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Bacillus cereus and *Citrobacter freundii* from *Lumbricus terrestris* Facilitate Vermicomposting and Improve Soil Characteristics

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ABSTRACT

Soil management is an effective way for increasing crop productivity especially in agriculture based countries like Pakistan.Vermicomposting is an ecofriendly approach theoughwhich earth worms mediate the process for improving soil fertility. The present study was undertaken for the improvement of soil fertility using microbes isolated from internal and external parts of *Lumbricus terrestris* Isolates were identified through different biochemical tests and morphological characterization. Bacterial strains were screened and those showing positive results for their adherence ability, autoaggregation and coaggregation response were used for the vermicomposting process. Both of these strains were inoculated for vermicomposting and whole of the setup was maintained for 60 days.The results showed that microbes were characterised and identified as *Bacillus cereus* and *Citrobacter freundii*. Both these strains showed ptimum growth at pH 7.0 and temperature 37°C. In the finally formed vermicompost water holding capacity, soil pH and aggregation changed positively by EG4. The results of the present finding showed positive impact of inoculation on vermicomposting in the presence of *L. terrestris*. This is beneficial for improving soil fertility and aggregation.

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Authors' Contribution
GQ performed the experimental work. SSQ and AWQ supervised the work. UR helped in data analysis. AAWQ wrote the manuscript.

Key words
***Bacillus cereus, Citrobacter freundii,* Coaggregation, Hydrophobicity, Autoaggregation, Vermicomposting.**

INTRODUCTION

The excessive use of pesticides, fungicides and herbicides for crop protection have killed the soil life leaving the soil barren (Aktar *et al*., 2009). Contemporary research is showing that the environment and soil health ultimately influences overall agricultural productivity. To over come the problem of soil deterioration and increase crop yield, sustainable agriculture in the form of organic farming is an ideal situation not interms of improving soil microbiological activity (Fliebach *et al*., 2007; Pagano *et al*., 2017). Organic farming system are thought to be the answer for the 'food safety, soil health and environmental damage' in future (DeLonge *et al*., 2016). A fertile soil helps the plants to maintain growth as well as supports microbial biodiversity. Microbial biodiversity in the soil is considered to be of great importance (Hartmann *et al*., 2015).

Vermicomposting is a technique which involves the interactions of earthworms with soil microorganisms. The process involves the activity of microorganisms for degradation of organic matter by fragmenting activity (Gong *et al*., 2017; Veeresh *et al*., 2013). Vermicomposts (VCs) are also significant for their ability of less nutrient leaching compared to fertilizers (Jouquet, 2011). Vermiculture scientists knew about the earthworm's role as waste manager and soil conditioner along with improving soil fertility (Sinha *et al*., 2009); moreover, these earthworms also harness the beneficial soil microbiota by converting waste organic material to a bemneficial product like vitamins, enzymes, plant growth hormones (Kalam and Ahmad, 2017).

Keeping their advantages, the study is aimed to develop a vermicompost with the help of native microbial communities for improving soil aggregation and fertility.

MATERIALS AND METHODS

Collection of earthworms

Earthworms *L. terrestris* were procured from the soil in the nearby agriculture land in the sterile plastic bottles having holes and containing 50 kg garden soil (suuplemented with compost) on 21st of March, 2016 early in the morning and maintained in laboratory conditions at 28°C with 70 % moisture till use. Microbial analysis was done after dissection.

Isolation of bacterial from L. terrestris

For isolation of bacteria associated with earthworms, worms were treated carefuuly, washed with sterile distilled

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water followed by surface sterilisation with 70 % ethanol and finally dissected out in Laminar air flow cabinet. Bacteria from the external surface of the *L. terrestris* were isolated by rubbing sterile swabs at the earthworm skin and spreading on the LB agar petri plates incubated at 37°C for 24 h. For isolation of bacteria from internal surface of worms, one gram part was taken from the internal part of dissected worm and serially diluted to 10^{-5} and spread on the LB agar petri plates (incubated at 37°C) for 24 h. The colonies with mucoidy appearance were further purified by quadrant streaking.

Bacterial characterisation

Bacterial strains were charactrised morphologically (Holt *et al*., 1994) and biochemically (Tittsler and Sandholzer, 1936). Further molecular identication based on 16S rRNA gene sequences was done using commercial services of ABI sequencing service Malaysia. Finally these obtained nucleotide sequence were used for construction of bacterial phylogenetic tree using neighbor joining method (Saitou and Nei, 1987) using MEGA 6.0 software (Tamura *et al*., 2013). The accession number of both strains were obtained after submission of sequences to gene bank.

Effect of different pH and temperatures on bacterial growth

Effect of different pH and temperature was recorded for both of the bacterial strains. For this LB-broth was adjusted at varying pHs and inoculated to make final cell densities 0.5 at 600 nm and incubated at 37°C for 24 h. Absorbance was checked after 24 h at 37°C. For studying the effect of different temperatures on bacterial growth, inoculated L-broth adjusted at varying temperature (at pH 7) was incubated at respective temperature conditions (4°C, 37°C and 45°C). Finally the absorbance was checked at 600 nm. The growth experiments were performed in triplicates.

BATH test assay

To determine the bacterial adherence to hydrocarbon surface hydrophobicity, affinity of mono cultures was evaluated using xylene as hydrocarbon following Rosenberg (1984). The cells were harvested (5000 x g for 20 min) from fresh culture and pellet was finally adjusted to 0.8 cell density using 3 ml of Phosphate Buffer Saline. Finally the absorbance of lower organic phase was recorded at 550 nm. The experiment was performed in triplicates and the values are presented as mean values. The hydrophobicity is expressed as a-b/a*100 where a is the initial cell concentration of aquous phase while b is the concentration of aquous phase after 15 min.

Autoaggregation and coaggregation assay

Bacterial autoaggregation and coaggregation of bacterial strains was determined following the method of Nyenje *et al*. (2012). From both cultures of IG1 and EG4, the coaggregating strain, equal volumes (1 mL each) were mixed and the OD (ODTot) of the mixture was recorded at 660 nm taken before incubation for 2 h. While, the tubes were centrifuged at 2,000 rpm (2 min) and the OD of the supernatant (ODs) was measured at same wavelength (660 nm). Bacterial coaggregation in IG1 and EG4 was determined using the following equation described in Nyenje *et al*. (2012).

Vermicomposting experimental set up

To study the effect of *L. terrestris* on soil aggregation, experimental setup for vermicomposting was performed in triplicates in large plastic buckets with 6 holes of 3 cm per bucket. Bacterial isolate (EG4) was used for inoculation to soil. The whole experimental setup was arranged for total duration of 60 days starting from 14th June 2016 till 15 August 2016 and placed in laboratory under shade conditions at 37°C. The experiment was conducted in two sets each in triplicate.

In first set, organic source in the form of cow dung. Different inoculum and organic sources like cow dung, fruit waste and garden waste were tested to find best result. Similiarly amount of organic material and monocultures was also optimized using different ratios (data not shown). Finally the cow dung (showing best results) in soil was supplemented in ratio 1:0.5, respectively. The inoculum was provided of purified monoculture (OD 0.8 at 600 nm) per gram weight of soil. Each pot was equilibriated with total 1kg of medium in all sets. Before the addition of *L. terrestris* (100 grams) to all sets, the medium was allowed to decompose for 15 days and finally worms were added to each set.

The experimental plan for both sets is: Set A (with organic source) comprised Control Bucket A, Soil; Bucket B, Soil with Dung; Bucket C, Soil with + *L. terrestris*; bucket D, Soil with Dung + Inoculum; Bucket E, Soil with Dung + Inoculum + *L. terrestris*. Set B without organic source comprised Bucket F, Soil + *L. terrestris*; Bucket G, Soil + *L. terrestris* + Inoculum; Bucket H, Soil + Inoculum.

Efficacy of the vermicompost soil was tested in terms of water holding capacity before and after the experiment while, with the development of vermicomposting, soil pH was determined after 15, 30,45 and 60 days intervals. When experimental time was over buckets were left empty and soil aggregates were analyzed using hand sorting and finally sieved using a sieve of mesh size 60.

Microscopic analysis of aggregates

After 60 days of experiment of vermicomposting, the soil aggregates were observed for the microscopy

and image analysis under stereomicroscope at 40 x magnification. The analysis of biofilm formed by bacterial isolates on soil aggregates was confirmed by staining the soil aggregates with Indian ink.

Statistical analysis

Data was analysed to check the correlation between autoaggregation, coaggregation and autoaggregation and hydrophobicity using SPSS Software 21.

RESULTS

Both bacterial strains isolated from the external and internal surfaces of earthworms were characterised morphologically and biochemally. Results showed that strain IG1 and EG4 showed off white colonies with circular form having entire margin, flat elevation while cell morphology revealed by straining results showed gram negative, spore forming non capsulated rods. Strain IG1 showed small colonies while EG4 were moderate in size (Table I). Biochemical characterization of bacterial siolates showed, Strain IG1 showed positive results for all biochemical tests except Litmus milk reaction while EG4 showed positive results for all the biochemical reactions (Table I).

Based on 16 S rRNA nucleotide sequence homology and phylogenetic analysis, the strain IG1 was identified as *Citrobactor freundii* with accession number KY435705.1, while EG4 as *Bacillus cereus* with assigned accession number KY435707.1 showing strong homology to their respective sequences (Fig. 1).

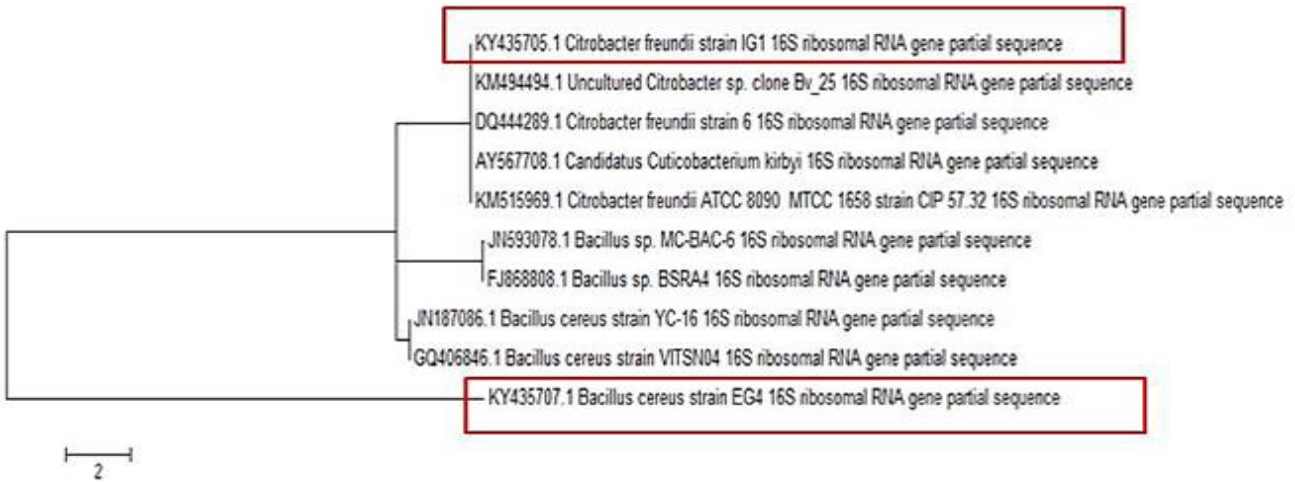

Fig. 1. Phylogenetic analysis of bacterial strains IG1 and EG4 with submitted sequences in genebank.

Table I.- Characterization of bacterial isolates.

Morphological characterization						Staining results			
Strains	**Size**	**Pigmentation**	**Form**	**Margin**	**Elevation**	**Simple staining**	**Gram staining**	**Spore staining**	**Capsule staining**
IGI	Small	Off White	Circular	Entire	Flat	Rod	Gram negative	Spore forming	No capsule
EG4	Moderate	Off White	Circular	Entire	Flat	Rod	Gram positive	Spore forming	Capsule forming

Strains	**Starch***	**Citrate utilization test**	**Litmus milk reaction**	**Methyle red test**	**Voges-Proskauer test**	**Catalase test@**
IGI	- ve	+ ve	Acid formation and reduction	+ ve	- ve	+ ve
EG4	+ ve	+ ve	Curd formation and reduction	+ ve	+ ve	+ ve

Starch: -ve, do not show clear zone around growth; +ve, show clear zone around growth. Citrate utilization test: +ve, blue color and growth; -ve, no growth and non-apperance of blue colour. Methyle red test: +ve, appearance of pink red color; -ve, pink color do not appear. Voges-Proskauer test: +ve, bubble formation; -ve, no bubbling.

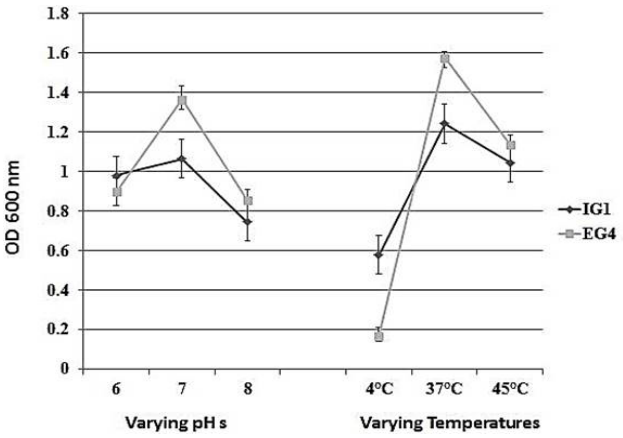

Fig. 2. Effect of pH and temperature on bacterial growth.

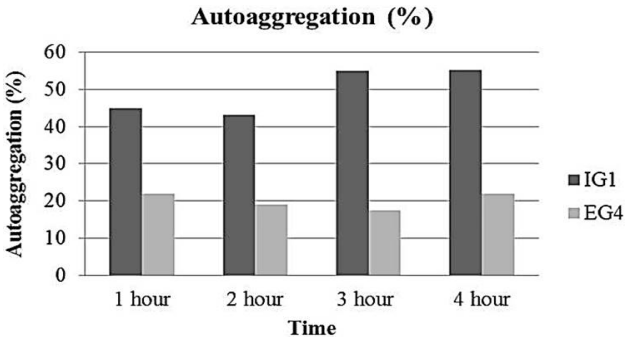

Fig. 3. Bacterial autoaggregation response in bacterial isolates.

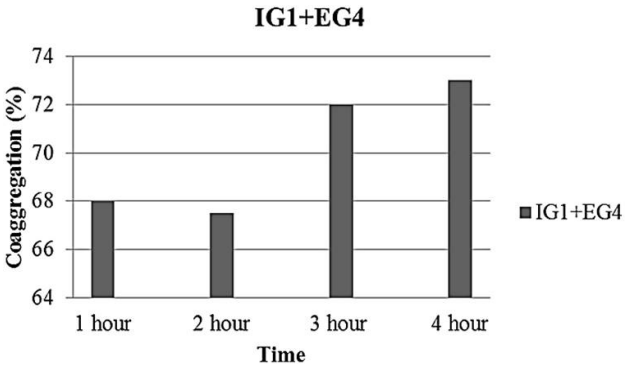

Fig. 4. Bacterial coaggregation assay between strains IG1 and EG4.

Bacterial aggregation: Coaggregation and autoaggregation

Bacterial autoaggregation response increased after 4 h of culture incubation. The maximum autoaggregation was recorded in IG1 strain as compared to strain EG4 (Fig. 3). Autoaggregation test of IG1and EG4 show increase in percentage after every hour of incubation. Coaggregation response of monocultures IG1 and EG4 showed maximum increase after 3rd and 4th hour of culture incubation. in percentage after every hour of incubation. EG4 increase in 2nd hour but decrease in 3rd hour and again increase in 4th hour (Fig. 4).

Bacterial hydrophobicity

The hydrophobicity percentage of both strains *i.e.,* IG1 and EG4 was determined and it was observed that hydrophobicity decreased in EG4 as compared to IG1 (Fig. 5).

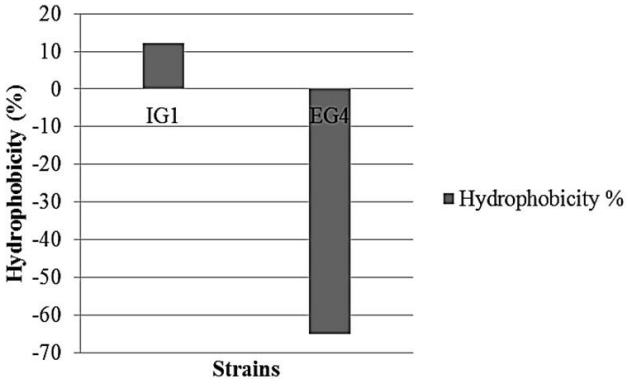

Fig. 5. Bacterial hydrophobicity Assay using xylene as an organic solvent.

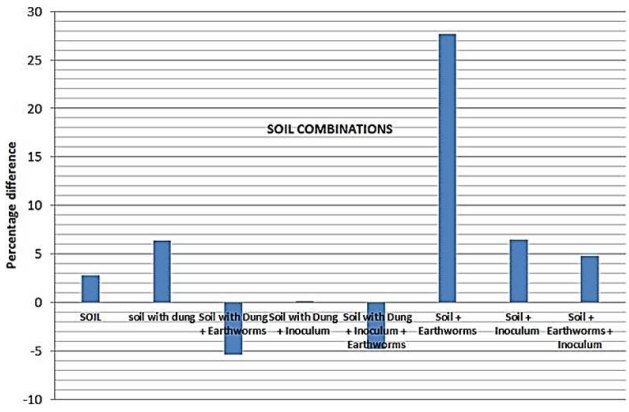

Fig. 6. Water holding capacity determination in vermicompost soil recorded at the start and end of experiment.

Effect of different physiological factors (pH and temperatures) on bacterial growth

While studying the effect of different pH and temperatures on bacterial growth, it was found that strain EG4 showed best growth as compared to strain IG1. Optimum pH for bacterial growth was 37°C while

optimum pH for bacterial growth was pH 7 (Fig. 7).

Water holding capacity

vermicompost soil was checked twice before and after the experimental. Water holding capacity recorded in vermicompost soil showed that set A where the cow dung was added in the soil, water holding capacity of the soil was relatively higher in soil supplemented with cow dung or the inoculum however, addition in set B was more pronounced where cow dung was not supplemented as a carbon source (Fig. 6).

pH values recorded at different time intervals

The pH of soil present in different buckets show change from basic to slightly acidic condition from neutral values recorded at different intervals of time (Fig. 7).

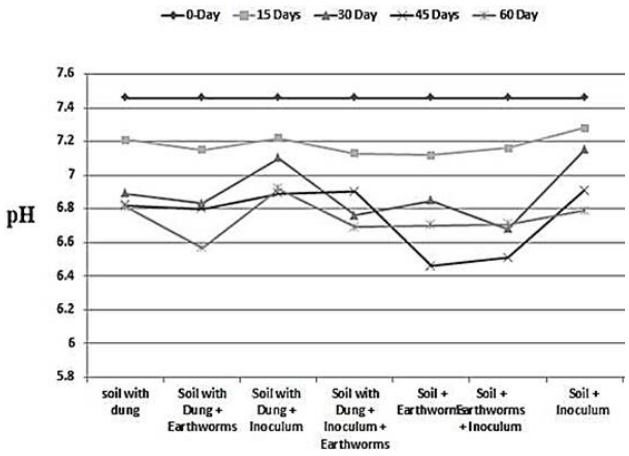

Fig. 7. Change in pH during the course of time in vermicompost soil.

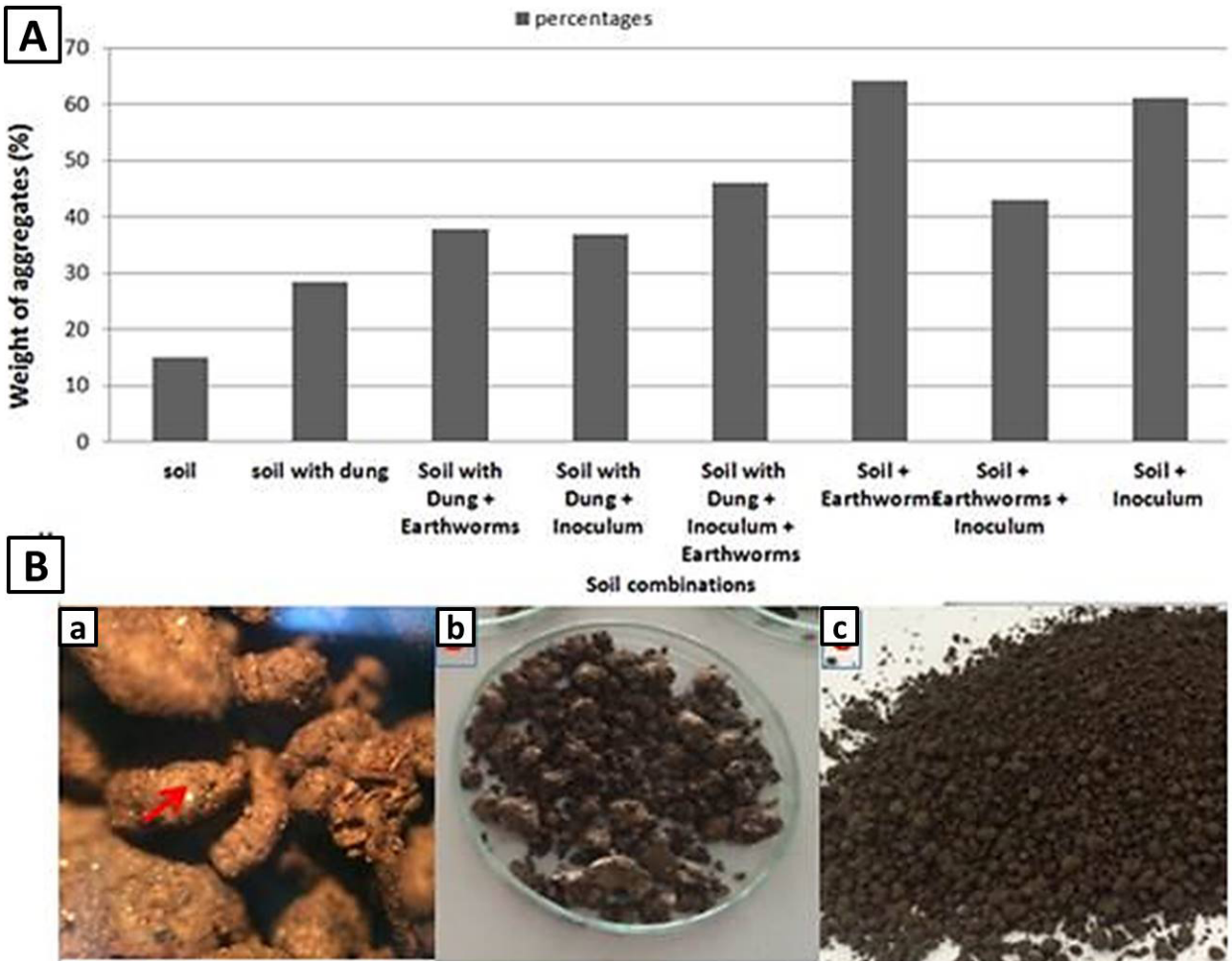

Fig. 8. **A,** Soil aggregates percentages developed in vermicompost soil; **B,** (a) image of soil aggregates observed in Stereo microscopic at 40 X magnification, (b) The soil aggregates had high moisture retention, (c) Water styable aggregates formed in inoculated soil combination of set B.

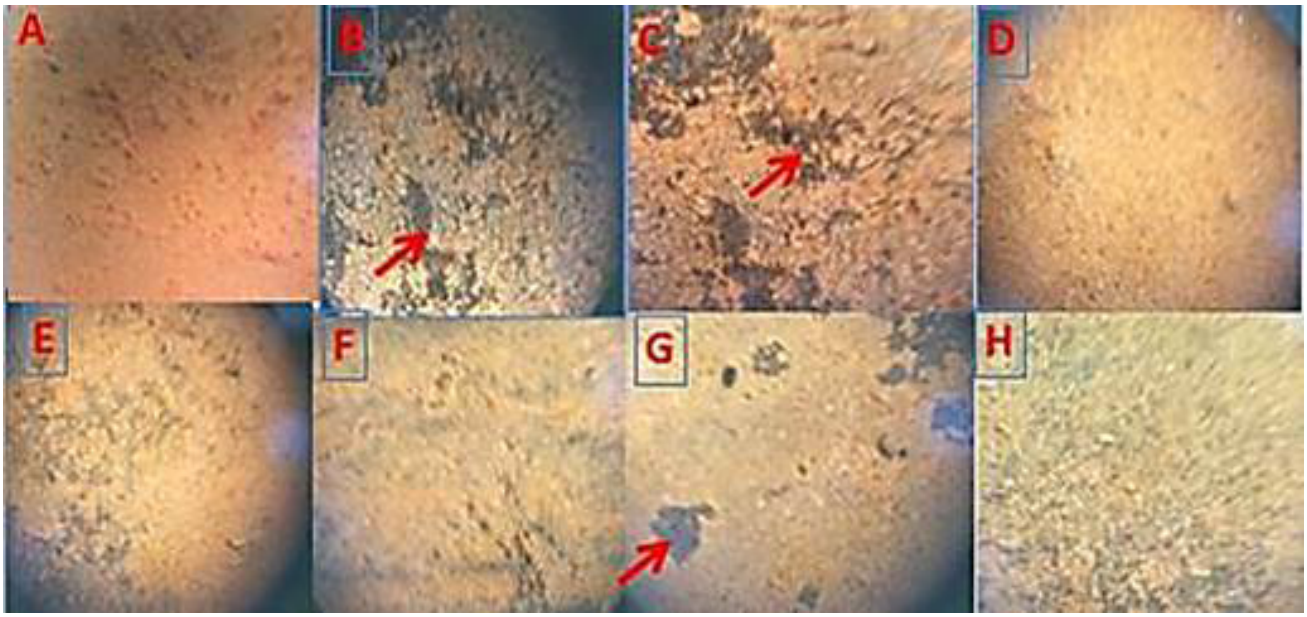

Fig. 9. Staining of soil aggregates using Indian ink. The arrows showed stained regions of aggregate.

Soil aggregates in vermicomposting experiment

Aggregates formed in the buckets at the end were determined by taking their weight. In general the weight of soil aggregates in set A were relatively less as compared to set B. The percentage weight of aggregates formed in different combination of soil from Set A and B were recorded. Maximum soil aggregates were recorded in combination where soil was either supplemented with earthworms (64.30 %) or inoculum (61.14 %) in set B as compared to control soil sample (18.30 %) (Fig. 8A, B).

Image analysis of soil aggregates from vermicomposting

Image analysis of soil aggregates were observed using stereomicroscope at 40 x magnification. The aggregate staining showed that there was a retention of indian ink after washing step of stained aggregates. The aggregates appeared bluish black with indian ink. Morever, the aggregates formed in combination from set B showed high moisture and dense clumps. Even after oven drying of aggregates resulted in stable soil aggregates as shown (Fig. 9).

DISCUSSION

Soil possesses varieties of microflora that is variably influenced by soil conditions. These soil microorganisms interact with other living communities in soil (Pagano *et al*., 2017). Earthworms stimulate soil fertility via bacterial polysaccharides enhance the decomposition of organic matter (Prabha *et al*., 2014). In the present study we dealt with the earthworms and bacteria associated with them for their response towards cells and soil aggregation. Strains were characterised using morphological, biochemical and molecular approach and identified as *Citrobactor freundii* (IG1) and *Bacillus cereus* (EGant 4). *C. freundii* is a soil bacterium and play a significant role in the conversion of nitrate to nitrite (Puchenkova, 1996). *B. cereus* species commonly associated with earthworms show anti helminthis product that inhibit soil pathogens (Valchovski *et al*., 2016). Bacterial autoaggregation and coaggregation assay was performed using mono cultures of IG1 and EG4 or in co cultures, respectively. assay was performed. Coaggregation of monocultures IG1 and EG4 showed gradual increase with the passage of time. The phenomenon of bacterial autoaggregation and coaggregation have great significance for the deveoplment of bacterial biofilm (Nyenje *et al*., 2012). These phenomenon are reported to mediate the development of biofilm formation by th boost of polymers with in bacterial species of same taxonomy. Moreover, these bacterial interactions are further facilitated by bacterial cell surface hydrophobicity (Basson *et al*., 2008). Autoaggregation showed positive correlation between strains IG1 and EG4 with increasing time duration (r=0.981). The coaggregation trend was significantly correlated (p-0.01; r-0.998) with IG1 in autoaggregation. However, non significant correlation was observed with strain EG4 (p=0.01; r-0.218). It has been reported that hydrophobic mutant strain of *Mycobacterium smegmatis* showed more dense biofilm formation as compared to hyfrophilic surfaces (Mazumder *et al*., 2010). When

correlation was determined between autoaggregation and hydrophobicity there was a positive correlation observed (p=0.01; r=0.76) in mono culture of strain IG1. Jankovic *et al*. (2012) reported that autoaggregation in probiotic bacteria directly correlates with adhesion and it is required for bacterial colonization. This is in line with our results for the strains IG1 isolated from the internal surface of *L. terrestris*, however, results of EG4 are contrary to this fact where autoaggregation and adherence are negatively correlated EG4 (p=0.01; r=-0.54). However, the biofilm formation is not always related to the aggregation abilities of bacterial strains as reported in *P. aeruginosa* by Jacobs and Chenia (2011) similar to our results where hydrophobicity is less in EG4 but the response of EG4 towards soil aggregation and vermicompost formation was positive as compared to IG1. These results also suggest that aggregation and hydrophobicity are not always the reliable criteria for evaluation of their adhesion characteristics because many factors interact with these phenomenons.

Vermicomposting is the process of compost formation by the aid of earthworms. Earthworms play an important role in the utilisation of waste organic matter and soil aggregation (Munnoli and Bhosle, 2008; Veeresh and Narayana, 2013). The vermicopost soil developed using inoculm of EG4 showed high water holding capacity as well as aggregate formation. Moreover, change in pH was recorded from basic to slightly acidic with the passage of time. This change in pH can be associated due to decomposition of organic material added in the soil. This change in soil pH might have favoured bacterial growth and soil aggregation as shown by the results. Earthworms or inoculum addition resulted in 64 % increment in aggregate formation as compared to non inoculated soil. maintain the soil structure and the cycling of plant nutrients (Gurav and Pathade, 2011). The earthworms feed on cellulosic waste and using enzymes secreted from their gut microbial flora ling in mutualism with eartworms similiarly, earthworms secrete mucus which maintain pH of surrounding between 6.5 to 7.5 needed to maintain soil micro flora (Aira and Domínguez, 2011). This is also clear in our findings where indian ink is used for staining of the cellular capsules without causing death of the cells. The stain showed dense clumps of dark regions showing presence of mucoidy regions of cells. Indian ink has been reported to be efficient in capsule staining as compared to other stains. Bacterial cells having high molecular weight polysaccharides show a tendency to virulence and biofilm formation however, the capsular material is not stained well with other dyes like crystal violet, methylene blue, or other simple stains (Breakwell *et al*., 2009). So present findings suggest that microbes islated from external surface of earthworm have pronounced improvement in soil aggregation as compared to isolate fom internal gut of *L. terrestris* . The use of these isolates as a biofertiliser can further improve crop yield in future, however these traits must be considerable for selection of isolates. Further research work to test the use of this strain as a biofertiliser is still in progress by Qurashi and co workers.

CONCLUSION

The present study showed that bacterial isolates from *L. terrestris* has ability to aggregate and helpful for improving the soil physiochemical properties like pH and water holding capacity. The results of this findings will be helpful for future selection of bacterial isolates showing these traits in vermiculture

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Statement of conflict of interest

The authors declare no conflict of interest.

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