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SCREENING OF SILVER-TOLERANT BACTERIA FROM A MAJOR PHILIPPINE LANDFILL AS POTENTIAL BIOREMEDIATION AGENTS

BADANIA PRZESIEWOWE BAKTERII ODPORNYCH NA DZIAŁANIE SREBRA Z DUŻEGO SKŁADOWISKA ODPADÓW NA FILIPINACH, MOŻLIWYCH DO WYKORZYSTANIA W BIOREMEDIACJI

Abstract: The field of microbial biotechnology has revolutionized the utilization of microorganisms to overcome the problems of environmental pollutions. The present study aimed to identify silver-tolerant isolates and screen their ability to synthesize silver nanoparticles for possible use as bioremediation agents. Seventeen bacterial isolates from soil collected from the Smokey Mountain landfill in Manila, Philippines, were found to tolerate 0.01 M $AgNO_3$ in the culture medium. Molecular and phylogenetic analyses using the 16S rRNA gene sequence identified the isolates as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus flexus*, *Bacillus thuringiensis*, *Alcaligenes faecalis*, *Achromobacter* sp. and *Ochrobactrum* sp. The formation of silver nanoparticles was evident in the change in color of the reaction mixtures, and was detected through UV-VIS spectroscopy with absorbance peaks at 250-300 nm and 400-450 nm. Scanning electron microscopy revealed the aggregation of diverse shapes of silver nanoparticles with sizes ranging from 70 to 200 nm. The best silver nanoparticle-synthesizing isolates were *Alcaligenes faecalis* and *Bacillus cereus*. The results denote the promising microbial technology application of the 17 silver-tolerant isolates in combating the adverse effects of metals and other pollutants in the environment.

Keywords: microbial biotechnology, bioremediation, silver nanoparticles, silver-tolerant bacteria, biosynthesis, landfill

Introduction

Silver has long been valued as a precious metal. Due to its unique properties of high thermal and electrical conductivity, silver is used in numerous applications in electronics, energy, chemical production, currency and jewellery, and in manufacturing industries.

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Silver is also incorporated in central venous catheters, endotracheal tubes, urinary catheters, wound dressings and numerous medical products to prevent nosocomial infections [1, 2]. In addition, because of its antimicrobial properties, silver is widely used in consumer products such as disinfecting sprays, cosmetics, fabrics, and household appliances [3]. However, the production, use, and disposal of products containing silver may result to increased amounts of silver in the environment [4]. Industrialization and anthropogenic activities have led to the inadvertent discharge of silver and other contaminants in landfills, effluents in groundwater and wastewater treatments [5]. Consequently, possible adverse effects of increased environmental exposure to environmental contaminants include damage of aquatic [6] and soil [7] organisms, the emergence of metal-resistant and antibiotic-resistant bacteria [8], and eventually, the impairment of human health [9, 10].

Growing social concern about environmental quality has been observed in the recent years. With a list of potential adverse effects of pollutants on public health and environment, the use of bacteria to remediate, degrade and/or utilize these hazardous materials has received increasing attention. Bacterial organisms are ubiquitous. They can be isolated from different substrates or from organisms having a symbiotic relationship with them. Therefore, there is a high possibility of isolating bacteria that are capable of degrading and/or utilizing pollutants in its habitat. Microbial biotechnology can be applied to utilize microorganisms to assess the well-being of ecosystems, transform pollutants into benign substances, and obtain an economically valuable product or activity [11, 12]. Hence, the field of microbial biotechnology extends its vast application in bioremediation in which bacteria are being utilized to tolerate pollutants in the environment and degrade or transform them into less toxic substances, and ultimately allow them to synthesize materials that can be used to resolve the crisis in environmental pollution.

A large number of researches highlight the benefit of bioremediation because it is economical, environmentally compatible and of high disposal ability for combined contaminants of organics and inorganics [13-15]. Bioremediation by biosorption of washing water from cotton fabric processing via silver nanoparticles with the bacterium *Chromobacterium violaceum* resulted to morphologically altered bacteria following the process, but a new culture was completely restored subsequently [16]. The process also allowed recovery of silver leached into the effluent for reutilization, avoiding any effect to the environment and reducing cost. The study made by Yang et al. [17] determined the ability of a native soil bacterium, *Stenotrophomonas* sp. strain YC1, to produce methyl parathion hydrolase (MPH), and eventually degrade organophosphates in a rapid degradation rate and broader substrate specificity. As such, the engineered strain showed potentials for in situ remediation of contaminated sites. Additionally, Jiang et al. [18] found that with the addition of *Bacillus thuringiensis* FQ1, the accumulation of cadmium increased with 14.29-97.67 and 95.07 % of phenanthrene was removed in the bacteria-fungi treatment with the spike of 500 mg/kg phenanthrene. The results of their study demonstrated the integrated remediation strategy of bacteria and fungi as an effective and promising method for soil bioremediation. Furthermore, the study of Zinicovscaia et al. [19] revealed the ability of a cyanobateria, *Nostoc linckia*, to synthesize amorphous selenium nanoparticles (SeNPs) both extracellularly and intracellularly. Scanning electron micrographs showed localization of SeNPs in the cyanobacterial surface after 24 h of incubation suggesting that that exopolysaccharide layer of the cyanobacteria played an important role in the accumulation of selenite ions. In addition, intracellular synthesis of SeNPs were attributed to the sulphate transport channels, thus, reducing the selenium into

its organic form through different mechanisms inside the cell. Moreover, *Escherichia*, *Citrobacteria*, *Rhodococcus*, *Klebsiella*, *Staphylococcus*, *Alcaligenes*, *Bacillus*, and *Pseudomonas* are the organisms that are commonly used in bioremediation [20-24]. Microbial biotechnology is now considered as the eco-friendly and cost-effective treatment technology for the elimination of pollutants mainly in water and soil [25]. Although bioremediation is preferred, sometimes the contaminants themselves become toxic to the bacteria involved in the process. These problems demand obtaining bacteria with high tolerance or resistance under extreme conditions and stable properties to attain an efficient bioremediation process. Nanomaterials may also be utilized in microbial biotechnology as they show remarkable abilities in recognizing pollutants in the environment, remediating toxic metals from aqueous environments as well as industrial effluents and generating less waste production for an environmentally sustainable society [26]. Thus, sanitary landfills are promising areas in which bacteria that are suitable for bioremediation purposes may be found. The Smokey Mountain is one of the largest landfills located in Manila, Philippines for over 40 years. The origin of the name comes from the smoke that originates from a mountain of all kinds of garbage producing chemical reactions. In line with the prevailing context, the Smokey Mountain is an abundant site for microorganisms that could have adapted sophisticated mechanisms to overcome the effects of contaminants in their environment. The present study aims to identify silver-tolerant bacteria from this landfill as potential bioremediation agents.

Materials and methods

Collection of soil samples

Soil samples were collected from Smokey Mountain in Manila, Philippines (14.6326° N, 120.9597° E) (Fig. 1). Smokey Mountain used to be a large landfill located in Tondo, Manila, Philippines by the seaside. It operated for more than 40 years, consisting of over two million metric tons of waste [27]. The site was turned into public housing for the residents living in the area surrounding the landfill when it was foreclosed in 1995 [28, 29]. The remaining landfill, which was not converted into housing facilities, is now covered with wild plants which emerged primarily due to the abundance of decomposing matters beneath the soil. Junk shops, coal making operations and scavenging of garbage are still evident in the area. It is important to note that although diversity of organisms thrives in the site, environmental contaminants may still be present.

Three sampling sites were established by quadrat method at 0, 50 and 100 m, respectively. The 0 m mark started at the point where diversity of plants in the mountain was evident. Surface soil samples (about 10-15 cm deep) of about 1 kg, were collected from the base of each uprooted plant sample. This was properly labelled and packed in polyethylene bags. Samples were sifted through 2 mm sieves and air dried for 3 to 5 days for further analyses.

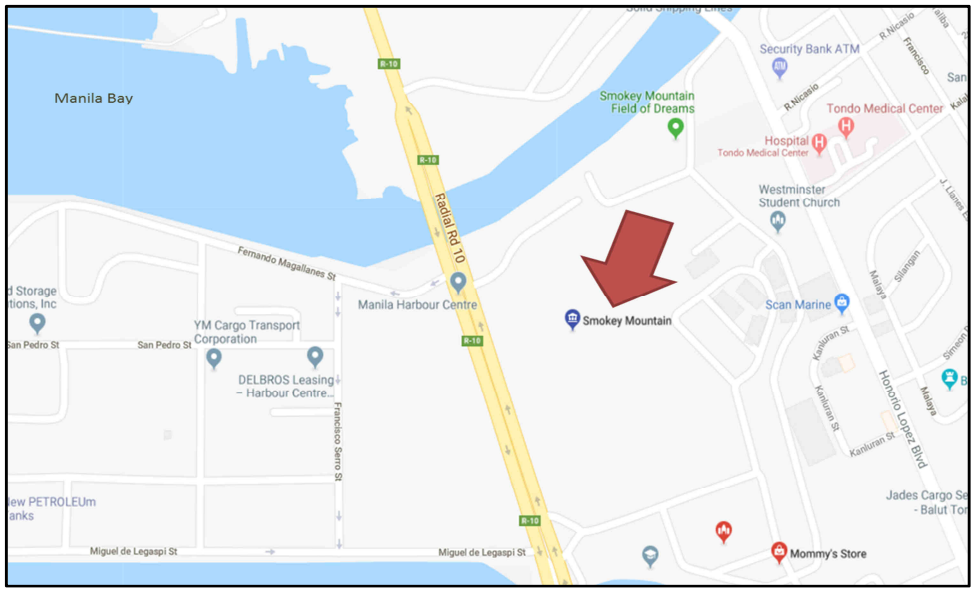

Fig. 1. Map showing the location of the sampling site
Source: https://www.google.com.ph/maps/search/smokey+mountain+housing/

Isolation of bacteria from soil

One gram of soil was added to 99 cm^3 distilled water. Serial dilution from 10^{-1} to 10^{-6} was prepared and 100 mm^3 of each dilution were plated onto brain heart infusion agar (BHIA) medium. The culture medium was amended with 0.1M $AgNO_3$ solution (1 % v/v) to assess the tolerance of the isolates to silver. All plates were incubated at 37 °C for 48 hours. Colonies were picked and were re-streaked on fresh plates with the same medium. Sub-culturing was done until pure cultures were obtained.

Morphological and biochemical characterization of the isolates

Bacterial cells were characterized based on their morphological features. Cell were stained using differential staining methods after 3 days of incubation. The different staining methods used were Gram staining, endospore staining (Schaeffer-Fulton method), capsule staining (India ink method) and acid fast staining (Ziehl-Neelsen staining technique). The biochemical tests included determination of the following characteristics: carbohydrate (Glucose, Sucrose and Lactose) fermentation, H_2S production, reactions in indole, methyl red, Vogues Proskauer, citrate utilization, urease, catalase, and oxidase tests. The isolates were identified following Bergey's Manual of Determinative Bacteriology [30].

Molecular identification of silver-tolerant isolates

Molecular identification of the isolates was carried out via amplification of the 16S ribosomal DNA (16S rDNA) fragment followed by direct sequencing.

DNA extraction

Bacterial DNA of the isolates was extracted by boiling method using Bio-Rad Instagene Matrix Kit (Bio-Rad, USA) following manufacturer's instructions. A single colony of each isolate was picked and suspended in 1 cm^3 sterile deionized distilled water in a microcentrifuge tube. The cloudy suspension was centrifuged for 1 min at 12000 rpm. The supernatant was removed and 100 mi^3 of InstaGene matrix were added to the bacterial pellet. This was vortexed and incubated at 56 °C for 15-30 min on a thermomixer (Eppendorf, USA), after which the tubes were placed in boiling water for 8 minutes. The crude DNA extracts were stored at –20 °C until further use.

DNA amplification

The 16S rDNA fragment was amplified by polymerase chain reaction (PCR) using 63F (5'CAGGCCTAACACATGCAAGTC3') and 1387R (5'GGGCGGWGTGTACAAGGC3') primers [31]. PCR amplification was performed using 1.0 mm^3 extracted DNA, 1X PCR buffer (KAPA Biosystems, USA), 0.3 μM of each primer, 0.3 mM of each dNTP, 0.025 U/mm^3 of Taq DNA polymerase (KAPA Biosystems, USA), 1.5 mM $MgCl_2$ and sterile distilled water added to a final volume of 50 mm^3 reactions. Amplification was carried out in a Corbett Palm-Cycler (Corbette, Australia) using the following program: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR products were electrophoresed in 1.5 % agarose gel containing GelRed™ (Biotium, USA), and the amplicons were visualized via a gel documentation system (Syngene DigiGenius, USA).

Gene sequence and phylogenetic analyses

Amplification products were sent to First BASE Laboratories (Kuala Lumpur, Malaysia) for sequencing. Forward and reverse sequences were processed and manually edited using Bioedit Sequence Alignment Editor Software 7.0 [32, 33]. Contigs were constructed using Bioedit, and were subjected to Basic Local Alignment Search Tool (BLAST) search via NCBI [34] to determine the most likely identity and designation of taxonomic units based on similarities. DNA alignments were done using MAFFT online software [35] and Gblocks program [36] to cure the poorly aligned regions, and to further select the conserved areas of the multiple sequence alignment.

Phylogenetic analyses of the 16S rDNA sequence data were conducted using Minimum Evolution method [37] to reveal the relationships of identified strains that were taxonomically similar with each other. The computer software MEGA 7 [38] was used to reconstruct the phylogenetic tree using the Tamura and Nei algorithm [37]. Confidence and reliability in the assignments of identity of the isolates were estimated using bootstrap analysis with 1000 replications [39]. MrBayes Software was used to compute for the marginal posterior probability of the 16S rDNA sequences of the 17 isolates among related taxa and assess the correctness of the generated cladogram.

Biosynthesis of silver nanoparticles

The bacterial isolates were grown in brain heart infusion broth at 37 °C for 24 hours, after which 100 mm^3 of 0.01 M silver nitrate ($AgNO_3$) solution (1 % v/v) were added to the cultures. The tubes were covered with aluminum foil to avoid photo-reduction of the

aqueous $AgNO_3$ solution. Furthermore, these reaction tubes were incubated in a rotating shaker set at 37 °C for 150 rotations per minute (rpm) for 24 hours. The synthesis of silver nanoparticles was monitored by visual inspection for a change in the color of the culture medium [40]. Negative controls included un-inoculated BHIB medium, BHIB medium inoculated with the isolates and BHIB medium with $AgNO_3$ solution. All experiments were done in triplicate.

Characterization of silver nanoparticles

The reduction of silver ions in the reaction mixtures was monitored using UV-visible spectral analysis (DU730 Life Science UV/Vis) in the range of 200-600 nm absorbance spectrum in steps of 1 nm, using a quartz cuvette with water as the reference. The best isolate was determined as the one exhibiting the darkest color after the silver incubation. This isolate was further analyzed through scanning electron microscopy (SEM) (Jeol JSM-5310, Jeol, Japan) operated at 10 to 15 kV. The sample was prepared by placing a drop of the solution on a 1 x 1 cm aluminum foil and was allowed to air-dry. Prior to SEM analysis, the sample was coated with gold to enhance the examination of the silver nanoparticles.

Results and discussion

Isolation of silver-tolerant bacteria

A total of 17 isolates obtained from the soil of Smokey Mountain were found to be tolerant to 0.1M $AgNO_3$. Six isolates were obtained from Station 1, five from Station 2 and six from Station 3. Silver ion is highly toxic to bacterial cells. It is usually found in consumer commodities due to its antimicrobial function. Recent studies have revealed that the antimicrobial properties of silver are due to its ionized form, Ag^+. This can attach to the surface of the cell membrane, interrupting its selective permeability and the metabolic pathway of the cell [41-43]. In addition, silver can cause damage to cells by interacting with thiol-containing proteins and DNA [44, 45]. Consequently, it binds to the DNA, intercalates between base pairs, and denatures the DNA, preventing its replication [44, 46].

However, bacteria have evolved tight resistance mechanisms that allow them to resist and tolerate the toxic effects of silver and other possible pollutants in their habitat. Bacterial silver resistance was first reported in 1975 in a strain of *Salmonella typhimurium* that caused an outbreak in a burns ward and resulted in the death of three people [47]. Gupta et al. [48] described the gene responsible for silver resistance, the *sil* operon, which was subsequently mapped to a 14.2 kilobase pairs region of the plasmid (pMG101) of this strain. This pMG101 gene also confers resistance to mercury, tellurite and to several antibiotics [49]. *Sil* operon codes for proteins that mediate silver resistance by restricting the accumulation of silver in the cell through a combination of silver sequestration in the periplasm (via SilE and SilF binding) and the regulator proteins SilSR active efflux [1]. This operon was found on plasmids that also contained antibiotic resistance genes [50, 51]. Furthermore, Sutterlin [52] pointed out that alteration of porins and activation of efflux pumps are the mechanisms that bacteria use when reacting to silver. Porins are channels in the bacterial cell membrane that act as highly selective barriers for protection from harmful compounds in the extracellular environment while providing sufficient nutrients to the cell [53]. Transcriptional down-regulation, change in the porin gene expression, abundance of these outer membrane proteins (OMP) and substitution of large to narrower porins may lead

to resistance of most bacteria. Reports have been made regarding antibiotic resistance acquired through loss or functional change of porins in a large number of organisms, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* [54-59]. In addition, the *sil* operon also codes for two efflux pumps SilCBA and SilP [51]. Efflux pumps are transport proteins involved in the extrusion of a variety of toxic compounds such as antibiotics, dyes, detergents, heavy metals, and organic substances to name a few [59]. Studies demonstrated that there is greatest expression of the Mex systems of *P. aeruginosa* and AcrB in *E. coli* when the bacteria are stressed due to unfavorable conditions in the environment [60, 61]. Strains of *Bacillus cereus group* have 390 to 455 transporters per strain, wherein 100 transporters are involved in the efflux pump system [62]. The silver-tolerant isolates obtained from the sampling site could have used a single or combined mechanisms that allowed them resist the toxic effects of silver.

Colonial, morphological and biochemical characteristics of the isolates

The colonial, morphological and biochemical characteristics of the microorganisms isolated from Smokey Mountain soil are shown in Tables 1 and 2, respectively.

Table 1

Colonial and morphological characteristics of silver-resistant isolates from Smokey Mountain soil

Isolate Code	Colonial traits	Cell shape	GR	EF	AFR	CF
1	Cream, circular, large, opaque, flat, smooth, waxy	bacillus	+	+	-	+
2	White, filamentous, large, granular, smooth, moist	bacillus, branching	+	+	-	+
3	Cream, large, opaque, flat, irregular, rough	bacillus	+	+	-	+
4	Yellow, circular, flat, semitranslucent, mucoid	bacillus, branching	-	-	-	-
5	Pale yellow, circular, opaque, flat, rough	bacillus	-	-	-	-
6	White, circular, large, opaque, flat, smooth, waxy	bacillus	+	+	-	+
7	Yellow, round, flat, semitranslucent, mucoid	bacillus	-	-	-	-
8	Cream, circular, large, opaque, flat, rough	bacillus	+	+	-	+
9	Yellow, circular, flat, semitranslucent, mucoid	bacillus	-	-	-	-
10	Cream, large, opaque, flat, irregular, rough	bacillus, branching	+	+	-	+
11	Cream, large, opaque, flat, irregular, smooth, waxy	bacillus, branching	+	+	-	+
12	Cream, circular, opaque, flat, rough	bacillus	+	+	-	+
13	Off white, translucent, mucoid, butyrous	bacillus	-	-	-	-
14	Cream, large, opaque, flat, irregular, smooth, waxy	bacillus	+	+	-	+
15	Yellow, circular, raised, smooth	bacillus, branching	+	+	-	+
16	Cream, circular, large, opaque, flat, smooth, waxy	bacillus, branching	+	+	-	+
17	Cream, circular, large, opaque, flat, smooth	bacillus, branching	+	+	-	+

GR - Gram staining, EF - Endospore formation, AFR - Acid fast reaction, CF - Capsule formation

Table 1 shows that all isolates were bacilli. Twelve of the isolates were Gram positive, spore-forming and encapsulated organisms, whereas five were Gram negative organisms. All isolates were non-acid fast organisms. Biochemical characterization results suggest that the isolates can be tentatively identified as *Bacillus* sp. (Isolate Nos. 1, 2, 3, 6, 8, 10, 11, 12,

14, 15, 16, 17), *Alcaligenes* sp. (Isolate Nos. 4, 7, and 9) *Ochrobactrum* sp. and *Achromobacter* sp. (Isolates 5 and 13, respectively).

Table 2

Biochemical characterization of the isolates

Isolate Code	Ind	MR	VP	Cit	Cat	Oxi	H_2S	CHO fermentation			Gas production	Suspected Organism
								Glu	Suc	Lac		
1	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
2	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
3	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
4	-	-	-	+	+	+	+	-	-	-	-	*Alcaligenes* sp.
5	-	-	+	+	+	+	-	+	-	+	-	*Ochrobactrum* sp.
6	-	-	+	+	+	-	-	+	+	+	-	*Bacillus* sp.
7	-	-	-	+	+	-	+	+	-	-	-	*Alcaligenes* sp.
8	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
9	-	-	-	+	+	+	+	-	-	-	-	*Alcaligenes* sp.
10	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
11	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
12	-	-	+	-	+	-	-	+	-	-	-	*Bacillus* sp.
13	-	-	+	+	+	+	-	+	-	-	-	*Achromobacter* sp.
14	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
15	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.
16	-	-	+	+	+	-	-	+	+	+	-	*Bacillus* sp.
17	-	-	+	+	+	-	-	+	+	-	-	*Bacillus* sp.

Ind - indole test, MR - methyl red, VP - voges proskauer, Cit - citrate, Cat - catalase, Oxi - oxidase, H_2S - hydrogen sulfide, CHO - carbohydrate, Glu - glucose, Suc - sucrose, Lac - lactose

Molecular identification of silver-tolerant isolates by 16S rRNA gene sequencing

Figure 2 shows the amplicons of the 16SrDNA of approximately 1.5 kilobase pairs of the silver-tolerant isolates, while Figure 5 presents the phylogenetic tree constructed using Minimum Evolution algorithm.

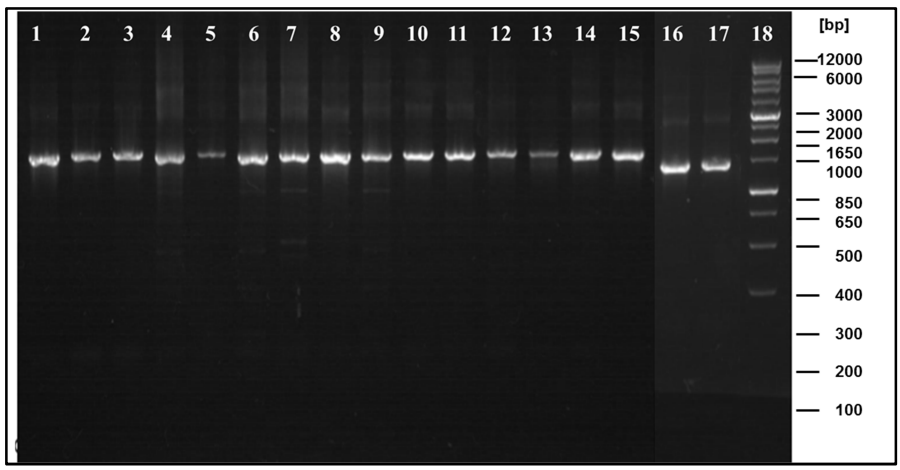

Fig. 2. 16S rDNA amplicons of the 17 silver-tolerant isolates (lanes 1-17) and the 1 kb DNA ladder (lane 18)

Pseudomonas aeruginosa was used as an outgroup. It is important to note that based on the phenotypic characterization, 12 out of the 17 isolates were found to be the Gram positive bacillus species, and 5 isolates were Gram negative. Thus, DNA sequences of possible species belonging to the groups, respectively, were included in the alignment database to determine the relatedness of the isolates with known species and be able to form a strong cladogram.

Table 3 presents the putative identity of the isolates and the bootstrap values with previously identified species based on BLAST search. All isolates possessed a 16S rDNA sequence with ≥ 90 % similarity to that of a previously characterized bacterial species. Accordingly, Bayesian inference was used to compute the marginal posterior probability of the 16s rRNA sequences of the 17 isolates among related taxa and assess the correctness of the formed cladogram (Fig. 4). Huelsenback and Ronquist [63] reiterated that the marginal posterior probability of the parameter (such as a phylogenetic tree) is based on the integration of all possible values of a parameter weighting each (sequences of the isolates and related taxa) by its probability. Hence, the posterior probability value that is closer to 1.0 indicates a positive correlation of the isolates with known species and the correctness of the clades in the phylogenetic tree. Although Isolates 4 and 7 seemed to outgroup in the phylogenetic tree using Minimum Evolution algorithm (Fig. 3), posterior probability value of 1.0 with *Alcaligenes faecalis* using Bayesian analysis (Fig. 4) suggests that the two isolates are related to it. The ≥ 0.7 posterior probability value among other isolates denotes that the isolates have a positive correlation with previously identified species. Thus, it can be inferred that the constructed phylogenetic tree is highly supported by strong matrix of aligned DNA sequences, branch lengths, and substitution parameters of the 17 silver-tolerant isolates associated with previously identified species [64].

Table 3

Percentage homology of the sequences of the 17-tolerant isolates and previously identified species

Isolate Code	**Taxonomic Identification based on 16s rDNA**	**Species with significant alignments**	**Bootstrap values [%]**
1	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
2	*Bacillus subtilis*	*Bacillus subtilis* JQ410787	98
3	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
4	*Alcaligenes faecalis*	*Alcaligenes faecalis* GQ426313	91
5	*Ochrobactrum* sp.	*Ochrobactrum* sp. KJ733941	99
6	*Bacillus* sp.	*Bacillus* sp. JN641291	96
7	*Alcaligenes faecalis*	*Alcaligenes faecalis* GQ426313	91
8	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
9	*Alcaligenes faecalis*	*Alcaligenes faecalis* GQ426313	99
10	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
11	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
12	*Bacillus thuringiensis*	*Bacillus thuringiensis* HE586326	92
13	*Achromobacter* sp.	*Achromobacter* sp. MF144503	94
14	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
15	*Bacillus cereus*	*Bacillus cereus* GQ409539	94
16	*Bacillus flexus*	*Bacillus flexus* LC189364	99
17	*Bacillus cereus*	*Bacillus cereus* GQ409539	94

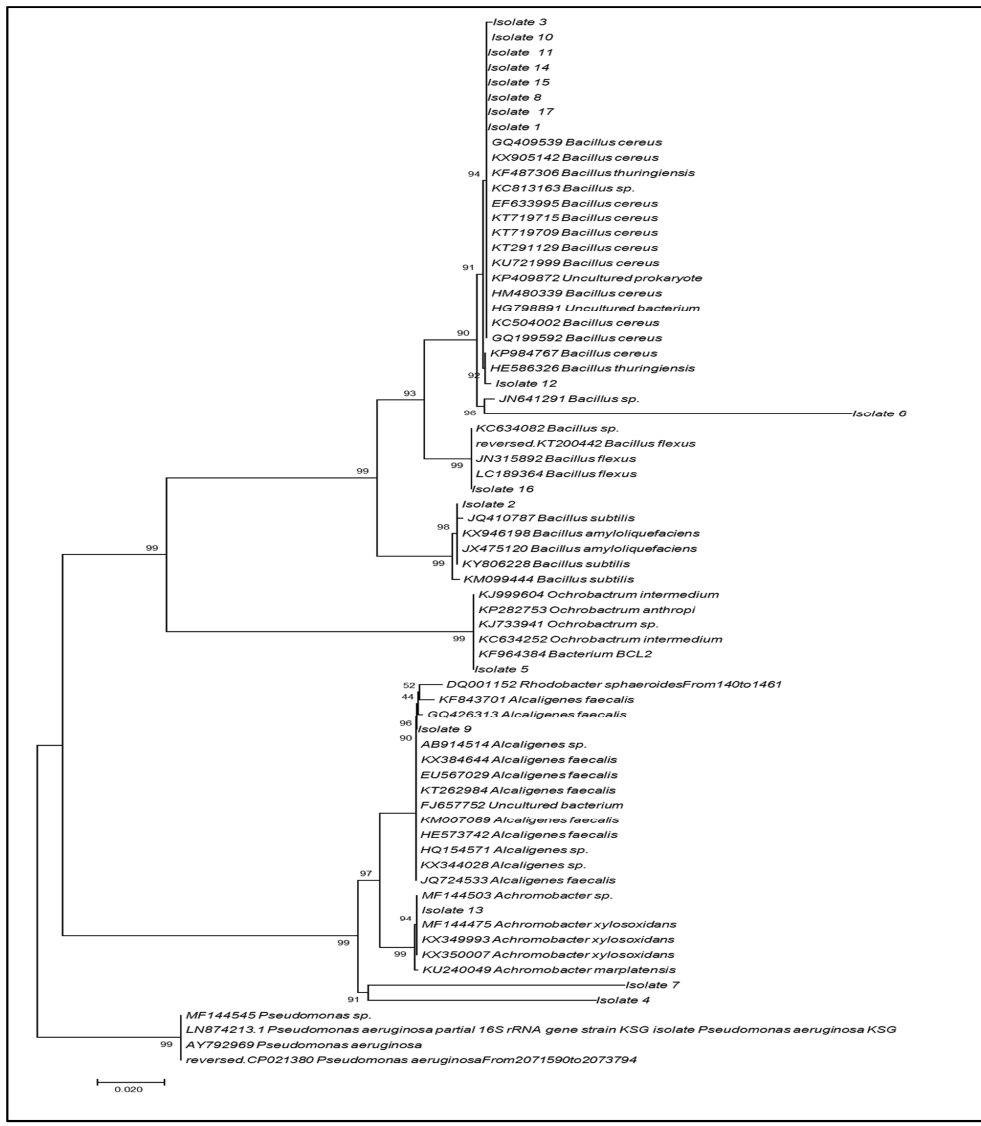

Fig. 3. Minimum-evolution phylogenetic tree constructed from alignment of 16S rDNA sequences of the 17 silver-tolerant isolates with related taxa

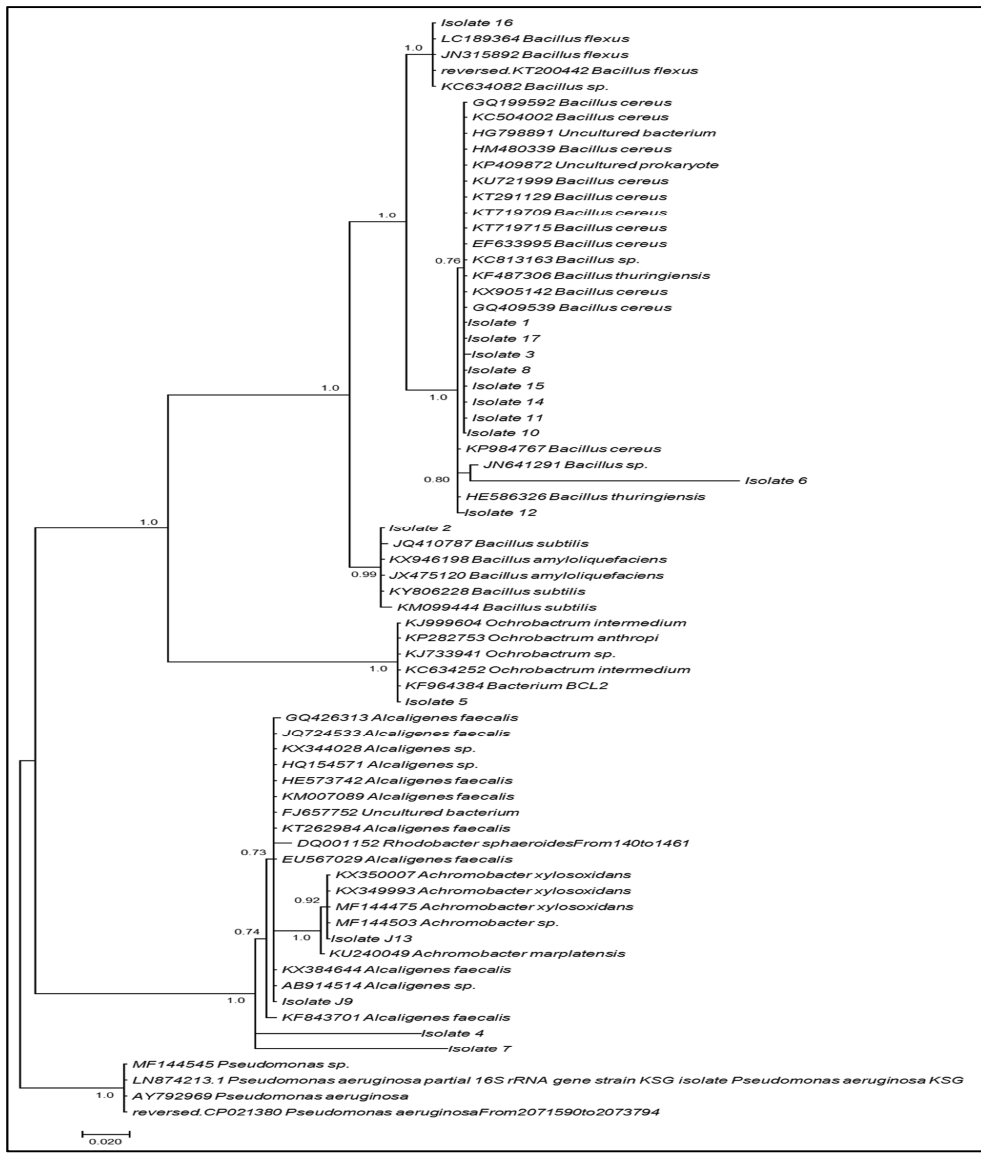

Fig. 4. Bayesian phylogenetic tree of the 17 silver-isolates. The numbers at the internal nodes indicate the marginal posterior probability that the clade is correct using the Bayesian inference

Biosynthesis of silver nanoparticles

The formation of silver nanoparticles by the reduction of $AgNO_3$ can be distinguished by the change in color of the reaction mixture [65]. In the present study, it was evident that silver nanoparticles were successfully synthesized by the isolates (Fig. 5). Remarkably, all isolates exhibited a change in color in the medium with Isolates 9 and 14 having the darkest

color (Fig. 5a). As the biosynthesis proceeded over 24 hours, the color changed from light yellow to reddish brown to dark brown. Thus, the intensity of color is related to nanoparticle production. The color changes are attributed to SPR (surface plasmon resonance) arising from the collective oscillation of free conduction electrons induced by electromagnetic field [66]. The inoculated BHIB tubes without $AgNO_3$ (Fig. 5b) became turbid after 24 hours of incubation indicative of bacterial growth. On the other hand, no color was observed in the control BHIB medium and control with $AgNO_3$ solution after the incubation period (Fig. 5c and 5d).

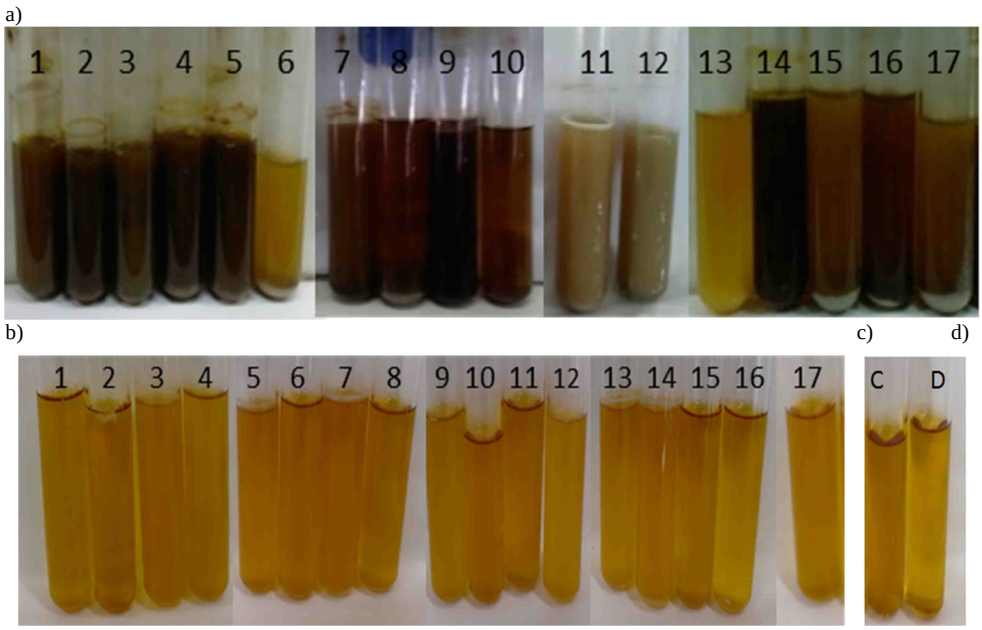

Fig. 5. Chromatic properties of cultures: a) growth of test organisms in brain heart infusion broth (BHIB) with 0.01M $AgNO_3$ solution; b) growth of test organisms in BHIB; c) uninoculated BHIB tube and; d) uninoculated BHIB with $AgNO_3$ solution after incubation

Characterization of silver nanoparticles

Preliminary characterization of the silver nanoparticles (AgNPs) synthesized by the isolates was done using UV-VIS spectrophotometer. The reaction mixture was scanned in the range of 200-800 nm. Figure 6 shows the visible spectra of the produced silver nanoparticles obtained from the reaction mixture of the 17 isolates. The visible spectral analysis showed that all isolates had maximum absorption peaks at around 250-300 and 400-450 nm. Reports suggest that the band located at 420-450 nm corresponds to the surface plasmon resonance of silver nanoparticles [67]. Furthermore, the observable absorption band at 250-300 nm can be attributed to the electron excitations of aromatic amino acids, such as tyrosine and tryptophan residues [68]. This observation suggests that extracellular proteins secreted by the isolates in the solution could be responsible for the reduction of silver ions into silver nanoparticles.

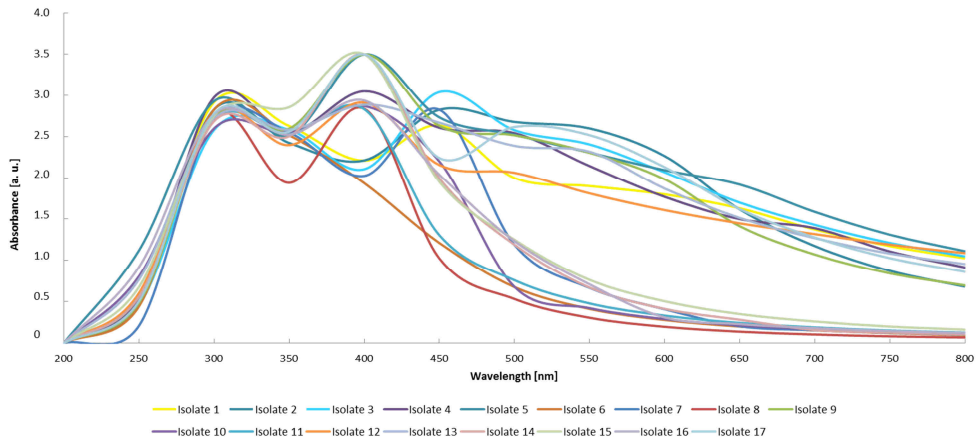

Fig. 6. UV-VIS spectra of the culture medium of the 17 isolates treated with 0.01M $AgNO_3$

The morphologies of AgNPs synthesized by the best isolates 9 and 14 were examined using SEM. Figure 7 presents the SEM images of AgNPs obtained by the reduction of $AgNO_3$ in the reaction mixtures. AgNPs were observed as irregularly shaped particles with sizes between 70 to 200 nm. The synthesized silver nanoparticles were kept at room temperature over many days. There was no observable variation in the color and UV-VIS spectra of the reaction mixtures even after 1 month. Thus, this indicates that the synthesized silver nanoparticles in the reaction mixture are stable over a long period of time.

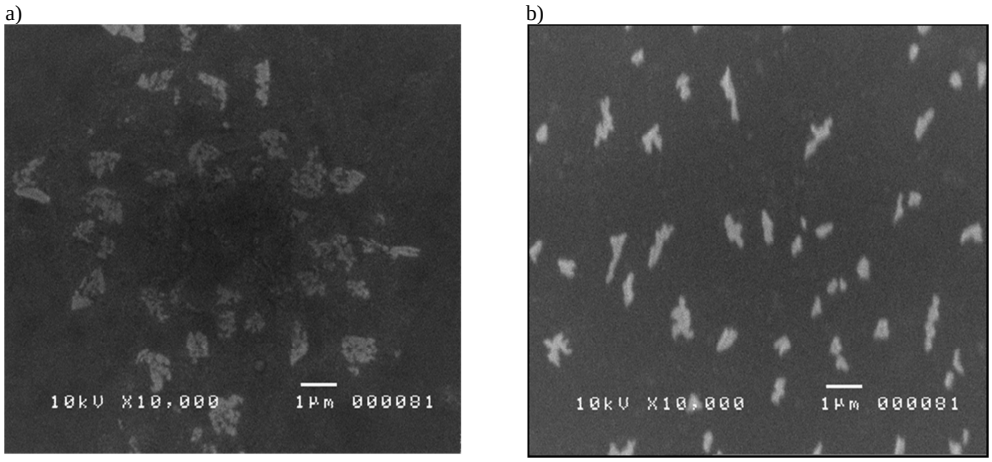

Fig. 7. SEM micrographs of the synthesized silver nanoparticles by Isolate nos. 9 (a) and 14 (b)

In the recent years, applications of microbial biotechnology for feasible solutions in environmental pollution have been adopted [69-73]. Nanotechnology presents a number of potential environmental benefits that could be divided into three categories: treatment and remediation, sensing and detection, and pollution prevention [74]. The scope of

nanotechnology to minimize pollution is in progress, and could potentially catalyze the most revolutionary changes in the environmental field [75]. Biosynthesis of nanoparticles using microorganisms has become a developing research area in microbial biotechnology, with various biological entities being employed in synthesis of nanoparticles constantly forming an alternative for conventional chemical and physical methods. Optimization of the processes can result in synthesis of nanoparticles with desired morphologies and controlled sizes, simple, cost-effective and environment-friendly. Bioremediation with the potential use of nanoparticles offers a promising approach for the degradation or elimination of toxic substances in the environment.

Conclusions

Seventeen bacteria identified as *Bacillus cereus*, *B. subtilis*, *B. flexus*, *B. thuringiensis*, *Alcaligenes faecalis*, *Achromobacter sp.* and *Ochrobactrum sp.* from soil of Smokey Mountain were found to be silver-tolerant. Hence, the ability to grow in culture medium amended with silver makes these isolates potential biological agents in remediating heavy metals and other pollutants in the environment. The synthesized silver nanoparticles can be further explored and utilized in numerous applications in bionanoremediation, diagnostics, antibacterial products, nanowires, and nanotoxicology researches.

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