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Isolation and Characterization of Mushroom Growth Promoting Bacteria from Spent Mushroom Compost of *Lentinula edodes*

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Abstract

The experiment was conducted from November, 2022 to June, 2023 in the laboratory of Department of Basic Sciences and in the Mushroom unit, Department of Plant Pathology of Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India, to study the isolation and characterization of mushroom growth promoting bacteria from spent mushroom compost of *Lentinula edodes*. The mushroom growth promoting bacteria (MGPB) stimulate mycelial growth and fruiting by enhancing nutrient elements concentration, protein and carbohydrate contents. The spent mushroom compost can be a good source of growth promoting bacteria for mushroom cultivation. For the present investigation, samples of spent mushroom compost (SMC) were collected from two locations of Solan i.e., Directorate of Mushroom Research, Chambaghat and University of Horticulture and Forestry (UHF), Nauni. A total of 45 bacterial isolates were isolated from SMC and purified for further studies. Out of 45 bacterial isolates, 28 were P-solubilizers, 29 were nitrogen fixers, 19 were HCN producers, 22 were siderophore producers and 23 were IAA producers. Out of 45 bacterial isolates only 3 isolates were selected based on multifarious PGPR traits. Among the selected bacterial isolates, maximum P-solubilization was shown by isolate DMR1-36, both qualitatively and quantitatively. Maximum siderophore production efficiency (SE%) was observed in isolate DMR1-36 on solid CAS medium as well as in case of liquid medium. Bacterial isolate DMR1-36 showed highest production of IAA. All three bacterial isolates showed maximum growth at temperature of 35°C, pH 7.0 and incubation period of 48 hours.

Keywords: *Lentinula edodes*, SMC, MGPB, 16srRNA, phosphate solubilisation

1. Introduction

Mushroom farming is a green enterprise since it recycles the waste from farms, animals, breweries and other sources while producing fruit bodies with unique nutritional and medicinal properties (Ansari et al., 2022). Mushroom is a fruiting body of fungi, most of the cultivated mushrooms belong to the phylum Basidiomycota, although some Ascomycota such as members from the genera Morchella or Tuber has also been successfully cultivated and commercially exploited (Liu et al., 2017; Rubini et al., 2014). Among basidiomycota, *Agaricus bisporus* is most widely cultivated species of edible mushrooms (Chauhan et al., 2012). Many different mushroom species have diversified chemical compositions and nutritional values. Apart from being excellent sources of crude protein, edible mushrooms have rich sources of dietary soluble fibre, essential minerals, complex polysaccharides, essential unsaturated fatty acids, vitamins B and secondary metabolites (Desisa et al., 2024). Mushrooms need substrate to grow like paddy straw, wheat straw, sawdust, and other agricultural residues that are rich in carbon and nitrogen sources. It has been reported that specific microorganisms required for crop to improve the growth and yield (Olanrewaju et al., 2017). Potent microbial population indeed plays a very crucial role in mycelia growth and other cultivation stages of mushroom including fructification. Various bacterial species belonging to particularly genus

Pseudomonas may play significant role in the artificial growing of edible mushrooms (Kumari and Narain, 2020).

Lentinula edodes (Shiitake mushroom) is a cultured edible fungus which is indigenous to Japan, China and other Asian countries with the temperate climate. It can grow in winter season and also round the year under controlled conditions. Bioactive compounds such as polysaccharides, purines, proteins (amino acids), fatty acids, polyphenols, and sterols in shiitake mushrooms have been shown to have high nutritional value and enhance human health by promoting anti-inflammatory, antioxidant, antitumor, antibacterial, and immunostimulatory effects (Xu et al., 2024). It may also lower blood pressure and serum cholesterol levels.

The occurrence of beneficial microorganisms with the substrates used for the cultivation of mushrooms stimulates the growth and primordial formation. Commercial production of *Lentinula edodes* is largely determined by the availability and utilization of cheap by- products which are agricultural wastes that are ideal and most promising substrates for cultivation (Alemu, 2014). Mushroom growth promoting bacteria (MGPB) promote the mycorrhizal growths, shortening soil composting procedure, improving nature of the substrate by secretion of secondary metabolites and help in mushroom fructification (Kadam et al., 2017).

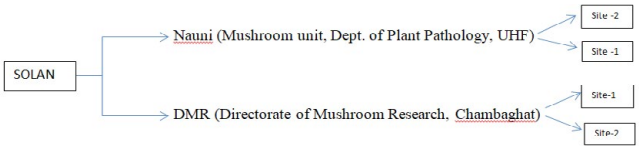
SMC (spent mushroom compost) is the residual compost waste generated after mushroom productions; SMC has many biological properties. The SMC is rich in bacterial diversity (Rana et al., 2025) and have the potency of soil, high water holding capacity and aeration (Hussain et al., 2023). SMC can enhance soil health by acting as biofertilizers and helping with bioremediation (Khalil et al., 2024). SMC contains a considerable amount of protein and carbohydrates and is rich in holocellulose as shiitake consumes 15%, 56% and 23% of hemicelluloses, cellulose and lignin content respectively (Kumar et al., 2022). The microbial communities play an important role as predictors of plant health because they improve plant fertilization and protection (Banerjee and Heijden, 2023). The bacteria within SMC are adapted to the harsh conditions of mushroom cultivation; they play a beneficial role in recycling compost nutrients. Therefore, isolation and characterization of microbial diversity with potential growth promoting traits in SMC are of prime importance for researchers and farmers. However, isolates from SMC have not been reported and tested for their potential effect on mushroom yield which may have better potential. Keeping in view the present status of knowledge, there is a need to screen bacterial diversity associated with SMC.

2. Materials and Methods

The present study was carried out between 2022-2023 (November, 2022 to June, 2023) in the laboratory of Department of Basic Sciences and in the Mushroom unit, Department of Plant Pathology of Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India entitled 'Isolation and characterization of mushroom growth promoting bacteria from spent mushroom compost of *Lentinula edodes'*. The studies were conducted to select best mushroom growth promoting rhizobacteria from spent mushroom compost and to study their response in the improvement of growth and yield of Shiitake mushroom.

2.1. Sample collection

The samples of spent mushroom compost (SMC) were collected from two locations of district Solan, Himachal Pradesh, these two locations were Directorate of Mushroom Research, Chambaghat and Mushroom farm, Department of Plant pathology, UHF, Nauni. The samples were collected from two different sites from each location. Therefore, there were total 4 sampling sites with three replications of each sample. The samples were collected in sterilized plastic bags and stored at 4°C in the laboratory of Department of Basic Sciences for isolation and carrying out the further analytical work.



2.2. Isolation and maintenance of bacterial isolates

One gram of sample (spent mushroom compost) was dissolved in 9 ml of sterilized distilled water and serial dilutions were made up to 10-8 under aseptic conditions. The serially diluted suspensions of samples were spread on pre-poured nutrient agar medium and on the selective media: Nitrogen free medium (Jensen, 1987) for nitrogen fixing activity, Pikovskaya's medium (Pikovskaya, 1948) for phosphate solubilizing ability as described by Rao (1999). The petri plates were incubated for 24-48 h at 35 ± 2°C temperature in incubator. The microbial count appeared on the plate was expressed as colony forming unit (cfu) gram ml^{-1} of sample (spent mushroom compost). The isolated cultures were purified by streak plate method and maintained on the slants of respective medium at 4°C in refrigerator. Bacterial cultures were maintained on nutrient agar at 4°C. Sub-culturing of bacterial cultures was done once in fortnight on respective medium at incubation temperature of 28±1°C.

2.3. Screening of bacterial isolates

The screening of the bacterial isolates for various plant growth promoting activities like P-solubilization, growth on N-free medium, siderophore production, HCN and auxin production (IAA) were performed by adopting the standard methods. The brief descriptions of these methods are as follows:

2.3.1. Phosphate solubilizing activity

Each of purified isolate were streaked in a straight line on PVK medium as described by Pikovskaya (1948) and was incubated for 72h at 30±2°C. Colonies showing solubilization halos (>0.1 mm diameter) were selected.

2.3.2. Siderophore production

The Chrome azurol S (CAS) plate assay method was used to measure siderophore production (Schwyn and Neilands, 1987). CAS (60.5 mg 50 ml^{-1} distilled water) was mixed with 10 mL iron solution to make sterilized blue agar (1 mM $FeCl_3.6H_2O$ in 10mM HCl). The hexadecyl trimethylammonium bromide (HDTMA)solution was produced by dissolving

72.9 mg HDTMA in 40 ml distilled water. As a result, 100 ml of CAS dye was made. 750 ml of nutrient agar was combined with 30.24 g of 1, 4 piperazine diethane sulphonic acid, and the pH was adjusted to 6.8 with 0.1N NaOH. It was autoclaved separately and thenmixed with Chrome azurol- S (100 ml) under aseptic conditions before being used in subsequent tests.

A bit of 72h old culture of each test bacterium was placed on blue coloured chrome-azurol-S agar (CAS) plates. Plates were incubated at 30±2°C for 24h and observed for production of orange halo around the bit.

% siderophore efficiency=(Z-C x 100)/C

where, Z-size of halozone and C-colony size

The halozone diameter around the colony was calculated by subtracting colony size from total size. Phosphate solubilization index (PSI) was measured using the formula (Premono et al., 1996).

2.3.3. HCN production

Bakker and Schippers (1987) approach was used to screen bacterial isolates for the generation of hydrogen cyanide (HCN). Bacterial cultures were streaked on King's B medium modified with 1.4 g l^{-1} glycine. Whatman No.1 filter paper strips were soaked in 0.5% picric acid in 2% sodium carbonate, then inserted in the lid of each petriplate, sealed with para film and incubated for 1 to 4 days at 35°C. The findings of the uninoculated control were compared. The colour of the filter paper on the plates was changed from yellow to orange brown.

2.3.4. Quantitative estimation of indole-3-acid

Bacterial cultures were grown in modified Luria Bertani broth amended with 5mm L-tryptophan, 0.065% sodium dodecyl sulphate and 1% glycerol 72 h at 35°C under shaking conditions. The cultures were centrifuged at 15,000 rpm for 20 minutes and supernatants were collected and stored at 4°C. The method described by Gorden and Palleg (1957) was used to determine the IAA equivalents i.e. 3 ml of supernatant was pipette out into test tube and 2 ml of Salkowski reagent (2 ml 0.5 M $FeCl_3$+98 ml 35% $HCLO_4$) was added to it. The tubes containing the mixture were left for 30 minutes (in dark) for colour development. Intensity of colour was measured spectrophotometrically at 535 nm. Similarly, colour was also developed in standard solution of IAA (10-100 µg ml^{-1}) and a standard curve was established by measuring the intensity of this colour.

2.4. Morphological, physiological and biochemical characterization of selected isolates

The most efficient bacterial isolates selected on the basis of plant growth promoting traits were subjected to morphological, physiological and biochemical characterization as per the criteria of Bergey's Manual of Systematic Bacteriology (Cappuccino and Sherman, 2002). Morphological characteristics of isolates including colony morphology, Gram's reaction and cell shape were investigated. Separate experiments were performed for optimization of conditions for growth of selected bacterial isolates. These were as under:

2.4.1. Effect of incubation period on growth of bacterial isolates

Five ml of nutrient broth was taken in test tubes and inoculated with 0.1 ml of 48hr old bacterial cell suspension (O.D. 1.0 at 540 nm). Each test tube was incubated for different time period (24h, 48h, 72h, 96h and 120h) and observed for turbidity. The optimum incubation period for growth was maintained for further experimentation.

2.4.2. Effect of temperature on growth of bacterial isolates

Five ml of nutrient broth was taken in test tubes and inoculated with 0.1 ml of 48 hold bacterial cell suspension (OD 1.0 at 540 nm). The optimum temperature for growth was selected on the basis of turbidity caused by the bacterial growth in test tube. Growth curves were drawn by growing the culture at various temperatures.

2.4.3. Effect of pH on the growth of bacterial isolates

Five ml of nutrient broth was taken in test tubes. The pH of medium was adjusted to the value of 3, 5, 7, 9 and 11 using 0.1 N NaOH or 0.1 N HCl. Each tube was inoculated with 0.1 ml of 48 h old bacterial cell suspension (OD 1.0 at 540 nm) of selected isolates. The experiment was carried out in triplicates. The pH suited for maximum growth was selected based on turbidity caused by the bacterial growth in test tube.

2.5. Biochemical characterization

2.5.1. Indole test

1% of tryptone broth was inoculated with a bacterial colony. Inoculated tubes were incubated at 37°C for 48 hours. After 48 hours of incubation, 1 ml of Kovac's reagent was added and then the tubes were shaken gently and allowed to stand for 20 minutes. The formation of the red coloration at the top layer indicates positive and yellow coloration indicates negative results.

2.5.2. Methyl red test

This test detects ability of microbes to oxidise glucose with production and stabilization of high concentration of acid end products. MR-VP broth was prepared, sterilized and inoculated with samples and incubated for 48 hours at 37°C. Following incubation, 5–6 drops of methyl red solution was added. Bright red colour change indicates positive result, red- orange colour indicates a weak positive result and yellow- orange indicates a negative result.

2.5.3. Citrate utilization test

Citrate is acted upon by enzyme citrase which produces

oxaloacetic acid and acetate. These are enzymatically converted to pyruvate and CO_2. During reaction, the medium becomes alkaline as the CO_2 combines with Na and H_2O to form sodium carbonate which is alkaline. Samples were inoculated on Simmon's citrate agar medium slants and incubated for 24-48h at 37°C. Positive result is indicated by blue colour slope and no colour change indicates a negative result.

2.5.4. Amylase production or starch hydrolysis test

The capacity of a microbe to break down starch is a criterion for determining its production of amylase. The bacterial colonies were inoculated in freshly prepared starch agar plates using a sterile inoculation needle by dot method and incubated at 37°C for 24–48h. Following the incubation period, the culture plates were flooded with Gram's iodine solution, and the plates were kept undisturbed for 5-10 minutes. Then, the iodine solution was carefully decanted from the plates, and any clean zone development surrounding the colonies was observed for a positive result.

2.5.6. Casein hydrolysis interpretation

Bacterial colonies were inoculated on nutrient agar with 1% casein and incubated at 37°C for 24h. Colonies forming clear zones shows the casein hydrolysis as positive result.

2.5.7. Catalase test

The purpose of the test is to determine whether a bacterium produces the catalase enzyme. The presence of catalase enables the breakdown of hydrogen peroxide into liquid water (H_2O) and oxygen gas (O_2), which results in the formation of bubbles, signifying a successful reaction. In this test, bacterial culture was mixed with a drop of hydrogen peroxide on a glass slide and observed for effervescence (bubble formation).

2.5.8. Urease test

Few drops of indicator phenol red added to the urea broth medium, a colour change from yellow to bright pinkish red is positive; lack of colour change is a negative result for urease.

2.5.9. Gelatin liquification test

The bacterial pure colonies were inoculated on gelatin agar media slants. These culture tubes were incubated at 25°C for one week. Liquification and solidification of gelatin media at 25°C showed positive and negative results respectively for this test.

2.6. Molecular Characterization of selected bacterial isolates by 16s rRNA sequencing

2.6.1. Genomic DNA extraction by conventional method (Sambrook et al., 1989)

Bacterial isolates were incubated overnight at 28° C in YEM broth. Five ml of overnight grown culture was centrifuged at 13,000 rpm for 1 min. Pellet was re-suspended by vortex in 500 µl of extraction buffer and 50 µl of 10% SDS. Then it was incubated at 65°C in water bath for 30 min until the sample lysate becomes clear. During incubation, tube was inverted at every 3 min. After incubation, 2 µl of RNAase A (50 mg ml^{-1}) was added to sample lysate, vortexed and incubated at room temperature for 5 min. To the lysate, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed well. The mixture was centrifuged at 10,000 rpm for 5min at room temperature resulting in the formation of two layers. Upper aqueous layer was transferred to eppendorf tube, and the extraction step was repeated. Sodium chloride (NaCl 5M, 1/10 volume) and absolute ethanol (2.5 volume) were added and incubated at-20°C overnight. The mixture was then centrifuged at 12,000 rpm for 20 min at room temperature and thereafter the supernatant was discarded. The DNA pellet so obtained was washed with 1 ml of 70% ethanol, dried to evaporate ethanol and finally suspendedin Tris-EDTA (TE) buffer.

2.6.2. Gel electrophoresis

The agarose was added in 100 ml of 1x TAE buffer and mixed by heating until the agarose solubilised completely. The slurry was cooled, and ethidium bromide was added in concentration of 0.5 µg ml^{-1}, mixed thoroughly and poured onto gel casting tray and comb was placed in gel. The sample of DNA was mixed with the gel loading dye (5:1 ratio) and slowly added into the gel submerged in 1xTAE gel running buffer. The electrophoresis was performed at 1- 5 V/cm^2 voltage until two dyes resolve and migrated the appropriate distance through the gel. After electrophoresis, the gel was observed under UV light and documented using gel documentation system (Bio-rad).

2.6.3. PCR amplification of 16S rDNA

PCR reaction was carried out in 20 µl reaction containing ~50 ng of template DNA, 20p moles of each primer, 0.2 mM dNTPs and 1U Tax polymerase (Genei, Bangalore) in 1xPCR buffer. Amplification was performed using thermo-cycler (multigene PCR system, Labnet). Reactions were cycled 35 times at 94°C for 3 min, 50°C for 30 sec, 72°C for 1.5 min followed by final extension at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel in 1xTAE buffer, run at 100V for 1 h and the size was determined by using 1 kb DNA ladder (Thermo Scientific).

2.6.4. Gel elution and dissociation

Gel elution was done by HiYield Gel/PCR DNA Extraction Kit. Agarose gel slice containing relevant DNA fragments was excised and extra agarose was removed to minimize the size of the gel slice.300 mg of the gel slice was transferred into a micro centrifuge tube. 500 µl of DF buffer was added to the sample and vortexed. Incubation was done at 55°C for 10-15 min until the gel slice gets completely dissolved. During incubation, the tubes were inverted at every 2-3 min and dissolved sample mixture was cooled at room temperature. A DF column was placed in 2ml collection tube. 800 µl of sample mixture (from above step) was applied into the DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube. 600 µl of wash buffer (ethanol added) was added into DF column

and centrifuged at 13,000 rpm for 30 sec. Flow through was discarded and DF column was placed back in 2 ml collection tube. It was again centrifuged for 3 min at 13,000 rpm to dry the column matrix. Dried column was transferred into a new micro centrifuge tube. 15-30 µl of elution buffer or distilled water was added into the centre of the column matrix. Stand for 2 min until elution buffer or distilled water was absorbed by the matrix. Centrifugation was done for 2 min at 13,000 rpm to elute purified DNA.

2.6.5. Sequencing and phylogenetic analysis

Representative bacterial isolates of each genotypic profile were chosen for 16S rDNA partial sequencing. Sequencing was performed from Eurofins lab, Bangalore, India, using both forward and reverse primers as mention above. The sequence alignment was done using the Clustal W (Thompson et al., 1994) and manually edited using the Bioedit package (Hall, 1999). The cladograms were constructed by neighbor-joining method (Saitou and Nei, 1987) with the Kimura-2-parameter model (Kimura, 1980) and were bootstrapped using the software programs in the MEGA 6.0 package (Tamura et al., 2013).

2.7. Statistical analysis

The data recorded on plant, soil and microbiological properties will be statistically analyzed by using MS-Excel and OPSTAT packages (Sheoran et al., 1998). The mean values of data will be used for the analysis of variance (ANOVA) as described by Panse and Sukhatme (1954).

3. Results and Discussion

3.1. Isolation and enumeration of bacteria associated with SMC

As represented in figure 1, the maximum bacterial count (6.69×10^6 cfu g^{-1} of substrate) was recorded in SMC sample from Directorate of Mushroom Research (DMR) Site -1 of district Solan. However, the minimum bacterial count (5.67×10^6 cfu g^{-1} of SMC) was recorded in SMC sample from Mushroom farm of University of Horticulture and Forestry, Nauni, district Solan. Based on bacterial growth on nutrient agar by calculating 10^6 cfu g^{-1} of substrate, total 45 bacterial isolates were selected. Out of 45 isolates, 10 isolates from Nauni Site (1) represented as NS1, 13 isolates from Nauni Site (2) represented as NS2, 14 isolates from Chambaghat Site (1) represented as DMR1 and 8 isolates from Chambaghat Site (2) represented as DMR2 were selected for further studies (Figure 1). Diverse microorganisms are present in the compost of button mushroom due to variation in composition of raw material used for compost preparation, time of sampling and environmental conditions (Yohalem et al., 1996; Siyoum et al., 2016; Bhardwaj, 2022). Xiong et al. (2016) reported that shifted environmental factors could reshape the composition of soil microbial community. The microbial abundance, composition and activity changes substantially during the composting process and are correlated with high microbial diversity and low activity in matured compost.

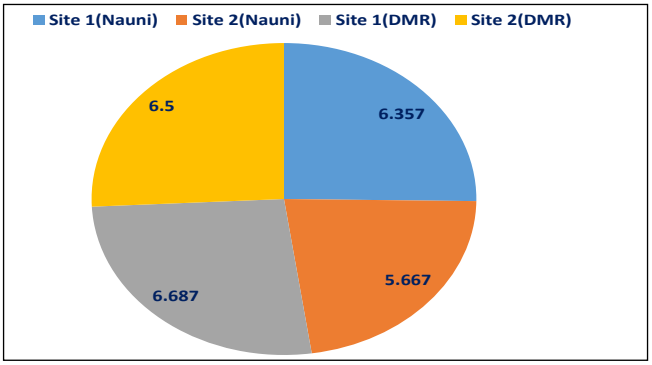


Figure 1: Comparative SMC microbial population (x10^6 cfu g^{-1} of substrate) from different sites

3.2. Screening of bacterial isolates for growth promoting traits

The forty-five selected bacterial isolates were screened for the growth promoting traits viz. IAA production, phosphate solubilization, nitrogen fixation ability, siderophore production and HCN production. As shown in Figure 2, out of 45 bacterial isolates, 23 bacterial isolates showed positive results for IAA production (51.11%), 28 bacterial isolates showed positive results for phosphate solubilization (62.22%), 29 isolates showed positive results for nitrogen fixing ability (64.44%), 22 isolates showed positive results for siderophore production (48.87%) and only 19 isolates showed positive results for HCN production (42.23%). It was found that the isolate DMR1-36 showed maximum growth promoting traits followed by NS2-23 and NS1-9. However, the isolates NS1-7, NS1-8, NS2-21, DMR1-25 and DMR1-30 did not show any growth promoting activities. The bacteria isolated from Site 1 and 2 of DMR showed high growth promoting traits followed by Site 1 of Nauni, whereas bacterial isolates from Site 2of Nauni exhibited low growth promoting traits. The variation in the composition of the compost, the environmental factors involved in mushroom growing and the timing of spent mushroom compost during sampling may have contributed to the difference in bacterial count at various sites.

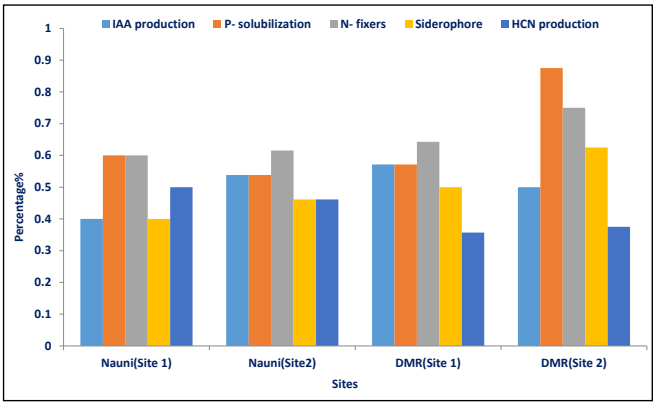


Figure 2: Percentage of isolates exhibiting growth promoting traits

3.2.1. Quantitative estimation of growth promoting traits in the selected bacterial isolates

The selected 45 bacterial isolates were further screened for quantitative estimation of phosphate solubilization, siderophore production and IAA production. Perusal of data presented in Table 1 and Plate 1 showed that all the 45 bacterial isolates were able to solubilise phosphate within the range of 62.41- 134 µg ml^{-1}. It was found that out of 45 isolates, maximum phosphate solubilization index and P- solubilization was observed in isolate DMR1-36 (3.17, 134 µg ml^{-1}), followed by isolate NS2-23 (2.93, 126.35µg ml^{-1}); whereas minimum was recorded in isolate DMR2-40 (1.63, 62.41µg ml^{-1}).The maximum P- solubilization (134µg ml^{-1}) of isolate DMR1-36 coupled with the decrease in the final pH from 7.0 (neutral) to 3.62 (acidic) of the medium. Bacterial isolate DMR1-36 showed maximum P- solubilization efficiency of 82.07% followed by NS2-23(76.87%) and NS1-9 (75.02%).

However, the minimum i.e. 20.93% was recorded for isolate DMR2-40. Phosphorus is one of the most important micronutrients which plays a major role in plants' growth and repair of cells and tissues. Phosphorus supports root development in various metabolic processes. Though plants are not able to absorb insoluble form of phosphorus, they gain the ability through the intervention of phosphatase enzyme which converts it into soluble form. Several PSBs have the ability to convert insoluble phosphate into soluble forms (H_2PO^{4-} and HPO_4^{2-}) bioavailable through acidification process, chelation and production of organic acids (Lebrazi et al., 2020). Present results are in line with Dawwam et al., (2013) who reported that 4 out of 7 selected bacterial isolates solubilized phosphate on PVK agar medium; isolate P31 showed highest phosphate solubilization efficiency of 350% and also showed the highest amount of soluble P (353.3 µg ml^{-1}) in liquid medium. Chauhan et al. (2014) reported 5 out of 30 bacterial isolates exhibited phosphate solubilization activity where isolate CKT3 showed maximum P- solubilization (210 µg ml^{-1}) accompanied by significant decrease in pH of the medium from neutral to acidic after 72 hours of incubation.

Table 1: Solubilization of tri calcium phosphate (TCP) by selected bacterial isolates in solid and liquid Pikovskaya's medium

Bacterial Isolates	P-solubilization in solid medium		P- solubilization in liquid medium (µg ml^{-1})	Final pH of supernatant
	P-solubilization index (PSI)	% P- Solubilization efficiency (% SE)		
NS1-2	2.13	54.96	69.95	4.15
NS1-3	2.04	61.93	118.83	5.32
NS1-4	2.42	22.84	64.85	5.13
NS1-9	2.92	75.02	115.41	3.50
NS1-10	2.13	27.61	65.00	3.60
NS2-14	2.15	24.20	48.95	4.11
NS2-17	2.05	66.14	65.80	4.22
NS2-22	2.16	67.67	74.72	4.15
NS2-23	2.93	76.87	126.35	4.03
DMR1-24	1.85	60.79	95.11	3.25
DMR1-25	1.73	71.23	84.12	4.63
DMR1-29	2.35	42.89	105.50	5.31
DMR1-31	2.72	37.12	81.75	5.13
DMR1-33	2.24	30.86	100.98	5.02
DMR1-35	2.15	57.04	109.90	4.51
DMR1-36	3.17	82.07	134.00	3.62
DMR2-40	1.63	20.93	62.41	4.12
DMR2-41	2.39	46.26	101.55	3.74
DMR2-43	2.53	73.58	109.20	5.02
DMR2-44	2.55	36.62	73.84	4.02
CD (p=0.05)	0.04	0.03	1.04	0.04

: PSI=Zone size + colony size/colony size; *: %solubilisation efficiency (%S.E.) = Z C/C×100; where C: colonydiameter; Z: Halozone Diameter C; ****: P-solubilized=T-C; where, T: P-solubilized in test; C: P-solubilized in control

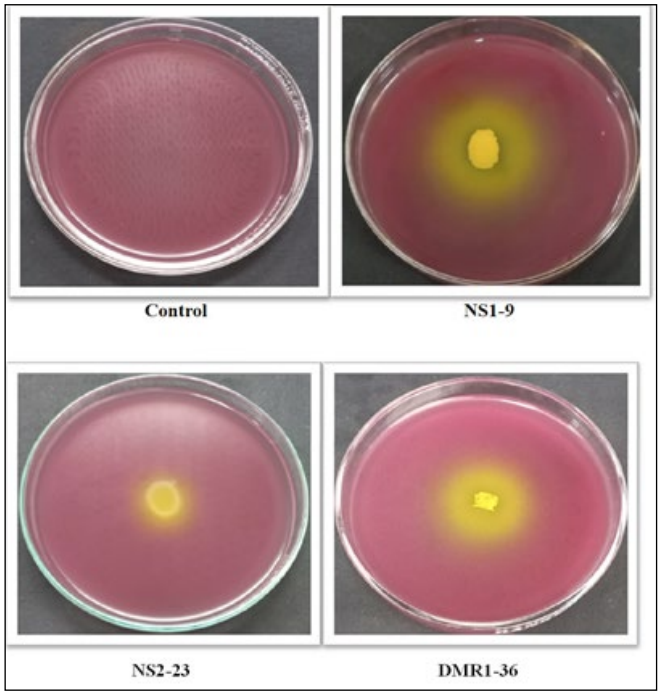


Plate 1: Phosphorus solubilisation by isolates NS1-9, NS2-23 and DMR1-36 on PVK media

Paul and Sinha (2013) found solubilization Index of 2.85 for *Pseudomonas aeroginosa* KUPSB12, using Pikovskaya agar plates. Clear zones are formed around the colonies, which is due to phosphate solubilization. Mohammad et al. (2018) reported that phosphate solubilization index for bacterial isolates were in the range of 2.5 to 3.2 on PVK medium. Dhiman et al. (2022) reported that P- solubilization in liquid assay for isolate DBD1 was 210 µg ml^{-1} and for isolate DRT5 was 190 µg ml^{-1} and P- solubilization efficiency for DBD1 was 41.17% and for DRT5 was 50 %.

3.2.2. Quantitative screening of siderophore producing bacterial isolates

The siderophore production efficiency of selected bacterial isolates was measured quantitatively based on bright zone

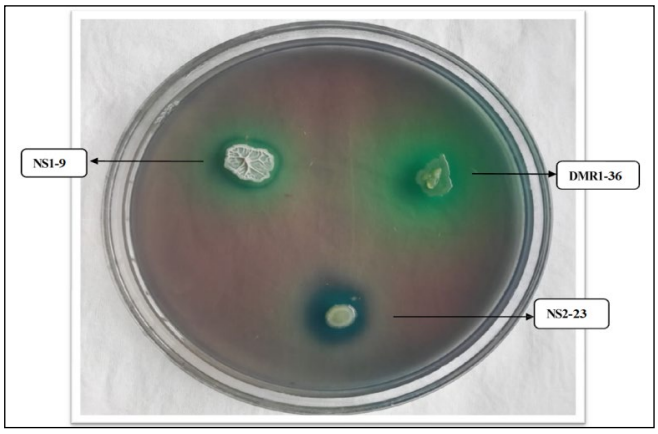


Plate 2: Siderophore production by selected isolates

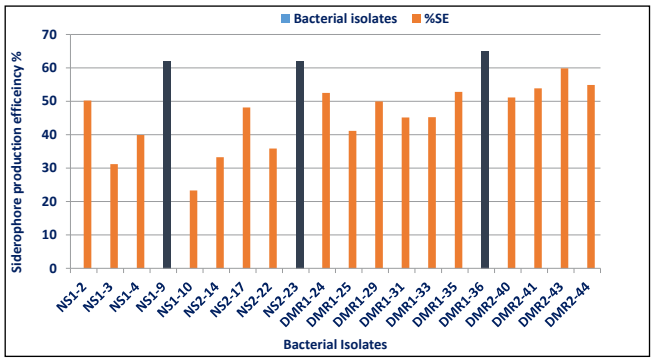


Figure 3: Siderophore (%) production efficiency by selected bacterial isolates

on CAS medium (Plate 2). Siderophore production efficiency significantly varied from 23.32% to 65.05% in all the selected bacterial isolates (Figure 3). The maximum (65.05%) siderophore efficiency was observed in isolate DMR1-36 on solid CAS media followed by isolate NS2-23 (62.14%); whereas the minimum siderophore efficiency was observed in isolate NS1-10 (23.32%).

Quantitative estimation of siderophore using CAS liquid assay of selected bacterial isolates revealed that maximum of 150.83 µg/ml siderophore production was recorded with bacterial isolate DMR1-36 with decrease in pH to 5.34. The minimum (65.37 µg ml^{-1}) siderophore production in liquid medium was recorded with bacterial isolate NS1-4 with decrease in pH to 4.12. Siderophores % efficiency in different tested bacterial isolates is illustrated in Figure 3. It is clearly observed from the figure that the three bacterial isolates, namely NS1-9, NS2-23 from Nauni sites and DMR1-36 from Chambaghat site showed high %age of siderophore production efficiency amongst 45 selected isolates. The availability of iron is quite limited due to very low solubility of the dominant ferric iron (Fe^{3+}) in soil and therefore it becomes unavailable to plants as micronutrient. Some bacteria have the capability to produce siderophores which are low molecular weight iron chelating compounds which chelate iron from mineral phases that can be taken up by energy dependent membrane transport mechanism and make it available to plants or bacterial cells. Ghazy and El-Nahrawy (2021) reported all the isolated bacterial strains exhibited an orange halo on CAS agar plate and the refore were considered positive for siderophore production which ranged from 1.62 cm (*B. subtilis*) to 0.36 cm (*B. licheniformis*). Ghadamgahi et al., (2022) reported that *Pseudomonas aeruginosa* FG106 showed siderophore production forming a clear halo zone of diameter 1.01 ± 0.21cm on the CAS medium.

3.2.3. Indole-3-acetic acid (IAA) production by selected bacterial isolates

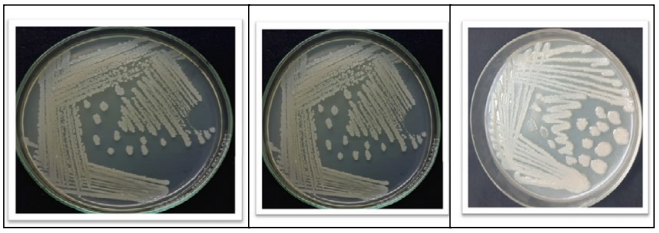
The data presented in Table 2 represents IAA production by selected bacterial isolates from spent mushroom compost. The IAA production by the bacterial isolates significantly varied from 23.91-67.24 µg ml^{-1} with the variation in final pH from 4.25 to 7.18. It was found that the bacterial isolate DMR1-36 produce maximum (67.24 µg ml^{-1}) amount of IAA

Table 2: IAA production by selected bacterial isolates

Bacterial Isolates	IAA production by Bacterial isolates (µg ml^{-1})	Final pH of supernatant
NS1-3	35.80	4.82
NS1-5	37.13	7.18
NS1-6	36.24	5.26
NS1-9	53.91	5.18
NS2-2	27.02	6.27
NS2-14	23.91	5.57
NS2-17	44.35	6.36
NS2-19	30.13	5.07
NS2-10	36.58	5.63
NS2-23	53.69	6.34
DMR1-24	48.80	5.65
DMR1-27	49.91	6.14
DMR1-31	26.80	7.15
DMR1-34	42.35	5.80
DMR1-36	67.24	6.78
DMR2-39	38.35	5.06
DMR2-41	46.14	4.25
DMR2-43	46.46	4.67
DMR2-44	47.03	5.23
CD (*p*=0.05)	0.03	0.04

with pH (6.78) followed by isolate NS1-9 (53.91 µg ml^{-1}) at pH 5.18. However, bacterial isolateNS2-14 showed minimum (23.91 µg ml^{-1}) IAA production with pH (5.57) after 48h of incubation at 35±2°C.

Indole acetic acid is a naturally occurring auxin in plants which control many physiological processes like cell enlargement and tissue differentiation. Bacterial auxins have the potential to change any of these processes by altering the plant auxin pool. It depends on the amount of IAA produced and the sensitivity of the plant tissue to change the level of IAA. Chaiharan and Lumyong (2011) reported 18.05% out of 216 bacterial strains produced IAA and the highest IAA producer among them (*Klebsiella* SN 1.1) produced 291.97±0.19 µg



GPB1 (NS1-9) GPB2 (NS2-23) GPB3 (DMR1-36)

Plate 3: Selected bacterial isolates streaked on NA plates

ml^{-1}. Bharucha et al. (2013) reported that all the 9 isolates of *P. putida* showed significant amount of IAA production in tryptophan supplemented medium: ranging from 26.4 to 591.8 µg ml^{-1}. Dawwam et al. (2013) reported that the seven bacterial isolates produced amounts of IAA that ranged from 0.6 to 10.73 µg ml^{-1}. Chandra et al. (2018) reported that IAA is synthesized by plants and microbes via number of pathways.

3.3. Morphological, physiological and biochemical characteristics of selected bacterial isolates

3.3.1. Morphological characteristics of selected bacterial isolates

Three isolates namely NS1-9, NS2-23 and DMR1-36 were selected based on maximum plant growth promoting traits (Plate 3) and were further characterized for morphological, physiological and biochemical characteristics.

Table 3 shows the morphological attributes of the three isolates namely NS1-9, NS2-23 and DMR1-36. Isolate NS1-9 was gram positive spherical shaped, lemon yellow colored, irregular form, convex elevation, entire margin, smooth surface, slimy texture with chained arrangement. NS2-23 was Gram negative, rod shaped; light yellow with irregular form, convex elevation, entire margin, smooth surface, slimy textured colony with clustered arrangement and DMR1-36 was Gram negative, rod shaped, creamy white in color, circular form, raised elevation, entire margin, rough surfaced, coarse texture with clustered arrangement. For capsule staining, only isolate NS2-23 gave positive result whereas the other two isolates showed negative results.

Khan et al. (2018) who characterized the form, elevation, margin, opacity and color of the selected bacterial isolates. A total of 14 bacterial strains were selected for Gram staining and out of them, only 11 bacterial strains were Gram negative

Table 3: Morphological characterization of selected bacterial isolates

Isolate	Colony color	Form	Elevation	Margin	Surface	Texture	Shape	Arrangement	Color	Gram's reaction	Capsule Staining
NS1-9	Lemon yellow	Irregular	Convex	Entire	Smooth	Slimy	Spherical	Chain	Purple	Positive	Negative
NS2-23	Light Yellow	Irregular	Convex	Entire	Smooth	Slimy	Rod	Cluster	Pink	Negative	Positive
DMR1-36	Creamy white	Circular	Raised	Entire	Rough/ granular	Coarse	Rod	Cluster	Pink	Negative	Negative

while the 3 strains were Gram positive. Vinayarani and Prakash (2018) reported that out of 50 isolates, 43 were gram negative while 7 were gram positive.

According to Bergey's manual of systematic bacteriology, the results of the biochemical and morphological tests showed that isolate NS1-9 was Gram positive coccus a shaped and may belong to *Arthrobacter*, *Glutamicibacter*, *Bacillus* sp., whereas isolate NS2- 23 and DMR1-36 were ram negative rod-shaped bacteria and may belong to Pseudomonas or *Klebsiella* sp.

3.3.2. Biochemical characteristics of selected bacterial isolates

Bacterial isolates NS1-9, NS2-23 and DMR1-36 were characterized by a series of biochemical tests and results were presented in (Table 4). All three isolates were negative for casein test whereas, positive for catalase, urease and citrate utilization test. IsolatesNS1-9and NS2-23 were positive for Voges Proskauer test whereas DMR1- 36 was negative. Only NS1-9 isolate was positive for starch hydrolysis and gelatin liquefaction. In case of biochemical characterization, the rhizobial isolate showed positive results for catalase test, urease test and ammonia production and negative for indole test, Voges Proskauer test, H_2S production, citrate test and casein test.

Table 4: Biochemical characterization of selected bacterial isolates

Bacterial Isolates	NS1-9	NS2-23	DMR1-36
Indole test	+	-	-
Methyl red test	+	-	+
Voges Proskauer test	+	+	-
Catalase test	+	+	+
Citrate utilization	+	+	+
Gelatin liquification	+	-	-
Starch hydrolysis	+	-	-
Casein hydrolysis	-	-	-
Urease test	+	+	+
Oxidase test	-	-	+

3.3.3. Physiological characterization of selected bacterial isolates

3.3.3.1. Effect of incubation period on growth of selected bacterial isolates

The results showed that maximum growth of the selected isolates NS1-9, NS2-23 and DMR1-36 was obtained at incubation period of 48 hours with the viable count of 42×10^6, 53×10^6 and 60×10^6 cfu g^{-1}, respectively (Figure 4). Initially all the isolates showed no growth between the period of 0 and 12 hours. Thereafter, all the three isolates showed increase in growth after 12 hours of incubation period. However, after 48 hours of incubation, a considerable decline in the growth and development of the selected bacterial isolates was seen.

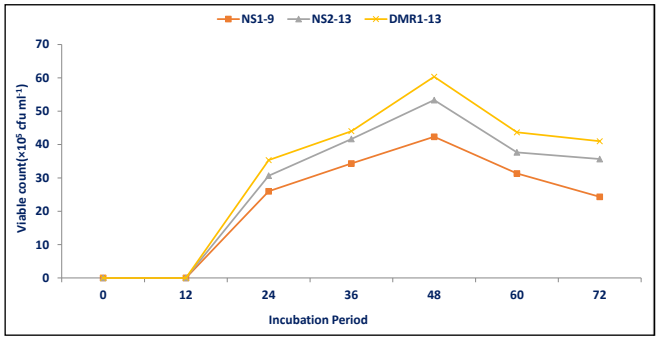


Figure 4: Effect of incubation period on viable count of selected bacterial isolates from SMC

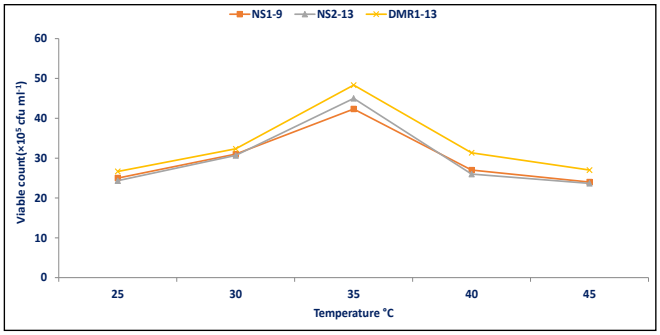


Figure 5: Effect of temperature on growth of selected bacterial isolates from SMC

3.1.1.2. Effect of temperature on growth of selected bacterial isolates

The data presented in Figure 5 shows the bacterial growth at different temperatures ranging from 25- 45°C. The three selected isolates, NS1-9, NS2-23 and DMR1-36, showed variation in growth at different temperatures. The maximum growth of all three isolates was recorded at 35°C with the viable count of 42×10^6, $45\times10^6$and 48×10^6 cfu ml^{-1} for isolate NS1-9, NS2-23 and DMR1-36, respectively. As the temperature raised above 35°C well marked decrease in growth of bacterial isolates was observed. Temperature, pH and incubation period are important factors for the growth and development of bacteria. Changes in temperature have the biggest effect on enzymes and their activity, with an optimal temperature that leads to the fastest metabolism and resulting growth rate. Temperatures below optimal will lead to a decrease in enzyme activity and slower metabolism, while higher temperatures can denature proteins such as enzymes and carrier proteins, leading to cell death.

Pawar et al. (2014) reported that the nodulating bacteria isolated from soybean root nodules showed good growth at temperature of 36° C. Zahid et al. (2015) reported all isolates were able to grow in temperature range of 25 to 40°C, and optimum temperature of 35°C was best for growth of the bacteria.

3.1.1.3. Effect of pH on the growth of selected bacterial isolates

Different pH values (5.0-9.0) significantly affected the growth

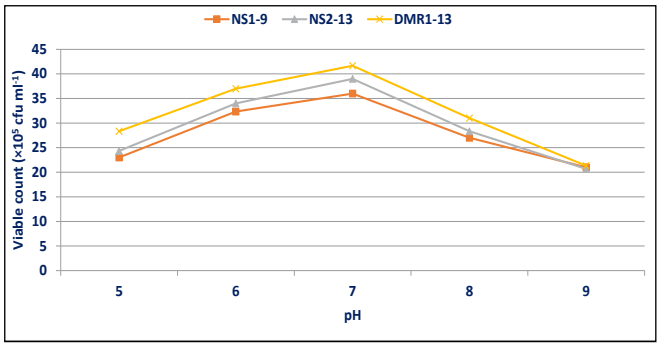


Figure 6: Effect of pH on growth of bacterial isolates from SMC

of selected bacterial isolates. Bacterial isolates showed poor growth below pH 5 and above pH 9.0. The maximum growth was recorded at pH 7.0 which was 36×10^6, 39×10^6 and 42×10^6 cfu g^{-1} for NS1-9, NS2-23 and DMR1-36, respectively (Figure 6).

The findings of the present study are in line with the study conducted by Rasul et al. (2012) reported that 29 of the isolated bacteria could grow in the pH range of 4.0 -10.0. Dinesh et al. (2015) who also reported that pH 7.0 is optimum for the growth of *Bacillus* and *Pseudomonas* sp.

3.4. Molecular characterization of bacterial isolates

The three most efficient growth promoting bacterial isolates viz., NS1-9 (GBP1) and NS2-23 (GPB2) and DMR1-36 (GPB3) were selected. These bacterial isolates exhibited all mushroom growth promoting traits and were selected for identification up to species level by molecular technique based on 16S rRNA sequencing. The DNA was extracted by conventional method (Sambrook et al., 1989) and purified DNA was amplified using Universal bacterial primers. The amplicon of expected size i.e. (1200 bp) was obtained.

The sequence data of the 16S rRNA of selected isolates were subjected to BLAST analysis. As 16S rRNA gene sequence provide accurate grouping of organism even at subspecies level it is considered as a powerful tool for the rapid identification of bacterial species (Jill and Clarridge, 2004). The sequence analysis of 16S rRNA revealed that strain GPB1 (Accession No. OR966873) showed maximum similarity of

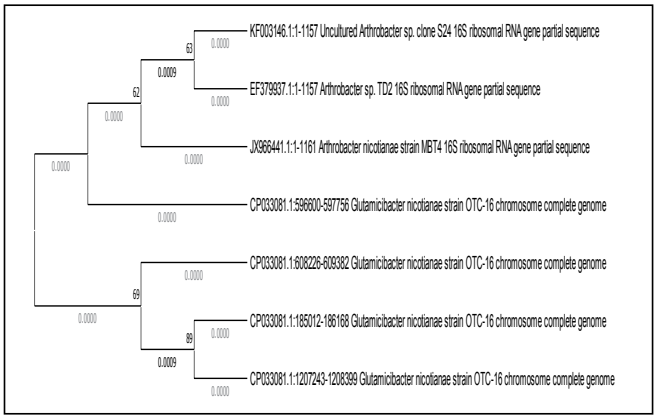


Figure 7: Neighbour joining tree based on relationship of bacterial isolates GPB1 with analyzed sequences

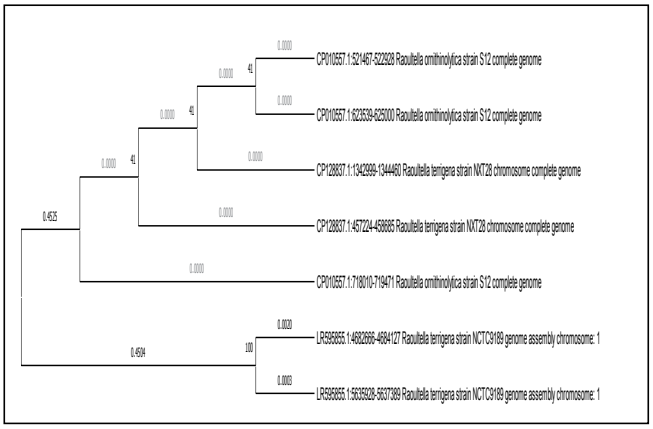


Figure 8: Neighbour joining tree based on relationship of bacterial isolates GPB2 with the analysed sequences

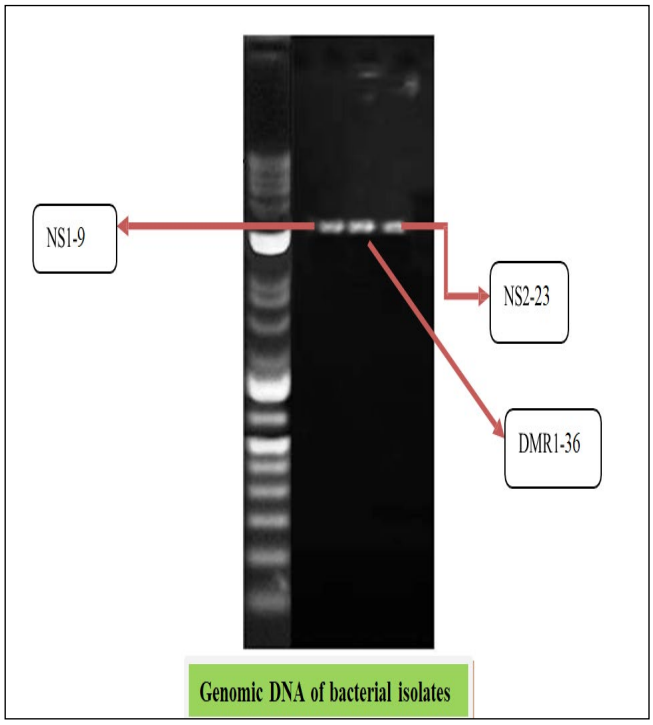


Plate 4: Molecular identification of bacterial isolates based on16S rRNA amplification

98.23% with *Glutamicibacter nicotinae* NBRC 14234 (T) and strain GPB2 (Accession No. OR966876) showed maximum similarity of 98.66% with *Raoultella terrigena* ATCC 33257 (T) whereas, strain GPB3 (Accession No. OR966870) showed maximum similarity of 98.19% with *Brucella pituitosa* strain ICMP 7355 (Figure 7 and 8). The phylogenetic analysis of 16S rRNA sequence of the isolates along with the sequence retrieved from the NCBI was carried out with MEGA X using the neighbor joining method with 1,000 bootstrap replicates (Plate 4).

4. Conclusion

Among the selected bacterial isolates, GPB1 was identified as

Glutamicibacter nicotinae, GPB2 was identified as *Raoultella terrigena and GPB3 as Brucella pituitosa* by 16S rRNA gene sequencing. From the present investigation, it can conclude that the spent mushroom compost can be good and efficient source of growth promoting bacteria that can be utilized for mushroom cultivation. Therefore, the bacterial isolates have potential to be used as biofertilizers which could enhance the productivity of mushrooms.

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