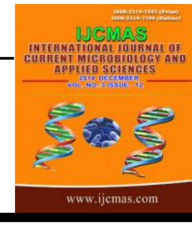

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Original Research Article

Active Presence of Cellulolytic Bacteria in Gut of *Eisenia foetida* reared on ZnO–NPs Spiked Substrates

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Keywords

Vermireactors, ZnO-NPs, *Eisenia foetida,* cellulolytic bacteria, Lignocelluloses, gut microbiome of earthworms.

A B S T R A C T

In present century nanotechnology is the emerging operating in all fields of science. Among all manufactured nanoparticles, Zinc oxide nanoparticles (ZnO-NPs) stand out as one of the most versatile materials because of their unique properties, functionalities and application. This manufactured particle has tremendous physical and chemical properties. It has catalytic efficiency as well antibacterial property, and is frequently used in household products including toothpaste, mouthwashes, ointments surface coatings, food packaging materials as antimicrobial agent. It is now increasingly used as nano-fertilizer to supplies nutrients to plants without generating harmful effect on soils as chemical fertilizer. Therefore, it is essential to understand their effect on 'earthworms' a manager of soil. With this aim fifteen bacterial strains of cellulolytic bacteria were isolated from gut of *Eisenia foetida* reared on ZnO-NPs spiked vermireactors. Results of biochemical and 16SrRNA gene sequence examinations shows that six strains belongs to *Bacillus* sp.; another five strains also belongs to sublines of *Bacillus;* others belongs to *Pseudomonas* sp. Sequences of the bacterial isolates have been deposited in NCBI to obtain accession numbers. Study suggests the gut micro-biomes of earthworms not affected with application of ZnO-NPs. Thus the application of Zn0-NPs may be safe in presence of earthworms in soil ecosystem.

Introduction

Among different metal oxide nanoparticles, zinc oxide (ZnO-NPs) have their own importance (Sabir *et al*., 2014) due to their vast area of applications, for example gas sensors, biosensor, cosmetics, storage, optical devices, window materials for displays, solar cell, and drug-delivery (Sawai *et al*., 1996; Song *et al*., 2006; Wang *et al*., 2008; Hung *et al*., 2001; Wang,2004).

Therefore, ZnO-NPs have attracted intensive research efforts for their unique properties and versatile applications in industrial as well household products in the form of optical materials, ultraviolet emitters, chemical sensors and nano-fertilizers. Raminoghadam *et al.* (2003) demonstrated high sensitivity for many toxic gases of bulk and thin films of ZnO-NPs. The growth and yield of food crops also can improve with application of these nanoparticles. Prasasd *et al*., (2012) treated seed of peanuts with ZnO-NPs (25nm) at 1000 ppm and recorded promoted seed germination, seedling vigor and plant growth and also proved to be effective in increasing stem and root growths in peanuts (Sabir *et al*., 2014). Batamanova *et al*. (2013) recorded average increase in wheat plant grown from seeds treated with metal oxide nanoparticles. The colloidal solution of zinc oxide nanoparticles is used as fertilizer. Selovanow and Zorin (2001) signified it as plant nutrient better than a fertilizer because it not only supplies nutrients for the plant but also receives the soil to an organic state without harmful factors of chemical fertilizers and also can be used in a very small amount as suggested by (Sabir *et al*., 2014).

It was also demonstrated that an adult tree requires only 40-50 kg of nano-fertilizers while an amount of 150 kg required for ordinary fertilizers. Even has great potential of economic importance, there is growing concerns to know about the toxicological and environmental effects of ZnO-NPs (Sabir *et al*., 2014). Sharma *et al*. (2009); Brayner *et al*. (2006); Franklin *et al*. (2007); Heinlaan *et al*. (2008); Wang *et al*. (2008) shown serious toxicity effect to bacteria, *Daphnia magna*, microalgae and even on human cells. The present study was undertaken to observe cellulolytic bacteria in gut micro biomes in earthworm *Eisenia foetida.* As earthworm is known as *key stone* species of soil ecosystem and their symbiotic or a mutualistic metabolic relationship is necessary to maintain terrestrial ecosystem (Kumar *et al*., 2010). They use secreted or membrane-bound digestive enzymes of associated gut micro-biome to mineralization or to degrade polymeric substances like cellulose, chitin (Howath, 2007). Therefore, is an essential to understand the activity of earthworm gut associated cellulolytic microbiomes on exposure of ZnO-NPs.

Materials and Methods

The research design to observe impact of ZnO-NPs in earthworms biomes was developed following published OECD guidelines and Unrine *et al.* (2010). Twenty clitellate adult earthworm *Eisenia foetida* weighing 0.30 ± 0.12 g each in three replicate exposure chambers containing 1 kg dry mass of artificial soil medium were chosen for test experiment. The soil medium consisted of 70% quartz sand, 10% peat moss and 20% kaolin. The pH was adjusted with the addition of a small amount of crushed limestone (Gupta *et al*., 2014). ZnO-NPs 50nm @ 5mg/kg mixed by homogenizer for 5 minutes and moisture content were maintained for 60%. Earthworm sub-samples were also taken at the beginning of the exposure from each exposure chamber. After 28 days of exposure, worms collected and washed thoroughly in running tap water followed by rinsing in distilled water. The complete intestine was dissected out and homogenized in autoclaved distilled water, containing 0.5 mm glass beads with vortex mixing for 5 min. The resulting suspension was serially diluted with water and used as inoculums. Cellulose-hydrolytic bacteria were isolated using Bushnell Hass medium (BHM) amended with carboxymethyl cellulase (CMC) as the sole carbon source. The CMC-amended with BHM medium consist of (g/l); CMC, 10; $MgSo_4.7H_2O$, 0.2; K_2HPo_4, 1; KH_2Po_4, 1; $NH_4NO_3$1; $Fecl_3.6H_20$, 0.05; $CaCl_2$, 0.02. After enrichment in CMC amended medium for more than five times, the inoculums (0.1 ml; successively diluted to 10-^5 times) were repeatedly streaked on BHM agar plates containing the amended CMC. After one week of incubation, the plates were stained by Congo red to observe cellulolytic activity of isolated strains. The cellulase activity of each culture was determined by measuring the zone of clearing on agar plate.

The individual colony having significant clear zone was selected and transferred to a fresh CMC-amended BMH medium; and was serially diluted 10-^{5} times and streaked over BMH agar plates repeatedly, and the bacteria were re-isolated. Through several such processes, fifteen pure bacterial cultures were obtained. Morphology of bacteria was observed in light microscope and biochemically characterized using Lab Manual Supplement, Pacarynuk (2008). Amplification and sequence analysis of the 16S rRNA gene was performed as described by Chen *et al*., (2008). The sequences of isolates were compared with other available sequences in the gene bank. The multiple sequence alignment including the fifteen cellulose-degrading strains and their close relatives were obtained using NCBI platform. The sequence identities were calculated using NCBI platform and obtained accession no with bioinformatics database.

Results and Discussion

Fifteen strains of cellulolytic bacteria were obtained from gut of earthworms reared on ZnO-NPs spiked substrates. The biochemical tests of isolated microbes are presented in table 1 and figure 1 and accession numbers obtained from NCBI database.

Results of biochemical and 16SrRNA gene sequence examinations shows that six strains belongs to *Bacillus* sp.; another five strains also belongs to sublines of *Bacillus;* others belongs to *Pseudomonas* sp. As ZnO-NPs are considered as antimicrobial agent, the presence of cellulolytic bacteria indicates the earthworm gut transform the property of antimicrobial property of ZnO-NPs. The presence of natural organic matter of gut of earthworms may reduce or even eliminate antibacterial property of ZnO-NPs (Li *et al.,* 2009).

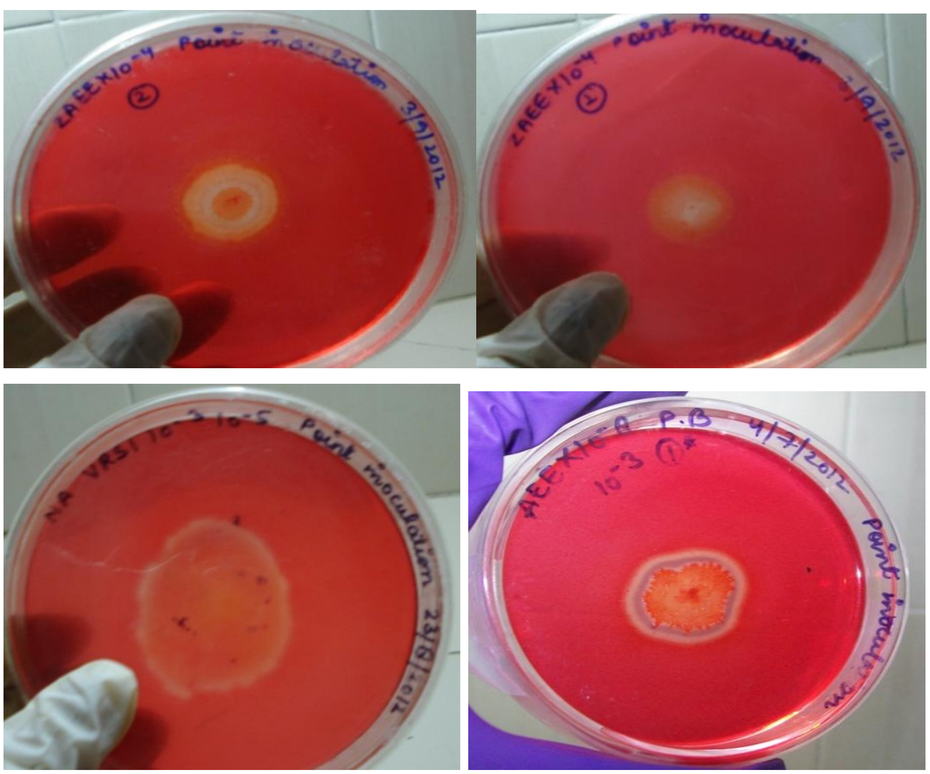
The organic matter of gut of earthworm may attach with ZnO-NPs either through the adsorption on the surface iron oxide as suggested by Gu *et al*. (1994) or ligand exchange between carboxyl/hydroxyl functional groups of humic acid and iron oxide surfaces. There may be hydrophobic interactions as suggested by Hyung *et al*.(2007) with available carbon based materials of organic matter of gut. Concentration of organic matter and their solution chemistry (Chen and Elimelech, 2008; Chen *et al*., 2006; Diegoli *et al*.,2008; Domingos *et al*., 2009) may also help to their attach with nanoparticles and to lose their integrity to behave as nanoparticles.

The particles firmly attached with organic matter may be due to charge of stabilizing layer of ingested organic matter. McKeon and Love (2008); Moreau *et al*. (2007) and Vamunu *et al*. (2008) observed large-molecular-weight biomolecules and biomacromolecules, including proteins, polypeptides, and amino acids, also affects the integrity of nanoparticles and it bind them into form of aggregates. As there are several report of toxicity of ZnO-NPs (Sharma *et al*., 2009; Brayner *et al*., 2006; Franklin *et al*., 2007; Heinlaan *et al*., 2008; Wang *et al*., 2008) beside their economic benefits. It is essential to find out way to nullify toxicity of nanoparticles after their use and release into soil. It is known that microorganisms play key role in organic matter decomposition in environment.

Table.1 Utilization of various carbon sources by bacterial isolates of *Eisenia foetida*

Sugars	IC 1	IC 2	IC 3	IC 4	IC 5	IC 6	IC 7	IC 8	IC 9	IC 10	IC 11	IC 12	IC 13	IC 14	IC 15
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-
Cellobiose	-	-	-	+	-	-	+	-	-	-	+	-	+	-	+
Dextrose	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+
Dulcitol	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Fructose	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-
Galactose	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-
Lactose	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-
Maltose	-	-	+	+	-	-	+	-	+	+	-	+	+	-	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Mannose	+	-	+	-	+	-	-	+	+	-	-	+	-	+	-
Melibiose	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Rhamnose	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+
Salicin	-	+	+	-	-	+	-	+	+	+	+	-	-	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Sucrose	-	+	+	+	-	-	-	+	+	+	-	+	+	+	+
Trehalose	-	-	-	+	+	-	-	+	+	-	-	+	+	-	+
Xylose	+	+	-	-	-	-	+	+	-	-	-	+	-	-	-

Fig.1 CMC test of selected bacterial isolates from EWs'gut exposed to ZnO –NPs spiked substrates



Brown *et al*., (2000) stated earthworms and microorganisms are interdependent and their interactions regulate the biogeochemistry of terrestrial soils through their direct influence on soil carbon dynamics, nutrient cycling and soil physical conditions. Clarke (1999) signified the evolutionary relationship between earthworm burrowing and feeding habits and the gut micro biome. The metabolic relationship of gut and associated biomes depends on environment of gut including natural organic matter and cellulolytic bacteria.

As gut micro-biomes of earthworms including natural organic matter may help to nullify the toxicity of ZnO-NPs, present finding suggests that active presence of bacteria in gut micro-biomes of earthworms reared over ZnO-NPs substrates proved the feasibility of earthworms to nullify toxicity of nanoparticles as antimicrobial reagent.

The back bone of third world economics, agriculture sector is facing various global challenges like climate changes, urbanization, sustainable use of resources and environmental issues (run off pesticides, fertilizers) etc. and the human population is increasing day by day therefore demand of food is growing rapidly. The population increases in world from current level of 6 billion to 9 billion by 2050 is expected (Chem and Yada *et al.*, 2011). Nanotechnology may play dominant role in agriculture production area and may modify the conventional agricultural practices in presence of earthworms without causing any adverse effects in soil ecosystem. Present study advocates the use of nano-fertilizer at large scale in presence of earthworms.

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