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Nitrate removal by electro-bioremediation technology in Korean soil

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ABSTRACT

The nitrate concentration of surface has become a serious concern in agricultural industry through out the world. In the present study, nitrate was removed in the soil by employing electro-bioremediation, a hybrid technology of bioremediation and electrokinetics. The abundance of *Bacillus* spp. as nitrate reducing bacteria were isolated and identified from the soil sample collected from a greenhouse at Jinju City of Gyengsangnamdo, South Korea. The nitrate reducing bacterial species were identified by 16 s RNA sequencing technique. The efficiency of bacterial isolates on nitrate removal in broth was tested. The experiment was conducted in an electrokinetic (EK) cell by applying 20 V across the electrodes. The nitrate reducing bacteria (*Bacillus* spp.) were inoculated in the soil for nitrate removal process by the addition of necessary nutrient. The influence of nitrate reducers on electrokinetic process was also studied. The concentration of nitrate at anodic area of soil was higher when compared to cathode in electrokinetic system, while adding bacteria in EK (EK + bio) system, the nitrate concentration was almost nil in all the area of soil. The bacteria supplies electron from organic degradation (humic substances) and enhances NO_3^- reduction (denitrification). Experimental results showed that the electro-bio kinetic process viz. electroosmosis and physiological activity of bacteria reduced nitrate in soil environment effectively. Involvement of *Bacillus* spp. on nitrification was controlled by electrokinetics at cathode area by reduction of ammonium ions to nitrogen gas. The excellence of the combined electro-bio kinetics technology on nitrate removal is discussed.

1. Introduction

Nitrate contamination in soil and water has become a topical issue concerned with environmental problem throughout the world since the 1970s. Nitrogen-containing compounds released into environment could cause serious problems, such as eutrophication of rivers [1,2] and causes disease called matheomoglobinemia and other health disorders such as hypertension [3], increased infant mortality [4], goiter [5], stomach cancer [6], cytogenetic defects [7] and birth defects [8]. The international drinking water quality standards recommended 50–100 mg/L as "acceptable" level for nitrate (NO_3^-) [9]. Physical and chemical processes such as reverse osmosis, ion exchange and chemical denitrification [10] have been developed for nitrate removal from water. An emerging technology for treating hazardous waste sites using *in situ* bioremediation methods is through the application of electric fields to transport the nutrients as well as bacteria [11]. The transport of nutrients and microorganisms could be achieved in heterogeneous or low hydraulic conductivity of soils by electric fields. This method can be used to stimulate bioremediation under aerobic or anaerobic conditions. In electrokinetic (EK) system, the following reactions take place at anode and cathode.

(1) Anodic reaction:

$$H_2O \rightarrow 2H^+ + 1/2O_2(g) + 2e^-$$

(2) Cathodic reaction:

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2(g)$$

Besides, pH of electrolytes and electroosmosis are the important parameters in EK system. On the removal of nitrate, the nitrate movement to and retention near the anode by an electrokinetic technique was studied in sandy soil by Eid et al. [12]. Cairo et al., [13] had shown that an electric field effectively moves nitrates over distances for upto 3 m in a soil at different concentrations. The pH gradient was strongly correlated with nitrate movement towards the anode. Eid et al. [14] and Manokararajah and Ranjan [15] also investigated on electromigration of nitrate in sandy soil and showed that the electrokinetic process effectively concentrated and retained nitrate close to the anode in the presence of hydraulic flow. Ottosen and Dalgard [16] have shown that NO_3^- can

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be removed from bricks in an applied electric DC field. Although EK system removes nitrate, high quantity of nitrate is accumulated at anodic area of the soil. The most versatile and widely used technology is biological denitrification [17,18]. Biological removal of nitrate is widely used in the treatment of domestic and complex industrial waste waters [19,20]. Kim et al. [21] noticed that the isolates (*Bacillus* sp.) required high C/N ratio with a high concentration of organic carbon and suggested that the strains can be used in a biological nitrification–denitrification process. The *Bacillus* strains were performed to convert ammonia to N_2 without formation of nitrous oxide under aerobic conditions. Hayatsu et al. [22] also noticed that *Bacillus* spp. involve in nitrification and denitrification process. Gabaldon et al. [23] tried to remove nitrate in wastewater of a metal-finishing industry by biological route. Methanol was added as a carbon source for denitrification which resulted the removal efficiency higher than 90%. Kim et al., [24] studied the nitrate removal without carbon source feeding by permeabilized *Orchrobactrum authropi* SY 509 using an electrochemical bioreactor. They used denitrifying enzymes to remove nitrate on working electrode. But no study has been carried out on the application of nitrate reducers in electrokinetic process.

The farmers world over apply synthetic fertilizers for increasing the production of food products in a short period. The addition of excess fertilizers makes the soil saturated with salt like nitrates. The excess nitrate may be hazardous to environment in particular for the human. The input of large amounts of nitrogen fertilizers to agricultural fields influences nitrification and denitrification processes. Hence, recent extensive research on nitrification and denitrification has been focused on the detection and identification of the dominant microorganism responsible for these processes in soil ecosystem. At this juncture, a suitable technology has to be identified for the removal of such man-made contaminants. The present study focuses on the removal of nitrate by the application of electrokinetics and biotechnology. An attempt was made by employing *Bacillus* strains for denitrification process to remove nitrate in the soil. Besides, electrokinetics was selected to accelerate the nitrate and ammonium removal process by electroosmosis. Starch was used as organic source to enhance the bacterial activity in the soil. It also focuses on the feasibility of nitrate removal by using *Bacillus* spp. with electrokinetic system.

2. Materials and methods

2.1. Bacterial enumeration and isolation from agriculture soil

Soil samples contaminated with nitrate were collected from a green house, Jinju city of Gyengsangnamdo. Soil samples were collected in a sterilized conical flask and transported by using icebox from the site to Korea Electrotechnology Research Institute (KERI)–microbiology laboratory. The collected samples were serially diluted (10-fold) using 9 ml of sterile distilled water-blanks. The samples were plated by pour plate technique. The nutrient agar medium and nitrate agar (Hi-media, Mumbai) were used to enumerate the heterotrophic bacteria and nitrate reducing bacteria respectively. The collected samples were serially diluted upto 10^{-14} dilution. One milliliter of the aliquot of appropriate dilution was pipette out into the sterile petri plate and 20 ml of respective medium was added into each petri plate. The prepared respective sterile medium was also poured into petri dishes. The plates were gently swirled so that the medium might be distributed evenly in the plate. Plates in triplicate were inverted and incubated at room temperature for 24 h. After 24 h the colonies were counted. The plates containing bacterial colonies with 30–300 numbers were selected for calculation. The bacterial colonies were expressed as colony forming units per gram (CFU/g). Morphologically dissimilar colonies of nitrate reducers were selected randomly from all plates and the isolated colonies were purified using appropriate medium by streaking methods. The pure cultures were maintained in nitrate agar slants for further analysis. The isolated bacterial cultures were identified by molecular techniques (16SrRNA gene sequencing). The strains were maintained at 4 °C to keep the microbial strain viable.

2.2. 16 S rRNA gene sequencing and phylogenetic analysis

The genomic DNA was isolated from thirty six isolates according to the procedure described by Ausubel et al. [25] and the small subunit 16S rRNA gene was amplified using the two primers 5′ AGAGTTTGATCCTGGCTCAG 3′ (*E. coli* positions 8 to 28); reverse primer: 5′ ACGGCTACCTTGTTACGACTT 3′ (*E. coli* positions 1477 to 1498). The purified PCR product, approximately 1.5 Kb in length was sequenced using five forward and one reverse primer as described earlier [26]. The deducted sequence was subjected to blast search for closest match in the database. A search of the GenBank nucleotide library for sequences similar to those determined was made by using BLAST, through the National Center for Biotechnology Information (NCBI) internet site (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene sequence of nitrate reducing *Bacillus* spp. EK1-EK36 were submitted in the Gene bank. The pair wise evolutionary distances were computed using the DNA DIST program with the Kimura 2 parameter model. The phylogenetic trees were constructed by using four tree making algorithms the UPGMA, KITSCH, FITCH and DNAPARS of the PHYLIP package [27]. The 16S rRNA sequences were aligned using multiple sequence alignment program CLUSTALW [28] and phylogenetic tree was plotted using NJPLOT [29] and PHYLODRAW [30] programs. A bootstrap analysis was performed to validate the reproducibility of the branching pattern.

2.3. Nitrate reduction test

Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. The nitrate reduction test was carried out by employing nitrate reduction kit (Fluka 73426).

2.4. Electrokinetic cell configuration

Fig. 1 shows the sketch of the laboratory EK reactor. The EK cell is made up of acrylic sheet with dimension $24 \times 4 \times 6\ cm^3$. It is divided into three compartments. The central one is for storing soil sample and the others two for working reservoir solution consisting of catholyte and anolyte. The soil sample was mixed with electrolyte solution (about 20% to 30% water content), then carefully stored in the central compartment. Titanium and graphite electrodes used as anode and cathode respectively were placed at each electrolytic compartment. To avoid soil leakage to the water reservoirs, a pair of nylon meshes [31] (mesh opening 149 μm) and a filter paper (Whatman No. 2) were placed between the soil sample and electrodes. The used soil for electrokinetic remediation was a kind of silty loam. DC power supply (3A-30 V, ED Laboratory, ED-245B) was used for impressing 20 V in EK system. Before starting the experiment, the EK cells were exposed for one hour in UV light for sterilization. The same types of cells were used for all the experiments. The cells were covered with UV sterilized polythene sheet to avoid the contamination. The sterility of all the systems was maintained carefully.

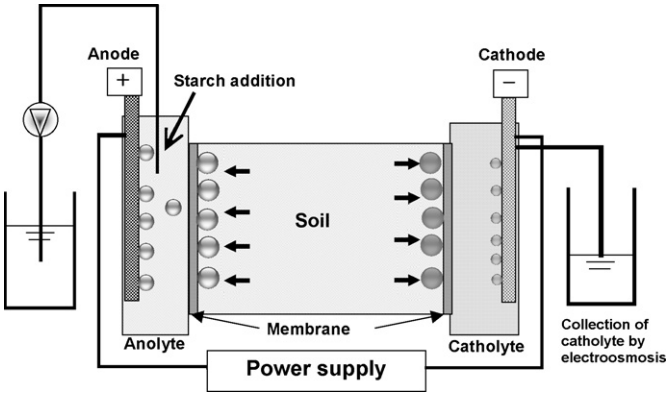

Fig. 1. Electrokinetic cell model.

2.5. pH, conductivity and electroosmosis measurements

The soil pH was measured according to the Korean standard test method (KSM ISO 10390: 2005). After finishing the EK experiments, pH and EC of the soil were estimated. The soil was dried in air and 5.0 g was mixed with 25 ml of distilled water in 50 ml vial. The mixture of soil and water was agitated with magnetic stirrer for 1 h at 300 rpm. After calibration with pH standard solution, the pH of soil was measured by pH meter (Istek Inc., Model 76P). Electrical conductivity (EC) was measured for the same ratio of mixture by employing EC meter (Istek Inc., Model 47 C). The average value of triplicate samples is presented. Electro osmotic flow was measured in electrokinetics with time by collection of excess of electrolyte from cathode chamber.

2.6. Nitrate removal in EK system

Before starting the experiment, the soil 400 g was dried at 100 °C for 24 h to kill all the bacteria present in the soil. The sterilized tap water by autoclave (about 20% to 30%) was mixed in the soil. The cell was also sterilized by UV light for 1 h and then sterilized tap water was used as electrolytes. The cell was maintained without any bacteria and voltage 20 V was impressed for seven days to remove nitrate. The room was also maintained free from any contamination. The sterility was checked by culturing the bacteria in the soil.

2.7. Nitrate removal by biological method

The identified species *Bacillus* spp. (21 isolates) were inoculated in nitrate broth and made as 24 h old mixed cultures. 2 ml of mixed culture (10^5 CFU/ml) was inoculated with 1% of sterilized starch as carbon source. The same soil was sterilized at 100 °C for 24 h and about 20% to 30% of bacteria- inoculated 1% starch in tap water was mixed in the soil. The soil was kept in incubator at 30 °C for seven days by employing EK cell. Every day, 1 ml of sterilized 1% starch solution was added on the surface of the soil to maintain the wet condition in biological system.

2.8. Nitrate removal by EK + bioprocess

The same soil was sterilized at 100 °C for 24 h and bacteria were inoculated in the soil along with 1% sterilized starch added tap water. The system was maintained between 28 °C and 30 °C by covering sterilized polythene papers. After 24 h of bacterial inoculation, the cell was used for EK experiment. 20 V was applied to the EK cell to remove nitrate in the soil along with bacteria. The EK + bio process was also carried out for a week. The sterilized 1% starch was used as anolyte for supplying of carbon in denitrification process.

Table 1
Original characteristics of soil used in the present study collected from Jinju.

Soil characteristics	Value
pH	5.6
EC (dS/m)	5.6
Organic (g/kg) (TOC)	25
P_2O_5 (mg/kg)	1384
Calcium (cmol/kg)	9.0
Potassium (cmol/kg)	2.40
Magnesium (cmol/kg)	2.1
Sodium (cmol/kg)	0.17
Nitrate (mg/kg)	268
Ammonium (mg/kg)	12

2.9. Nitrate estimation

The collected samples after completion of EK process were air-dried and 10 g was put in the 100 ml flask and 2 M potassium chloride was added in the flask with soil. The mixture of the soil was agitated for 30 min and the filtered solution was obtained with filter paper (Whatman No. 2). The solution was used for the estimation of nitrate and ammonium in the soil by using QuAAtro auto analyzer (BRAN + LUEBBE, Germany).

3. Results and discussion

The researchers are concentrating towards nitrate removal from water resources, aquaculture ponds or aquaria, soil and industrial wastes [32]. Nitrates were removed by biological techniques [23,33–39], hydraulic gradient [15], chemical methods [40,41], electrokinetics [12,13,16,42–44] and biofilm- reactor [45]. In the present study, electro -biokinetic was employed to remove nitrate in the soil collected from a vinyl house.

Table 1 shows the chemical characteristics of soil collected from Jinju vinyl house, South Korea. Organic content of the soil was 25 g/Kg. The calcium, potassium, and magnesium were 9 cmol/Kg; 2.4 coml/Kg and 2.1 cmol/Kg respectively. The nitrate concentration was 268 mg/Kg and ammonium was 12 mg/Kg. The soil contained 8.7×10^6 CFU/g of heterotrophic bacteria and 5.5×10^5 CFU/g of nitrate reducing bacteria.

Genomic DNA was extracted from all the nitrate degrading bacterial isolates. Amplification of gene encoding for small subunit ribosomal RNA of identified species was done using eubacterial 16S rDNA primers. The 16S rDNA amplicons derived from nitrate reducers was cloned in pTZ57R/T vector. The recombinant plasmid (pACE4, harboring 16S rDNA insert) was partially sequenced. The sequence obtained was matched with the previously published sequences available in NCBI using BLAST. Sequence alignment and comparison revealed more than 98% similarity with all bacterial isolates. The nucleotide sequence data have been deposited in GenBank under accession number mentioned in Table 2. The phylogenic trees were constructed to find out the evolutionary interrelationship among various bacterial species present in the environment. Phylogenetic tree of 16S rRNAs have shown that the species of same genera have been aligned in a same branch. The results which obtained in this study led to the conclusion that the evolutionary relations exhibited by the isolates are such that these organisms belong to the same eubacterial group (Fig. 2). The boot strap values of 100 indicates the branching pattern which was confirmed in all resamplings and the scale bar represents 0.1 estimated neucleotide change per sequence position. Kim et al. [21] also noticed dominating strains of heterotrophic *Bacillus* in Korean soil. In Korea, a process (BioBeol Bacillus)–(Korean patent No. 151928) known as an advanced wastewater treatment system in which *Bacillus* strains have predominated. It was reported that this process is able to

Table 2
List of nitrate reducers and nitrate reduction capability of nitrate reducers collected from soil.

No	Strain no.	Name of bacteria with accession numbers	BLAST hit with % in parenthesis	Nitrate reduction capability
1	EK 1	*Bacillus* sp. EU910221	*Bacillus* sp. Q1 16S ribosomal RNA gene, partial sequence EU236732 (99)	–
2.	EK 2	*Bacillus* sp. EU910222	*Bacillus* sp. 096002 16S ribosomal RNA gene, partial sequence EF522805 (99)	+
3	EK 3	*Bacillus oleronius* EU910223	*Bacillus oleronius* strain ATCC 700005 16S ribosomal RNA gene, AY988598 (99)	+++
4	EK 4	*Bacillus megaterium* EU910224	*Bacillus megaterium* partial 16S rRNA gene, isolate SUF4 AJ880767 (98)	–
5	EK 5	*Paenibacillus sp* EU910225	*Paenibacillus* sp. PALXIL08 16S ribosomal RNA gene, DQ407282 (98)	+++++
6	EK 6	*Bacillus* sp. EU910226	*Bacillus* sp. PN13 16S ribosomal RNA gene, partial sequence DQ523735 (99)	–
7	EK 7	*Bacillus* sp. EU910227	*Bacillus* sp. WN559 16S ribosomal RNA gene, partial sequence DQ275174 (99)	+
8	EK 8	*Paenibacillus sp* EU910228	*Paenibacillus* sp. PALXIL08 16S ribosomal RNA gene, partial sequence DQ407282 (98)	+++++
9	EK 9	*Bacillus* sp. EU910229	*Bacillus* sp. SP80 16S ribosomal RNA gene, partial sequence FJ404758 (99)	–
10	EK 10	*Paenibacillus sp* EU910230	*Paenibacillus* sp. SAFN-035 16S ribosomal RNA gene, partial sequence AY167800 (99)	+++
11	EK 11	*Bacillus* sp. EU910231	*Bacillus* sp. 3477BRRJ 16S ribosomal RNA gene, partial sequence FJ215800 (99)	+++
12	EK 12	*Paenibacillus sp EU910232.*	*Paenibacillus* sp. SAFN-035 16S ribosomal RNA gene, partial sequence AY167800 (99)	+++
13	EK 13	*Bacillus cereus* EU910233	*Bacillus cereus* strain CMG528-03J 16S ribosomal RNA gene, partial EU622832 (99)	+++++
14	EK 14	*Bacillus pycnus* EU910234	*Bacillus pycnus* gene for 16S rRNA, partial sequence AB271739 (99)	–
15	EK 15	*Bacillus cereus* EU910235	*Bacillus cereus* strain BAC-B2 16S ribosomal RNA gene, partial sequence DQ884352 (99)	+++++
16	EK 16	*Bacillus* sp. EU910236	*Bacillus* sp. SP80 16S ribosomal RNA gene, partial sequence FJ404758 (99)	–
17	EK 17	*Bacillus pumilus* EU910237	*Bacillus pumilus* strain CT3 16S ribosomal RNA gene, partial sequence EU660356 (99)	–
18	EK 18	*Paenibacillus* sp. EU910238	*Paenibacillus* sp. 6495m-C2 partial 16S rRNA gene, isolate 6495 AJ509004 (99)	+++
19	EK 19	*Bacillus megaterium* EU910239	*Bacillus megaterium* strain ST53 16S ribosomal RNA gene, partial FJ386540 (99)	+
20	EK 20	*Brevundimonas sp* EU910240	*Brevundimonas* sp. WPCB153 16S ribosomal RNA gene, partial seq FJ006909 (99)	–
21	EK 21	*Bacillus* sp. EU910241	*Bacillus* sp. BD-85 16S ribosomal RNA gene, partial sequence AF169519 (98)	–
22	EK 22	*Bacillus* sp. EU910242	*Bacillus* sp. SP80 16S ribosomal RNA gene, partial sequence FJ404758 (99)	–
23	EK 23	*Bacillus* sp. EU910243	*Bacillus* sp. I_GA_W_11_8 16S ribosomal RNA gene, partial sequence FJ267543 (99)	–
24	EK 24	*Bacillus* sp. EU910244	*Bacillus* sp. C_W 16S ribosomal RNA gene, partial sequence EU835742 (99)	–
25	EK 25	*Bacillus* sp. EU910245	*Bacillus* sp. 3439ABRRJ 16S ribosomal RNA gene, partial sequence FJ215794 (99)	+++
26	EK 26	*Bacillus* sp. EU910246	*Bacillus* sp. MH03 16S ribosomal RNA gene, partial sequence AY690697 (99)	–
27	EK 27	*Bacillus subtilis* EU910247	*Bacillus subtilis* gene for 16S rRNA, partial sequence AB364963 (99)	+++++
28	EK 28	*Bacillus cereus* EU910248	*Bacillus cereus* isolate NH5-1 16S ribosomal RNA gene, partial sequence EF690422 (99)	+++++
29	EK 29	*Bacillus firmis* EU910249	*Bacillus firmus* strain CM21 16S ribosomal RNA gene, partial sequence EU660344 (99)	+++
30	EK 30	*Bacillus* sp. EU910250	*Bacillus* sp. SP80 16S ribosomal RNA gene, partial sequence FJ404758 (99)	–
31	EK 31	*Bacillus pumilus* EU910251	Bacillus pumilus strain BG-B26 16S ribosomal RNA (rrs) gene partial, EU869253 (99)	+
32	EK32	*Bacillus cereus* EU910252	*Bacillus cereus* strain U25-3 16S ribosomal RNA gene, partial sequence EU531543 (99)	+
33	EK 33	*Bacillus* sp. EU910253	*Bacillus* sp. PGBw2 16S ribosomal RNA gene, partial sequence EU162022 (99)	–
34	EK 34	*Lysinibacillus sp.* EU910254	*Lysinibacillus* sp. R-31030 partial 16S rRNA gene, strainR-31030, AM910293(99)	+++
35	EK 35	*Bacillus* sp. EU910255	*Bacillus* sp. 3406BRRJ 16S ribosomal RNA gene, partial sequence FJ215787 (98)	++++++
36	EK 36	*Paenibacillus sp.* EU910256	*Paenibacillus* sp. 2 16S ribosomal RNA gene, partial sequence AY745242 (98)	+

remove nitrogen and phosphorous as well as organic matter efficiently [46].

The nitrate reducing capability of all bacterial strains were tested and presented in Table 2. Among 36 strains, 21 isolates were identified as nitrate reducers. *Bacillus* sp. (EK 2), *Bacillus oleronius* (EK 3), *Paenibacillus sp.* (EK 5), *Bacillus* sp. (EK 7), *Paenibacillus* sp. (EK 8), *Paenibacillus* sp. (EK 10), *Bacillus* sp. (EK 11), *Paenibacillus* sp. (EK 12), *Bacillus cereus* (EK 13), *Bacillus cereus* (EK 15), *Paenibacillus* sp. (EK 18), *Bacillus megaterium* (EK 19), *Bacillus* sp. (EK 25), *Bacillus subtilis* (EK 27), *Bacillus cereus* (EK 28), *Bacillus firmis* (EK 29), *Bacillus pumilus* (EK 31), *Bacillus cereus* (EK32), *Lysinibacillus* sp. (EK 34), *Bacillus* sp (EK 35) and *Paenibacillus* sp. (EK 36) were nitrate reducers. *Bacillus* species are either obligate or facultative aerobes. Investigation of aerobic metabolism of *Pseudomonas* and *Bacillus* species had revealed many interesting features of assimilatory nitrate reduction to ammonia [47,48]. Tiedje [49] reported that *Bacillus* species could carry out anaerobic dissimilatory reduction of nitrate to ammonia. However, *Bacillus* species is capable of using nitrate and nitrite as the alternative electron acceptors [36].

Figs. 3 and 4 show the pH and conductivity of the soil during nitrate removal process in EK and EK + bio system. It can be seen that in both the systems, the pH of the soil nearer the anode was about 3.0 and at cathode was 7.7. The pH of anolyte and catholyte was 2.0 and 10.1 respectively in EK system. In EK + bio system, the pH of anolyte and catholyte was 2.1 and 10.2 respectively. It is due to the production of large quantity of H^+ at anolyte and OH^- production at catholyte. There is no much significant difference in pH between EK and EK + bio system. Reddy and Chinthamreddy [50] noticed that the pH of the soil decreases to 2–3 near the anode and increases to 8–12 near the cathode in EK system. The conductivity value was 4.2 dS/m in soil near the anode while in EK with bacterial system the EC value was 1.1 dS/m. There was no significant difference on conductivity of soil near the cathode in both EK and EK + bio system. It can be expected that the reduction of EC at anode in EK + bio system may be due to the consumption of nitrate. Generally, the nitrate reducers require organic compounds for growth. The organic compounds serve as sources of carbon and energy. Subsequently, they use nitrate (NO_3^-) as terminal electron acceptor during respiration throughout the soil where the possibility of nitrate accumulation at anodic area is lower in EK + bio system. Hence EC value was lower in anodic area of EK + bio system.

The electroosmotic flow in EK and EK + bio system is presented in Fig. 5. The electro osmotic flow in EK and EK + bio system increases with time. The highest osmotic flow at 168 h was about 90 ml in EK system and slightly increased value of about 95 ml in EK + bio system. It indicates that there is a possibility of movement of H^+

Table 3
Enumeration of nitrate reducers in EK and EK + bio system.

System	Cathode soil	Anode soil	Anolyte	Catholyte
EK + bio	6.8×10^{15} CFU/g	3.2×10^4 CFU/g	6.3×10^2 CFU/ml	7.2×10^8 CFU/ml
EK	TLTC*	TLTC*	TLTC*	TLTC*

*TLTC: too low count.

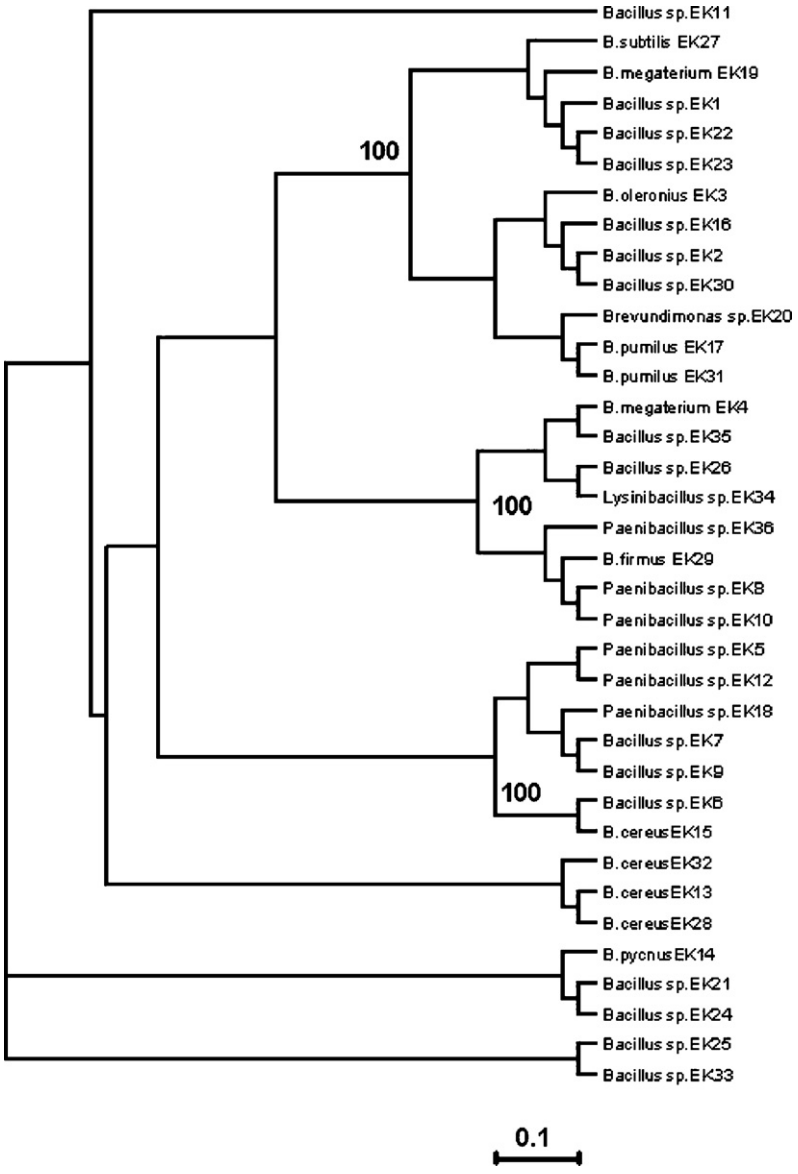

Fig. 2. Phylogenetic tree of nitrate reducers collected from JinJu soil.

and O_2 from anodic area to cathodic surface. It may be expected that H^+ and O_2 may involve on the nitrate removal process in the presence of bacterial activity.

Bacterial enumeration of EK-bio and EK system are presented in Table 3. After finishing the experiment, the nitrate reducers were enumerated. The bacterial count was higher at cathodic area of soil (6.8×10^{15} CFU/g) when compared to anodic area (3.2×10^4 CFU/g) of the soil. Catholyte also contained higher number of bacteria (7.2×10^8 CFU/ml) compared to anolyte (6.3×10^2 CFU/ml). Even though bacterial count was high at cathodic area of soil, the availability of cations may be higher which may increase the EC of the system. It can be explained that the EC value depends upon the availability of the bacterial activity in EK + bio system. DeFlaun and Condee [51] reported that the bacteria has unidirectional movement towards positive electrode (anode) in an electric field in trichloroethylene removal of electrokinetics at neutral pH. Besides, they observed that the recovery of cells at pH 8.5 was approximately three orders of magnitude higher than at pH 6.5 and approximately six times higher than at pH 7.5. The effect of pH on transport rate was also tested by the same group, with in a range of pH values that are optimal for most bacteria. At pH 5.5, the negative charge

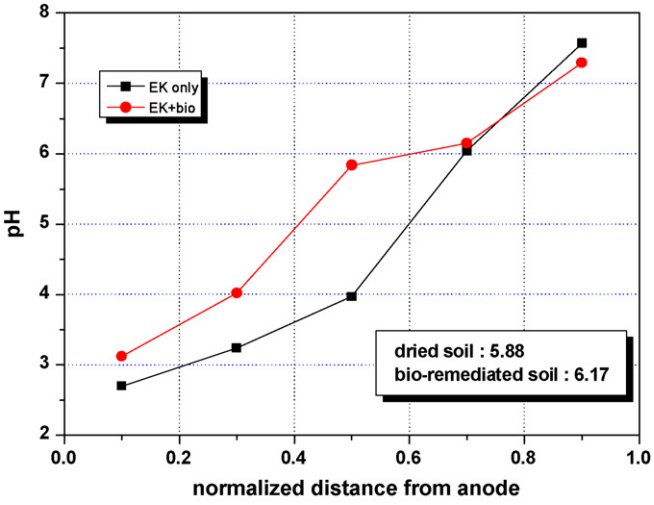

Fig. 3. pH profile of the soil after completing EK system and EK + bio system.

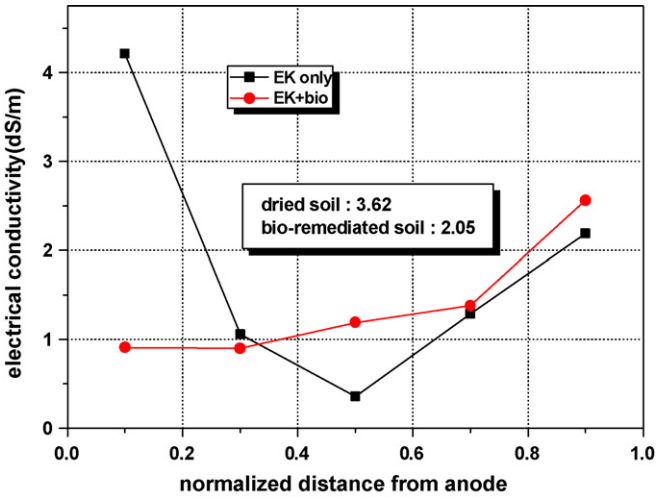

Fig. 4. Electrical conductivity of the soil section at variable locations.

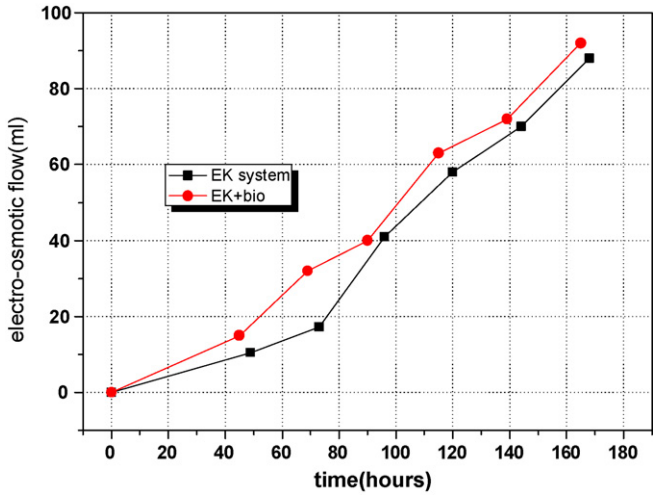

Fig. 5. Electroosmotic flow with time for EK and EK + bio system.

on the cell might have been reduced to the point where the cells were moving very slowly in the electric current. Alternatively, the low pH might have resulted in a net neutral or positive charge on the cells, causing them to stop or move in the opposite direction toward cathode. Net positive charges have been measured in cells held at pH 2. Hence it is possible to change the charges of bacteria with free available cations like calcium and magnesium at low pH anodic area of the soil which may accumulate in more number at high pH of cathodic area.

The estimated nitrate and ammonium values from anode to cathode and the average values of nitrate and ammonium in all the systems viz. dried soil, bio-remediated, EK system and EK + bio are presented in Figs. 6–8. In EK system, nitrate value in the soil near the anode was 120 mg/kg whereas at cathodic soil, the nitrate value was almost nil. The movement of NO_3^- through the soil column was significantly influenced by the development of a pH gradient [13]. The nitrate value in EK + bio system was almost nil at all the points. The nitrate value was in the range between 18 mg/kg and 23 mg/kg in the presence of bacteria alone (without EK). It indicates that bacteria reduced nitrate in the soil uniformly. In biological system, ammonium ion was higher in the range between 58 mg/kg and 70 mg/kg when compared to other systems. In EK system, the ammonium values were in the range between 4 mg /Kg and 15 mg /Kg from anode to cathode. While adding bacteria in EK system, the ammonium ion increased in all the points when compared to EK system. The initial average value of nitrate and ammonium in the dried soil was 268 mg/kg and 20 mg/kg respectively. The value of nitrate (23 mg/kg) was reduced and ammonia increased (70 mg/kg)

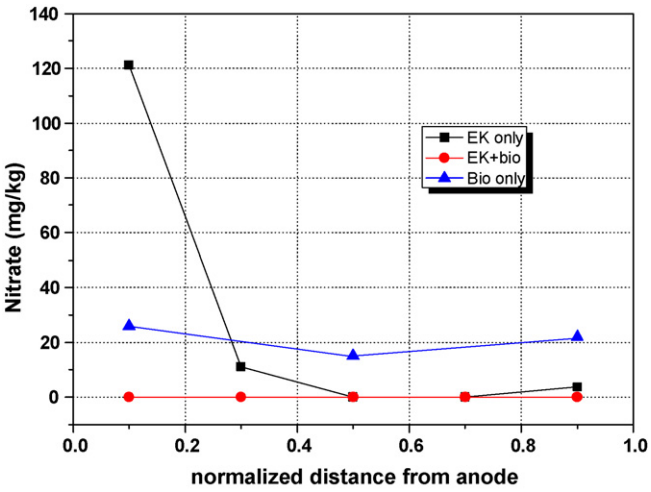

Fig. 6. Nitrate concentration with variable distances from anode.

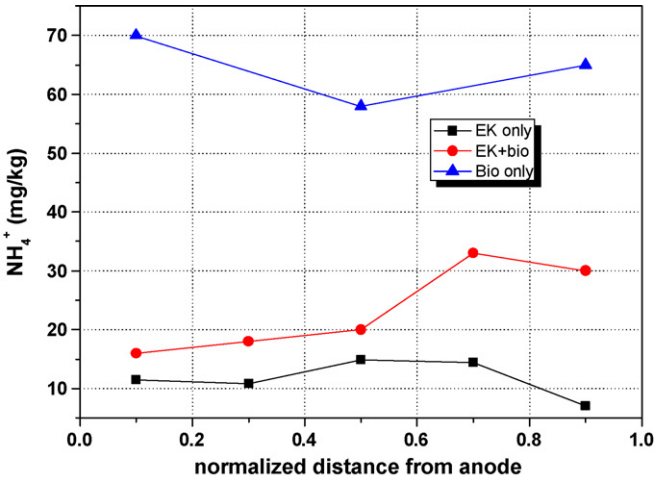

Fig. 7. Ammonium concentration in soil at variable distances from anode.

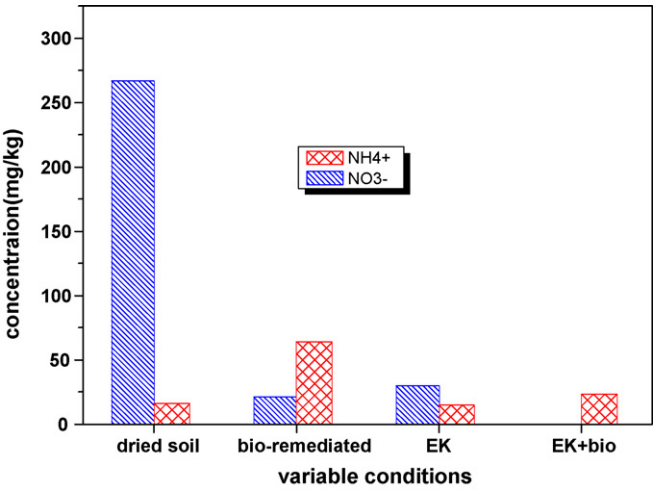

Fig. 8. Average value of ammonium and nitrate in various systems.

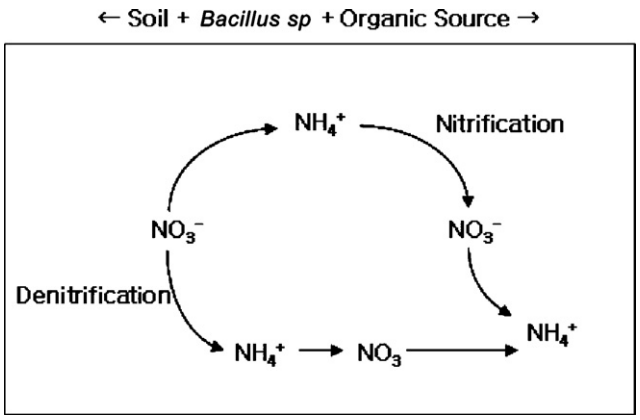

Fig. 9. Nitrate removal process by bacteria.

by bacteria in bio-remediated system. In EK system, the average value of nitrate and ammonium was 40 mg/kg and 20 mg/kg respectively. While adding bacteria, in EK system, nitrate was completely removed and ammonium was about 24 mg/kg. The results indicate that bacteria enhance ammonium ions and EK reduces ammonium ions significantly.

Nitrogen is very common and found in many forms in the environment viz. inorganic forms including (NO_3), nitrite (NO_2), ammonia (NH_3) and nitrogen gas (N_2). NO_3^- is highly soluble and is stable over a wide range of environmental conditions. The conversion of NO_3^- by bacteria called as denitrification process is represented below

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2\text{gas}[52].$$

The redox form of the reaction is given as

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O.$$

In the present study, *Bacillus* species have been selected for denitrification process. *Bacillus* spp. can use NO_3^- as their primary electron acceptor in aerobic condition when low oxygen availability restricts their metabolism [53]. Ghafari et al. [32] also explained the following reaction as denitrification process in soil environment.

$$2NO_3^- + 6H_2O + 10e^- \rightarrow N_2 + 12OH^-$$

Ammonia is found in two forms – the ammonium ions (NH_4^+) and dissolved, unionized (no electrical charge) ammonia gas (NH_3). The reaction between the two forms is shown by this equation

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$$

The form of ammonia changes easily when pH changes. As pH increases, H^+ concentration decreases, and OH^- concentration increases. This makes the equation to shift to left, increasing the amount of aqueous NH_3. When, the pH is below 8.75, NH_4^+ predominates [54].

Nitrification process is also a biological process, oxidation of ammonia with oxygen into nitrite [55]. Nitrate will be formed as the final product in nitrification process.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

$$NO_2 + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

The nature of the carbon source determines the route of nitrate reduction. Indeed, competition between denitrification and dissimilatory nitrate reduction to ammonium in media with low dissolved oxygen concentrations seems to be largely controlled by the nature of the electron donor [49]. Hence, it is very essential to remove both nitrate and ammonium ions in the soil. In the present study, 1% starch was used as organic source which can be utilized by bacteria. The initial addition of dissolved carbon and nitrate ratio was approximately about 23.7: 1. The distribution of C:N ratio in soil depends upon the osmotic flow of EK. Besides, it was believed that the concentration of phosphorous and TOC were required for the growth of denitrifying microorganisms. Since the soil collected from Jinju soil has about 1384 mg/kg of phosphate (Table 1), the nitrate reduction efficiency is higher in the presence of denitrifiers [56] along with EK system. Schipper and Vojvodic-Vukovic [35] suggested that the nitrate removal is dependent on the continued supply of organic carbon to denitrifying bacteria. They demonstrated that the available carbon in this denitrification wall could support denitrification and nitrate removal for at least five years.

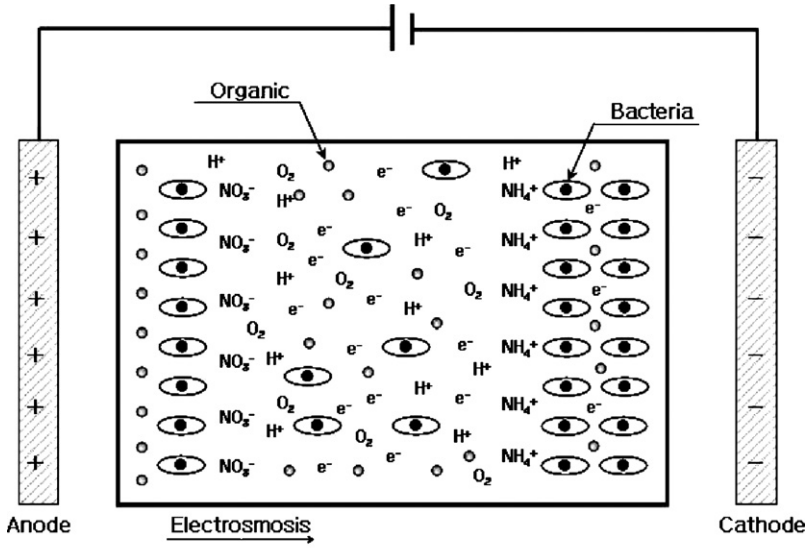

Fig. 10. Nitrate removal by EK + bio process.

Rajakumar et al. [36] used glucose, starch, cellulose, sucrose and acetic acid as organic source in nitrate removal process by aerobic *Pseudomonas* sp. and *Bacillus* sp. in laboratory and they suggested that starch was the best organic source in nitrate reduction.

The present authors also believe that the supply of rich H^+ by electroosmosis from electrokinetics encourages the denitrification process as follows.

$$2NO_3^- + 2H^+ + 5H_2 \rightarrow N_2 + 6H_2O$$

It can also possible that H^+ from anode attracts NO_3^- and subsequently bacteria supplies electrons by degradation of organic source.

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O(\text{humic substance or by bacteria})$$

The dissimilatory reduction of nitrate to ammonium is also possible in the presence of *Bacillus* spp.

$$NO_3^- + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O[49]$$

The nitrate level was higher at anodic area in EK system which indicates the movement of nitrate to anodic side and supports with the observation made by Eid et al. [12] and Cairo et al., [13]. But NO_3^- level was zero due to denitrification by EK + bio when compared to initial level (268 mg/kg). Subsequently, oxygen also can be entered into the soil by electroosmosis process which converts NH_4^+ ions to N_2.

$$NH_4^+ + 0.75O_2 \rightarrow 0.5N_2 + 1.5H_2O + H^+$$

It can be claimed that EK + bio removes nitrate significantly in soil by electro osmosis along with biological denitrification. The formation of NH_4^+ was higher in biological system when compared to EK-bio and EK. Since O_2 is not available in biological system, the conversion of ammonium to nitrogen is not possible. Hence, the ammonium value is higher in biological system. It is also possible that the available ammonium ions can be oxidized into nitrate by *Bacillus* sp. through "nitrification" process. The nitrification process is the disadvantage aspect in biological removal of nitrate. The acidic water (H^+) movement from anode by electroosmosis may react with NH_3 and converted as NH_4^+. It can be explained that since NH_4^+ is attracted by OH^- ions, the soil at cathode contained higher amount of NH_4^+ ions in EK + bio system. Due to the high pH at cathode, the ammonium ions can be converted as ammonia gas which will be released at cathodic area. At the same time, NH_4^+ can be reduced by electron supplied by bacteria (organic source).

$$2NH_4^+ + 2e^- \rightarrow 2NH_3 + H_2$$

It is also possible that *Bacillus* sp. can tolerate in the presence of alkaline pH and also encourages the conversion of NH_4^+ to free NH_3 which supports the observation made by Wiley and Stokes [57]. A mechanism for nitrate reduction in the combined process of EK + bio system has been proposed in the present study (Figs. 9 and 10). Hence, it can be claimed that bacteria and electroosmosis by EK convert nitrates to ammonium ions and ammonium ion is converted into ammonia gas at cathodic area in EK-bio combined system.

4. Conclusions

21 strains were identified as nitrate reducers in Jinju-vinyl house. The dominant genus *Bacillus* spp. present in the soil were used to remove the nitrate in the soil through electrokinetic process. The concentration of nitrate at anodic side of the soil was higher than that in cathodic region of EK system. In EK + bio system, the nitrate concentration was almost zero and gave about 100% efficiency. Bacterial degradation on organic source (humic substances) and transportation of hydrogen and oxygen by electro osmosis accelerate the nitrate reduction process in the soil. Subsequently, EK process suppresses the nitrification process (conversion of ammonium to NO_3^-) by attracting NH_4^+ to the cathodic surface and converting into ammonia gas at high pH. The authors strongly believe that the basic data will be useful to develop EK-biotechnology on removal of nitrates in agricultural soil environment.

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