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Research Journal of Agricultural Sciences
Volume 14; Issue 02 (Mar-Apr 2023); pp 559–564

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Full Length Research Article

Unraveling the Plant Benevolent Traits of Bacteria Isolated from the Aqueous Vermicompost

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Abstract

Vermicompost extract is a decomposition product obtained by the various species of worms mainly earthworms. It plays crucial role to the field of agriculture and used in organic farming since it contains essential components like enzymes, macro and micronutrients, phytohormones and many important microorganisms. With this background, the study was aimed at isolation of microorganisms from aqueous extract of vermicompost. Thirty strains were isolated based on cultural characteristics which were then qualitatively analyzed for the capability to produce metabolites like ammonia, auxins, phosphatase, amylase, cellulase and protease. The microorganisms producing these end products can be used as inoculants in plants in ecofriendly agricultural practices as these metabolites and enzymes are reported to promote the growth of the plants. Out of thirty isolates, three potential strains namely V8, V11 and V30 were selected and by 16S rRNA sequencing identified as *Stenotrophomonas maltophilia, Pantoea agglomerans* and *Mesorhizobium muleiense.* The strains isolated have not been reported widely as plant growth promoting agents. So further studies can be targeted on developing these microbial strains as biofertilizers using the standard methods of bioformulation development. From the study it can be concluded that vermicompost extract is a good source of beneficial microbial strains in addition to the essential nutrients. Hence it can be used as a biofertilizer which is economically and environmentally suitable for the soil and sustainable agriculture.

***Key words*:** Vermicompost extract, Biofertilizer, Bioformulation, Sustainable agriculture, 16S rRNA sequencing

Due to the shrinkage in land for cultivation and rapid population explosion it has become necessary to increase the global food supply. This was possible due to the advent of "green revolution" which came as a miscellaneous blessing to mankind. There was an increase in the food production at the cost of declining the nutritional quality and soil fertility over a period of time. The beneficial microorganisms that help to replenish the soil get killed by this approach. To add on they also have an adverse effect on the health of human beings. The increasing cost and negative impacts of synthetic fertilizers and pesticides emphasizes on the biological methods of crop protection and cultivation. It can be achieved through natural farming technologies that include the use of manure, microbial inoculums and composts.

Due to rapid urbanization, large amounts of organic wastes are dumped in the environment which poses a threat by polluting the atmosphere due to the release of gases like ammonia, contaminating groundwater and posing serious health issues. These problems can be overcome by converting the waste into vermicompost which is more efficient than the conventional composting techniques. Vermicomposting is a biological process mediated by earthworms and the microflora associated with it. A variety of domestic wastes like vegetable wastes, garden waste, agricultural litter, industrial waste from breweries, municipality are used for this process [1-2]. Vermicompost is used extensively in organic farming due to its rich source of nutrients. It has been reported that the aqueous extract of vermicompost has wide spectrum of activity as a nutrient resource and pesticidal activity. Living microorganisms present in the aqueous extract of vermicompost induce disease resistance as well as stimulate nutrient uptake and plant growth [3-4]. But these bacteria have not been studied widely for their plant growth promoting ability [5-6].

These microorganisms can support growth of the plants either directly or indirectly. The mechanisms include ammonia assimilation, phosphate solubilization, production of auxins, production of hydrolytic enzymes and antibiosis. Examples include *Bacillus*, *Pseudomonas*, *Serratia*, *Arthrobacter*, *Micrococcus*, *Flavobacterium*, *Chromobacterium*, *Agrobacterium*, *Hyphomycrobium*, free-living nitrogen-fixing bacteria and members of the family *Rhizobiaceae*. The strategy of applying these microorganisms to agriculturally significant

Received: 01 Jan 2023; Revised accepted: 25 Mar 2023; Published online: 24 Apr 2023

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Citation: Josephine CMR, Prasad R, Santhosh BN, Jackson M, Karthik K. 2023. Unraveling the plant benevolent traits of bacteria isolated from the aqueous vermicompost. *Res. Jr. Agril. Sci*. 14(2): 559-564.

crops as inoculants is gaining popularity since it has a variety of benefits, such as the significant decrease of the use of agrochemicals, improved soil health, cytotoxic activity against phytopathogens, and increased crop yield [7-8].

With this background, the present study was focused on isolating and characterizing benevolent microorganisms from aqueous vermicompost extract, evaluating its plant growth promoting ability by performing *in vitro* biochemical tests and identifying the potential strains through 16S rRNA sequencing. The isolated microbial strains would serve as promising candidates for ecofriendly and sustainable agricultural practices.

MATERIALS AND METHODS

Sample collection

The samples were collected from the vermicompost unit located in Kristu Jayanti College, Bengaluru, India. The samples were collected from a 75-day old compost. Sampling was done at different depths of the compost and placed in sterile sealed plastic bags until further processing.

Vermicompost extract preparation and isolation of microorganisms

Vermicompost samples collected was mixed with sterile distilled water and kept in dark. After 72 hours, the supernatant fraction was collected which was the source to isolate beneficial microorganisms. Vermicompost extract sample contains enzymes, nutrients, vitamins, easily soluble minerals and numerous bacterial populations (including PGPR strains). Serial dilutions of the aqueous extract were prepared in 0.85% saline and 0.1 ml aliquots of (10^{-1} to 10^{-5}) dilutions were plated to nutrient agar (NA) and incubated at 28 °C for 2days. Single colonies were picked and further streaked onto nutrient agar plates to obtain pure cultures.

Qualitative screening of the isolates for production of secondary metabolites

Test for the presence of ammonia

One of the crucial intermediates in the nitrogen cycle is ammonia [9]. The strains were inoculated into peptone water and allowed to grow for 24 hours at 37 °C. Ammonia was after incubation by adding Nessler's reagent to the tubes. Ammonia was present when the colour changed from yellow to brown [10].

Indole acetic acid production

Bacterial cultures were cultivated in nutrient broth containing L-tryptophan (2 mg/mL) at a temperature of 28 °C for 48 to 72 hours while being constantly stirred at 120 rpm. After incubation, Salkowski's reagent (50 mL, 35% perchloric acid, 1 mL, 0.5 M FeCl3 solution) was added with 2 mL of the supernatant, 2 drops of orthophosphoric acid, and 4 mL to detect IAA. The development of pink colour in the tubes substantiates that IAA is present [11].

Phosphate solubilization

The strains were inoculated in 0.4% bromophenol blue-containing modified Pikovskaya's agar [12] and incubated at 37 °C for 48 hours. The colonies were surrounded by a yellow hydrolysis zone that demonstrated phosphate was hydrolyzed.

HCN production by bacterial isolates

HCN is a secondary volatile metabolite produced by certain bacterial strains which is efficient in controlling the weeds and pests [13]. Modified glycine agar was used which acts as a substrate for HCN production. The Whatman filter paper no. 1 was placed on top of the plate, sealed, and incubated for 5–6 days at 30 °C in a solution of 2% NaOH and 0.5% picric acid. HCN production was indicated by the filter paper turning from yellow to brown.

Production of hydrolytic enzymes

PGPRs are known to produce different hydrolytic enzymes which are effective in controlling different pests and diseases [14].

α-Amylase and protease production

Starch agar plates were used for amylase activity and inoculated with the test bacterial strains. A zone of hydrolysis seen after adding Lugol's iodine to the plates indicated the production of amylase. Similarly for protease assay, skim milk agar plates were used and observed for inhibition zones after incubation.

Cellulase activity

Cellulose hydrolyzing microorganisms were tested for clear zone development around the colonies on the agar plates containing the medium supplemented with carboxymethylcellulose as only source of carbon. After incubation time, CMC plates are flooded with 0.1% Congo red solution and allowed to stand for 15min at room temp, then washed with the help of 1M NaCl to check for the zone of clearance around the colonies.

Phenotypic characterization of the microbial isolates

The phenotypic characteristics of the isolated bacterial strains were determined according to Bergey's Bacteriology Determinative Manual [15]. The microorganisms were differentiated by performing Gram's staining as per the standard procedures [10]. Biochemical tests such as production of indole, acidic and non-acidic end products, cytochromeoxidase, catalase, citrate utilization were performed.

Molecular identification of the potential isolates by 16S rRNA sequencing

Genomic DNA was isolated by following alkaline lysis method. The 16S rRNA primers namely (F)5'AGTTTGATCCTGGCTCAG3' and (R) 5'ACGGCTACCTTGTTACGACTT3' were used for the reaction. Amplification reactions contained 50 ng genomic DNA, 1 × Taq DNA polymerase buffer, 1 U Taq DNA polymerase, 1.5 mM MgCl2, 0.2 mM of each dNTP, and10 pM of each primer. PCR was conducted at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, 1 min at 50°C, and 2 min at 72°C, with an extension of 72°C for10 min. The samples were sent for sequencing. The sequences were aligned CLUSTAL W developed by [16]. The multiple distance matrix obtained wasthen used to construct phylogenetic trees using neighbor joining method of [17]. These sequences were phylogenetically compared from closely related genera, retrieved from the NCBI GenBank BLAST program [18]. Phylogenetic trees were constructed by using Mega 7.0 software (Molecular Evolutionary Genetic Analysis).

RESULTS AND DISCUSSION

Isolation of microorganisms from vermicompost extract

A total of 30 microbial isolates were selected based on the cultural characteristics which were stored as 60% glycerol

stocks for further analysis. The isolates were named as V1-30 (Fig 1).

Fig 1 Spread plate and pure cultures of representative bacterial genera

Ammonia production

Ammonia is the intermediate of biological nitrogen fixation (BNF) in which the process starts from decline of nitrogen gas to ammonia and this can be used by microorganisms for the plant growth. Out of 30 microbial strains tested, 21 showed a positive result by developing a yellow to brown color upon adding Nessler's reagent (Fig 2).

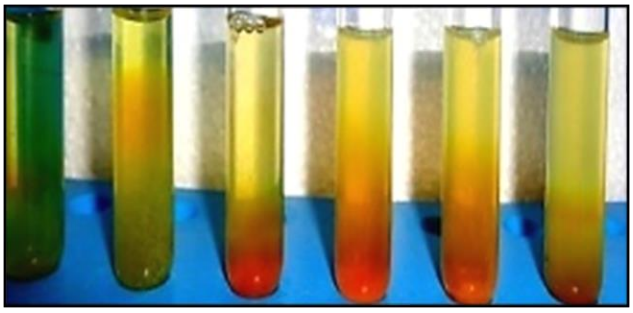

Fig 2 Results depicting ammonia production

Indole acetic acid production

IAA is known to stimulate cell division and differentiation in plants. When Salkowski's reagent was added to the culture supernatant, 12 isolates showed a pink color, indicating IAA production (Fig 3).

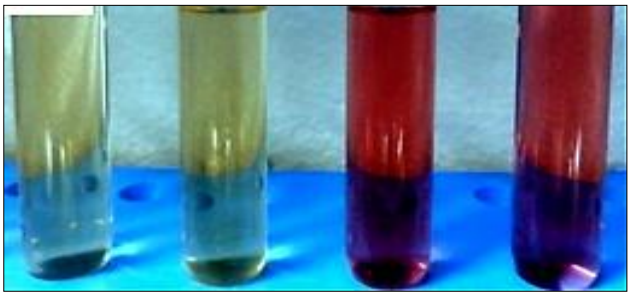

Fig 3 Results of IAA productionn

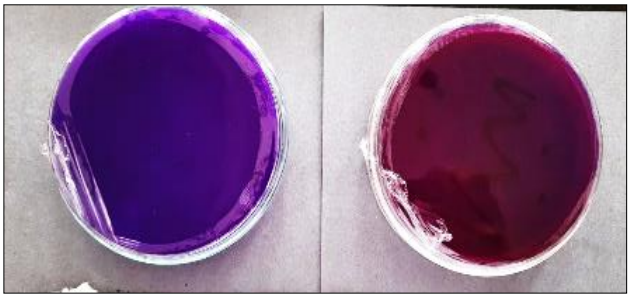

Fig 4 Results of phosphate solubilization in modified Pikovskaya's agar

Phosphate solubilization

Ten isolates showed a good zone of clearance in the modified Pikovsakya's agar plates. The zone of clearance seen after incubation was due to the enzymes released by the microorganisms which degrade calcium triphosphate present in the media.

HCN production

The HCN production of PGPR strains can be identified by growing culture in media containing glycine with filter paper diluted in picric acid at top. Picric acid under alkaline condition undergoes reduction with HCN to form potassium isopurpurate which is indicated by the formation of brown precipitate which marks the production of HCN. Five isolates answered positive for the same.

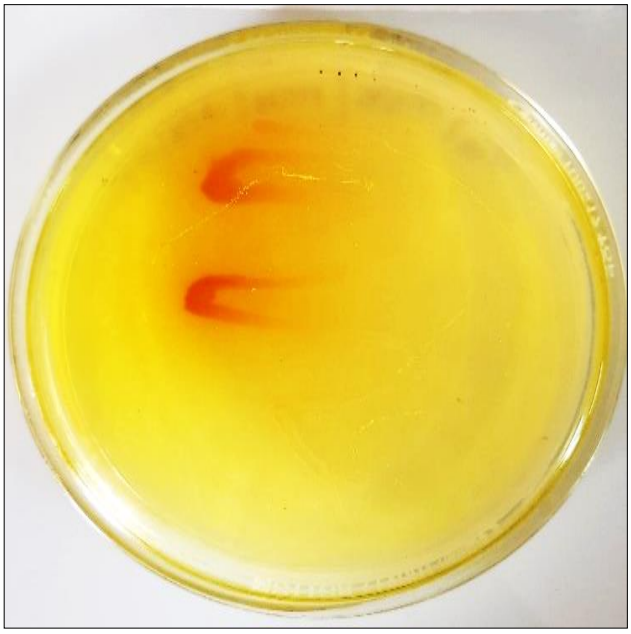

Fig 5 Results of HCN production test

Production of hydrolytic enzymes

Protease activity

From the 30 strains tested, 15 were positive exhibiting clear zone of hydrolysis on skim milk agar. Proteolytic bacteria use enzymes caseinase to hydrolyse casein and form soluble nitrogenous compounds.

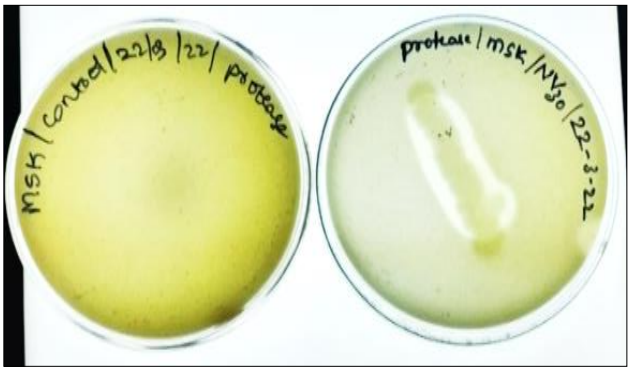

Fig 6 Results of protease activity

Amylase activity

From 30 isolates, 12 were positive which is evident by a clear zone of hydrolysis after addition of Lugol's iodine. Amylase breaks down starch through hydrolysis and clear halo will appear around the colonies of amylase positive species.

Fig 7 Amylase activity

Fig 8 Cellulase activity

Cellulase activity

Cellulase activity was determined by a clear zone of clearance shown on CMC agar around the colonies. From the 30 isolates, 5 isolates were positive which showed a clear zone after adding congo red.

Phenotypic characterization of the microbial isolates

Out of 30 isolates, 10 isolates which showed maximum positive results for PGPR tests were shortlisted for identification by morphological and biochemical methods. The results of the same are tabulated in (Table 1).

Table 1 Morphological and biochemical characterization of selected bacterial isolates

Strain	Gram's staining	Indole test	Methyl red test	Voges Proskauer test	Citrate test	Catalase test	Oxidase test
V_3	-	+	-	-	+	+	+
V_5	-	+	-	-	+	+	+
V_8	-	-	-	-	+	+	-
V_{11}	-	-	-	+	+	+	-
V_{12}	+	+	-	-	+	+	-
V_{15}	-	+	+	-	+	+	-
V_{22}	+	+	-	-	+	+	+
V_{24}	+	+	-	+	+	-	+
V_{26}	+	-	+	-	+	-	+
V_{30}	-	+	+	-	-	+	+

16S rRNA sequencing

Based on the biochemical tests carried out, three isolates which showed maximum response to all the tests performed, namely V_8, V_{11}, V_{30} were identified by 16S rRNA sequencing. On the basis of phylogenetic analysis of 16S rDNA partial sequences, it was found that V_8 was closely related to *Stenotrophomonas maltophilia*, V_{11} related to *Pantoea agglomerans*and V_{30} to *Mesorhizobium muleiense*. Phylogenetic analyzes of the strains based on the neighbor-joining method are shown in (Fig 9-11).

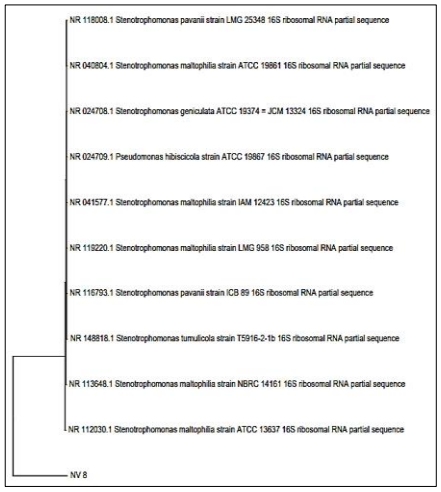

Fig 9 Phylogram of 16S rRNA gene sequences showing the relationship among strain V_8 and representatives of some related taxa

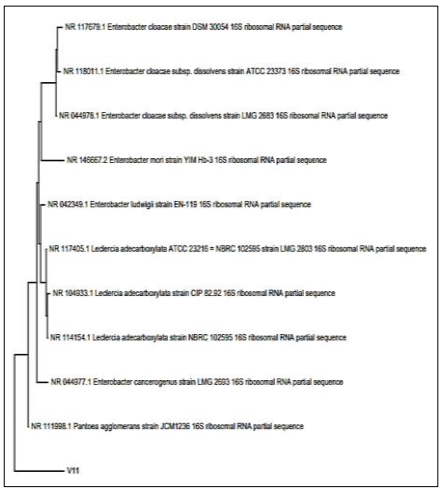

Fig 10 Phylogram of 16S rRNA gene sequences showing the relationship among strain V_{11} and representatives of some related taxa

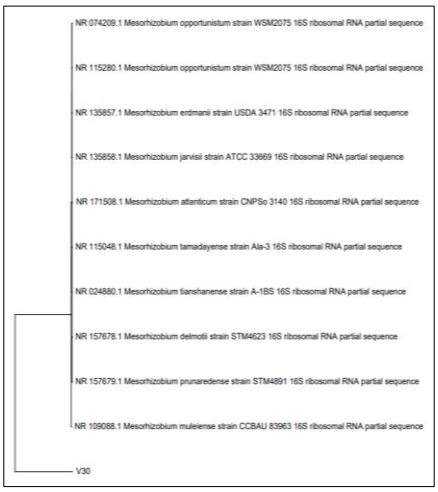

Fig 11 Phylogram of 16S rRNA gene sequences showing the relationship among strain V_{30} and representatives of some related taxa

Agricultural practices are indiscriminately reliant on chemical fertilizers that have raised concern regarding soil pollution, adverse effects to mankind. It is appropriate to replace chemical fertilizers with ecofriendly substitutes such as organic farming, vermicomposting and other soil enrichment techniques [19-20]. Recently many researches have been conducted on vermicomposting, but not much emphasis is given on the aqueous extract of vermicompost and its associated microorganisms. These microorganisms are efficient in producing various derived metabolites which aid in plant growth and development [2]. Hence, the present work was targeted on isolating beneficial bacteria from vermicompost extract and harnessing the plant growth promoting and biocontrol ability of these strains. These bacteria promote plant growth hydrolyzing the insoluble reserves of nutrients [21], nitrogen fixation [22], production of growth hormones such as IAA [23] and producing hydrolytic enzymes such as chitinase, β-1,3-glucanase, fluorescent pigments and cyanide [24]. This paper covers various mechanisms employed by the beneficial microorganisms isolated from vermicompost extract. Out of the 30 microbial strains isolated, 21 showed a positive result to ammonia production, 12 strains tested positive for IAA production, 10 were identified as phosphate solubilizers, 5 strains showed HCN production. 15 strains were able to

hydrolyze casein by synthesizing protease, 12 strains synthesized the enzyme amylase and 5 were able to hydrolyze cellulose. Ammonia a major intermediate in biological nitrogen fixation is produced by various microorganisms thereby making it available to plants [25-26]. Several bacterial genera like *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Mesorhizobium* are reported to fix atmospheric nitrogen [27]. Indole acetic acid is the most common phytohormone that is produced by the beneficial bacteria that promote plant development and plant-bacterial interactions [28]. It was reported that the production of indole acetic acid by *Streptomyces fradiae* NKZ-259 increased plant growth and reduced pest population [29]. The strain *Pseudomonas simiae* MB-751 produces indole acetic acid, which plays a role in the control of *M. incognita* and improves plant growth [30]. Phosphorus, also after nitrogen, is an important nutrient for plants, but mainly occurs in a form that is difficult for crops because it does not dissolve and these microbes are known to hydrolyze this insoluble phosphate by chelating it and making it available to plants [31]. They play an important role in providing usable form of P to the plants without affecting the soil health [32]. There are several volatiles described to date [33] and HCN is one of them. HCN production play a role in disease suppression, for instance, strains of *Pseudomonas fluorescens* is reported to suppress black root rot of tobacco [34]. Another mechanism of action of these microorganisms is the production of enzymes, for example, cellulase, lipase, protease, peroxidase, and phosphatases that enhance plant growth [35]. Based on the *in vitro* qualitative screening of the isolates, three strains which showed maximum response to the tests performed, namely V_8, V_{11}, V_{30} were analyzed by 16S rRNA sequencing.

Results from the GenBank database indicated that the isolates belonged to the phylum Proteobacteria. Strain V_8 was closely related to *Stenotrophomonas maltophilia* [36]. Various species of *Stenotrophomonas* have previously been reported to be able to promote plant growth [37]. The V_{11} strain was identified as *Pantoea agglomerans*, a rod-shaped Gram-negative bacterium found in soil that has adapted to live with plants [38-39]. Certain strains belonging to this species are agronomically important for plant growth promotion and biocontrol [40] and for their ability to produce IAA [41]. Strain V_{30} was found to be related to *Mesorhizobium muleiense*, which has not been widely studied as a plant growth promoter. To our knowledge, this is the first study of *Mesorhizobium muleiense* isolated from vermicompost extract. A recent Indian study highlighted *Mesorhizobium* sp. and *Pseudomonas aeruginosa* with a 32% increase in grain yield compared to the uninoculated control [42]. To conclude, this is one of the few studies on isolation of beneficial microorganisms from aqueous vermicompost extract and elucidating its plant growth promoting ability to be used in ecofriendly agricultural practices.

CONCLUSION

To conclude, it can be deciphered that the aqueous extract of vermicompost acts as a potential source of beneficial novel microorganisms that stimulate plant growth and protect the plants against several phytopathogens. Future studies can be focused on developing standard bioformulations using this beneficial microflora which can be used as bioinculants for sustainable agriculture.

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