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Acidic Neutralization by Indigenous Bacteria Isolated from Abandoned Mine Areas

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Abstract: Soil acidification has been a serious problem in abandoned mine areas, and could be exacerbated by acid deposition with the release of mine wastes. In this study, three different indigenous bacterial consortia were isolated from abandoned mines in South Korea, from which the potential for acid neutralization of microorganisms was evaluated. They were all able to neutralize acidity within 24 h in the liquid nutrient medium. Moreover, a strong positive correlation ($R = +0.922$, $p < 0.05$) was established between the ammonium ion (NH_4^+) production yield and the resulting pH, indicating that NH_4^+ served as an important metabolite for biological neutralization. *Serratia liquefaciens*, *Citrobacter youngae*, *Pseudescherichia vulneris*, and *Serratia grimesii* had higher acid neutralization ability to generate NH_4^+ by the metabolism of nitrogen compounds such as carboxylation and urea hydrolysis. Therefore, acidic soils can be expected to be ameliorated by indigenous microorganisms through in situ biostimulation with the adequate introduction of nitrogenous substances into the soil environments.

Keywords: neutralization; soil acidification; biological amelioration; indigenous bacteria

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1. Introduction

Soil acidification, defined as a decrease in the soil pH to below 5.5, is a serious problem that damages ecosystems, making it difficult to maintain nutrient balance and biodiversity [1]. Acidified soils reduce soil productivity and plant growth yield through the loss of basic cations (i.e., Ca^{2+}, Mg^{2+}, K^+, and Na^+) and dissolution of acidic cations (i.e., Al^{3+} and Mn^{2+}). Moreover, soil acidification causes the leaching of toxic heavy metals, which contributes to the severe pollution of the neighboring environments [2–5]. In particular, high concentrations of metals associated with fine precipitates, including metals adsorbed to colloidal hydrous ferric oxides [6] and metals [7] in the receiving waters can pose a hazard to some aquatic organisms, even if the acidic precursors (e.g., acid mine drainage) are mitigated [8].

Acid deposition from atmospheric acid rain and fertilizer overuse contributes intensively to soil acidification [9–11]. Moreover, most of the abandoned mine areas have been left unattended for a long time without any other action, so the acidification has been aggravated by the discharge of mine wastes, such as acid mine drainage and mine ore [12–14]. The problem is worsening in Korea because of the recent acceleration of forest soil acidification. Furthermore, most soils in Korea are derived from granite and granite gneiss as the parent materials, which are more likely to be acidic than calcareous shale or limestone [15]. Therefore, it is essential to develop sustainable acid soil restoration technology.

Conventional technologies using alkaline chemicals, such as calcium carbonate ($CaCO_3$), lime (CaO) [16], and phosphogypsum ($CaSO_4 \cdot 2H_2O$) [17], have been applied to ameliorate acidic soil. However, repeated and excessive use of these chemical ameliorants could cause serious problems, including re-acidification and hardening of the soils, nutrient imbalance caused by the accumulation of salts, and an increase of mineral nutrients [16–19].

Biological soil remediation is a methodology for promoting economically environmental conservation. Some microorganisms in soil have adapted to be tolerant to acidic conditions through various mechanisms that may play an important role in changing the soil pH [20–22]. For example, several bacteria could increase the pH of the surrounding environment by producing ammonia by the deamination and decarboxylation of nitrogenous substrates that are broken down to urea and amino acid to maintain the cellular pH homeostasis [23–25]. For instance, acid-tolerant bacteria of *Bacillus cereus* isolated from the acidic soil of a tea plantation secrete urease to neutralize the pH [26]. *Lactobacillus acidophilus* has been shown to respond to acid stress through well-organized metabolisms associated with decarboxylation reactions in the biomolecular analysis [27]. In addition, *Klebsiella* sp. and *Raoultella* sp. remediated the soil spiked by acid mine drainage derived from pyrite oxidation, increasing the concentration of NH_4^+ while simultaneously raising the pH [28]. It has been reported that sulfate-reducing bacteria that are tolerant to low pH and high concentrations of arsenic and ferrous can neutralize pH to promote sulfate reduction while producing alkalinity [29].

In biological soil restoration approaches, biostimulation promotes the growth of indigenous microorganisms by supplying rate-limiting nutrients (e.g., nitrogen, phosphorous, oxygen, and other electron donors) in contaminated soil [30]. It is widely used to enhance the decomposition efficiency of various organic contaminants [31–33]. Nitrogen fertilizer has been utilized to restore soils from acidification [34] and facilitate the circulation of nitrogen [35]. Furthermore, the addition of organic anions (e.g., oxalate, malate, and citrate) along with urea fertilizer significantly increased soil pH and decreased soil exchangeable acidity through their decarboxylation reactions [36].

Soil acidification generally occurs over a wide area. Therefore, the use of indigenous microorganisms that have survived competition between various physicochemical environmental factors and microorganisms is an efficient method for neutralizing acidic soil [30,37]. To implement biostimulation for the neutralization of acidic soil more appropriately, it is necessary to investigate the distribution and acid neutralization ability of the indigenous microbial consortium in the target-contaminated area. Therefore, this study evaluated the feasibility of acid neutralization using indigenous microorganisms collected from three abandoned mine areas in South Korea. In addition, the main mechanisms of acid neutralization were elucidated through the biochemical analyses of acid-neutralizing bacterial strains.

2. Experimental Procedure

2.1. Soil Sampling and Characterization

Acidic soil (Y1) or heavy metal contaminated soil samples (C1 and C2) were collected near an abandoned mine area located in Yong-in Province, Gyeonggi-do (37°13′39.34″ N, 127°07′48.08″ E, Figure 1a) or Cheonan Province, Chungcheongnam-do, South Korea (36°54′33.5″ N, 127°14′59.7″ E for C1, Figure 1b; 36°54′00.8″ N, 127°15′33.8″ E for C2, Figure 1c), respectively. The soil samples were taken from five locations in a sampling point and mixed into a single plastic bag. Subsequently, they were air-dried at room temperature and filtered through a 2 mm standard sieve.

Soil samples were characterized according to standard analytical protocols. The pH was measured using a pH meter (BP3001, Trans Instrument, Singapore) after mixing 5 g of soil and 25 mL of distilled water followed by leaving it for an hour. The moisture content was determined by weighing the samples before and after drying at 105 °C until a constant weight was reached. Organic content was determined using the Tyurin titrimetric method [38,39]. For that, 0.5 g of soil sample was oxidized with 10 mL of potassium dichromate solution ($K_2Cr_2O_7$, 0.4 N) by heating on a hot plate. After boiling for 5 min, it was cooled down to room temperature, and 100 mL of distilled water, 5 mL of phosphoric acid (85%), and 5 drops of diphenylamine indicator were added to the digested solution. Excess dichromate was measured by titration with a solution of ammonium ferrous sulfate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.2 N). It was assumed that the dichromate reduced during the

oxidation process was equal to the organic carbon (C) present in the sample. The percentage of organic content was calculated by multiplying the organic carbon by the Van Bemmelen factor of 1.724 [40]. Meanwhile, the ammonium nitrogen (NH_4^+-N) content was measured using the Kjeldahl digestion method [41]. To extract the NH_4^+-N from the soil, 10 g of soil sample was mixed with 50 mL of potassium chloride solution (KCl, 2 M) for 30 min and filtered using a qualitative filter paper (No. 2, Whatman™, Maidstone, UK). The filtrate was heated through the Kjeldahl distillation device with the addition of 0.2 g of magnesium oxide (MgO), and the vaporous product was condensed in 10 mL of boric acid solution (H_3BO_3, 2% (*w*/*v*)). After that, NH_4^+-N content was determined by titration by H_2SO_4.

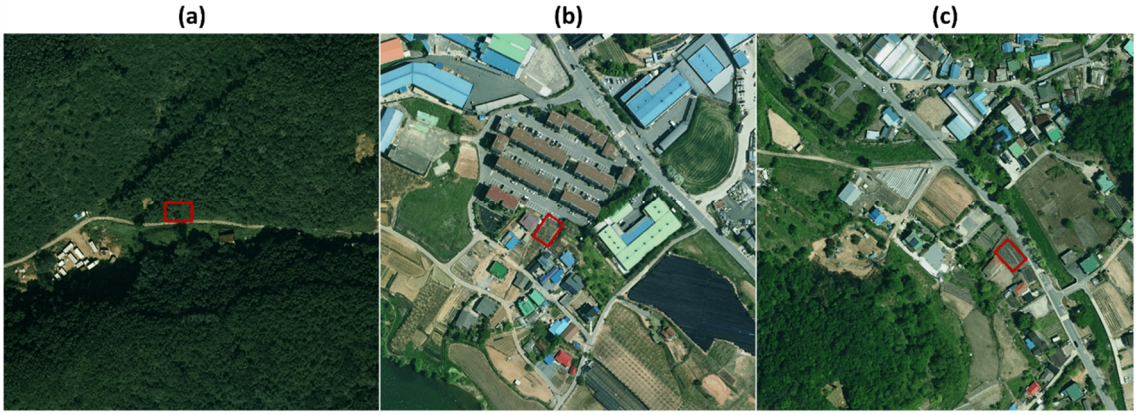

Figure 1. Sampling sites of (**a**) Y1, (**b**) C1, and (**c**) C2, respectively.

Particle size distribution analysis was carried out according to the Hydrometer method [42,43]. For that, an air-dried soil sample was soaked in 100 mL of a dispersing agent, a sodium hexametaphosphate solution ($(Na(PO_3)_6)$, 5% (*w*/*v*)) for 18 h. Then, it was vigorously mixed for 5 min and transferred to the measuring cylinder. The final volume was adjusted to 1 L by compensating with distilled water, after which it was homogenized by repeatedly inverting it in a cylinder with a cap. Thereafter, the clay fraction was determined by measuring the specific gravity immediately and after 10 h using a hydrometer (DK-713, measurement range 0.995–1.050, Daekwang Inc., Seoul, Korea). The soil suspension was sieved using a standard sieve with a mesh size of 270 mm, and the remainder was weighed to estimate the sand fraction after drying for 24 h. The silt fraction was finally calculated by excluding the fractions of sand and clay.

The concentrations of heavy metals (arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn)) in C1 and C2 soil samples were determined using an inductively coupled plasma–optical emission spectroscopy (ICP–OES, Optima 7300DV, Perkin Elmer, Waltham, MA, USA) according to the standard methods [41]. Apart from this, a high-throughput next-generation sequencing (NGS) technique was used to analyze the diversity of the indigenous microbial community in each soil sample.

2.2. Microcosm Acid Neutralization Test

The acid neutralization potential was evaluated using the indigenous bacterial consortiums isolated from each soil sample. They were isolated by adding 5 g of a soil sample to 50 mL of NaCl solution (0.85% (*w*/*v*)) and mixed for 15 min at 250 rpm using a funnel shaker (MMV-1000W, EYELA, Tokyo, Japan). Subsequently, 2 mL of the extract was transferred to 100 mL Difco™ Nutrient Broth (NB, Franklin Lakes, NJ, USA) of pH 7.0, incubated at 30 °C, and shaken at 160 rpm for 2 days. The bacterial consortium was cultivated in NB medium until the optical density at 600 nm (OD_{600}) reached up to 1.10 ± 0.05, and they were then harvested by centrifugation at 14,000× *g* for 5 min followed by washing twice with a sterile 0.85% (*w*/*v*) NaCl solution. The bacterial cells were then transferred to 500 mL Erlenmeyer flasks containing 300 mL of NB medium, which was acidified to pH 4, 4.5, and 5 by adding a 4 M sulfuric acid (H_2SO_4) and 4 M sodium hydroxide (NaOH) solution.

They were incubated for 6 days at 30 °C while being agitated continuously at 160 rpm in a shaking incubator (VS-8480SF, Vision Scientific Co., Ltd., Daejeon, Korea). At the same time, the control was set up in the same manner without adjusting the pH using an acidic or alkaline reagent. The microbial biomass, dehydrogenase activity, pH, and ammonium ion (NH_4^+) concentration were monitored daily during the acid neutralization process.

The OD_{600} of the microbial biomass was measured using a UV-Vis spectrometer (UV-3300, Humas, Korea), while the dehydrogenase activity was determined using an INT assay [44]. For the INT assay, 0.5 mL of a bacterial suspension was centrifuged at 10,000× *g* for 5 min, after which the supernatant was decanted. A 0.5 mL aliquot of 0.2% (*w*/*v*) INT (iodonitrotetrazolium chloride, TCI, Tokyo, Japan) solution (in 200 mM phosphate buffer, pH 7.6) and 0.1 mL of 1% (*w*/*v*) sucrose (Daejung Chemicals, Korea) solution were added to the bacterial cell pellet. After incubation at 30 °C for 30 min, 50 µL of HCl (35%, Samchun Chemicals, Seoul, Korea) and 1 mL of 1,4-dioxane (Sigma, Saint Louis, MI, USA) were added to block the enzymatic activity and simultaneously extract the INT formazan produced. The dehydrogenase activity was determined by the absorbance at 481 nm.

The aliquot was centrifuged at 1000× *g* for 10 min, and the resulting supernatant was used to determine the pH and NH_4^+ concentration. In this study, pH was measured using a pH meter (BP3001, Trans Instrument, Petro Centre, Singapore), while NH_4^+ concentration was determined using the Nessler reagent colorimetric method (Tyagi et al., 2009). For that, the 50-fold diluted supernatant was reacted with the Nessler reagent (HACH, Ames, IA, USA), from which its absorbance was measured comparably at 425 nm against the reagent blank. The observed absorbance was finally converted to the NH_4^+ concentration (mM) over a calibration curve drawn by NH_4Cl.

2.3. Monitoring Microbial Community during the Test

The microbial community has been monitored because it might be altered from the initial acidic conditions of the medium and the degree of acid neutralization during the period test. The bacterial cultures obtained from the acid neutralization test were spread over Difco™ Nutrient Agar (NA, BD, Franklin Lakes, NJ, USA) plates. After incubating for 24 h at 30 °C in a static incubator (VS-1203PIN, Vision Scientific Co., Ltd., Daejeon, Korea), the grown colonies were sorted by color, shape, and size, and the total number of colonies was counted as CFU/mL. The colonies were inoculated individually into 1 mL NB medium and stored in a deep freezer (−80 °C) as a glycerol stock for further use.

For microbial identification, the genomic DNA was extracted from the isolated single cell pellet using the HiGene™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) according to the manufacturer's instructions. The 16S rRNA fragment was then amplified by a polymerase chain reaction (PCR) with a 16S bacterial universal primer set of 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′, synthesized by Bioneer (Daejeon, Korea)) and BioFact™ *Taq* DNA polymerase kit (BIOFACT, Korea). PCR was performed in a thermal cycler (Professional Basic Thermocycler, Biometra Ltd., USA) with an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR product was purified using a HiGene™ Gel and PCR Purification system (BIOFACT, Daejeon, Korea). Partial 16S rRNA sequences were analyzed using an ABI 3730XL DNA Analyzer (Applied Biosystems, Waltham, MA, USA). Each bacterial strain was identified by comparison with the Gene Bank databases using the Basic Local Alignment Search Tools (BLAST) of the National Center for Biotechnology Information (NCBI).

2.4. Acid Neutralization Potential of Single Bacterial Strain

The acid neutralization potential for each bacterial strain during an acid neutralization test was compared. Before the test, the bacteria were aseptically incubated in fresh NB medium of pH 7.0 at 30 °C, 160 rpm for 24 h. The grown bacterial strains were transferred individually to an acidic NB medium, adjusted to pH 4.5, and incubated at 30 °C for

48 h. After incubation, the OD_{600}, pH, and NH_4^+ concentrations were measured, as mentioned above.

2.5. Biochemical Characterization

The biochemical properties of each bacterial strain were characterized using API 20E, API 20NE, and API ZYM kits (BioMe'rieux, Marcy-I'Étoile, France) according to the manufacturer's instructions. In the meantime, their urease activity was determined using Christensen's Urea Agar (Peptone 1 g/L, Dextrose 1 g/L, NaCl 5 g/L, KH_2PO_4 2 g/L, Urea 20 g/L, Phenol red 0.012 g/L, Agar 15 g/L) [45]. Single bacterial isolates were spread over Christensen's Urea Agar plates and incubated for 48 h. The urease expression was examined throughout the color change from yellow (acidic) to purple-pink (alkaline) of phenol red, depending on the condition of the medium.

3. Results and Discussion

3.1. Physicochemical and Biological Characterization of the Soil Samples

Table 1 lists the physicochemical properties including pH, moisture content, organic matter content, NH_4^+-N concentration, and particle size distribution of soil samples along with the concentration of heavy metals. The Y1 soil sample collected from a hillside located in Yongin was acidic at pH 4.51, while the C1 and C2 soil samples collected from farmland near the abandoned mine area in Cheonan were neutral at pH 7.8 and 7.1, respectively. Conversely, the concentration of ammonium nitrogen (NH_4^+-N) was higher in the acidic soil Y1 with 2.69 mg/kg than that in the other soils that were in neutral pH. It could be presumed that the microbial metabolism capable of producing NH_4^+ was further activated by an indigenous bacterial consortium, resulting in the accumulation of NH_4^+-N in an environment with a continuous supply of acidic precursors.

Table 1. Physicochemical properties of the soil collected from the abandoned mine areas.

		Y1	C1	C2
Soil depth		Topsoil	Subsoil	Topsoil
pH		4.5	7.8	7.1
Moisture content (%)		25.15	9.92	10.61
Organic content (%)		2.17	0.18	0.34
NH_4^+-N (mg/kg)		2.69	2.34	1.99
Particle size distribution	Sand (%)	73.40	86.26	85.62
	Silt (%)	19.71	9.62	6.91
	Clay (%)	6.88	4.12	7.47
Heavy metals	As (mg/kg)	-	97.66	56.08
	Cd (mg/kg)	-	0.81	1.19
	Cu (mg/kg)	-	4.5	35.8
	Pb (mg/kg)	-	24.2	32.1
	Zn (mg/kg)	-	32.2	252.6

In the meantime, C1 and C2 soil samples were found to be contaminated with a variety of heavy metals. Among them, arsenic was detected at 97.66 mg/kg and 56.08 mg/kg in C1 and C2 soils, respectively, exceeding the national soil pollution standard (i.e., 25 mg/kg or more). In addition, zinc was found at a rather high concentration of 252.6 mg/kg in C2 soil, but the concentration did not exceed the suggested contamination level. Other heavy metals were measured to be below the contamination levels in C1 and C2 soils, and it was confirmed that heavy metal contamination was not severe in Y1 soil.

The microbial communities differed according to the soil samples (Figure 2). In Y1 soil, the dominant genera were found with *Rhodanobacter* spp. (19.34%), *Paraburkholderia* spp. (16.26%), and *Natranaerobaculum* spp. (12.94%), as shown in Figure 2a. *Pseudomonas* spp. was the predominant genus (C1: 82.91%, C2: 87.37%) in soils contaminated with arsenic

(Figure 2b), suggesting that they had a similar microbial community structure at the genus level.

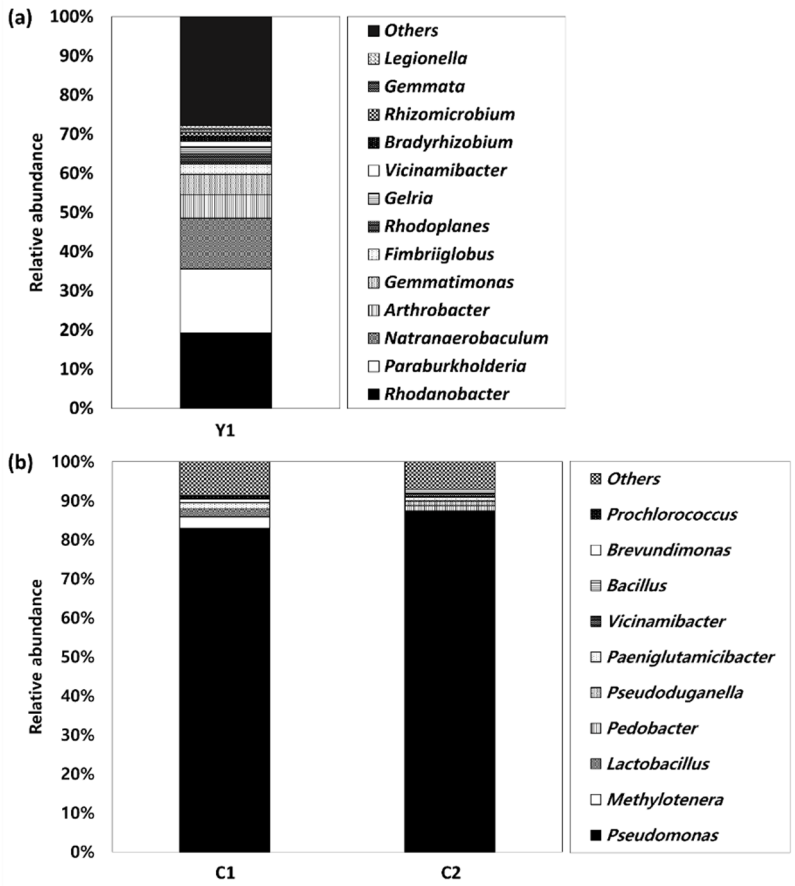

Figure 2. Microbial community structures at the genus level of bacterial consortia isolated from (**a**) Y1 soil sample and (**b**) C1 and C2 soil samples.

3.2. Acid Neutralization by the Isolated Bacterial Consortia

The isolated consortia of indigenous bacteria could survive above pH 4.5 and effectively neutralize the acidity (Figure 3), irrespective of the original soil conditions. At an initial pH of 5, regardless of the type of microbial community, bacterial growth started immediately after inoculation with concomitant acid neutralization. In contrast, the bacterial strains proliferated in the initial pH 4.5 after some acclimation to the environmental stress (i.e., low pH). The growth of microorganisms and the increase in pH were accelerated after 2 h in Y1 and C1, whereas the C2 bacterial consortium reached equivalent levels after 6 h. Hence, the C2 consortium has slower growth because it has a longer acclimation time to acidic conditions than the other consortia. Subsequently, they grew rapidly after adapting to environmental changes, so the acidity was neutralized concomitantly with the proliferation of microorganisms and enzymatic activity (i.e., dehydrogenase activity). Complete neutralization from an initial pH of 4.5 and 5 took 24 h and 8 h for Y1, respectively. In the case of C1, neutralization was completed within 12 h and 6 h at an initial pH of 4.5 and 5, respectively. Similarly, in the case of C2, 12 h and 6 h were also needed to neutralize an initial pH of 4.5 and 5, respectively, although it took a longer acclimation time than C1 soil in the beginning. This suggests that all three indigenous bacterial consortia successfully neutralized the acidic medium to pH 7 or higher within 24 h. In addition, as incubation time was increased, the pH increase rate gradually decreased as the bacterial growth was slowed down, as shown in Figure 3. It did not exceed pH 8.5 while maintaining a neutral pH range. It was also found that pH no longer increased after 14 days of stabilization. This neutralization pattern has been consistently confirmed in our previous studies [28,46].

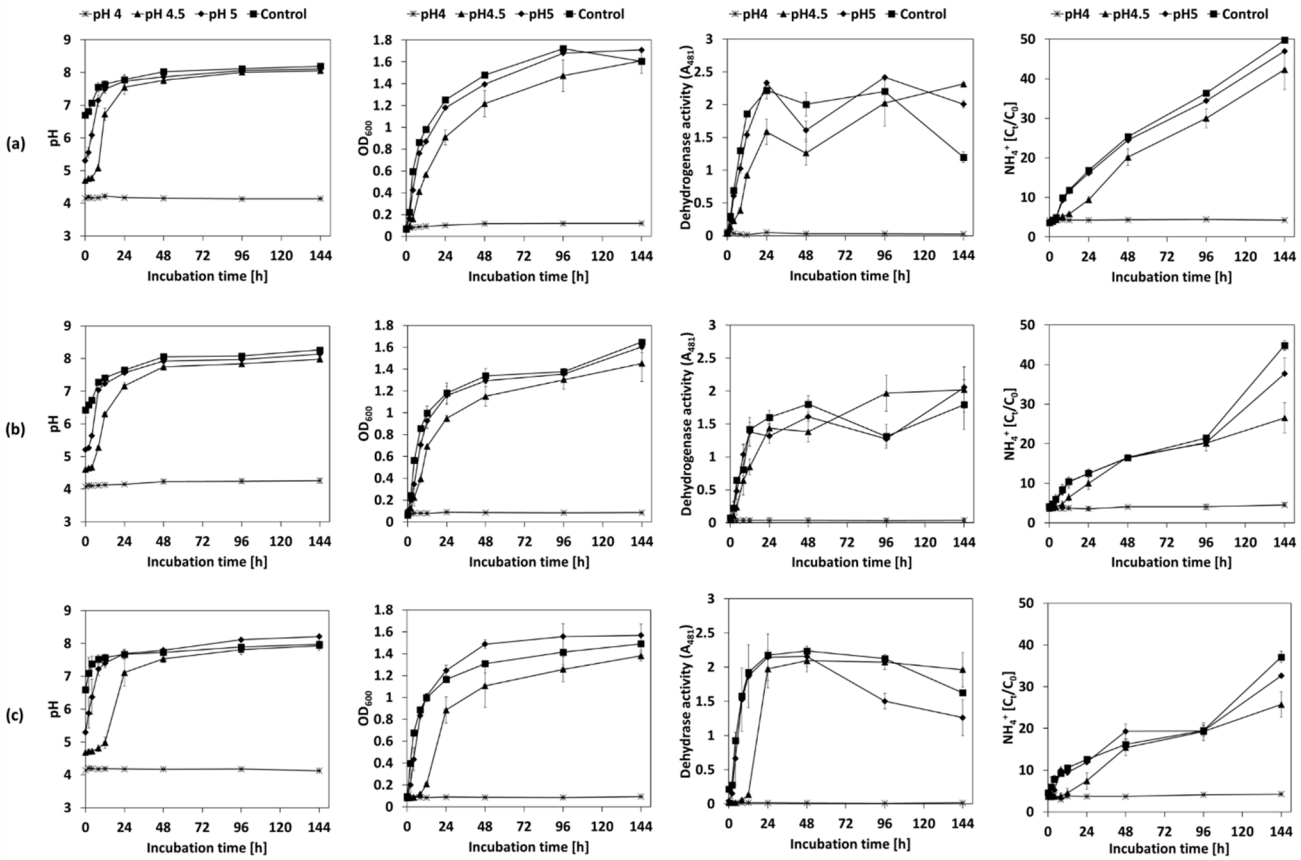

Figure 3. Acid-neutralizing ability of bacterial consortium of (**a**) Y1, (**b**) C1, and (**c**) C2 in the acidic NB medium for six days under the different pH conditions (i.e., pH 4, 4.5, 5, and control). Each column exhibits the temporal variation in pH, optical density at 600 nm (OD_{600}), dehydrogenase activity as of absorbance at 481 nm (A_{481}), and NH_4^+ production yield, respectively. The yield of NH_4^+ production also increased continuously except for the initial pH 4, suggesting that the microbial metabolism may be due to NH_4^+ production during acid neutralization. Indeed, a strong positive correlation was established between the hydroxyl ion (OH^-) concentration and the amount of NH_4^+ produced with a correlation coefficient (R) of 0.922 ($p < 0.05$, Figure 4), regardless of the types of bacterial consortium. That is, the NH_4^+ concentration increased with increasing pH, indicating that NH_4^+ generation is closely related to acid neutralization. It could be suggested that NH_4^+ served as an important metabolite, contributing to acid neutralization. Meanwhile, the NH_4^+ production yield was the highest by the isolated from Y1, which is consistent with the fact that a large amount of NH_4^+-N was accumulated in Y1 soil, as mentioned above.

In this regard, in acidic environments, some microorganisms consume excess protons to maintain pH homeostasis for their survival in acidic environments [47]. Specifically, in these acid-resistant systems, microorganisms consume protons from nitrogenous compounds such as urea and amino acids to produce alkaline substances (e.g., NH_4^+) [48]. In this study, the NB medium is composed mainly of peptones and beef extracts that can supply abundant nitrogenous compounds that contribute to ammonia production [49]. Consequently, the isolated bacterial consortia can neutralize acidity by ammonium-associated metabolism. Therefore, acidified soil could be ameliorated using indigenous microorganisms through the introduction of appropriate nitrogenous substances such as nitrogen fertilizer and biochar [34,35,50] as an alternative nutrient medium.

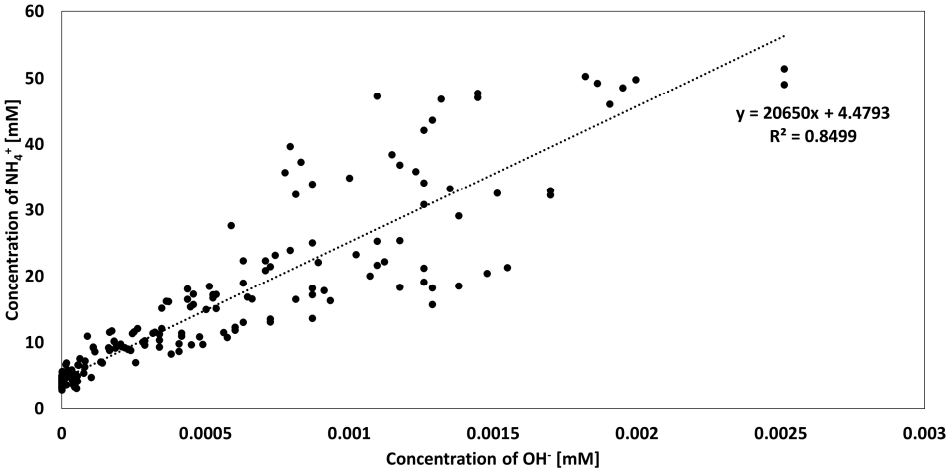

Figure 4. Relationship between the concentrations of hydroxyl ion (OH^-) and ammonium ion (NH_4^+) during acid neutralization using the isolated bacterial consortia.

3.3. Monitoring Microbial Community during Incubation

In this study, the changes in microbial community distribution were evaluated to determine if the consortia were maintained and to identify the major bacterial strains capable of acid neutralization. Each bacterial consortium consisted mainly of four or five bacterial strains (Table 2). The bacterial species were identified as *Serratia* spp., *Pseudomonas* spp., *Pseudescherichia* sp., *Atlantibacter* sp., *Bacillus* sp., *Salmonella* sp., *Citrobacter* sp., *Aeromonas* sp., *Leclercia* sp., and *Stenotrophomonas* sp. In the Y1 consortium, *Bacillus toyonensis* (YNA-1), *Serratia grimesii* (YNA-2), *Pseudomonas protegens* (YNA-3), and *Atlantibacter hermannii* (YNA-4) formed as colonies on the NA plates depending on the degree of neutralization (Figure 5a). *Serratia grimesii* (YNA-2) was the dominant species in the Y1 consortium with a higher growth rate under acidic conditions. In addition, *Atlantibacter* sp. (YNA-3) was observed in the early stages of neutralization under an initial pH of 4.5, while *Bacillus* sp. (YNA-1) was found at the initial pH of 5. For the C1 consortium (Figure 5b), *Pseudescherichia* sp. (CNA-1) and *Citrobacter* sp. (CNA-3) were the main species that contributed to the acid-neutralization process. *Salmonella* sp. (CNA-2) survived at pH > 5. *Pseudomonas* sp. (CNA-4) and *Serratia liquefaciens* (CNA-5) grew from when neutralization was complete. On the other hand, for the C2 consortium (Figure 5c), *Serratia grimesii* (CNB-1) and *Pseudomonas protegens* (CNB-2) predominantly survived under acidic conditions and contributed to the neutralization of acidity. *Leclercia* sp. (CNB-4) grew under acidic conditions (initial pH 4.5), but they were immediately colonized by the other strains after 24 h of incubation because of their slower growth or lower competitiveness under neutral conditions.

3.4. Acid Neutralization Properties of the Single Bacterial Strains

The potential of acid-neutralization of single strains was evaluated by inoculating them individually in an acid medium adjusted to pH 4.5. Of these, 11 bacterial strains increased the pH by an average of 41% within 48 h compared to the initial pH (Figure 6), suggesting that they have acid resistance and neutralization properties. It showed that the YNA-2, CNA-1, CNA-3, and CNB-1 strains had a high neutralizing ability, consistent with the results of previous experiments. On the other hand, CNA-5 and CNB-4, which showed low growth rates in the heterogeneous consortium, had higher growth rates in the single strain culture to accomplish acid neutralization. Conversely, despite both strains being able to neutralize the acidity, they remained vulnerable to nutritional competition for growth over other isolates in the consortium. YNA-3, CNA-2, CNA-4, CNB-2, and CNB-5 strains also have high acid-neutralizing properties under acidic conditions in pH 4.5. In contrast, the growth rates of the three bacterial strains, YNA-1, YNA-4, and CNB-3, were very slow at pH 4.5, which was consistent with the previous results, showing that they are barely found at pH > 5.

Table 2. Identification of acid-neutralizing bacterial strains in the Y1, C1, and C2 consortia by 16S rRNA gene sequencing analysis.

Isolates		Species	Identities	Gaps
Y1	YNA-1	*Bacillus toyonensis*	1471/1473 (99%)	1/1473 (0%)
	YNA-2	*Serratia grimesii*	1459/1461 (99%)	1/1461 (0%)
	YNA-3	*Pseudomonas protegens*	1465/1465 (100%)	0/1465 (0%)
	YNA-4	*Atlantibacter hermannii*	1441/1466 (98%)	1/1466 (0%)
C1	CNA-1	*Pseudescherichiavulneris*	1447/1457 (99%)	0/1457 (0%)
	CNA-2	*Salmonella enterica* subsp.	1418/1441 (98%)	0/1441 (0%)
	CNA-3	*Citrobacter youngae*	1459/1490 (98%)	0/1490 (0%)
	CNA-4	*Pseudomonas plecoglossicida*	1470/1474 (99%)	4/1474 (0%)
	CNA-5	*Serratia liquefaciens*	1479/1484 (99%)	4/1484 (0%)
C2	CNB-1	*Serratia grimesii*	1453/1455 (99%)	0/1455 (0%)
	CNB-2	*Pseudomonas protegens*	1443/1443 (100%)	0/1443 (0%)
	CNB-3	*Aeromonas taiwanensis*	1451/1461 (99%)	2/1461 (0%)
	CNB-4	*Leclercia adecarboxylata*	1461/1484 (98%)	3/1484 (0%)
	CNB-5	*Stenotrophomonas maltophilia*	1475/1493 (99%)	2/1493 (0%)

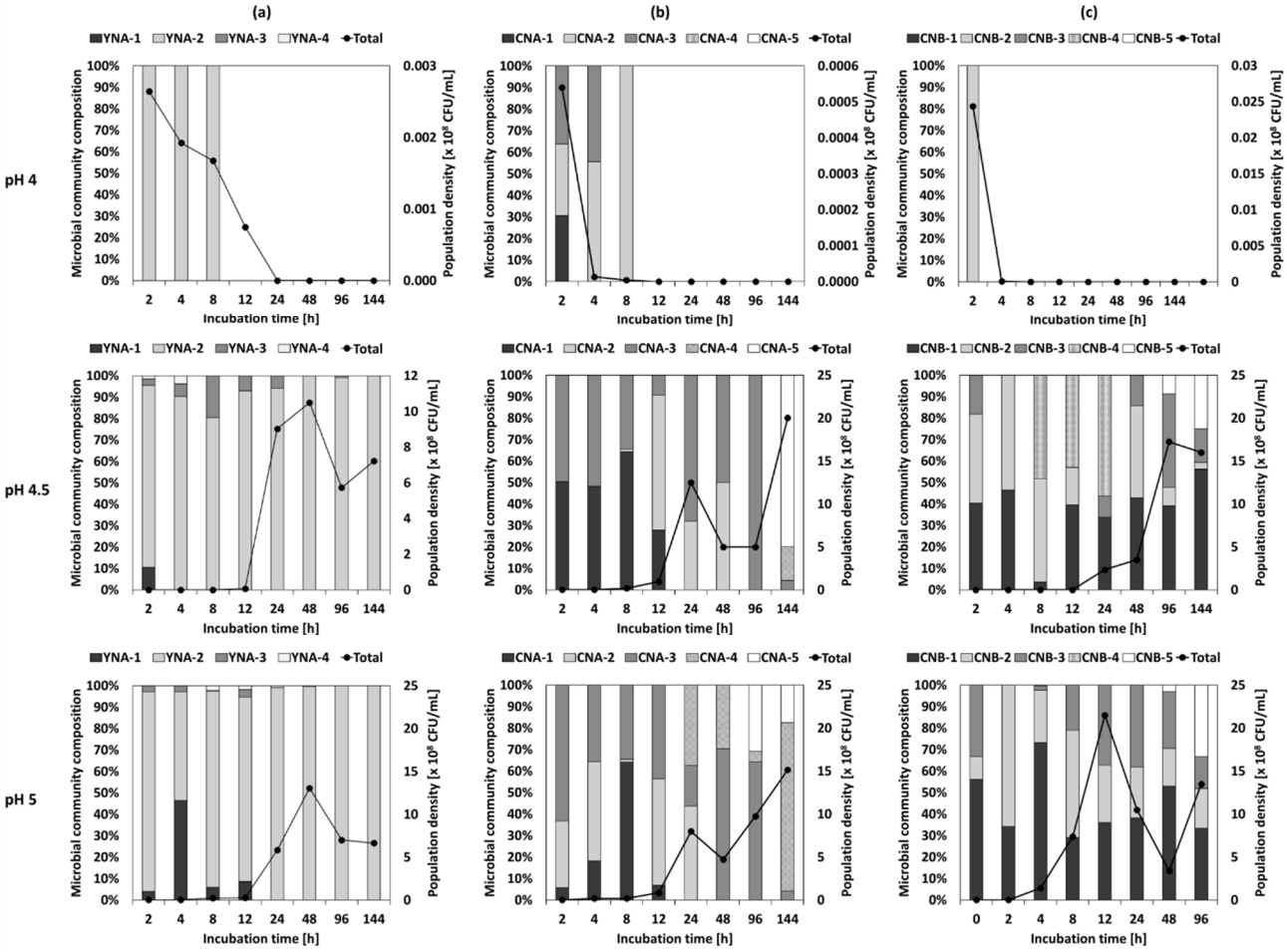

Figure 5. Distribution of the bacterial strains (bar type) and variation of population density (marked line graph) of the (**a**) Y1, (**b**) C1, and (**c**) C2 bacterial consortium with an incubation time of acidic neutralization process at an initial pH of 4, 4.5, and 5, respectively.

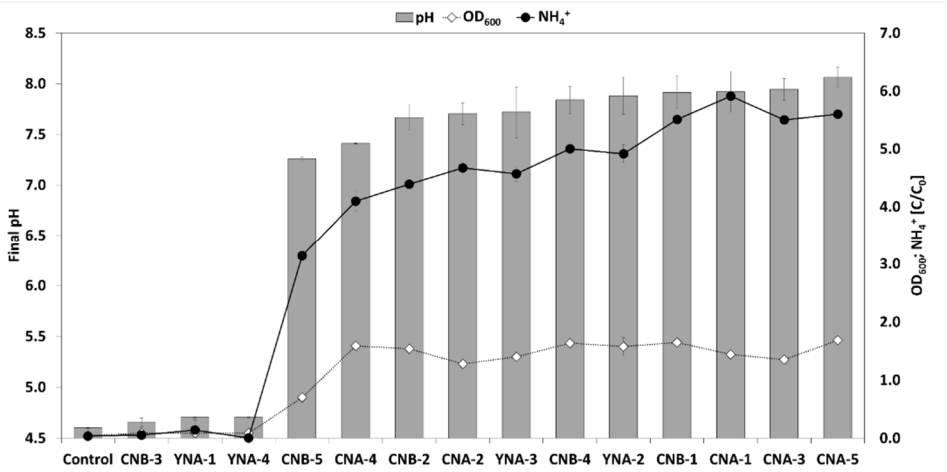

Figure 6. Comparison of the acid neutralization properties of the final pH (bar graph), OD_{600} (dashed line), and normalized concentration of ammonium ion (NH_4^+) (solid line) for single bacterial strains after 48 h of the acid-neutralization test. Control was an acidified medium without bacterial inoculation.

Individual bacterial strains of each consortium grew competitively according to the initial conditions and degree of acid neutralization as they had different acclimation times to acidic conditions. In addition, these changes in the microbial community made it possible to identify which types of microorganisms with large contributions to acid neutralization could be taken. Nevertheless, detailed mechanisms of bacterial acid neutralization could not be predicted precisely because of the complex interactions between microorganisms in the consortium. In this study, additional experiments were carried out to determine the neutralization properties of each bacterial strain, which has been discussed in the following sections.

3.5. Acid-Neutralizing Mechanism of Bacterial Strains

The microbial metabolism for acid neutralization could be predicted based on the biochemical properties of each bacterial strain. The primary metabolism is considered as an NH_4^+ production in utilizing nitrogenous compounds under acidic conditions. This microbial NH_4^+ production occurs mainly by deamination and decarboxylation through the formation of alkaline products and the consumption of protons [24,48]. It also serves as a major mechanism of bacterial resistance to acidification.

Several metabolic pathways are involved in NH_4^+ production and H^+ consumption, including urease, arginine deaminase, and decarboxylase. First, urea hydrolysis is a mechanism that converts urea to ammonia (NH_3) and carbon dioxide (CO_2), which can be found in a wide range of microorganisms as one of the most representative ammonia production mechanisms [51,52]. The ammonia produced consumes protons to become ammonium ions (NH_4^+) that neutralize protons. In this study, five bacterial strains (YNA-3, CNA-3, CNA-4, CNB-2, and CNB-5) showed a change in substrate color when expressing urease in Christensen's Urea Agar assay, as shown in Table 3.

Second, the arginine deaminase (or arginine dihydrolase) system can produce citrulline and ammonia through the breakdown of arginine. The resulting citrulline is converted to ornithine and carbamoyl phosphate by ornithine carbamoyltransferase (OTC), and finally mineralized to NH_3 and CO_2 by carbamate kinase (CK) [53,54]. The results of the API 20E/20NE test showed that YNA-3, CNA-1, CNB-2, and CNB-4 strains positively activated arginine dihydrolase (Table 3), which could neutralize acids via the arginine deiminase (ADI) pathway.

Third, the decarboxylase of some amino acids, such as lysine, ornithine, and histidine, functions at low pH under extracellular conditions and contributes to pH maintenance by importing and consuming protons through decarboxylation [27,55,56]. Decarboxylation has

been reported to produce ammonia and the formation of biogenic amines [49]. As a result of biochemical analysis, the three bacterial strains of YNA-2, CNA-5, and CNB-1 belonging to *Serratia* species can generally express lysine decarboxylase and ornithine decarboxylase, whereas CNA-2 (*Salmonella* sp.) can express only lysine decarboxylase (Table 3). In addition, the glutamate decarboxylase (GAD) system is also important for acid resistance to survive in acidic environments [57]. GAD catalyzes the α-decarboxylation reaction of glutamate to produce γ-aminobutyric acid and carbon dioxide, which ultimately consumes protons [58]. Among the GAD systems, the glutaminase YbaS/GlsA converts glutamine to glutamate and ammonia [59]. It can be expressed in various bacteria [59,60], including *Escherichia coli*, *Pseudomonas* spp., and *Serratia* spp. Therefore, it implied that the selected bacterial strains belonging to the *Pseudomonas* spp. and *Serratia* spp. can express glutaminase.

Table 3. Biochemical properties of acid-neutralizing bacterial strains using API 20E, API 20NE, and API ZYM and the urease activity.

	YNA-2	CNA-1	CNA-2	CNA-5	CNB-1		YNA-3	CNA-4	CNB-2	CNA-3	CNB-4	CNB-5
API 20E						**API 20NE**						
β-galactosidase	+	+	+	+	+	Reduction of NO_3^- to NO_2^-	-	+	+	+	-	+
Arginine dihydrolase	-	+	-	-	-	Indole production	-	-	-	-	-	-
Lysine decarboxylase	+	-	+	+	+	Fermentation	-	+	+	+	-	+
Ornithine decarboxylase	+	-	-	+	+	Arginine dihydrolase	+	-	+	-	+	-
Citrate utilization	+	-	-	+	+	Hydrolysis (β-glucosidase)	+	+	+	+	-	+
H_2S production	-	-	-	-	-	Hydrolysis (Protease)	+	-	+	+	+	-
Tryptophan deaminase	-	-	-	-	-	β-galactosidase	-	+	+	+	-	+
Indole production	-	-	+	-	-	Assimilation						
Acetoin production	+	-	-	+	+	D-glucose	+	+	+	+	+	+
Fermentation/oxidation						L-arabinose	-	+	+	+	-	+
D-glucose	+	+	+	+	+	D-mannose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	D-Mannitol	+	+	+	+	+	+
Inositol	+	-	-	+	+	N-acetyl-glucosamine	+	+	+	+	+	+
D-sorbitol	-	-	-	+	-	D-maltose	-	+	+	+	-	+
L-rhamnose	-	+	+	-	-	Potassium gluconate	+	+	+	+	+	+
D-sucrose	+	-	-	+	+	Capric acid	+	+	+	-	+	-
D-melibiose	-	+	-	-	-	Adipic acid	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	Malic acid	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	Trisodium citrate	+	+	+	+	+	+
Oxidase	-	-	-	-	-	Phenylacetic acid	-	+	-	-	+	-
Nitrate reducibility	+	+	+	+	+	Oxidase	+	-	-	+	+	-
API ZYM						**API ZYM**						
Alkaline phosphatase	+	+	+	+	+	Alkaline phosphatase	+	+	+	+	+	+
Esterase (C4)	+	+	+	+	+	Esterase (C4)	+	+	+	+	+	+
Esterase Lipase (C8)	+	-	+	+	+	Esterase Lipase (C8)	+	+	+	+	+	+
Lipase (C14)	+	-	-	-	-	Lipase (C14)	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	Leucine arylamidase	+	+	+	+	+	+
Acid phosphatase	+	+	+	+	+	Acid phosphatase	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+
β-glucuronidase	+	+	+	+	+	β-glucuronidase	-	+	+	+	+	+
α-glucosidase	+	-	-	+	+	α-glucosidase	+	-	-	-	-	+
β-glucosidase	-	-	-	-	-	β-glucosidase	-	-	-	-	+	-
Urease *	-	-	-	-	-	**Urease ***	+	+	+	+	-	+

* Urease activity determined by the Christensen's Urea Agar Assay.

Overall, the bacterial strains isolated from abandoned mine soils can neutralize the acidity of the medium by generating ammonia from nitrogen compounds through several specific metabolisms, such as urea hydrolysis, arginine deaminase system, and amino acid decarboxylation.

4. Conclusions

This study examined the feasibility of the eco-friendly acid neutralization process using various types of indigenous bacterial consortia isolated from acidic soil or heavy metal-contaminated soils in abandoned mine areas. All bacterial consortia could successfully neutralize the acidified medium within 24 h, whose reaction was strongly correlated with NH_4^+ production. Each bacterial consortium consisted mainly of four to five bacterial strains, which were identified as *Pseudomonas* spp., *Serratia* spp., *Citrobacter* sp., and *Leclercia* sp., with strong acid resistance and neutralizing properties. These microorganisms can neutralize acidity by producing ammonia through urea hydrolysis, arginine deaminase system, and amino acid decarboxylation. This suggests that the biological neutralization of acidic soil using these microbial metabolisms can effectively remediate soils with ap-

propriated bio-stimulation (e.g., supplementation of nitrogen compounds) under various environmental conditions.

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