compared to other nitrogen sources for strain growth.

(a)



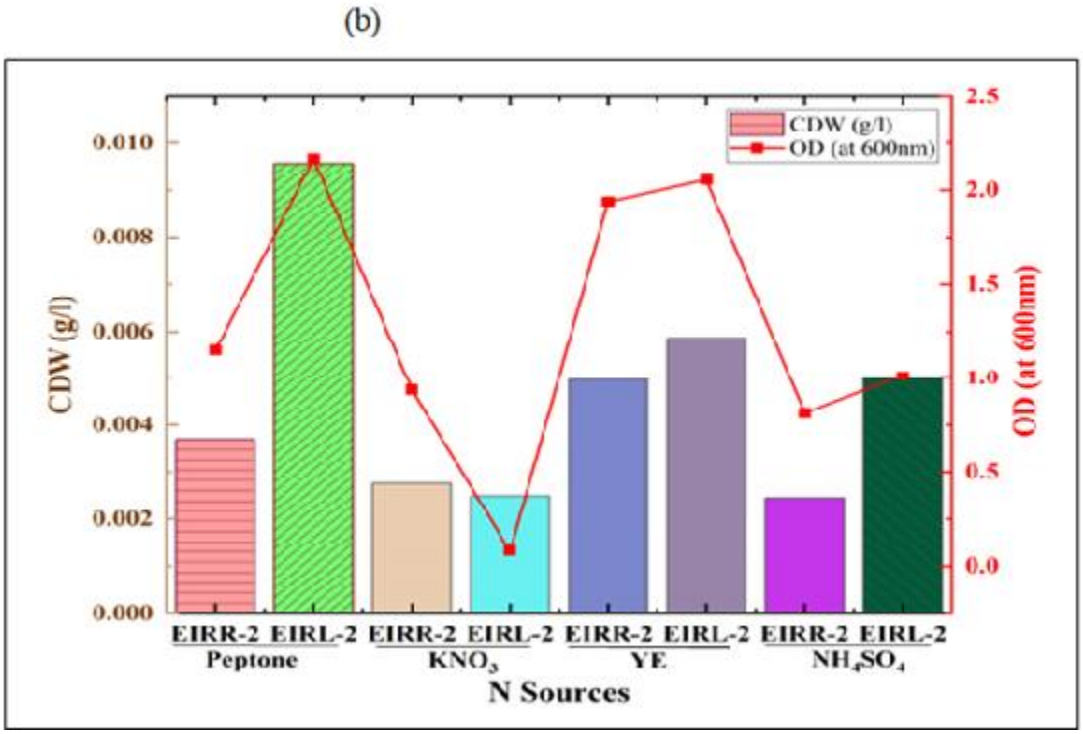


Figure 12 (a) Effect of various carbon sources against CDW (g/L) and OD_{600nm} for *S. Liquefaciens* EIRL-2 and P. *vulgaris* EIRR-2. (b) Effect of various nitrogen sources against CDW (g/L) and OD_{600nm} for *S. Liquefaciens* EIRL-2 and P. *vulgaris* EIRR-2.

4.5.4 Effects of NaCl Concentration (Mm/ml) on bacterial isolates

The two isolates grew on different salt concentrations. *P. vulgaris* EIRR-2 exhibited maximum growth on 1M of NaCl with maximum CDW (g/l) (0.07417) and OD_{600nm} (1.3993). This bacterial isolate was probably salt tolerant because it grew at higher concentrations. *S. liquefaciens* EIRL-2 grew on the lowest salt concentration (0.05M NaCl) with maximum CDW (g/l) (0.007) and OD_{600nm} (2.0727). Abdulkarim *et al.* (2009) reported that *E. coli* and *S. aureus* have optimum growth under NaCl concentration. Thus, they show that the higher the NaCl concentration, the lesser the bacteria will reach optimal growth. Increasing salt concentration decreases the growth rate due to the osmotic effect on the bacteria. In our findings, we can observe that *S. liquefaciens* EIRR-2 shown salt-tolerant and *P. vulgaris* EIRL-2 grew well under lower salt concentrations (Figure 13).

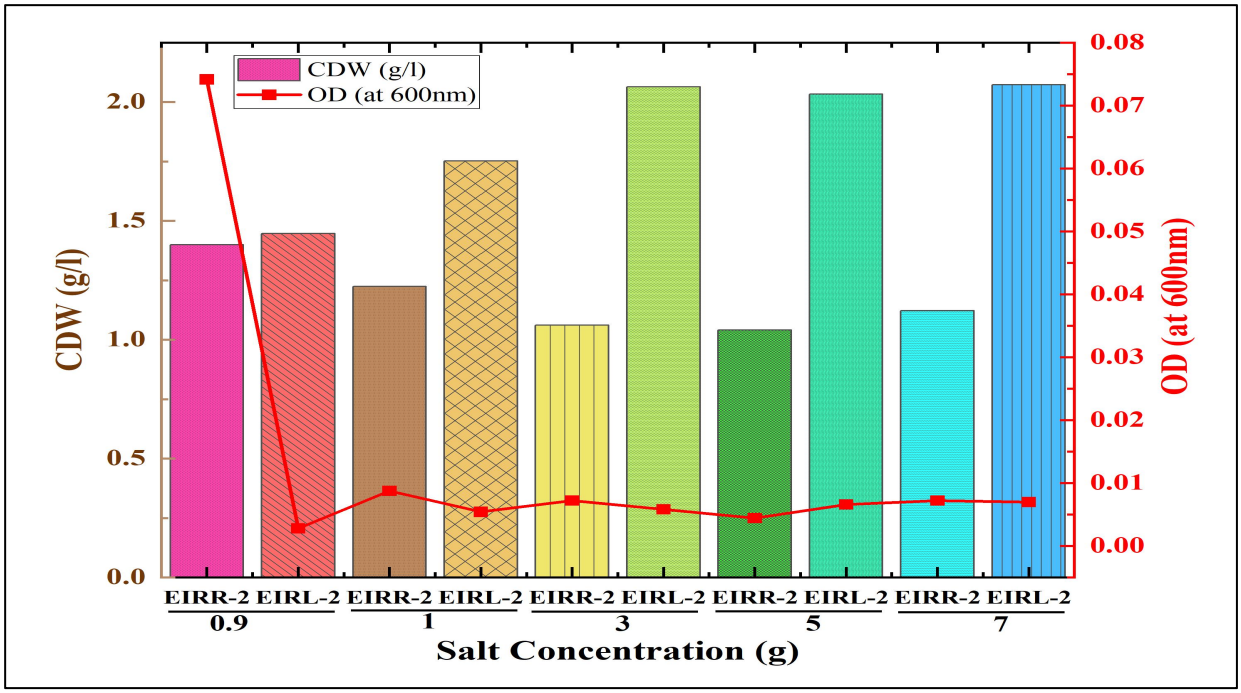


Figure 13 Effect of various salt concentrations against CDW (g/L) and OD_{600nm} for *P. vulgaris* EIRR-2 and *S. Liquefaciens* EIRL-2 isolates

4.6 Evaluation of antimicrobial activities

The extract exhibited inhibitory activity against all the test organisms. Highest antibacterial activity was recorded against the *Pseudomonas aeruginosa* strain with 14 mm zone of inhibition from the crude extract with concentration of 1 g/ml. The isolate EIRL2 *Proteus vulgaris* strain from which the crude metabolite was extracted from showed high levels of resistance as it covered the inhibition zone completely after being inhibited for about 10-12 hours. (Figure 14). Sugiyama (2015) explains that antibiotic-producing microorganisms can protect themselves from the fatal effects of their product; this is called self-resistance. This trait of antibiotic-producing organisms is essential in understanding the similarity and the difference between drug resistance and self-resistance mechanisms, which in turn is significant in developing novel therapeutic drugs. Syed and Rekha (2019) evaluated the crude extract's antibacterial activity against *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Proteus vulgaris*, and *Salmonella typhi,* where they found the extract showing zones of inhibition against all the test bacteria. Nxumalo *et al.* (2020) reported that crude metabolite extracts of endophytic bacteria Isolate one strain *Pseudomonas aeruginosa* CP043328.1 demonstrated significant

inhibitory ability against *E. coli*, *P. mirabilis*, *B. cereus* and *S. aureus*. The extract was mainly effective on *S. aureus* with a 31±1.25mm inhibition zone diameter.

Table 3 Antibacterial activity of isolates bacteria

	Antimicrobial		**Activity**	
	Zone of	inhibition of	ethyl acetate extracts	(mm)
PA	R1	R2	+	-
EIRR-2	15	14	28	6
	12	13	27	6
	13.5	13.5	27.5	6
EIRL-2	13	12	28	6
	15	15	27	6
	14	13.5	27.5	6

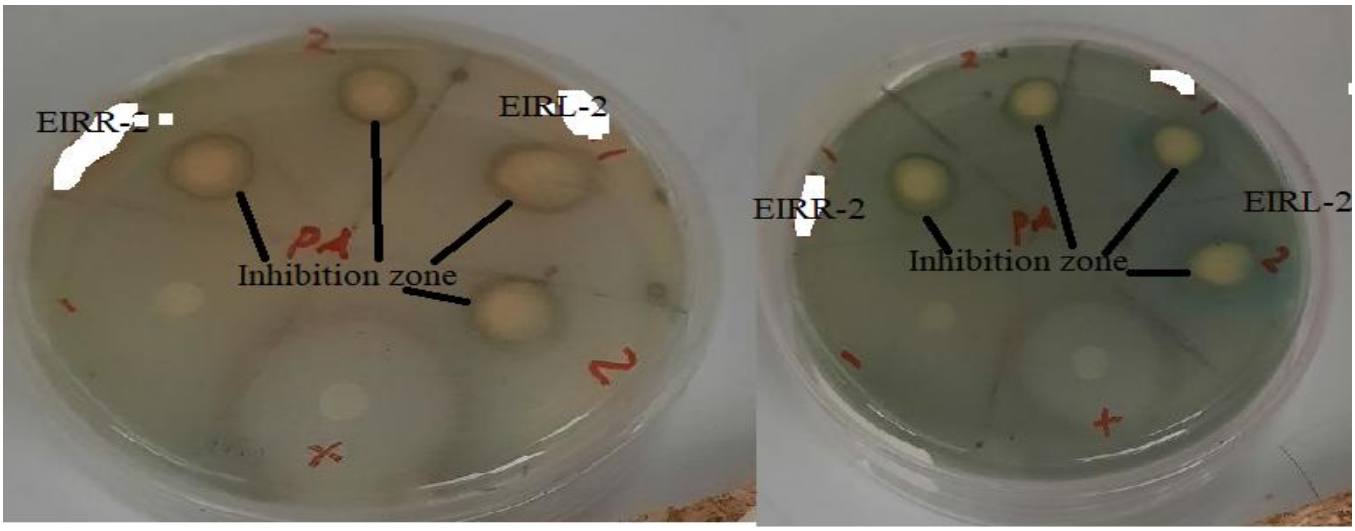


Figure 14 Antibacterial activity of *P. vulgaris* EIRR-2 isolate from root part of *R. nepalensis* and *S. Liquefaciens* EIRL-2 against (P. aeruginosa)

5. Conclusions and Recommendations

5.1. Conclusions

This study successfully isolated eight endophytic bacterial isolates from the leaf, stem, and root parts of Rumex nepalensis plant

- The emerged endophytic bacteria were purified and identified by biochemical methods and MALDI-TOF MS as belonging to *C. skazakii* EIRS-2, *C. skazakii* EIRS-3, *and Skazakii* EIRR-3, *P.vulgaris* EIRR-2, *Stutzeri* EIRL-1*, Serratia liquefaciens EIRL*-2, and *S. liquefaciens* EIRS-1.
- The isolates produced the enzymes amylase, cellulase, catalase and protease, which can be used as novel sources of essential enzymes.
- The isolates were good phosphate and zinc Solubilizers, as demonstrated by the formation of clearing zones on agar plates containing insoluble phosphate and zinc. Of the isolates, 75% were able to solubilize insoluble phosphate, whereas 50% were able to solubilize insoluble zinc, showing the ability of the isolates to be used as bio-fertilizers to reduce the impact of chemical fertilizers added in phosphate and zinc-deficient agricultural fields by being used as bio-inoculants for plants.
- Evaluation of growth conditions for two representative isolates showed that temperature of 30°C -32°C with a pH range of 7.5 and glucose and Mannitol as sole sources of carbon about peptone and yeast extract as sole nitrogen sources under optimal growth conditions for the isolates.
- Ethyl acetate secondary metabolite extract of the hyper-producer isolate *Proteus vulgaris* EIRL-2 from which the crude metabolite was extracted yielded 1g/500 ml of oil extracts. The crude extracts exhibited inhibitory activity against three test organism's *B. subtilis, E. coli,* and *Pseudomonas aeruginosa*, and the isolate EIRL-2 itself showed resistance against its secondary metabolite.

5.2. Recommendations

Based on the findings of the research the following recommendations are made:

- The medicinal value of the plant was not studied in this section to its full right leaving a gap in the additional study
- The endophytic isolated bacteria should have to be taken from various topography for the further investigations.

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Appendices

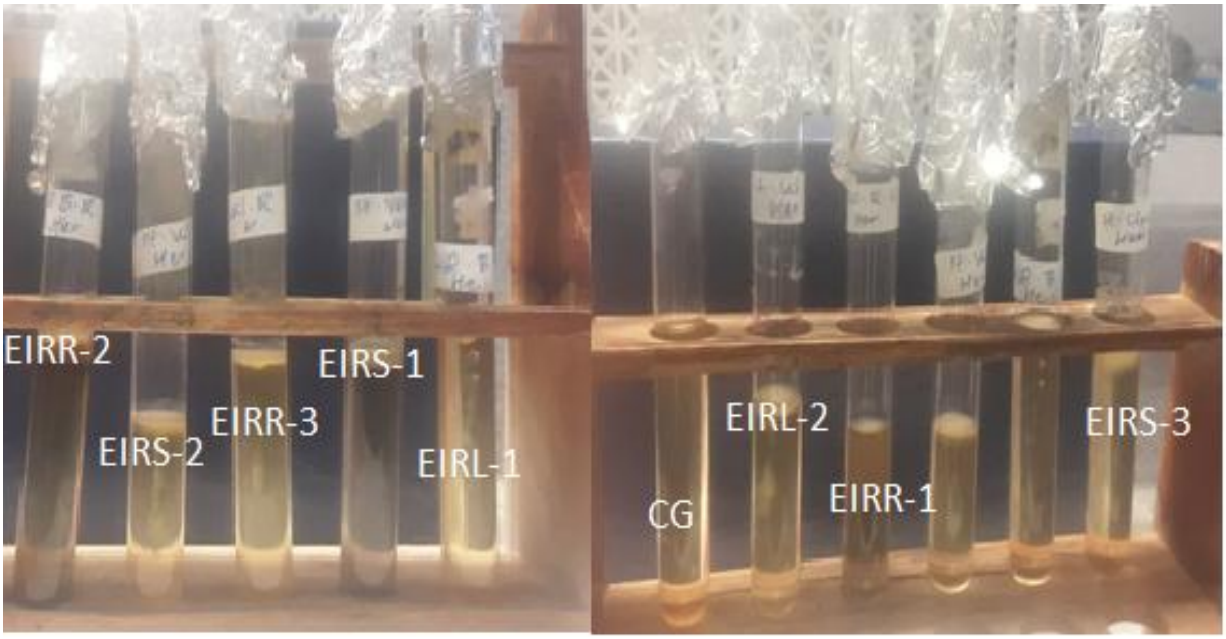

Figure A_1. Motility test against certain of the recent endophytic bacterial isolates. *P. vulgaris* EIRR-2, *C. skazakii* EIRR-3, *S. liquefaciens* EIRS-1. *C. Skazakii* EIRS-2, *C. Skazakii* EIRS-3, *P. stutzeri* EIRL-1, and *S. Liquefaciens* EIRL-2

Figure A_2. Biochemical test

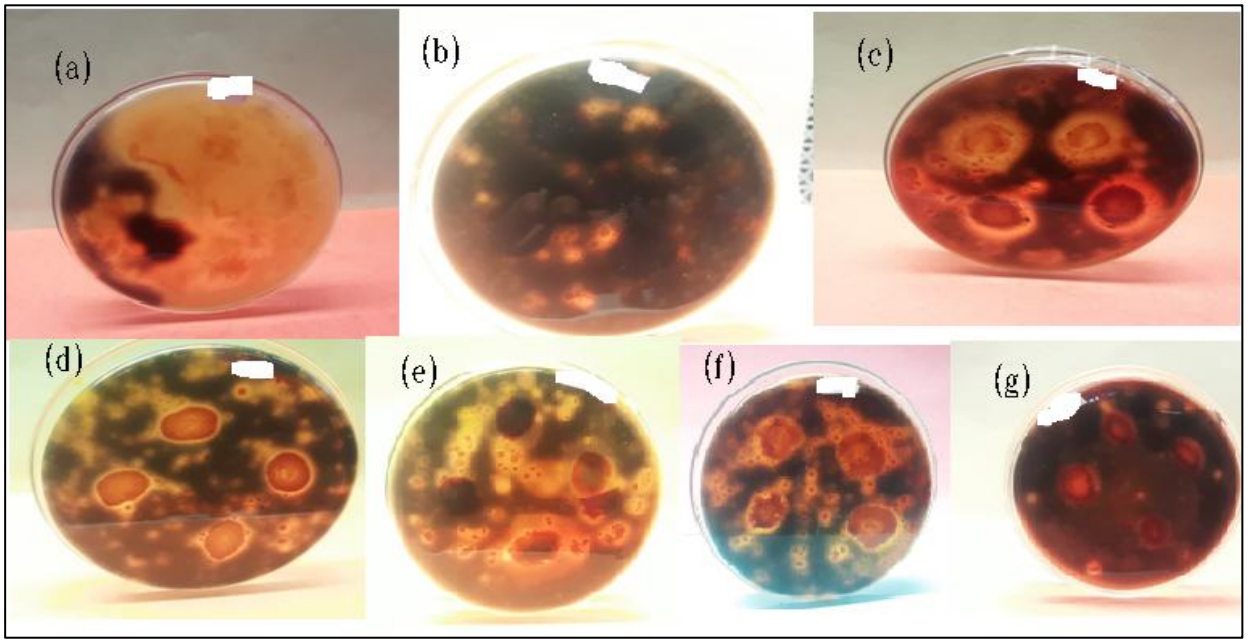

Figure A_3. Amylase test (a) EIRL-1 *pseudomonas stutzeri* strain (b) EIRR-3 *Cronobacter scazaki* strain (c) EIRS-1 *Serratia Liquefaciens* strain (d) EIRS-2 *Cronobacter scazaki* strain (e) EIRS-3 *Cronobacter scazaki* strain (f) EIRR-2 *Proteus vulgaris* strain (g) EIRL-2 *Serratia Liquefaciens* strain which were isolated from root, stem and leaf of *R.nepalensis*.

Table A_1: Mean, SD, minimum and maximum values of CDW (g/L) against *S. Liquefaciens* EIRL-2

CDW (g/L) against *S. Liquefaciens* EIRL-2								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) at pH6	3	.005367	.0031005	.0017901	-.002335	.013069	.0023	.0085
CDW (g/L) at pH7	3	.007400	.0020224	.0011676	.002376	.012424	.0059	.0097
CDW (g/L) at pH7.5	3	.025467	.0211984	.0122389	-.027193	.078126	.0110	.0498
CDW (g/L) at pH8	3	.023767	.0182234	.0105213	-.021503	.069036	.0061	.0425
Total	12	.015500	.0153689	.0044366	.005735	.025265	.0023	.0498

Table A_2 Significant test at 0.05 confidence interval, Post Hoc test, and Multiple Comparisons against *S. liquefaciens* EIRL-2 for CDW (g/L) for various pH value

Multiple Comparisons						
Dependent Variable: CDW (g/L) against *S. Liquefaciens* EIRL-2 LSD						
(I) CDW (g/L) against S. Liquefaciens EIRL-2 for various pH values	(J) CDW (g/L) against *S. Liquefaciens* EIRL-2 for various pH values	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) against S. Liquefaciens EIRL-2 for various pH6	CDW (g/L) against *S. Liquefaciens* EIRL-2 for various pH7	-.0020333	.0115121	.864	-.028580	.024514
	CDW (g/L) against *S. Liquefaciens* EIRL-2 for various pH7.5	-.0201000	.0115121	.119	-.046647	.006447
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH8	-.0184000	.0115121	.149	-.044947	.008147
CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH6	.0020333	.0115121	.864	-.024514	.028580
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7.5	-.0180667	.0115121	.155	-.044614	.008480
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH8	-.0163667	.0115121	.193	-.042914	.010180
CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7.5	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH6	.0201000	.0115121	.119	-.006447	.046647
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7	.0180667	.0115121	.155	-.008480	.044614
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH8	.0017000	.0115121	.886	-.024847	.028247
CDW (g/L) against S. Liquefaciens EIRL-2 for various pH8	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH6	.0184000	.0115121	.149	-.008147	.044947
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7	.0163667	.0115121	.193	-.010180	.042914
	CDW (g/L) against S. Liquefaciens EIRL-2 for various pH7.5	-.0017000	.0115121	.886	-.028247	.024847

Table A_3: Mean, SD, minimum and maximum values of CDW (g/L) against *P. vulgaris* EIRR-2

CDW (g/L) against *P. vulgaris* EIRR-2								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) against *P. vulgaris* EIRR-2 at pH6	3	.004667	.0015308	.0008838	.000864	.008469	.0035	.0064
CDW (g/L) against *P. vulgaris* EIRR-2 at pH7	3	.006833	.0021962	.0012680	.001378	.012289	.0043	.0082
CDW (g/L) against *P. vulgaris* EIRR-2 at pH7.5	3	.008400	.0013748	.0007937	.004985	.011815	.0072	.0099
CDW (g/L) against *P. vulgaris* EIRR-2 at pH8	3	.007200	.0016093	.0009292	.003202	.011198	.0055	.0087
Total	12	.006775	.0020249	.0005845	.005488	.008062	.0035	.0099

Phosphate solubilization				
Type of isolate	Name of isolate	Colony diameter in cm	Clear zone in cm	solubilization index in cm
EIRR-1	NI	2.5	2.7	1.93
EIRR-3	*Cronobacter skazakii*	2	2.2	1.91
EIRS-1	*Serratia liquefaciens*	0.8	1.1	1.73
EIRS-3	*Cronobacter skazakii*	1	1.2	1.83
EIRS-2	*Cronobacter skazakii*	0.8	1.1	1.73
EIRL-2	*Serratia liquefaciens*	1.5	1.8	1.83
EIRL-1	*Psedomonas stutzeri*	0.9	1.1	1.82

Table A_4 Significant test at 0.05 confidence interval, Post Hoc test, and multiple Comparisons against *P. vulgaris* EIRR-2 for CDW (g/L) for various pH value

Multiple Comparisons						
Dependent Variable: CDW (g/L) against *P. vulgaris* EIRR-2 LSD						
(I) CDW (g/L) against P. vulgaris EIRR-2 for pH values	(J) CDW (g/L) against P. vulgaris EIRR-2 for pH values	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) against P. vulgaris EIRR-2 at pH6	CDW (g/L) against P. vulgaris EIRR-2 at pH7	-.0021667	.0013932	.159	-.005379	.001046
	CDW (g/L) against P. vulgaris EIRR-2 at pH7.5	-.0037333*	.0013932	.028	-.006946	-.000521
	CDW (g/L) against P. vulgaris EIRR-2 at pH8	-.0025333	.0013932	.107	-.005746	.000679
CDW (g/L) against P. vulgaris EIRR-2 at pH7	CDW (g/L) against P. vulgaris EIRR-2 at pH6	.0021667	.0013932	.159	-.001046	.005379
	CDW (g/L) against P. vulgaris EIRR-2 at pH7.5	-.0015667	.0013932	.293	-.004779	.001646
	CDW (g/L) against P. vulgaris EIRR-2 at pH8	-.0003667	.0013932	.799	-.003579	.002846
CDW (g/L) against P. vulgaris EIRR-2 at pH7.5	CDW (g/L) against P. vulgaris EIRR-2 at pH6	.0037333*	.0013932	.028	.000521	.006946
	CDW (g/L) against P. vulgaris EIRR-2 at pH7	.0015667	.0013932	.293	-.001646	.004779
	CDW (g/L) against P. vulgaris EIRR-2 at pH8	.0012000	.0013932	.414	-.002013	.004413
CDW (g/L) against P. vulgaris EIRR-2 at pH8	CDW (g/L) against P. vulgaris EIRR-2 at pH6	.0025333	.0013932	.107	-.000679	.005746
	CDW (g/L) against P. vulgaris EIRR-2 at pH7	.0003667	.0013932	.799	-.002846	.003579
	CDW (g/L) against P. vulgaris EIRR-2 at pH7.5	-.0012000	.0013932	.414	-.004413	.002013
*. The mean difference is significant at the 0.05 level.						

Table A_1: Effects of various nitrogen sources on EIRR-2 isolate against CDW (g/L) and OD $_{600nm}$

EIRR-2	**Biomass CDW g/L**				**OD600nm**			
N-sources	R1	R2	R3	AV	R1	R2	R3	**Av**
peptone	0.0033	0.0033	0.0045	0.0037	1.148	1.147	1.148	1.1476
KNO3	0.0024	0.0027	0.0032	0.002766	0.937	0.938	0.940	0.93833
YE	0.0049	0.0052	0.0048	0.004966	1.943	1.944	1.937	1.94133
NH4so4	0.0019	0.0033	0.0022	0.002466	0.820	0.818	0.816	0.818
EIRL-2	Biomass CDW g/L				OD600nm			
N-sources	R1	R2	R3	AV	R1	R2	R3	Av
peptone	0.0051	0.0061	0.1752	0.0095733	2.168	2.169	2.169	2.16866
Kno3	0.0028	0.0022	0.0025	0.0025	0.080	0.082	0.081	0.081
YE	0.0058	0.0056	0.0062	0.005866	2.058	2.052	2.051	2.05366
NH4so4	0.0038	0.0082	0.003	0.0050	1.011	1.012	1.013	1.012

Table A_2 Effects of various carbon sources on EIRR-2 isolate against CDW (g/L) and OD $_{600nm}$

EIRL-2	Biomass CDW g/L				OD_{600nm}			
C-sources	R1	R2	R3	AV	R1	R2	R3	Av
Glucose	0.0085	0.0097	0.0082	0.0088	1.463	1.466	1.462	1.4636
Mannitol	0.0093	0.0061	0.0064	0.00726	1.432	1.434	1.429	1.4316
sucrose	0.0018	0.0024	0.0026	0.002266	1.32	1.32	1.31	1.32
molasses	0.0003	0.0002	0.0022	0.0009	1.279	1.281	1.280	1.28
Wheat bran	0.0009	0.0032	0.0038	0.002633	0.781	0.782	0.783	0.782
bagasse	0.0006	0.005	0.008	0.004533	1.037	1.026	1.025	1.02933

Table A_3: Table A_2 Effects of various carbon sources on EIRR-2 isolate against CDW (g/L) and OD_{600nm}

EIRR-2	Biomass CDW g/L				OD600nm			
C-sources	R1	R2	R3	AV	R1	R2	R3	Av
Glucose	0.0068	0.0073	0.0075	0.007200	1.433	1.435	1.435	1.434
Mannitol	0.0088	0.0077	0.0064	0.00763	1.459	1.460	1.460	1.4596
Sucrose	0.0042	0.0059	0.0049	0.00500	1.264	1.265	1.265	1.26466
Molasses	0.008	0.0022	0.0010	0.0037333	1.34	1.36	1.33	1.34333
Wheat bran	0.0022	0.0022	0.0030	0.002466	1.311	1.318	1.313	1.314
Bagasse	0.0018	0.006	0.0008	0.002866	0.465	0.466	0.466	0.4656

Table A_4 Effects of various temperature ranges on EIRR-2 isolate against CDW (g/L) and OD $_{600nm}$

Temperature	Biomass CDW g/L				OD_{600nm}			
	R1	R2	R3	AV	R1	R2	R3	Av
25	0.0012	0.0049	0.0036	0.0032	1.205	1.203	1.204	1.204
25	0.0033	0.0048	0.0038	0.0038	1.734	1.736	1.736	1.735
30	0.0093	0.0098	0.0067	0.0086	1.936	1.939	1.944	1.93966
30	0.0074	0.0085	0.0080	0.0079	2.105	2.107	2.107	2.106
32	0.0052	0.083	0.0056	0.0063	1.758	1.757	1.757	1.757
32	0.0085	0.0073	0.0061	0.0073	2.106	2.107	2.105	2.106
35	0.0023	0.0046	0.0038	0.003566	1.629	1.628	1.629	1.628
35	0.0074	0.0057	0.0042	0.0057	1.891	1.893	1.893	1.892
37	0.0019	0.0006	0.003	0.0018	1.010	1.011	1.012	1.011
37	0.0062	0.0019	0.0048	0.0043	1.811	1.810	1.811	1.810

Table A_5 Effects of various pH ranges on EIRR-2 and EIRL-2 **isolates against CDW (g/L) and OD $_{600nm}$**

S.N.	Isolates	CDW (g/)				OD600nm			
		R1	R2	R3	AV	R1	R2	R3	AV
6	EIRR-2	0.0064	0.0035	0.0041	0.0047	1.861	1.862	1.861	1.861
6	EIRL-2	0.0053	0.0085	0.0023	0.0054	2.006	2.004	2.006	2.0053
7	EIRR-2	0.008	0.0043	0.0082	0.0068	2.255	2.258	2.255	2.256
7	EIRL-2	0.0066	0.0059	0.0097	0.0074	2.001	2.003	2.006	2.0033
7.5	EIRR-2	0.0081	0.0072	0.0099	0.0084	2.190	2.187	2.187	2.188
7.5	EIRL-2	0.0498	0.0156	0.011	0.0254	2.368	2.368	2.368	2.368
8	EIRR-2	0.0055	0.0087	0.0074	0.0074	2.157	2.157	2.157	2.157
8	EIRL-2	0.0425	0.0061	0.0227	0.024	2.001	1.999	2.000	2.000

Table A_6 **Effects of various salt conc. (Mm/ml) EIRR-2 and** EIRL-2 **isolates against CDW (g/L) and OD** $_{600nm}$

Strain codes	NaCl con	OD 600nm				CDW			
		R1	R2	R3	Av	R1	R2	R3	Av
EIRL-2	1	1.445	1.445	1.449	1.446333333	0.0024	0.0024	0.0036	0.0028
	0.5	1.747	1.754	1.755	1.752	0.0053	0.0056	0.0054	0.005433
	0.3	2.06	2.07	2.06	2.063333	0.0071	0.0060	0.0043	0.005833
	0.1	2.033	2.034	2.034	2.033666667	0.0050	0.0094	0.0054	0.0066
	0.05	2.072	2.072	2.074	2.072666667	0.0072	0.0070	0.0069	0.0070
EIRR-2	1	1.395	1.397	1.406	1.399333	0.0097	0.0036	0.0054	0.074166667
	0.5	1.226	1.226	1.222	1.224666667	0.0084	0.0102	0.0064	0.008766667
	0.3	1.060	1.063	1.063	1.062	0.0039	0.0071	0.0062	0.007233333
	0.1	1.036	1.042	1.044	1.040666667	0.2135	0.0055	0.0039	0.004433333