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Isolation and Screening of Agriculturally Important Bacteria (PGPR) from Organic Sources of Nutrient (Panchgavya, Jeevamrit and Farm Yard Manure) for Future Use

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Agriculture is an essential component to maintain the human health but due to sudden increase in population the demand has increased and to meet the requirement the use of chemical fertilizer has also increase. The continuous use of chemical fertilizer and pesticides degrade the quality and fertility of soil and also reduce the yield of various crops. The organic sources such as panchgavya, jeevamrit and Farm Yard Manure consists load of beneficial microorganisms that promote the plant growth, yield and promote natural resources. The present study was carried out by isolating the bacteria from organic sources (panchgavya, jeevamrit and FYM) because minute amount of

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bacterial culture is suffient to prepare large one according to need and can be stored for longer time as compare to organic input. The samples of panchgavya, jeevamrit and FYM were collected from different location of Himachal Pradesh. A total of 32 bacterial isolates from panchgavya, 43 from jeevamrit and 59 from FYM were isolated and screened for multiple plant growth promoting traits. Only 4 bacterial isolates from panchgavya, 2 from jeevamrit and 2 from FYM were preferred on the basis of maximum plant growth promoting traits like P-solubilization, Nitrogen fixer, Siderophore production, HCN production, IAA production and Antifungal activity. From the selected bacterial isolates 67.78% were P- solubilizers, 60.37% were Nitrogen fixer, 62.37% were Siderophore producer, 34.29% HCN producer, 45.88% IAA producer and 78.22% show antagonism against *Pythium graminicolum* and 74.81% show antagonism against *Collectotrichum capsici*. Hence these bacterial isolates have potential to act as biofertilizer for environment friendly sustainable agriculture systems and alternative to harmful chemicals.

Keywords: Organic sources of nutrient; microorganism; biocontrol; PGPR; plant growth; biofertilizers.

1. INTRODUCTION

The agriculture and food security mainly depends upon the fertility status of soil to sustain the life in all forms. The microorganisms present in the soil play an important role in plant growth promotion and health [1]. The continues uses of chemical fertilizers and pesticides in the agricultural fields leads to degradation of soil quality and fertility, thus agricultural land with fertile soil is decreasing day by day [2]. Nowadays isolation of microorganism from various traditional organic inputs like panchagvya, jeevamrutha and Farm Yard Manure plays important role in the expansion of sustainable agriculture and benefit the society by socially, commercially and environmentally. The organic inputs which are used in organic farming system are the fermented product of liquid formulations obtained from cow and used for plant growth promotion [3]. These organic inputs, like panchgavya, jeevamrit and FYM are used as widely conventional way to protect plants by acting as biocontrol agent and soil microorganisms from harmful chemical fertilizers and pesticides [4]. Panchagavya is the mixture of five ingredients (dung, urine, milk, curd, Jaggery and ghee) obtained from cow. Jeevamrit is also prepared from cow dung, urine, gram flour, organic soil and jaggery by fermentation. Farmyard manure (FYM) is a mixture of decomposed urine, dung, litter, and remaining from roughages and fodder served to animals. An ideal-decomposed FYM contains 0.5–1.5% N, 0.2–0.4% P_2O_5, and 0.5–1.0% K_2O.

Panchagavya and jeevamrit are the richest sources of beneficial microorganisms which sustain, stimulate the plant growth and help in getting better vegetative growth and also good quality yield [5]. The interest of scientist has been increased toward isolation of microorganism from panchagavya, jeevamrit and FYM for the enhanced growth of various crops in organic agriculture from last few years. Devakumar et al., [6] and Srinivas et al., [7] have reported the survival of many beneficial microorganisms viz., nitrogen fixers, phosphorus solubilizers, actinomycetes and fungi in panchgavya, jeevamrutha and FYM. These organic inputs contain beneficial microorganisms, predominately lactic acid bacteria, *Bacillus* and *Pseudomonas*, certain fungi such as *Aspergillus* and Yeast (*Saccharomyces cerevisiae*). Hence the microorganism present in the organic sources of nutrient such as panchgavya, jeevamrit and FYM have potential as biofertilizer can be used for enhanced growth of various crops and maintains the fertility of soil by natural means.

2. MATERIALS AND METHODS

2.1 Collection of Samples

The samples of panchgavya, jeevamrit and FYM were collected from Hamirpur, Solan and Una district of Himachal Pradesh. From each site two subsites were selected for sample collection. The samples were collected in plastic bags and placed in the Microbiology laboratory, Deptt. of Basic Science, College of Forestry, Dr YS Parmar University of Horticulture and Forestry, Nauni-Solan. Panchgavya and Jeevamrit was also prepared in laboratory mixing the fresh cow urine, cow dung, cow milk, cow curd and cow ghee from local cow farm and placed in sterile containers and stored in refrigerator for further uses.

2.2 Method of Preparation of Panchgavya

2.5 kg of fresh cow dung was taken and mixed thoroughly with 250 g of cow's ghee and kept in

a mud pot. The mixture was kept for 2 days and mixed twice a day. On the 4th day, the cow's urine, milk curd and Jaggery was added. This solution was kept for another 9 days, shaked twice a day for about 20 minutes to provide aerobic fermentation which facilitates microbial growth and activity. On the 10^{th} day the panchgavya solution was ready and was covered with a muslin cloth to prevent the contamination caused by insect and flies [8].

2.3 Method of Preparation of Jeevamrit

5 kg of fresh cow dung was mixed with 5 ltr of cow urine, 1 kg of jaggery, 0.5 kg of gram flour and 0.5 Kg of organic soil in pot. The mixture was kept for 7 days and mixed twice a day with wooden stick for about 20 minutes to provide aerobic fermentation which facilitates microbial growth and activity. On the 8^{th} day the jeevamrit solution was ready and covered with a muslin or fine cloth to prevent the contamination caused by flies [8].

2.4 Isolation of Microorganisms from Panchgavya, Jeevamrit and FYM

Isolation was carried out by serial dilution method in which 1ml of panchgavya and jeevamrit whereas 1g of FYM sample was inoculated in test tube containing 9 ml of distill water. The samples were diluted up to 10^{-8} concentration. 0.1 ml of diluted sample was taken and spread on prepoured nutrient agar plate. After spreading the plates were incubated at 37°C temperature for 24 hours in the incubator for the growth of colonies. The isolated colonies that showed diverse morphological variation was selected and inoculated separately in 5 ml of nutrient broth and incubated at 37ºC for 24 hours. After incubation, it was again plated in nutrient agar plates to check the purity of isolates. After checking the purity of isolates, they were streaked on the slants and were stored in refrigerator for further use.

Bacterial isolates were screened out for the production of hydrogen cyanide (HCN) as per method described by Bakker and Schippers [9]. Bacterial cultures were streaked on prepoured plates of King's B medium amended with 1.4 g l^{-1} glycine. Whatman No.1 filter paper strips were soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate and were placed in the lid of each petriplates and plates were sealed with parafilm and were incubated at 35 ± 2°C for 1-4 days. Uninoculated control was kept for comparison of results. Plates were observed for change in color of filter paper from yellow to orange brown.

2.4.1 Screening of bacterial isolates on the basis of plant growth promoting traits

Plant growth promoting rhizobacteria (PGPR) were selected on the basis of plant growth promoting traits like P-solubilization, growth on N-free medium, siderophore, HCN, auxin production and antagonism against *Pythium aphanidermatum* and *Collectotrichum capsici* (major fungal pathogens of turmeric) were performed by adopting standard protocol. These methods are described as follow:

Phosphate solubilizing activity: Each of purified isolate were streaked in a straight line on PVK medium as described by Pikovskaya [10] and was incubated for 72 h at 30 ± 2°C. Colonies showing solubilization halos (>0.1mm diameter) were selected.

Nitrogen fixing activity: Each of the purified isolate were streaked in a straight line on Jensen's medium and was incubated for 72 to 120 h and the plates showing growth of bacteria in the form of bacterial colony were selected.

Siderophore production: Siderophore production was detected by CAS plate assay method [11]. Sterilized blue agar was prepared by mixing CAS (60.5 mg/50ml distilled water) with 10 ml iron solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). This solution was slowly added to hexadecyltrimethyl ammonium bromide (HDTMA) solution was prepared by dissolving 72.9 mg HDTMA in 40 ml distilled water). Thus, 100 ml CAS dye was prepared. 750 ml nutrient agar was mixed with 1, 4 piperazine diethane sulphonic acid (30.24 g) and pH 6.8 was adjusted with 0.1N NaOH. It was autoclaved separately and then mixed with Chrome azurol-S (100 ml) under aseptic conditions and then the plates were prepared for further experiments. A bit of 72 h old culture of each test bacterium was placed on prepoured blue coloured chrome-azurol-S agar (CAS) plates. Plates were incubated at 30 ± 2°C for 24 h and observed for production of orange halo around the bit.

$$\text{Per cent siderophore efficiency} = \frac{Z-C}{C} \times 100$$

Where,

Z = Size of halozone
C = colony size

HCN production: Bacterial isolates were screened out for the production of hydrogen cyanide (HCN) as per method described by Bakker and Schippers [9]. Bacterial cultures were streaked on prepoured plates of King's B medium amended with 1.4 g/l glycine. Whatman No.1 filter papers strips were soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate and were placed in the lid of each petriplate sealed with parafilm and were incubated at 35 ± 2^{o}C for 1 - 4 days. Uninoculated control was kept for comparison of results. Plates were observed for change in colour of filter paper from yellow to orange brown.

Indole Acetic Acid (IAA) production: Bacterial cultures were grown in modified Luria Bertani broth amended with (5 mM L- tryptophan, 0.065% sodium dodecyl sulphate and 1% glycerol) 72 h at 35^{o}C under shaking conditions. The cultures were centrifuged at 15,000 rpm for 20 minutes and supernatant were collected and stored at 4^{o}C. The method described by Gorden and Palleg [12] was used to determine the IAA equivalents; 3 ml of supernatant was pipette into test tube and 2 ml Salkowski reagent (2 ml 0.5 M FeCl3 + 98 ml 35% HClO4) 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.

2.4.2 Antagonistic activity of bacterial isolates against test fungus

Agar streak plate method was used to test the efficacy of bacterial isolates against the test fungus. A loop full of 48 h old culture of each isolate were streaked a little below the centre of the prepared MEA petri plate and incubated at 30±2^{o}C for 24 h to check contamination. Mycelial disc of 5 days old culture of the test fungal pathogen (*Fusarium oxysporum* and *Rhizoctonia solani*) was placed separately on one side of the streak in each plate.

$$I = \frac{C-T}{C} \times 100$$

Where,

I = Per cent growth inhibition
C = Growth of fungus in control
T = Growth of fungus in treatment

2.5 Morphological, Physiological and Biochemical Characterization of Selected Bacterial Isolates

The most efficient bacterial isolates selected on the basis of plant growth promoting traits and *in vitro* antagonistic activity were subjected to morphological, physiological and biochemical characterization as per the criteria of Bergey's Manual of Systematic Bacteriology [13].

2.5.1 Morphological characterization

Morphological characteristics of isolates including colony morphology, Gram's reaction and cell shape were investigated.

2.5.2 Physiological characterization

Separate experiments were performed for optimization of conditions for growth of selected rhizobial isolates as given below:

Effect of pH on the growth of bacterial isolates: 5 ml of nutrient broth was taken in test tubes. The medium was adjusted to various pH (3, 5, 7, 9 and 11) using 0.1 N NaOH or 0.1 N HCl as the case may be. 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 on the basis of turbidity caused by the bacterial growth in test tube.

Effect of temperature on growth of bacterial isolates: 5 ml of nutrient broth was taken in test tubes and inoculated with 0.1 ml of 48 h old 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.

Effect of incubation period on growth of bacterial isolates: 5 ml of nutrient broth was taken in test tubes and inoculated with 0.1 ml of 48 hr old bacterial cell suspension (O.D. 1.0 at 540 nm). Each test tube was incubated for different time period (24 h, 48 h, 72 h, 96 h and 120 h) and observed for turbidity. The optimum incubation period for growth was maintained for further experimentation.

2.5.3 Biochemical characterization

The selected bacterial isolates were tested for biochemical traits like Indole test, Methyl red

Test, Citrate utilization, Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Hydrogen sulphide production, Catalase test, Voges Proskauer test, Urease test, Carbohydrate fermentation, Ammonia production and Cellulase test as per the criteria of Bergey's Manual of Systematic Bacteriology [13].

2.6 Statistical Analysis

The data collected on various parameters under laboratory condition were subjected to statistical analysis as per methods outlined by Gomez and Gomez [14]. The Critical Difference at 5 per cent level was used for testing the significant differences among the treated means.

3. RESULTS

3.1 Isolation of Bacteria from Panchgavya, Jeevamrit and FYM

A total of 27 bacterial isolates (14 from FYM, 5 from panchgavya and 8 from jeevamrit) were selected from different organic sources panchgavya on nutrient agar medium from different locations of Himachal Pradesh (Table 1 and Plate 1).

3.2 Screening of Bacterial Isolates for Multifarious Plant Growth Promoting Traits

On the basis of multifarious plant growth promoting traits i.e. P-solubilization, growth on nitrogen free medium, siderophore production, IAA, HCN production and antagonism against major fungal pathogens i.e. *Pythium aphanidermatum* (causal organism of Rhizome rot) and *Collectotrichum capsici* (causal organism of leaf spot) only best 3 bacterial isolate (1 from panchgavya, 1 from jeevamrit and 1 from FYM) were selected and again purified on the NA medium and preserved for further use (Plates 2-5).

3.3 Morphological Characterization of Selected Bacterial Isolates

On the basis of morphology and gram reaction out of selected 3 bacterial isolates, 2 isolates were gram positive rod and 1 bacterial isolate was gram negative rods so these selected bacterial isolates may belong to the *Bacillus* and *Pseudomonas* sp. (Table 2).

3.4 Physiological Characterization of Selected Isolates

The physiological characterization of selected bacterial isolates revealed that all the isolates were able to grown on a broad temperature range of 25 - 45°C but optimum temperature for the growth was found to be 35°C on the basis of maximum OD at 540 nm. The selected isolates were able to grow on the different pH range of 3.0 - 11.0 and with the maximum growth at the nearly neutral 7.0 pH. Similarly different incubation periods (24 - 96 h) were tested for the selected bacteria and found maximum growth at 24 - 48 hrs of incubation on the basis of OD at 540 nm as presented in Figs. 1-3.

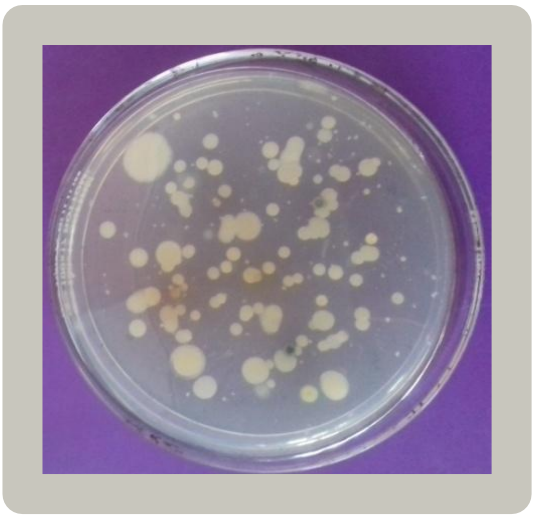


Plate 1. Isolation of bacteria on NA media

3.5 Biochemical Characterization of Selected Isolates

All the selected three bacterial isolates were positive for Methyl red test, starch hydrolysis, Gelatin hydrolysis, Catalase test, Casein hydrolysis and fermentation of glucose. However, all the isolates were negative for Indole test, Citrate utilization and hydrogen sulphide (H2S) production (Table 3).

4. DISCUSSION

Isolation and screening of microorganisms from panchgavya, jeevamrit and FYM are new or less exploited source and can be used as alternative way to produce efficient biofertilizer for sustainable agriculture. During the study panchgavya and jeevamrit were prepared by

mixing the five different products of cow like cow dung, cow urine, cow milk, cow ghee, cow curd, gram flour, organic soil and jaggery in the laboratory condition. Similar findings were recorded with Raghavendra et al, [15] and Devkumar et al., [16] who also prepared the panchagavya and jeevamrit from different by product of cow. Since the organic inputs such as panchgavya, jeevamrit and FYM are an enriched source of the microbial diversity, sufficient amount of microorganisms were isolated from the panchgavya, jeevamrit and FYM by serial dilution method on nutrient agar medium. The results of present study are in agreement with those of Sharma and Singh, [17] who isolated the maximum number of bacterial population was ranged from $60.5x10^{-4}$ to$175x10^{-4}$ cfu/ml and minimum concentration was exhibited in dilution 10^{-6} which ranged from $23.5x10^{-6}$ to $80.5x10^{-6}$. The selected isolates were gram positive or negative rods, grow best at a temperature of 35±2°C, 24-48 hrs of incubation and at neutral pH on the basis of morphological, physiological and biochemical characterization. Similar results were observed with Sharma & Singh, [17] and Teo & Teoh, [18] who studied a mixture of both gram positive and negative bacteria and rods and some bacilli species from panchgavya, jeevamrit and FYM. On the basis of morphological, physiological and biochemical characterization by Bergey's Manual of systematic bacteriology the selected bacterial isolates were tentatively identified as *Bacillus* sp. and *Pseudomonas* sp [19,20].

Control **PGPR1, PGPR2** **PGPR3**

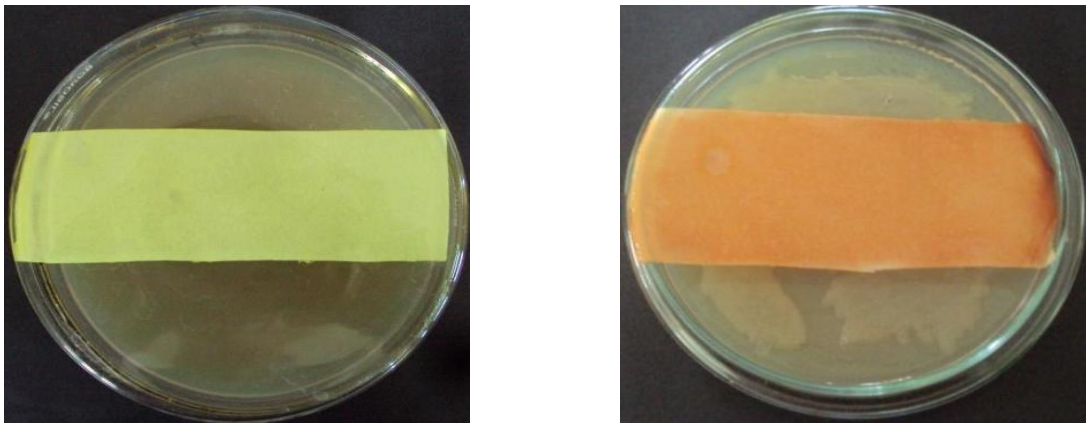
Plate 2. Phosphate solubilization by different bacterial isolates on PVK media

Control **PGPR1, PGPR2** **PGPR3**

Plate 3. Siderophore production by bacterial isolates PGPR1, PGPR2 and PGPR3 on CAS medium

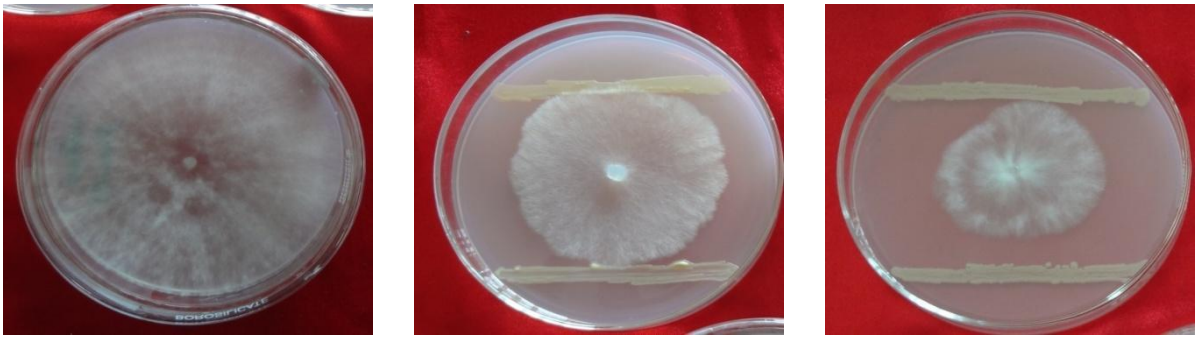
Table 1. Isolation of bacteria from panchgavya, jeevamrit and FYM

Organic source (OS)	Locations		
	Microbial count 10^8 cfu/g/ml on NA media		
	Hamirpur	Una	Solan
FYM	92.44	107.46	78.26
Panchgavya	59.66	97.79	79.36
Jeevamrit	88.80	89.94	84.21
CD(0.05)	3.63		



Control **HCN production by selected bacterial isolates**

Plate 4. HCN production by bacterial isolates PGPR1, PGPR2 and PGPR3 on king's medium



Collectotrichum capsici **PGPR1 and PGPR2** **PGPR3**

Pythium aphanidermatum **PGPR1, PGPR2 and PGPR3**

Plate 5. Inhibition against *Collectotrichum capsici* and *Pythium aphanidermatum* by selected bacterial isolates

Table 2. Morphological characterization of selected bacterial isolates

Bacterial isolates	Form	Elevation	Margin	Surface	Gram's reaction	Shape
PGPR1	Irregular	Flat	Undulate	Rough	+	Rods
PGPR2	Circular	Flat	Lobate	Rough	-	Rods
PGPR3	Irregular	Raised	Curled	Smooth	+	Rods

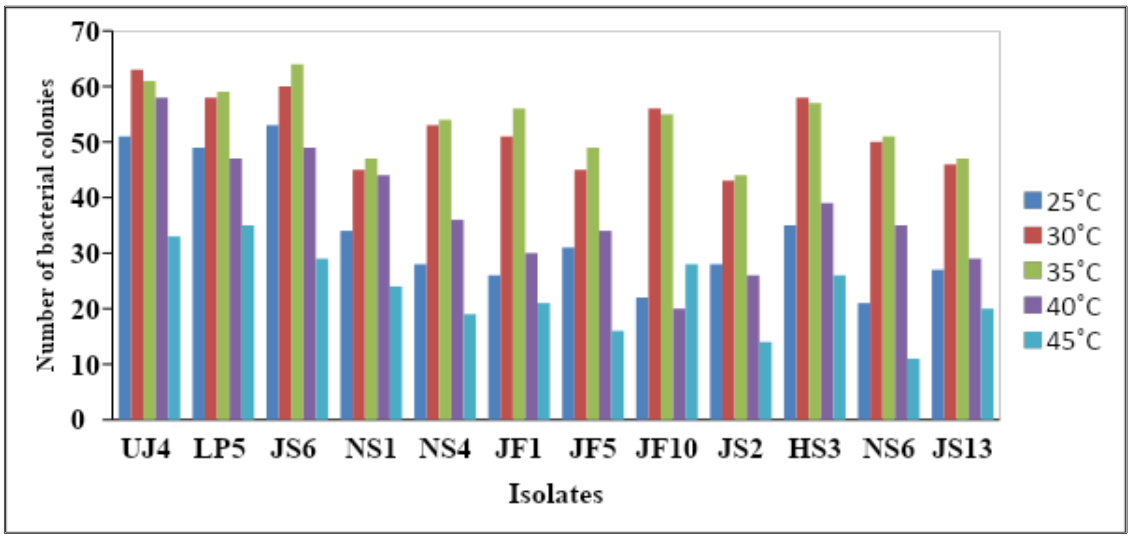


Fig. 1. Effect of different temperature on the growth of selected bacterial isolates

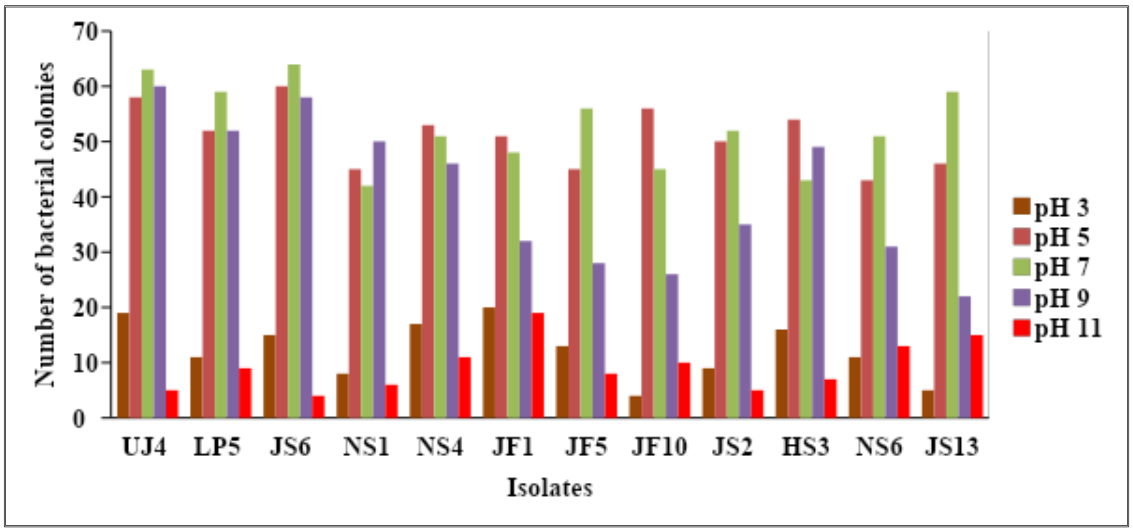


Fig. 2. Effect of different pH on the growth of bacterial isolates

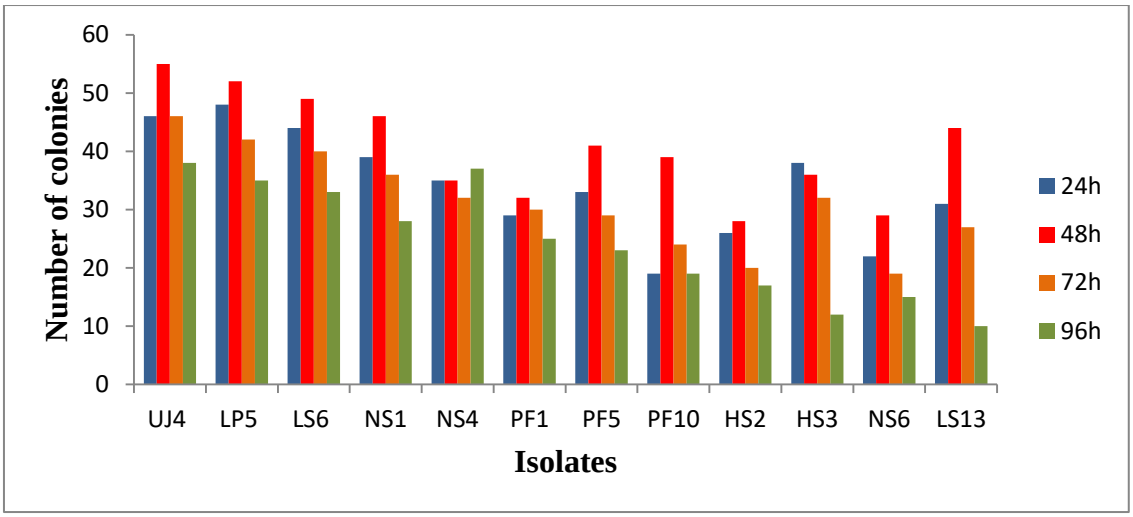


Fig. 3. Effect of incubation period on the growth of selected bacterial isolates

Table 3. Biochemical characterization of selected bacterial isolates

Test	Bacterial isolates		
	PGPR1	PGPR2	PGPR3
Indole test	-	-	-
Methyl red test	+	+	+
Citrate utilization test	+	-	-
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Hydrogen sulphide production	+	-	-
Catalase test	+	+	+
Fermentation of Glucose	+	+	+
Voges Proskauer test	+	+	+
Casein hydrolysis	+	+	+

5. CONCLUSION

It is concluded from the present investigation that preparation of panchgavya, jeevamrit was very easy and cheap process. Being a richest source of microbial diversity, isolation and characterization of bacterial isolates from these sources are new and less exploited. The bacterial isolates were isolated from panchgavya, jeevamrit and FYM by standard method and were further screened for various plant growth promoting activities. Only three bacterial isolates were selected on the basis of maximum plant growth promoting traits and tentatively identified as *Bacillus* sp. and *Pseudomonas* sp and preserved for further use in net house and field trial on various crops. The selected bacterial isolates can be used as biofertilizer for enhanced production of crop and to maintain the fertility of soil by natural means. The use of bacterial isolates may minimize the use of chemical fertilizer in near future.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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