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Presumptive characterization of selected bacterial isolates and evaluation of their hydrolytic enzyme activity

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Abstract
Leguminous crops producing pulses which are interacted whit *Rhizobium* and plant growth-promoting rhizobacteria (PGPR). This interaction promotes the growth and seed yield of pulse crops by fixing atmospheric nitrogen. *Rhizobium* forms nodules with leguminous crops to increase the nitrogen fixation capacity. Different presentive tests are oxidative, catalase, fermentative, methyl red and citrate for *Rhizobium* determination also BIOLOG GEN III microplate was used to presumptive determination of species of selected bacterial isolates by the carbon utilization pattern and these bacterial isolates showed production of different hydrolytic enzymes such as Amylase, Cellulase, Chitinase, Pectinase, and Lipase. These properties play important role in nodule formation which help in nitrogen fixation and the growth and development of the plant. Due to this property our isolated bacterial use as a biofertilizer.

Keywords: *Rhizobium*, Leguminous, BIOLOG, and hydrolysis

Introduction
Bacteria secreted many hydrolytic exoenzymes that play an important role in contributing to the host metabolism as well as for their various applications in industrial and agricultural processes (Hossain, T. J., *et al*., 2020) [8]. These extracellular hydrolytic enzymes, carry out a wide range of catabolic and biotransformation reactions which are distinct from but complement the metabolic processes of the host (Krishnan *et al*., 2015; Banerjee and Ray, 2017; Egerton *et al*., 2018; Rowland *et al*., 2018) [9, 10, 11, 12]. Many articles are already reported on the purification and characterization of these hydrolytic exoenzymes such as protease, lipase, amylase, cellulase, and chitinase, among others, from the GI bacteria of various freshwater and marine fishes (Ray *et al*., 2012) [13]. Cellulase and hemicellulose activity was seen in temperate (infective and noninfective) and tropical strains (infective) of *Rhizobium.* Hydrolytic enzymes were determined by a cup-plate assay. The presence of cellulase and hemicellulase was confirmed by viscometric assay. Application of enzymes in *Rhizobium* are many (Martinez-Molina, E., *et al*., 1979) [14]. Biofertilizers include all the beneficial soil bacteria that can either be symbiotic or free-living. They have together been stated as plant growth-promoting rhizobacteria (PGPR). These classes of microorganisms are inherited with multiple traits that directly or indirectly promote plant growth promotion activities. They fix atmospheric nitrogen, solubilize insoluble soil phosphorus, potassium, and zinc making it available to the plants necessary for their proper growth and development (Gaby and Buckley, 2012; Singh *et al*., 2012; Xie, 1998) [15, 16, 17] synthesize siderophores that help in iron sequestration (Saha *et al*., 2016); All four strains hydrolyzed lipase and Catalase enzyme. *Rhizobium trifolii* and *Rhizobium phaseoli* utilized citrate and in the utilization of carbon sources, fast-growing strains were able to utilize carbon in comparison to slow-growing.

Materials and Methods
1. Presumptive test of *Rhizobium*
a. Catalase test
This test was performed by taking 2-3 drops of 3% H_2O_2 on a clean grease-free glass slide and a single colony was mixed with the help of an inoculation loop. Immediate formation of gas bubbles was considered a positive test.

b. Methyl red test
Inoculate MRVP broth with a pure culture of the organism and incubate at 35-37 °C for a minimum of 48 hours in ambient air. Add 5 or 6 drops of methyl red reagent per 5 mL of broth. Observe the color change in the broth medium.

c. Oxidation fermentation test
Inoculate many tubes of test medium contain (Sodium chloride: 5.0 g, Di-potassium phosphate: 0.3 g, Peptone: 2.0 g, Bromthymol blue:0.03 g, Agar: 3.0 g, Glucose: 10 g, Water: 1000 ml with the identified bacterial strains using a toothpick by stabbing "halfway to the bottom" of the tube. Cover one tube of each pair with a 1 cm layer of sterile mineral oil or liquid paraffin (it creates an anaerobic condition in the tube by preventing diffusion of oxygen), leaving the other tube open to the air. Incubate both tubes at 35 °C for 48 hours (slow-growing bacteria may take 3 to 4 days before results can be observed).

d. Citrate test
A well-isolated colony is taken from an 18–24-hour culture with a sterile inoculating loop. The citrate agar petri dish is inoculated by streaking the surface of the plate. The plate should be streaked back and forth with the loop or the inoculating stick. The petri dish is then incubated aerobically at 35-37 °C for up to 4 days. The petri dish should be examined daily for 4 days before discarding the result as a negative. The color change, if present, is observed.

2. Biochemical property of *Rhizobium*
a. Lipase production
Inoculate bacterial strains drop on, medium contains Casein 15.0gm, Vitamin K 1 10.0 gm, Sodium Chloride 5.0gm, Papaic Digest of Soybean Meal 5.0gm, Yeast Extract 5.0gm, L-Cystine 0.4gm, Hemin 5.0gm, Egg Yolk Emulsion 100.0ml, Agar 20.0gm. Take a loopful of the test organism and streak it as a straight line on the plate. Incubate anaerobically in a gas pack jar immediately after streaking and transfer into the incubator maintained at 35-37ºC for 24-48 hours for anaerobes and aerobes incubate the plate at 35-37 ºC for 24-48 hours. Examine the plate for the formation of an iridescent sheen.

b. Starch hydrolysis test
Few drops of Gram's iodine solution were added to the two days old culture of an isolate grown on the starch agar plate. The dark blue colour developed due to the formation of the starch iodine complex. The clear area around the streaked culture was considered positive and it indicated the degradation of starch due to the production of amylase enzyme.

c. Protease Production
Inoculate bacterial strains drop on, medium contains Casein 15.0gm, Vitamin K 1 10.0 gm, Sodium Chloride 5.0gm, Papaic Digest of Soybean Meal 5.0gm, Yeast Extract 5.0gm, L-Cystine 0.4gm, Hemin 5.0gm, Egg Yolk Emulsion 100.0ml, Agar 20.0gm. Take a loopful of the test organism and streak it as a straight line on the plate. Incubate anaerobically in a gas pack jar immediately after streaking and transfer into the incubator maintained at 35-37ºC for 24-48 hours for anaerobes and aerobes incubate the plate at 35-37ºC for 24-48 hours. Examine the plate for the formation of an iridescent sheen.

d. Cellulase production
The collected bacteria were inoculated in cellulose agar media composed of KH_2PO_4 0.5 g $MgSO_4$ 0.25 g cellulose 2.0 g agar 15 g gelatin 2 g and distilled water lL and at pH 6.8–7.2. Confirmation of cellulose-degrading ability of bacterial strains was performed by streaking on the cellulose Congo-Red agar media with the following composition: KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1 L and at pH 6.8–7.2. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing a discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies [W. J. Lu, *et al*., 2004.], and only these were taken for further study.

e. Chitin solubilization
Rhizobium and PGPR strains were tested for their Chitin solubilization activity on a YEMA medium supplemented with a colloid of chitin. To detect chitin solubilization, plates were spotted with 10µl of exponentially grown *Rhizobium* and PGPR strains. These plates were incubated at 28 °C for 5-7 days (Yanni *et al*., 2001). The radial area of the solubilization clear zone including colony was measured and chitin solubilization activity was expressed in mm radial area solubilized h^{-1}

f. Gelatine solubilization
Gelatine liquefying potentials of all pure cultures were tested in Petri dishes containing (g/L) gelatine 120.0 peptones 5.0; beef extract 3.0. Aliquots of medium were dispensed into Petri dishes the number of which corresponded with the number of pure cultures obtained from the isolation experiments spread or drop on a plate and incubated at 28°C for 2-7 days in a BOD incubator. After incubation, it observes the bacteria show a white smear around the bacterial colony and shows positive activity.

3. Carbon source utilization by Biolog omnilog system
Identification of isolated endophytic bacterial strains can be performed by the new GEN III microtitre Plate™ test panel of the Biolog system. The GEN III microplates enable testing of Gram-negative and Gram-positive bacteria in the same test panel. The test panel provides a " Phenotypic Fingerprint" of microorganisms which can be used to identify them to a species level. The test panel contains 71 carbon sources and 23 chemical sensitivity assays. GEN III dissects and analyzes the ability of the cell to metabolize all major classes of compounds, in addition to determining other important physiological properties such as pH, salt and lactic acid tolerance, reducing power, and chemical sensitivity. All the reagents applied were from Biolog, Inc. (Hayward, CA, USA). Fresh overnight grown cultures of the strains were used. Bacterial suspensions were prepared by removing bacterial colonies from the respective medium plate surface with a sterile cotton and agitating it in 5 ml of 0.85% saline solution. Bacterial suspension was adjusted in IF-A to achieve a 90–98% transmittance (T90) using a Biolog turbidimeter. The bacterial suspension (150µL) was dispensed into each well of a Biolog GEN III microplate. The plates were incubated at 45°C in an Omnilog Reader/Incubator (Biolog). After incubation, the phenotypic fingerprint of purple wells is compared to the Biolog's extensive species library.

Table 1: Substrates (71 carbon sources and 23 chemical) are present in the 96 wells of GEN III Micro Plate (for both Gram-negative and Gram-positive bacteria) of the Biolog Microstation system

A1 Negative control	A2 Dextrin	A3 Maltose	A4 D-trehalose	A5 D-cellobiose	A6 Gentobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive control	A11 pH-6.0	A12 pH-5.0
B1 D-Raffinose	B2 D-Lactose	B3 D-Melibioe	B43- Methyl D-glucoside	B5 D- Salicin	B6 N-Acetyl D-glucosamine	B7 N-Acetyl D-mannosamine	B8 N-Acetyl D-galactosamine	B9 N-Acetyl D-neuraminic acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α -D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium lactate	C11 Fusidic acid	C12 D –serine
D1 D-Sorbitol	D2 D-Mannitol	D3 L-Arabitol	D4 Myo-Inositol	D5 Glycerol	D6 D-Glucose 6 phosphate	D D- fructose 6 phosphate	D8 D- aspartic acid	D9 D -serine	D10 Troleandomycin	D11 Rifamycin	D12 Minocycline
E1 Gelatin	E2 Glycl-L-proline	E3 L-alanine	E4 L-Arginine	E5 L-Aspartic acid	E6 L-Glutamic acid	E7 L-Histidine	E8 L-Pyroglumatic acid	E9 L- Serine	E10 Lincomycin	E11 Guanidine hydroclanle	E12 Niaproof 4
F1 Pectin	F2 D galacturonic acid	F3 L galactonic acid galactone	F4 D gluconic acid	F5 D glucoronic acid	F6 Glucuronamide	F7 Mucic acid	F8 Quinic acid	F9 D saccharic acid	F10 Vancomycin	F11 Tetrazolium violet	F12 Tetrazolium Blue
G1 Hydroxy phenyl acetic acid	G2 Pyruvic acid methyl ester	G3 D- Lactic Acid Methyl ester	G4 L-Lactic acid	G5 Citric acid	G6 a Ketoglutaric acid	G7 D-Malic acid	G8 L- Malic acid	G9 Bromo succinic acid	G10 Nalidixic acid	G11 Lithium chloride	G12 Potassium tellurite
H1 Tween 40	H2 G-Amine N butyric acid	H3 a Hydroxyl butyric acid	H4 β-Hydroxyl butyric aci	H5 a ketobutyric acid	H6 Acetoacetic acid	H7 Propionic acid	H8 Acetic acid	H9 Formic acid	H10 Aztreonam	H11 Sodium butyrate	H12 Sodium broamate

Results

1. Presumptive test and biochemical characterization of *Rhizobium*

Root nodules of the leguminous plants from the farmer field of the north-eastern part of Varanasi, Mirzapur, Jaunpur, Basti, Gorakhpur, Maharajan, and Allahabad of Utter Pradesh, India, were collected and 42 bacterial isolates are isolated. The best-grown strains (N25, N30, N39, N40, and N42) were selected and further experiments were performed on selected bacterial strains. Different biochemical tests such as Catalase test, Methyl red test, Oxidation fermentation test, Citrate test, Lipase production, Starch hydrolysis test, Protease test, Cellulase test, Chitinase test, Gelatinase test N_2-fixation test, and HCN production test of these strains were performed in YEM agar media or YEM broth.

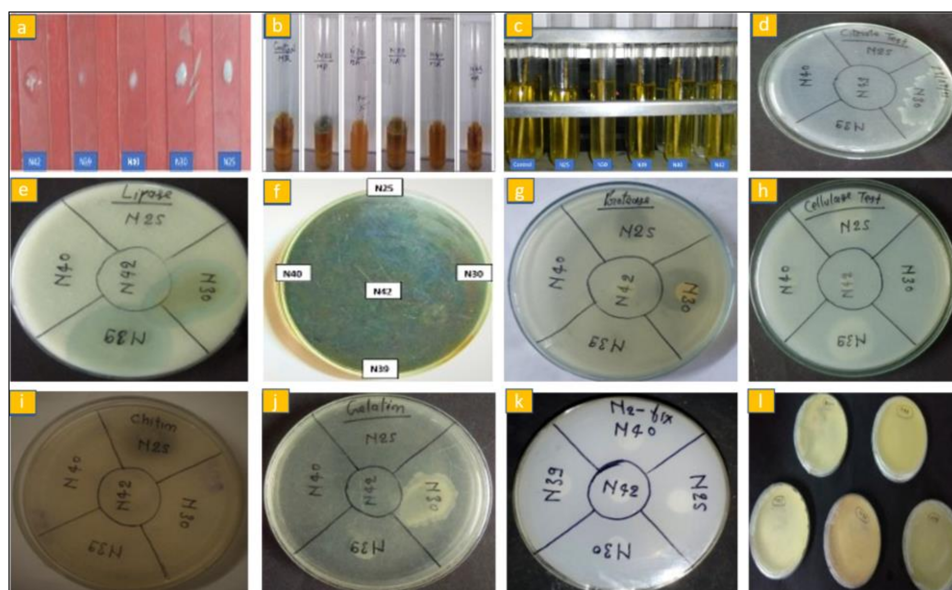

Fig 1: Different presumptive and biochemical tests. (a). Catalase test. (b) Methyl red test. (c) Oxidation fermentation test. (d) Citrate test. (e) Lipase production. (f) Starch hydrolysis test. (g) Protease test. (h) Cellulase test. (i) Chitinase test. (j) Gelatinase test (k) N_2- fixation test (l) HCN production test of selected *Rhizobium* strains

Table 2: Different presumptive and biochemical tests shown by selective *Rhizobium* strains

***Rhizobium* strains**	**Catalase test**	**Methyl red test**	**Oxidation fermentation test**	**Citrate test**	**Lipase production**	**Starch hydrolysis test**	**Protease production**	**Cellulase test**	**Chitinase test**	**Gelatinase test**	**N_2-fixation test**	**HCN production**
N25	+	-	-	-	-	-	+	+	+	-	+	-
N30	+	-	-	+	+	+	+	-	-	+	+	+
N39	+	-	-	-	+	-	-	+	-	+	+	-
N40	+	-	-	-	-	-	-	-	-	-	+	-
N42	+	-	-	-	-	-	-	-	-	-	+	-

a) **Catalase test:** Selected bacterial strains were tested *in-vitro* for catalase activity by the use of hydrogen peroxide (H_2O_2) on a clean slide. All *Rhizobium* (N25, N30, N39, N40, and N42,) strains tested showed positive catalase activity. The best catalase activity was depicted by the *Rhizobium* strains N30 followed by N25, N39, N40, and N42. (Fig.1(a) and Table 2)

b) **Methyl red test:** All selected *Rhizobium* (N25, N30, N39, N40, and N42) strains were tested in methyl red solution in a test tube in *in-vitro* condition, None of the selected *Rhizobium* strains gave a positive result. (Fig. 1(b) and Table 2).

c) **Oxidation fermentation test:** Fermentation property of selected bacterial strains can determine. This test was performed in the test tube none of the strains showed growth in fermentation media which indicate that all selected strains were negative. (Fig. 1(c) and Table 2).

d) **Citrate test:** All five (N25, N30, N39, N40, and N42) *Rhizobium* strains were tested *in-vitro* for citrate utilization activity on citric acid supplemented medium. Only N30 *Rhizobium* strains showed citrate utilization activity. (Fig.1(d) and Table 2).

e) **Lipase production:** The *Rhizobium* strains were tested for lipid utilization activity in *in-vitro* conditions, supplemented with egg yolk in the medium. Only two *Rhizobium* strains N30 and N39 out of five strains showed lipase production activity which indicated that both secrete lipase enzymes. (Fig. 1 (e) and Table 2).

f) **Starch hydrolysis test:** The starch is hydrolyzed by the amylase enzyme produced by a different type of micro-organism. Selected *Rhizobium* strains were tested for positive and negative utilization activity in *in vitro* conditions, supplemented with starch in a YEMA medium. In the test, only N30 *Rhizobium* strains showed positive results which indicated that strain N30 can produce amylase enzyme. (Fig. 1(f) and Table 2).

g) **Protease test**: Casein is a protein that is digested by enzyme proteases secreted by some microorganisms. All selected *Rhizobium* (N25, N30, N39, N40, and N42) strains were tested for the protease activity in a Petri plate supplemented with casein protein. In the experiment, only two *Rhizobium* strains N30 and N25 showed positive results out of five strains which indicated that both strains produce a protease enzyme. (Fig.1(g) and Table 2).

h) **Cellulase test:** All five (N25, N30, N39, N40, and N42) selected *Rhizobium* strains were tested *in-vitro* for cellulose utilization activity on cellulose supplemented media. Two *Rhizobium* strains N25 and N39 out of five strains showed cellulose utilization activity which is an indication of secretion of cellulase enzymes by both strains. (Fig.1(h) and Table 2).

i) **Chitinase test:** All the five selected *Rhizobium* strains were tested for chitin utilization activity in *in-vitro* condition, supplemented with chitin in YEMA medium. Only one *Rhizobium* strain N25 showed chitin solubilization activity which is an indication of chitinase enzyme secretion by this strain. (Fig. 1 (i) and Table 2).

j) **Gelatinase test:** Gelatine is a collagen protein that is digested by the gelatinase enzyme secreted by some microorganisms. All selected *Rhizobium* (N25, N30, N39, N40, and N42) strains were tested in a Petri plate supplemented with gelatine in a YEM agar medium. Only two *Rhizobium* strains N30 and N39 showed positive results for gelatine hydrolysis which means that both the strains can produce gelatinase enzymes. (Fig.1 (j) and Table 2).

k) **N_2- fixation test:** Selected bacterial strains were tested *in-vitro* for N_2- fixation activity by the use of asbeys agar medium. All *Rhizobium* (N25, N30, N39, N40, and N42,) strains tested showed positive N_2-fixation activity. (Fig.1(k) and Table 2).

l) **HCN production test:** All five (N25, N30, N39, N40, and N42) selected *Rhizobium* strains were tested *in-vitro* for HNC production activity. One *Rhizobium* strain N30 out of five strains showed HCN production activity. The color changed from yellow to light pink. (Fig.1 (l) and Table 2).

3. Study the carbon utilization of selected bacterial strains using BIOLOG GEN III microplate

The Biolog equipment is used for the identification of aerobic and anaerobic bacteria, yeasts, and fungi, allowing us to optimize our processes, obtaining more information related to the microorganisms under study with high-quality results. It contains 71 carbon sources, 23 chemical sensitivity tests and one negative control, and one positive control was chosen to optimally differentiate bacteria. It can also be used to assess microbial community function. In my research best-grown strains (N25, N30, N39, N40, and N42) were selected, and further experiments were performed on selected bacterial strains by the use of GEN III microtiter pates.

Fig 2: Carbon utilization pattern of selected bacterial isolates

Fig 3: Carbon utilization by N25, N30, N39, N40, and N42 showed strong color intensity; Data obtained using Heat map analysis

Heat map formed based on carbon utilization pattern using GEN III microplate of Biolog Microstation system has been presented in (Fig.2). The heat map described the color intensity formed by the log-transformed value of absorbance of substrate present in the 96 wells of the GEN III microplate. The heat map presents color intensity from high (red) to low (dark green). All the five bacterial strains formed three major colors one is; a dark red color that showed maximum utilization of constitute present in this well, the second color is Light yellow/pink color that showed moderate utilization of constituent and the third is dark green color showed less utilization of constituent in the well.

Heat map analysis of N25 isolates

The heat map formed by *R.* strains (N25) showed three major colors. These *Rhizobium strains* showed a strong red color

instance in Tetrazolium violet followed by vancomycin, Tetrazolium blue, Potassium tellurite, Aztreonam, pH-5, pH-6, 1% NaCl, 4% NaCl, Fusidic acid, Rifamycin, Minocycline and 1% Sodium lactate which depicted their higher metabolic potential of *R. pusense* towards these carbon substrates and different stress tolerance property. Gelatin, α- D-glucose, D-sorbitol, pectin, hydroxyphenyl acetic acid, tween 40, gentobiose, D-turanose, stachyose, D-lactose, D- melibiose, D- salicin, D-fructose, inosine, D-mannitol, D- arabitol, D-glucose 6 phosphate, troleandomycin, rifamycin, Glycyl- L-proline, L-alanine, L-arginine, L-aspartic acid, Citric acid, L-glutamic acid, L-histidine, L-pyroglumatic acid, D- serine, lincomycin, Niaproof 4, L-galactonic acid lactone, D-gluconic acid, D- glucoronic acid, Mucic acid, quinic acid, D –sacchric acid, Vancomycin, L-lactic acid, L-malic acid, potassium tellurite, G- amine –N –butyric acid, and positive control, stachyose, N-acetyl neuramic acid, inosine, L-serine, D-saccharic acid, Bromo-succinic acid, formid acid, N-acetyl neuramic acid, D-saccharic acid, Bromo-succinic acid, D-Galactose, D-Sorbitol, hydroxyphenyl acetic acid, D-mannose, Pyruvic acid methyl ester, G- Amine N butyric acid, L-alanine, L-galactonic acid lactone, D-galactose which depicted their utilization by *R.* strains (N25) bacterial strains less and avarase. (Figure 3. A)

Heat map analysis of N30 isolates

Tetrazolium violet, Sodium butyrate, Potassium tellurite, tetrazolium blue, rifamycin, pH-6, Positive control 1% NaCl, 1% sodium lactate, lincomycin, vancomycin, and L-Glutamic acid wells showed dark red color intensity and indicate a high level of metabolism with *Rhizobium* strains (N30), while others showed lower intensity by *Rhizobium* strains (N30). The color pattern of N30 differed from bacterial strains N25, N39, N40, and N42. *Rhizobium* strains (N30) showed light yellow/pink color intensity representing average/medium/moderate metabolization with raffinose, D-sorbitol, D-mannitol, L-arabitol, L-galactonic acid galactone, a-hydroxy butyric acid, Myo-inositol, glycerol, D-glucose 6-phosphate, D-fructose 6-phosphate, D-malic acid, N-acetyl D-mannosamine, Stachyose, lithium chloride, guanidium hydroplane, and 4% NaCl while N30 showed dark green color which indicates less or no metabolization with Negative control, dextrin, gelatin, hydroxyphenyl acetic acid, tween40, pyruvic acid methyl ester, D-lactic acid methyl ester, a-ketobutaric acid, 3-methyl glucose-fucose, l-fucose, N-acetyl D-muramic acid, D-saccharic acid, pH-5.0, D-serine, Minocycline, and sodium bromate. (Figure 3. B)

Heat map analysis of N39 isolates

The color pattern of N39 differed from *Rhizobium strains* N25, N30, N40, and N42. *Rhizobium strains* N39 showed dark red color indicating a high level of metabolism with Tetrazolium violet, D-sorbitol, hydroxyphenyl acetic acid, D-mannitol, L-arabitol, L-alanine, Myo-inositol, L-lactic acid, N-acetyl D-glucosamine, Sucrose, N-acetyl glucosamine, iconic acid, L-malic acid, Acetic acid, Inosine, L-serine, Bromo succinic acid, nalidixic acid, Terazolium blue, and potassium tellurite. *Rhizobium* strains N39 showed light yellow/pink color representing average/medium/moderate metabolization with raffinose, pectine, tween40, dextrin, D-lactose, D-mannose, glacial L- proline, D-galacturonic acid, G-amino N-butyric acid, D-melibiose, D-sorbitol, L-galactonic acid galactose, b-hydroxybutyric acid, L-glutamic acid, Glucuronamid, l-histidine, propionic acid, inosine, L-serine, p-6, and rifamycins, Also N30 showed light green color which indicates less or no metabolization with Negative control, dextrin, gelatin, pyruvic acid methyl ester, hydroxybutyric acid, D-lactic acid methyl ester, a-ketobutaric acid, 3-methyl glucose, fucose, L-fucose, 1% sodium lactate, 4% NaCl, fusidic acid, lithium chloride, sodium butyrate, N-acetyl D-muramic acid, D-saccharic acid, pH-5.0, D-serine, Minocycline, and sodium bromate. (Figure 3. C)

Heat map analysis of N40 isolates

Pectine, Tween 40, Dextrin, D-lactose, Maltose, L-alanine, a-hydroxy butyric acid, Myo-inositol, D-cellobiose, glycerol, D-glucose 6-phosphate, sucrose, Propionic acid, Acetic acid, D-serin, Formic acid, Positive control, pH-6, Sodium butyrate, 8% NaCl, Tetrazolium blue, and Sodium bromate wells showed dark red color indicates the high level of metabolism with *Rhizobium* strains (N40). N40 *Rhizobium strains* showed different color patterns with bacterial strains N25, N30, N39, and N42. *Rhizobium* strains (N40) showed light yellow/pink color representing average/medium/moderate metabolization with a-D glucose, gelatine, galacturonic acid, pyruvic acid methyl ester, G-amino n butyric acid, D-melibiose, B-hydroxy butyric acid, Glucoramide, Bromo succinic acid, 1% NaCl, Nalidixic acid, guanidium hydroclane, lithium chloride, pH-6, Minocycline, and potassium tellurite. D-mannose, D-mannitol, Flycial L-proline, D-fructose, L-arabitol, Myo-inositol, D-gluconic acid, 3-Methyl glucose, L-aspartic acid, D-glucuronic acid, N-acetyl d-glucosamine, D-fructose, L-glutamic acid, Mucic acid, L-raffinose, D-aspartic acid, inosine, D-saccharic acid, Bromo succinic acid, 1% sodium lactate, Lincomycin, 4% NaCl, and D-serin with N40 *Rhizobium strains* showed light green color which indicates less or no metabolization. (Figure 3. D)

Heat map analysis of N42 isolates

The color pattern of N42 differs from bacterial strains N25, N30, N39, and N40. *Rhizobium strains* (N42) showed dark red color indicating a high level of metabolism with L-lactose, Maltose, D-fructose, D-cellobiose, D-glucuronic acid, Mucic acid, D-tyrosinase, L-rhamnose, Lpyroglumatic acid, Bromo succinic acid, Positive control, 1% NaCl, and Potassium tellurite. *Rhizobium* strains (N42) showed light yellow/pink color representing medium metabolization with glucose, D-sorbitol, L-alanine, L-arginine, 3-methyl glucoside, D-salicin, sucrose, N-acetyl manosamin, L-fucose, D-fructose 6-phosphate, D-malic acid, L-serine, and Niaroof 4. N30 *Rhizobium strains also* showed a light green color which indicates less or no metabolization with Negative control, dextrin, gelatin pyruvic acid methyl ester, hydroxybutyric acid, D-lactic acid methyl ester, a-ketobutaric acid, 3-methyl glucose, fucose, L-fucose, 1% sodium lactate, 4% NaCl, fusidic acid, Lithium chloride, sodium butyrate, N-acetyl D-neuraminic acid, D-saccharic acid, pH-5.0, D-serine, Minocycline, and sodium bromate. (Figure 3. E)

Table 3: Presumptive identification of Bacterial isolates by using BIOLOG GEN III library database

Sr. No.	Bacterial isolates	Presumptive identification of isolates by BIOLOG GEN III plate
1.	N25	*Rhizobium radiobacter,*
2.	N30	*Rhizobium radiobacter*
3.	N39	*Paenibacillus mendelii*
4.	N40	*Rhizobium radiobacter*
5.	N42	*Rhizobium radiobacter*

Discussion

Plant root and rhizospheric soil are rich in microorganism which contains many species of *Rhizobium* that play important role in plant growth and development. These bacteria showed many properties, including degradative enzymes (Cellulase, Pectinase, Chitinase, Lipase, Protease, etc.). Plant growth-promoting pseudomonads produce IAA, HCN, siderophore, and P-solubilization. Pseudomonas, Bacillus, and *Rhizobium* genera have, the ability to change their metabolism in response to the phosphorus available for cellular growth. (Gyaneshwar, P., *et al*., 2002, & Rodriguez, H., 1999) [3, 4]

Hydrogen peroxide is produced from dis-mutation by catalase activity which plays an important role in the symbiosis (Orikasa *et al.,* 2010) [19]. Root nodulating bacteria are highly susceptible to hydrogen peroxide (Ohwada *et al.,* 1999) [20]. Methyl red test showed the ability of bacteria to utilize glucose and convert it to a stable acid like lactic acid, and acetic acid (McDevitt, S. 2009) [1]. The bacterial species that showed positive methyl red tests were identified as Rhizobium, Enterobacter & Bacillus sp. Four bacterial strains were capable of growth on methyl red, (Adedayo, O., *et al*., 2004) [2]. *Rhizobium* isolated from fenugreek gives positive methyl red test (Panwar *et al*., 2012) [21]. In my research, all bacterial strains showed a negative methyl red test. Aerobic bacteria species metabolize carbohydrates oxidatively, which can be determined by the oxidative-fermentative test. In the present study, all strains (N25, N30, N39, N40, and N42) were found to be aerobe or facultative anaerobe and metabolized carbohydrate oxidatively. A similar finding also reported the oxidative & fermentative nature of rhizobia isolated from maize (Perez *et al*., 2019). The citrate test determines the ability of the bacteria to be capable of fermenting citrate. The *Rhizobium phaseoli* and *Rhizobium trifolii* showed a positive result for citrate utilization while, *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* showed a negative citrate test (Datta *et al*., 2015) [22]. *Rhizobium* spp. (N30) and showed a positive (+) citrate utilization test and the rest of the bacterial strains showed negative results. Lipase is an enzyme capable degrade lipid in the culture or plant roots. One out of five bacterial strains *Rhizobium* spp. (N30) were found to be positive (+) lipase producers' activity. A similar finding showed mycotoxin patulin degradation enzymatic by an extracellular lipase from *Ralstonia* (He *et al*., 2022). A similar result also reported extracellular lipases produced by Bacillus *spp*. and Pseudomonas *spp*. (Hasan *et al*., 2018) [23]. This result is in agreement with previous findings of researchers.

Biolog GEN III plates are used to characterize microorganisms and allowed for the comparison of the functional diversity of bacterial strains (Woźniak, M., *et al*., 2019) based on the ability to oxidize various carbon sources and identification of bacterial species including Gram-positive and Gram-negative. GEN III library database identified N25 as *Rhizobium radiobacter*, N30 as *Rhizobium radiobacter*, N39 as *Paenibacillus mendelii*, N40 as *Rhizobium radiobacter*, N42 as *Rhizobium radiobacter*, Several workers have documented their findings to better understand. Strains ES2 (Stenotrophomonas maltophilia), ZR1 (*Novosphingobium resinovorum*), ZR3 (*Delftia acidovorans*) and ZR4 (D. *acidovorans*) showed the highest metabolic activities on the tested substrates. Whereas, ES4 (*Brevundimonas* sp.), ES7 (*Brevundimonas* sp.), and ER1 (*Comamonas koreensis*) were characterized by the lowest utilization of all carbon sources (Wozniak, M., *et al*., 2019) [24]. *Paenibacillus castaneae, Chryseobacterium indoltheticum, Pseudomonas fluorescens, Acinetobacter johnsonie, Mycobacterium flavescens, Ralstonia pickettii, Acinetobacter schindleri, Microbacterium maritypicum (Chojniak*, J., *et al*., 2015) [5].

Conclusion

Based on the results conclude the selected bacterial strains are *Rhizobium* isolated from the root of leguminous crops. That bacterial belonged to proteobacteria were identified as *Rhizobium.* They help in plant growth-promoting activity by (starch, lipid, cellulose, chitin, Protein, and Gelatin). Biolog's microarrays were used for identification and characterization of selected bacterial strains which did not exactly match. Although attention was given to presenting the GEN III microplate as a novel tool for identification of strain-based different carbon sources could not yield a concrete result. One out of five bacterial strains N30 (*Rhizobium spp*.) showed the best performance in all maximum tests.

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