

Amending coral soil using exopolysaccharide from salt-tolerant *Bacillus velezensis* TSD5 bacteria from an atoll in Vietnam

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ARTICLE INFO

Article history:

Received on: 25/02/2025

Accepted on: 03/06/2025

Available Online: 25/07/2025

Key words:

Exopolysaccharide, *Bacillus velezensis*, coral sand, water retention capacity, reduce soil salinity

ABSTRACT

Salinity and drought are significant abiotic stresses affecting plant growth and productivity. To convert arid and saline coral sandy soil in the Vietnamese coral archipelago into arable land, this study isolated and evaluated the salinity tolerance and exopolysaccharide (EPS) production of *Bacillus velezensis* TSD5 strain from the coral archipelago in Vietnam. EPSTSD5 was estimated to have a molecular weight of 1.09×10^5 Da based on its intrinsic viscosity, with monosaccharide components consisting of glucose, rhamnose, and mannose (of which mannose is the majority). The results of FT-IR, EDX, and zeta potential analysis showed that EPSTSD5 contains many hydrophilic and negatively charged functional groups, in addition to having a very negative zeta potential, giving it good water-holding capacity (1793%) and the ability to absorb salts and cations. Under NaCl stress, EPS production was significantly stimulated with increased polysaccharide and protein content, thereby enhancing cellular stress tolerance. Adding EPS or the *B. velezensis* TSD5 strain to coral sand soil improved aggregate formation and water retention while reducing free salt concentration in the soil. The highlight of this study is the analysis from the structural characteristics of EPS to experiments that clearly demonstrate the role of the *B. velezensis* TSD5 strain in water retention and salinity reduction in coral sandy soil through its water-holding capacity, aggregation formation, and salt absorption ability of EPS. These findings provide an important premise for the development of the *B. velezensis* TSD5 strain to improve arid and saline soils into arable land, as well as expand environmental treatment directions.

1. INTRODUCTION

Exopolysaccharides (EPS) are natural polymers with many critical biological functions. EPS has been shown to have important properties such as antioxidant [1], cholesterol-lowering [2], antitumor [3], antiviral [4], and immunomodulatory [5] activity. Furthermore, it has been discovered that EPSs improve soil quality and help retain nutrients in the soil, particularly by mitigating environmental factors, which in turn benefits plant health and various abiotic stress situations [6]. When it comes to the production of microbial biofilms, EPS is crucial. Biofilms and interactions between plants and bacteria are essential for several rhizosphere processes, including carbon sequestration and nutrient cycling [7]. A matrix of extracellular EPS surrounds biofilm-secreting bacteria, helping to shield them from antibacterial substances, including antibiotics, UV light, heavy metals, and so on [8]. Additionally, rhizobacteria colonize and establish themselves, thanks

in part to these EPS. They can produce EPS to protect themselves and thereby protect plants against adverse environmental conditions. EPS can be produced by plants, algae, fungi, and bacteria.

Because EPS plays an important role in overcoming environmental problems, it has attracted many studies. The addition of EPS-producing bacterial strains has helped reduce soil salinity and improve saline soil [9,10]. EPS-producing bacteria have also shown the ability to protect plants from drought stress by enhancing soil aggregation and increasing soil water retention capacity. Notably, the density of rhizosphere microorganisms that promote plant growth also increased, which plays an important role in improving soil structure [11]. In addition, the applications of heavy metal pollution remediation from EPS-producing microorganisms are also noteworthy. *Halomonas* sp. produces EPS to help convert As^{3+} into a less toxic form As^{6+} , helping to biologically restore As-contaminated rice soil [12]. *Bacillus* sp. produces EPS to help adsorb and reduce the content of heavy metals Cd and Pb, precipitate $Fe_2Pb(PO_4)_2$, $Cd_2(PO_4)_2$, and Pb_2O_3 , and increase microaggregates in the root soil. *Bacillus* sp. added to the soil helps enrich the functional bacterial community in the microaggregates, thereby preventing the adsorption of Cd and Pb by lettuce [13].

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Bacillus species are considered significant rhizobacteria that promote plant growth because they produce a wide range of physiologically active secondary metabolites that may prevent the growth of harmful rhizospheric microorganisms and plant diseases [14–16]. Because *Bacillus* species can develop endospores that withstand heat exposure and desiccation and may be processed into stable, dry powders with extended shelf lives, they are preferred in agricultural systems [17,18]. Exopolysaccharide biopolymers produced by microorganisms are essential for the environment.

The floating islands of the coral archipelago in Khanh Hoa province, Vietnam, have an average height above the water of about 3–5 m with a severe climate. The sun, wind, frequent thunderstorms, lack of fresh water, and numerous island chains devoid of vegetation characterize the archipelago's extremely harsh environmental and climatic characteristics [19]. Furthermore, because coral sand soil has been replenished by being pulled out from the seabed, it frequently possesses qualities that are detrimental to plant growth, such as being arid, unable to retain water, and rapidly losing nutrients.

EPS-producing *Bacillus velezensis* strains have shown antioxidant [1], anticancer [3], stimulation of rhizosphere microbial community and plant growth promotion through soil amendment, effective improvement of soil nutrient composition, protection of soil from acidification [20], enhancement of salt tolerance, and increased phosphate uptake by plants [21]. However, studies combining structural analysis with experiments to demonstrate the role of EPS as well as *B. velezensis* strain in soil are still unclear, especially on dry, saline coral sand soil. This study, through analyzing the properties of EPS to predict some functions of EPS and by experimental application on coral sand soil that needs to be improved to reconfirm the predictions about the water retention, salinity reduction, and soil aggregation of EPS and culture fluid of the EPS-producing *B. velezensis* TSD5 strain. These findings contribute to clarifying the role and mechanism of EPS as well as the EPS-producing *B. velezensis* strain in improving dry, saline coral sand soil, thereby opening up many directions for application of this bacterial strain on many different types of soil.

2. MATERIALS AND METHODS

2.1. Coral Soil Samples Collection

Coral soil was collected from an atoll in the coral archipelago of Khanh Hoa province, Vietnam. Culture medium: Lysogeny Broth (LB) (g/l): Pepton 10, Yeast extract 5, NaCl 5, pH 7. LB agar: LB medium supplemented with 15 g/l agar.

2.2. Isolation of a Salt-Tolerant Bacterial Strain Capable of Producing EPS

Weigh 10 g of soil sample and add it to 90 ml of sterile physiological saline. Then, continue diluting from concentration 10^{-1} to 10^{-7} . Take 150 μ l of the dilution and spread it on an LB agar plate supplemented with 3% NaCl, then incubate at 30°C for 72 hours. Shiny colonies with smooth and slimy surfaces will then be selected and checked using the String test method. EPS-producing colonies will produce mucus; the oozing mucus can be seen with the naked eye [22] or use the circular end of the implant to stretch the colony out, elongating into fibers >5 mm is considered positive, capable of producing EPS [23].

Testing the salt tolerance of bacteria: The salt tolerance of the bacterial strain was conducted based on [24,25]. The selected bacterial strain was cultured in LB agar medium supplemented with 0%–20% NaCl and incubated at 30°C for 46 hours. Tested at salt concentrations at which the bacterial strain could grow.

2.3. 16S rRNA Gene Amplification and Phylogeny Analysis

Bacterial strains were cultured on LB agar medium containing 3% NaCl, then incubated at 30°C for 24 hours and observed for colony morphology and mucus production ability. Cells were Gram stained and observed under an optical microscope to determine Gram staining ability, endospore formation ability, and cell shape and size. The bacterial strain was also molecularly identified by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified through the following PCR cycling profile: initial denaturation at 95°C for 3 minutes, followed by 35 cycles each at 94°C for 45 seconds, at 55°C annealing for 45 seconds, and at 72°C extension for 45 seconds, followed with final elongation at 72°C for 10 minutes. A PCR reaction was performed in a 25 μ l volume comprising 2.5 μ l of template DNA, 12.5 μ l of 2X Taq Master mix, 1 μ l of each primer, and 8 μ l of deionized water, and the size of the amplified fragments was evaluated by 1% gel electrophoresis. Pairs of primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [26] were used to amplify the 16S rRNA gene. The amplified samples were sent for sequencing. Bacterial isolates were further confirmed at the species level by BLAST search using the GenBank/EMBL database using the BLAST tool and building a phylogenetic tree using MEGA XI software in the maximum-likelihood method. These sequences have been deposited in GenBank under accession number PP69439.

2.4. Cultivation of Bacterial Strain

The colony was pre-cultured in 5 ml of LB medium, shaking at 200 RPM at 30°C overnight. The purpose of this step is to increase the bacterial population. Then, 5% of the pre-culture medium was transferred to two 100 ml Erlenmeyer flasks containing 30 ml of LB medium without and with 3% NaCl. Shake the flask at 30°C, at 200 RPM for 48 hours. The post-fermentation fluid was measured for OD600 to determine cell growth ability and EPS extraction to evaluate the EPS biosynthesis ability of the bacterial strain.

2.5. Extraction of Exopolysaccharide

The EPS extraction process was conducted based on the method of [1]: A 20 ml aliquot of the culture was diluted 2-fold by volume in distilled water, then heated at 100°C for 10 minutes to inactivate enzymes involved in EPS degradation. After centrifugation at 8,000 RPM for 20 minutes, the supernatant was filtered through a 0.22 μ m membrane filter to remove the remaining cells and mixed with two times the volume of cold absolute acetone (4°C), kept at 4°C overnight. Collect the precipitate by centrifugation at 8,000 RPM for 20 minutes and wash twice with acetone to remove impurities. To remove small-sized impurities (simple sugars, salts), crude EPS was dissolved in distilled water and dialyzed (14kDa cut-off dialysis membrane, Himedia, Mumbai, India) in water every 8 hours—water exchange. After 40 hours of dialysis, the sample was kept at –25°C for 16 hours, then lyophilized. Weigh the obtained dry EPS.

2.6. Characterisation of Exopolysaccharide

EPS after extraction is determined for total sugar content using the phenol sulfuric acid method according to Duboi's method [27] and quantified total protein using the Bradford method [28].

Scanning electron microscopic images of EPS were taken on a JSM-SEM IT200 (JEOL, Japan). The composition ratio of elements, a critical aspect of our research, was determined with high precision by the EDS Dry SD60 detector in the JSM-IT200 electron microscope based on the X-ray energy dispersion spectroscopy (or energy dispersion spectroscopy) method with the effective method ZAF

quantitative adjustment. The dynamic laser scattering method determined the particle size distribution and zeta potential distribution of the sample. EPS was dissolved in sterile distilled water at 1 mg/ml concentration to determine the zeta potential [29].

Estimation of EPS molecular weight: 50 mg EPS was dissolved in 100 ml of 0.1M NaNO₃ solution for 24 hours [stock solution 0.05% (w/v)]. The stock EPS solution was diluted in 0.1M NaNO₃ solution to concentrations of 0.025%; 0.0125%; 0.00625%; 0.003125%; and 0.0015625%. Then, the flow time of the solvent (0.1M NaNO₃ solution) and the EPS solutions were measured using a Cannon-Fenske viscometer at 25°C, from which the graph of the dependence of the reduced viscosity on the EPS concentration was constructed and the intrinsic viscosity ($[\eta]$) was determined [30,31]:

$$[\eta] = \lim_{c \rightarrow 0} (\eta_{sp} / c)$$

$[\eta]$ is the intrinsic viscosity; c is EPS concentration, η_{sp} is specific viscosity; reduced viscosity $\eta_{red} = \eta_{sp} / c$.

From there, estimate the EPS molecular weight based on the Mark-Houwink formula: $[\eta] = KM^\alpha$, in which M is the EPS molecular weight to be calculated, K and α are constants depending on the nature of the solvent and temperature, and α usually has a value in the range of 0.5–0.8 [31].

FT-IR: FT-IR spectra were recorded on an Impac-410 FTIR instrument using KBr crystal compact discs as described previously [32] to determine the functional groups of EPS.

Determination of monosaccharide components of EPS: 20mg of freeze-dried EPS was hydrolyzed with 3 ml of 2M trifluoroacetic acid for 2 hours at 120°C. The sample was then lyophilized and diluted in sterile distilled water, centrifuged at 10,000RPM for 10 minutes, and filtered through a 0.22 μ m membrane filter before HPLC analysis. The monosaccharide composition of EPS was analyzed using an Agilent 1,260 HPLC system equipped with a Hi-Plex Ca column (300 $\times$ 7.7 mm), an RI detector, and distilled water as the mobile phase. The flow rate was set to 0.6 ml/minute with a run time of 25 minutes. The hydrolyzed EPS sample, along with Sigma-Aldrich standard sugars, was injected into the column. The retention times of the sample were compared to those of the standards.

2.7. Exopolysaccharide's Water Holding Capacity

Water holding capacity (WHC) is the amount of water retained in 1 g of dry EPS. It is the sum of bound, hydrodynamic, and physically trapped water. Conduct experiments based on the WHC determination by weight change method [33,34]. 50 mg of EPS was added to a 2 ml Eppendorf tube containing 1 ml of distilled water, mixed for 1 minute, kept still every 15 minutes, shaking for 5 seconds, repeated four times, and then centrifuged 5,000RPM at 4°C for 15 minutes. Decant the supernatant to separate the water not held by EPS. Use absorbent paper to absorb all the water remaining on the outside, then weigh the EPS after hydrating. The water-holding capacity of EPS is calculated according to the following formula:

$$WHC (\%) = \frac{m_{after}}{m_{initial}} \times 100$$

2.8. Water Holding Capacity of Soil Treated with EPS and Strain TSD5

Sample preparation

Used crushed coral sand soil (≤ 2 mm). The mixed soil consists of crushed coral sand with the addition of 13% substrate and 3%

bentonite (*). The substrate has ingredients according to the weight ratio: 3 coconut fibers: 3 smoked husks: 2 sugarcane mud: 2 sugarcane ash.

Design experiments based on publications

Exp.1. 50 g of coral sand soil with 10 ml of distilled water added.

Exp.2. 50 g of mixed soil and 10 ml of distilled water.

Exp.3. 50 g of mixed soil was added to 10 ml of TSD5 strain culture solution (10^9 cells/ml).

Exp.4. 50 g of mixed soil supplemented with 10 ml of distilled water and 70 mg of crude EPS.

Exp.5. 50 g of mixed soil supplemented with 10 ml of distilled water and 140 mg of crude EPS.

Five corresponding soil samples in the above five experiments were determined the water holding capacity, drainage rate, and aggregation ability. Proceeded based on the "Droplet Counting Method" of Vardharajula and Shaik [35] Brischke and Wegener [36] with modifications:

Step 1: Determine the soil drainage rate

25 ml of water was added to each soil sample of the above 5 experiments, mixed for 1 minute, and left for 2 hours. Whatman filter paper with a diameter of 125 mm was placed at the bottom of the funnel (funnel rim diameter was 70 mm) and moistened with distilled water so that the filter paper adhered closely to the funnel. The funnel is placed on the measuring cylinder. The prepared test sample was transferred to the funnel and spread evenly, adding 25 ml of distilled water to the funnel. Calculate the time it takes for 20 ml of water to flow out of the funnel, then calculate the drainage rate (ml/second).

Step 2: Determine the soil aggregation capacity and water holding capacity of the soil

The soil in the step 1 experimental funnels was drained of water. 5 mg of each sample was taken to take an SEM image to observe the surface and evaluate the ability of EPS to agglomerate in the experimental soil samples. The remaining soil is placed on a glass dish of known mass and weighed. The dish containing the soil sample was dried at a temperature of $103^\circ\text{C} \pm 2^\circ\text{C}$ and weighed again. The WHC of the soil samples was calculated as follows:

$$WHC (\%) = \frac{m_{soil,saturated} - m_{soil,0}}{m_{soil,0}} \times 100$$

WHC is the water holding capacity of the soil, expressed in %; $m_{soil,saturated}$ is the mass of saturated soil, in g; $m_{soil,0}$ is the mass of dried soil, in g.

2.9. Evaluation of The Salinity Reduction Potential of Free Water In Soil

Five experiments were designed with mixed soil samples (*):

Exp.1: 50 g of mixed soil and 10 ml of distilled water

Exp.2: 50 g of mixed soil and 10 ml of 3% NaCl solution

Exp.3: 50 g of mixed soil, 70 mg of EPS, and 10 ml of 3% NaCl solution

Exp.4: 50 g of mixed soil, 0.3 g of NaCl, and 10 ml of LB medium

Exp.5: 50 g of mixed soil, 0.3 g of NaCl, and 10 ml of TSD5 strain culture solution in LB medium (10^9 cells/ml).

Each experiment was repeated 3 times.

In each experiment, mix well and let stand for 24 hours, then mix with water in a ratio of 1:5 (soil: water, w/w), shake for 1 hour, and put into a funnel lined with Whatman filter paper to collect the water flowing out. Measured EC of the collected water samples using a Total Meter EZ-9909SP electrical conductometer.

2.10. Statistical Analysis

All experiments were repeated three times. Statistical differences were determined by One-way ANOVA using Minitab Statistical Software. Differences were considered statistically significant when $p < 0.05$ according to the Terkey method.

3. RESULTS AND DISCUSSION

3.1. Salt Tolerance of Bacterial Strain TSD5

Strain TSD5 can grow on LB agar medium supplemented with 0%–12.5% NaCl (Fig. 1). At a concentration of 15%, the strain grew very poorly, and colonies were much smaller at lower NaCl concentrations. Strain TSD5 cannot grow at 20% NaCl concentration. This is a high result compared to some publications on salt-tolerant strains such as three strains of three species: *Bacillus paramycoides*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus*, which can tolerate salt from 2% to 10% [24]. EPS-producing *Rahnella aquatilis* JZ-GX1 is tolerant of 0%–9% NaCl and grows well at 3% concentration [25]. *Virgibacillus dokdonensis* VITP14 bacteria has salt tolerance at NaCl salt concentrations of 0%–10% [29]. However, the salt tolerance of strain TSD5 is still lower than strain *Bacillus subtilis* LR-1, which can grow in 160 g/l NaCl (16%) environment [37].

3.2. Identification of Bacterial Strain

The bacterial strain TSD5 has been morphologically described and molecularly identified (sequencing of the 16S rRNA gene segment). The colony is opaque white in shape, has a shiny surface, and has mucus on the edges (Fig. 2a). Under the microscope, the strain's cells are rod-shaped, gram-positive, 2–3 μm , and capable of producing endospores (Fig. 2b). The sequenced 16S rRNA gene segment of bacterial strain TSD5 is 1422 bp in length. The partial 16S rRNA of TSD5 strain has been deposited in the GenBank with accession number PP694391. The genes were analyzed and aligned with corresponding genes on GenBank to create a phylogenetic tree, as depicted in Figure 2c. The isolated strain TSD5 has the 16S rRNA gene sequence most similar to *B. velezensis* strain NR116240 (99.8%). The genus *Bacillus* has been

published to have salt-tolerant strains such as *Bacillus megaterium* NCT-2 [38], *B. paramycoides*, *B. amyloliquefaciens*, and *B. pumilus* [24]. *B. subtilis*, *B. megaterium*, *B. cereus*, *B. amyloliquefaciens*, *B. pumilus*, *Bacillus licheniformis*, *B. endophyticus*, *B. atrophaeus*, *B. safensis*, *B. mojavensis* [39]; and produce EPS such as *B. velezensis* KY498625 [40], *B. proteolyticus* A27 [41], *B. licheniformis* DM-1 [42] or has both salt tolerance and EPS production such as *B. subtilis* LR-1 [37], *Bacillus tequilensis*, and *Bacillus aryabhatai* [43]. *Bacillus* species are assessed to have good metabolism/genome and spore formation and are highly resilient to abiotic stresses, helping to enhance plant resistance to saline soils [39]. The functional groups of EPS can help bind and complex with Na^+ in soil and thus reduce plant exposure to this ion under saline conditions [39]. According to TRBA 466 of the European Union, *B. velezensis* belongs to Biosafety Level 1 and can be used without restriction in agriculture and the environment.

Bacillus velezensis strains are known for their ability to improve soil contaminated with heavy metals [44], enrich the soil microbiota, improve the efficiency of soil nutrients, solubilize inorganic phosphorus, fix nitrogen, produce indole-3-acetic acid, and tolerate salt well [20]. These are rhizosphere microorganisms with the ability to form biofilms that enhance symbiotic cooperation with soil microorganisms, promote growth, inhibit disease, and reduce stress on plants [45,46].

3.3. Characterisation of Exopolysaccharide

Table 1 shows that strain TSD5 grows and produces EPS well at NaCl concentrations of 0%–3%. The strain grows better in the environment without NaCl (OD600 = 10.53). However, under NaCl stress conditions, this bacterial strain promotes EPS production better (1.25 g/l), especially since the total sugar and total protein content in EPS are also 2 times higher than in the condition without NaCl supplementation. The differences here are all statistically significant, $p < 0.05$. Increased EPS production under salt stress conditions resulted in increased cell viability, as EPS plays an important role in biofilm formation. Biofilm formation increases the resistance of EPS-producing cells [47], and also increases soil aggregation, water, and nutrient uptake, which helps microorganisms in the biofilm, as well as plants, to withstand adverse environmental conditions [9].

The SEM results on Figure 3a,b show that the EPS sample has a structure consisting of discrete particles, 1–20 μm in size from the medium without NaCl added (Fig. 3a), while the EPS from the medium supplemented with 3% NaCl consists of particles covered by a layer of glue that helps bind the components in the sample (Fig. 3b).

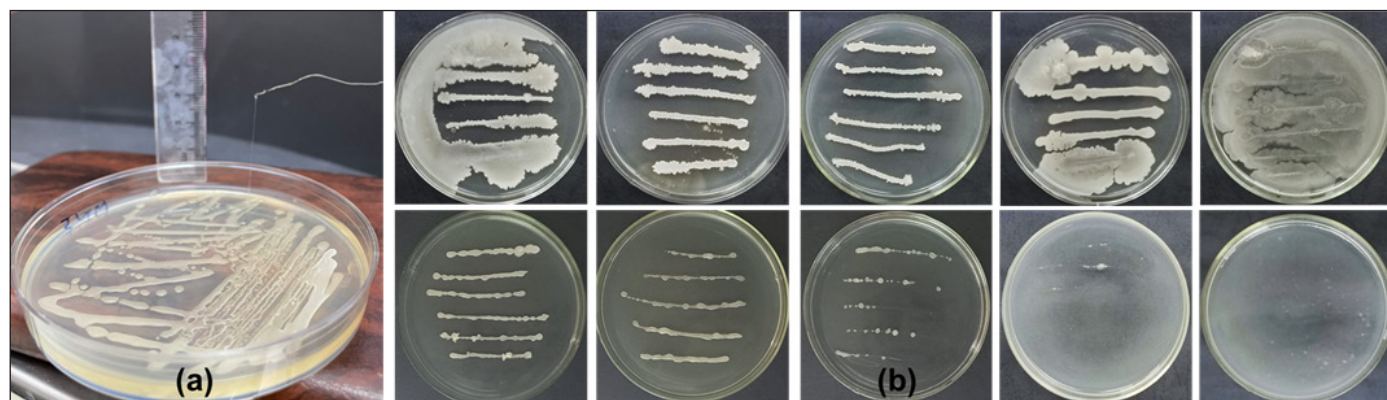


Figure 1. Growth ability strain TSD5 on LB agar medium without and with different concentrations of NaCl after 46 hours incubation at 30°C.

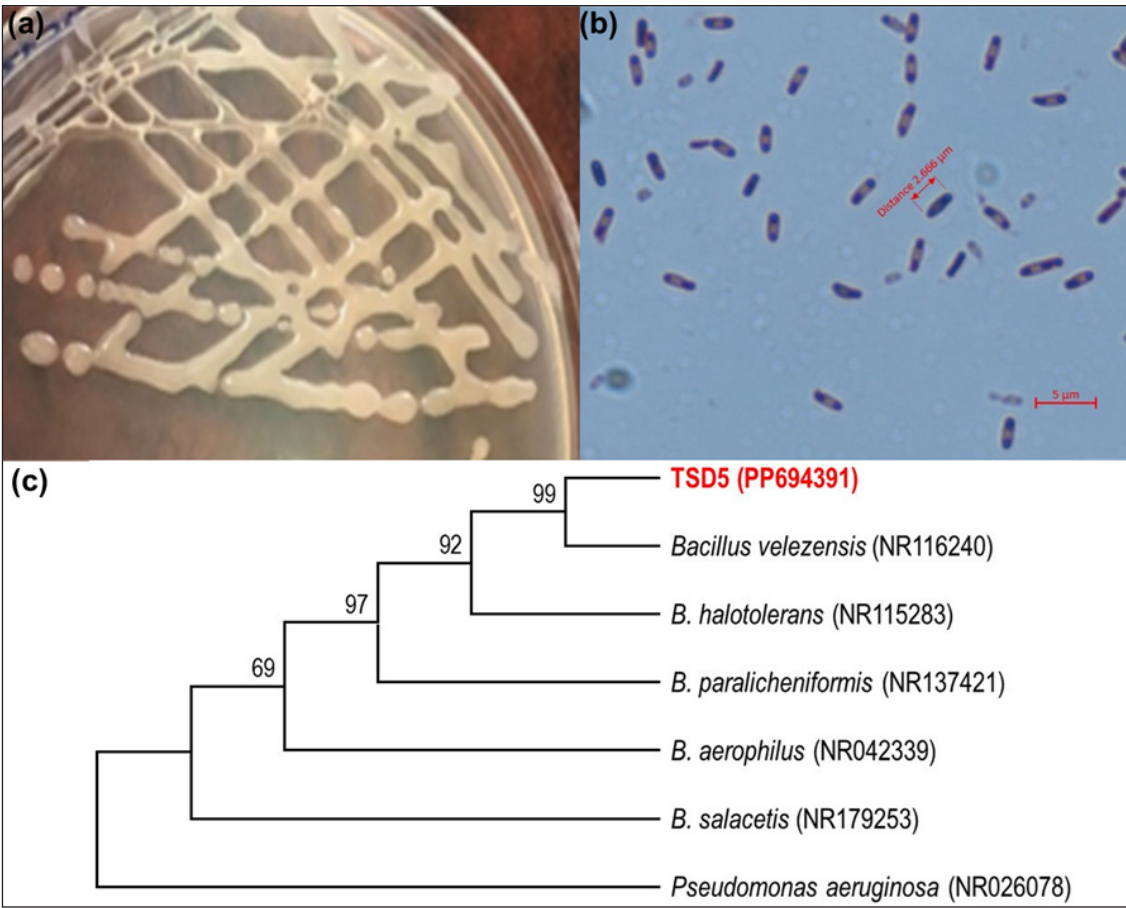


Figure 2. The digital image of colony (a), electron microscopic image of cell (b), and Phylogenetic tree was built from the 16 rRNA gene sequence-related strains on GenBank (c) of strain TSD5.

Table 1. Effect of NaCl stress conditions on growth and EPS production of bacterial strain TSD5.

Medium LB	OD600	EPS content (g/l)	Total sugar (% of EPS)	Total protein (% of EPS)
without NaCl	10.53 ± 0.41	1.02 ± 0.09	19.68	8.65
3% NaCl	8.92 ± 0.37	1.25 ± 0.10	41.95	27.83

Values are presented as mean ± SD; $n = 3$, $p < 0.05$.

This is because EPS from the NaCl-stressed environment has a much higher polysaccharide and protein content (more than 2 times) than the environment without NaCl salt, thereby increasing the viscosity and colloidal properties of EPS [9]. The elemental composition of EPS was detected by the EDX spectrum in Figure 3c,d. The EPS sample from a NaCl-free environment includes 11 elements C, N, O, Na, Mg, P, S, Cl, K, Ca, and Zn, with 2 main elements C and O accounting for 32.44% and 42.9% by mass, respectively (Fig. 3c). However, EPS separated from NaCl-containing medium did not appear Zn and Ca, and only had 9 elements, including C (32.80%) and O (40.40%) (Fig. 3d). The results show the effect of NaCl on the ion adsorption capacity of EPS. In addition, Na and Cl content only account for 0.73% and 0.56%, respectively, in EPS from the NaCl-free environment, while accounting for a larger amount in EPS from the salt-added environment, 8.40% and 2.04%, respectively. It has been shown that when the environment is salty, EPS increases the adsorption of both Na and Cl, helping to reduce the free NaCl concentration in the solution (reducing salinity). EPS or EPS-producing bacterial strains that help reduce soil salinity have been reported by some [9,10].

The presence of cations such as Ca, Mg, Na, Ca, Zn, and K shows the ability of EPS to adsorb cations. This is explained by the fact that EPS is a polyanion with a highly charged zeta potential of -30.1mV (Fig. 4a), as it contains negatively charged functional groups and thus has the ability to adsorb cations [48,49]. S and P are also present in EPS, which are anionic functional groups thought to be attached to the sugar ring to help transfer these negative charges in EPS [29], helping to bind cations [50]. Furthermore, S present in EPS is also a characteristic feature of marine bacteria [10,29]. The EPS sample of *B. velezensis* TSD5 had a zeta potential of -30.1mV , indicating anionic properties can adsorb cations. EPS reported a wide range of negative zeta potentials: -3.44mV in *Enterococcus* sp. F2 [34]; *Paenibacillus tarimensis* REG 0201M -35.2mV [49]; -33.83mV in *B. velezensis* P1 [51].

EPS molecular weight is estimated based on intrinsic viscosity. Based on the graph in Figure 4b, the intrinsic viscosity $[\eta]$ was determined to be 0.6514 dl/g , combined with the Mark-Houwink formula: $[\eta] = \text{KM}^\alpha$, in which $\alpha = 0.687$, $K = 2.259 \times 10^{-4}$ [31], from here the molecular weight of EPS was determined to be $1.09 \times 10^5\text{Da}$. Compared with

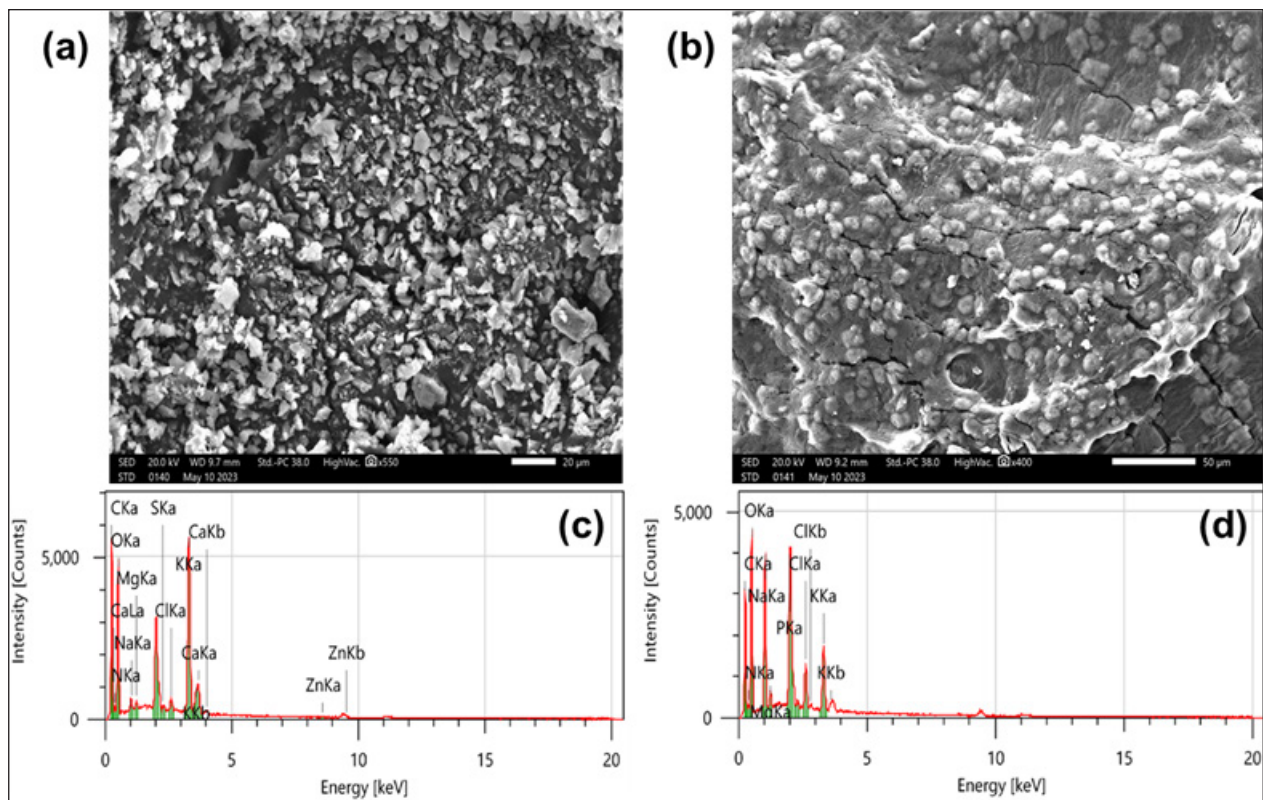


Figure 3. SEM images and EDX spectrum of EPS samples cultured in an environment without added NaCl salt (a, c) and in an environment supplemented with 3% NaCl environment (b, d).

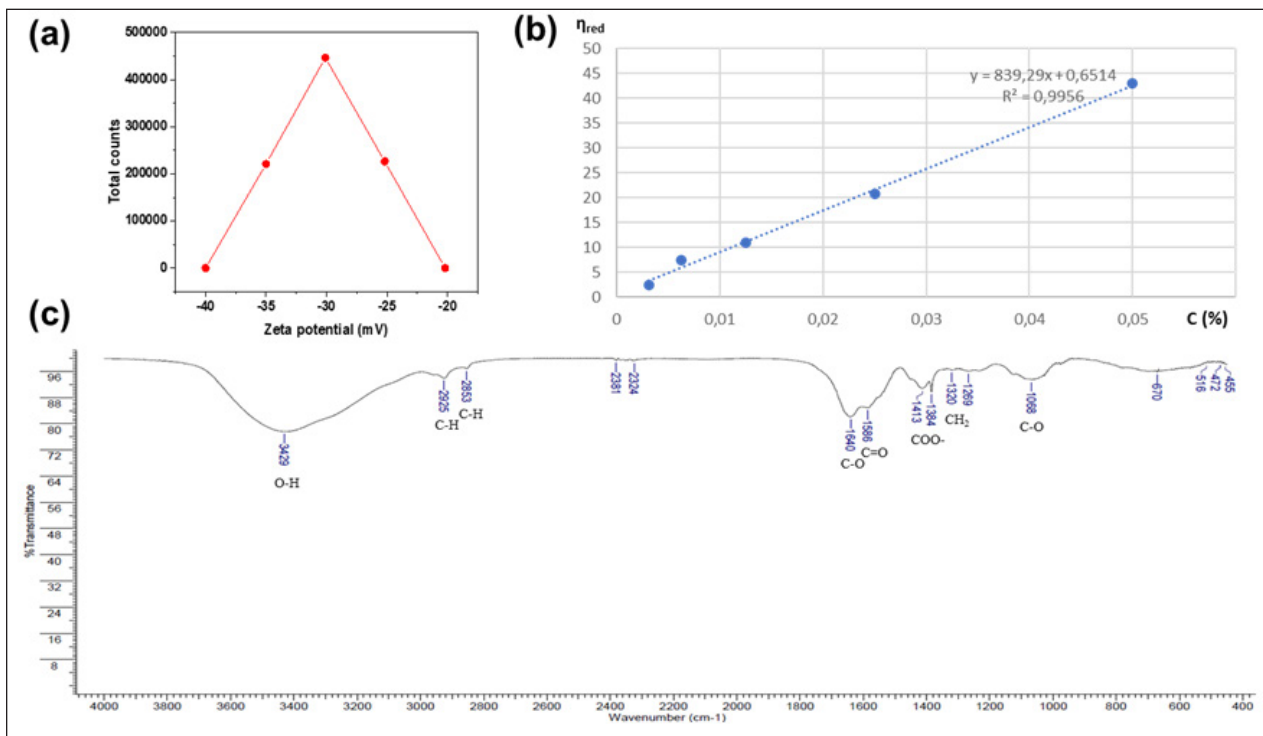


Figure 4. Zeta potential distribution of EPSTD5 (a); Dependence of reduced viscosity on EPS concentration in 0.1M NaNO₃ solution at 25°C (b); FTIR spectrum with main functional groups of EPS TSD5 (c).

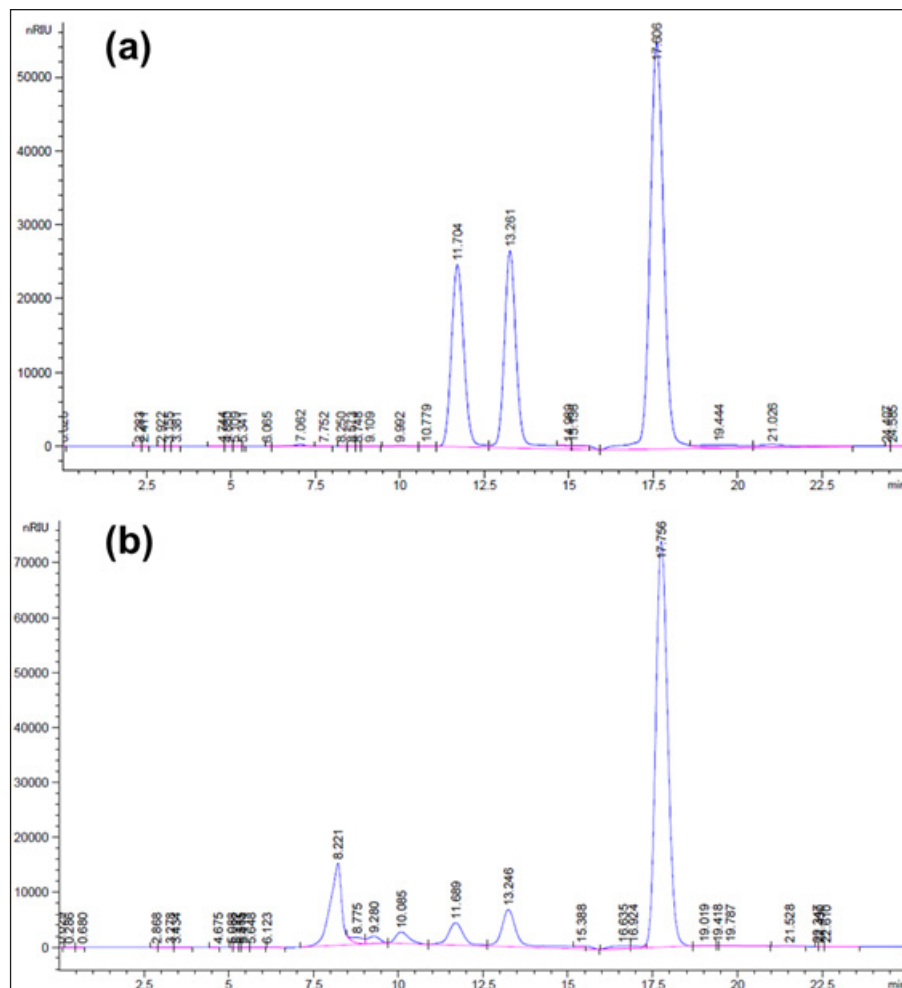


Figure 5. HPLC results of standard monosaccharides (a) and hydrolyzed EPSTSD5 sample (b).

previous publications on the molecular weight of EPS from *B. velezensis* strains, this result is equivalent to EPSKY471306 [40]; lower than the mass of EPS-SN-1 reaching 2.21×10^5 Da [52], but higher than EPSF6 reaching 2.7×10^4 Da [53] and MHM3 (1.145×10^4 Da) [54]. The intrinsic viscosity of EPS in strain TSD5 was 0.6514 dl/g, higher than that in *Virgibacillus salarius* BM02 at 0.12–0.54 dl/g [55] but lower than that in *B. megaterium* at 5.2 dl/g [56]. However, intrinsic viscosity is also greatly influenced by the environment and culture conditions [55]. The higher the intrinsic viscosity, the longer and more complex the polymer chains are, resulting in higher strength and stiffness.

FT-IR spectrum (Fig. 4c) provided further evidence for the presence of carbohydrates and proteins in EPS. The broad spectral bands characteristic of the presence of polysaccharides were the bands from 4,000 to 2,500 cm^{-1} [57], 1,200–900 cm^{-1} [58]. Specifically, the maximum peak band at 3,429 cm^{-1} is characteristic of the abundant hydroxyl (-OH) group in polysaccharides [57,52], and the weak bands at 2,925 cm^{-1} and 2,853 cm^{-1} are attributed to the stretching of the C-H group in the methylene group [59], the peak at 1,068 cm^{-1} represents the presence of the C-O group [58]. The spectral band 1,700–1,200 cm^{-1} is characteristic of protein [58], in which the peaks 1,640 cm^{-1} , 1,586 cm^{-1} are the signs of C=O [52] and C=O [3] groups, respectively, the peaks 1,413 cm^{-1} and 1,384 cm^{-1} indicate the presence of carboxyl group (COO-) [40], besides the band 1,500–1,320 cm^{-1} is also assigned to methylene group (-CH₂) [32]. The infrared spectrum of EPS in some strains of *B. velezensis* also gave similar

results with the appearance of a large band of O-H with an absorption peak at about 3,000 cm^{-1} such as 3,387.19 cm^{-1} [52], 3,428.76 cm^{-1} [40]; in addition, the C-H stretching groups peaked at about 2,928.24 cm^{-1} [52]; the corresponding C-O group peaked at 1,640 cm^{-1} [52]; the carboxyl group peaked near 1,447–1,380 cm^{-1} [40]; in addition, the region 1,200–950 cm^{-1} (also known as the fingerprint region) is characteristic for each polysaccharide [40].

HPLC results (Fig. 5) recorded the presence of monosaccharides glucose: rhamnose: mannose with a ratio of 1.00: 1.83: 14.20 in the EPSTSD5 hydrolyzed sample, in which mannose accounted for the majority. This result is quite different from some other publications on EPS from *B. velezensis* strains containing monosaccharides such as fructose and glucose [1]; glucose, mannose, and galactose [40]; mannose (63.52%) and glucose [3]; glucuronic acid, glucose, fructose, and rhamnose (4.00: 2.00: 1.00: 0.13) [54]. This is explained by the fact that the EPS biosynthesis process in bacteria is quite complex, occurring through many pathways, each of which shares a number of key conserved enzymes. In addition, environmental conditions also affect the structure and branching of EPS [60].

Thus, through analysis of the properties of EPS, it is shown that this is a heteropolymer consisting of monosaccharides mannose, rhamnose, and glucose, with a molecular weight of about 1.09×10^5 Da, has a negative zeta potential (capable of cation adsorption), contains hydrophilic functional groups, and has the ability to adsorb metals

such as carboxyl, and hydroxyl. To increase the ability to withstand salinity stress, strain TSD5 increases EPS production as well as increases the protein and sugar content in EPS to create more colloidal EPS and adsorb more Na, and Cl than in non-saline conditions. These are the properties that help EPS retain water and reduce salinity.

3.4. Exopolysaccharide's WHC

The WHC test results in Figure 6a show that the EPS size increases with time after soaking in water for 1–2 hours. The data in Figure 6b show similar results, with WHC reaching 993.33% after 1 hour of soaking in water. The result is higher than some WHC reports of EPS from *Lactobacillus kefiranoferiens* ZW3 reaching 496.00% [61], EPS-F2 from *Enterococcus* sp. F2 is 882.5%. EPS after 2 hours of soaking in water for WHC reached 1,740% (Fig. 6b), and the weight increased to 0.87 g (initial dry EPS amount was 50 mg), 1.75 times higher than after 1 hour of soaking in water. The test increased to 4 hours of soaking in water showed that WHC increased insignificantly compared to after 2 hours ($p > 0.05$), proving that after 2 hours, the hydrated EPS has reached its maximum value (saturated water), so soaking in water for longer but the WHC did not increase significantly. WHC is an essential indicator of EPS; the higher the WHC, the better the water retention ability, and the less water is separated. Therefore, EPS from the TSD5 bacterial

strain has a high water retention capacity, promising to bring good water retention capacity to the soil. WHC is one of the functional properties of EPS that is greatly influenced by factors such as molecular weight, particle size, ionic form, and composition of the polysaccharides [33,1]. This property is due to EPS containing high levels of polysaccharides, especially under salt-stress conditions. Polysaccharides are hydrophilic in nature, contributing to the formation of gel networks [62], while proteins are hydrophobic [63] and can interact with polysaccharides through covalent interactions. Non-covalent protein/polysaccharide interactions are important for colloidal stability and soil aggregation ability [62]. Polysaccharides contain various hydrophilic functional groups, such as carboxyl, hydroxyl (shown in the FT-IR analysis results), and phosphate groups [64].

The above test proved that EPSTSD5 has a good water retention capacity (1740%) as predicted when analyzing EPS characteristics, promising to help increase the water retention capacity of the soil.

3.5. Water Holding Capacity of Soil Treated with EPS and Strain TSD5

Figure 7A shows that the coral sand soil sample (Exp.1) has the highest water drainage rate (0.94 ml/second), 18.8 times higher than the worst-drained mixed soil sample in Exp.2. The coral sand

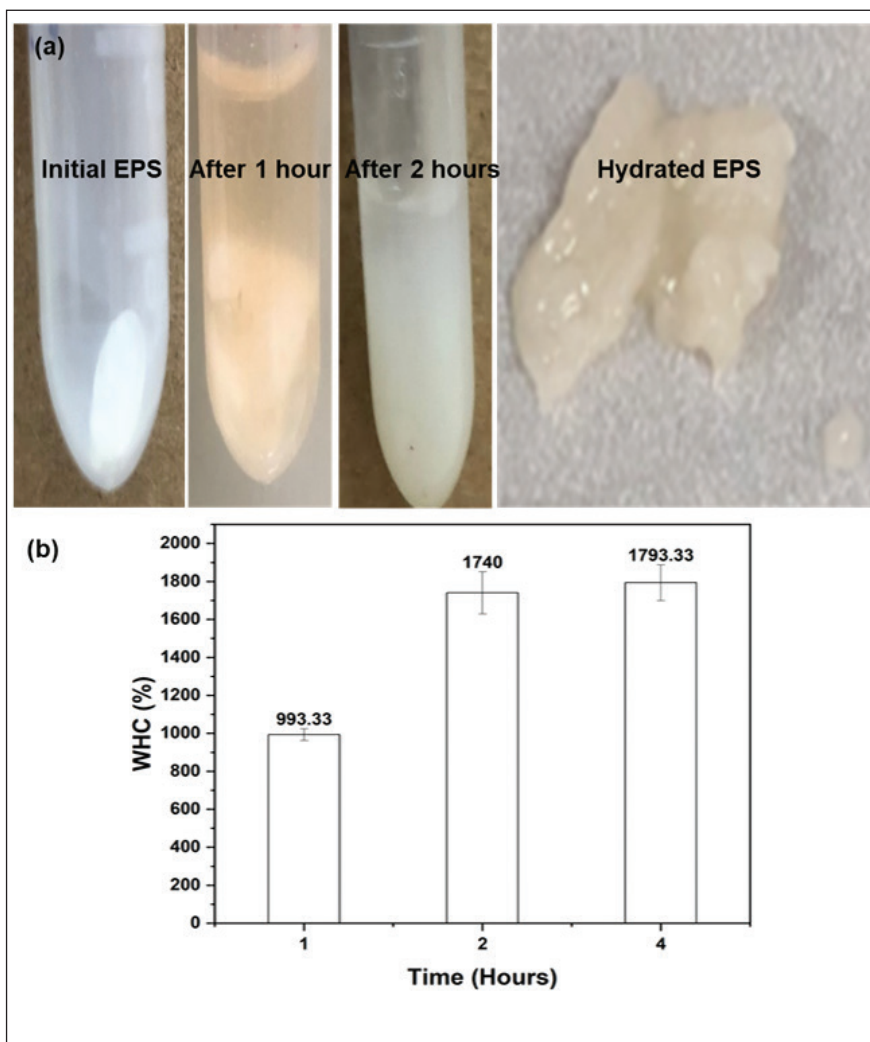


Figure 6. Image (a) and graph (b) of water holding capacity of EPSTSD5 initially and after 1–4 hours of hydration. Values are presented as mean $\pm$ SD; $n = 3$.

sample (Exp.1) drained quickly due to its loose structure, so water easily drains through the gaps between sand grains. However, in the mixed soil sample (Exp.2), bentonite particles were added that have the ability to expand to help seal the gaps between the sand grains, helping to retain water for the longest time. In Exp. 3,4,5, the drainage rate is 2.4–3.6 times higher than sample Exp.2 due to the addition of EPS/EPS fermentation solution that helps bind soil components together to form aggregates [35]. There are gaps between the agglomerated particles to help water circulate and drain better in soil samples without EPS addition [65]. The EPS content in Exp.5 is twice as high as in Exp. 4, so it can create more agglomerates, thus better drainage capacity. However, in practice, the drainage rate in Exp. 5 was higher than that in Exp. 4 (Fig. 7A), but this difference was not statistically significant ($p > 0.05$). Figure 7B shows that the WHC in Exp. 4 and 5 with added EPS/EPS-producing strain culture broth was significantly higher than that in the samples without added EPS in Exp. 1 and 2 ($p < 0.05$). The WHC in Exp.5 (EPS content 140 mg) is higher than in Exp.4 (70 mg EPS) and 1.35 times higher in Exp.2 (mixed soil, no EPS), 1.96 times higher than with Exp.1 (coral sand sample). The WHC in Exp.2 is higher than Exp.1 due to the addition of bentonite to retain water. Exp.3–5, in addition to bentonite, also adds EPS/culture solution of the TSD5 strain that produces EPS; EPS has the ability to retain water, so WHC is higher than in Exp.2, $p < 0.05$.

Visual observation in Figure 7C and D shows that, after soaking in water for 2 hours, sample Exp.2 has a clear layer separation phenomenon by weight, with a white layer (coral sand) underneath

and a layer of light organic medium in the upper layer (black). In Exp.3–5, the layer separation phenomenon is significantly improved, and the black substrate has been mixed evenly with sand. Because in Exp.3–5, EPS/culture fluid containing EPS is added, it helps bind the soil components together to form aggregates [35], making it more difficult to layer. The SEM results (Fig. 7E) contribute to confirming the above argument. On the soil samples supplemented with EPS or the culture solution of EPS-producing strain TSD5 (Exp.3–5), there was a phenomenon of agglomeration of particles together, forming larger blocks, with no or very few single small particles left as on Exp.1 and 2 without EPS. Agglomeration creates gaps between particles, allowing water to circulate better. Therefore, Exp. 4 and 5 (Fig. 7A) had a faster drainage rate than on Experiment 2 (without EPS). The above agglomeration phenomenon is due to EPS clinging to the soil surface through hydrogen bonding, cation bonding, anion adsorption, and van der Waal forces. Soil agglomeration improves soil structure and provides soil stability [9].

The above experiments have proven that EPSTSD5 and the TSD5 strain fermentation solution have the ability to bind soil components, and increase aggregation and water retention capacity for soil as previously predicted.

3.6. The ability to reduce soil salinity of EPS and TSD5 strain

The data in Figure 8 show that when treating soil with EPS or culture fluid of the EPS-producing TSD5 strain, the EC in the collected water samples (free water) was reduced by about half. This is explained by

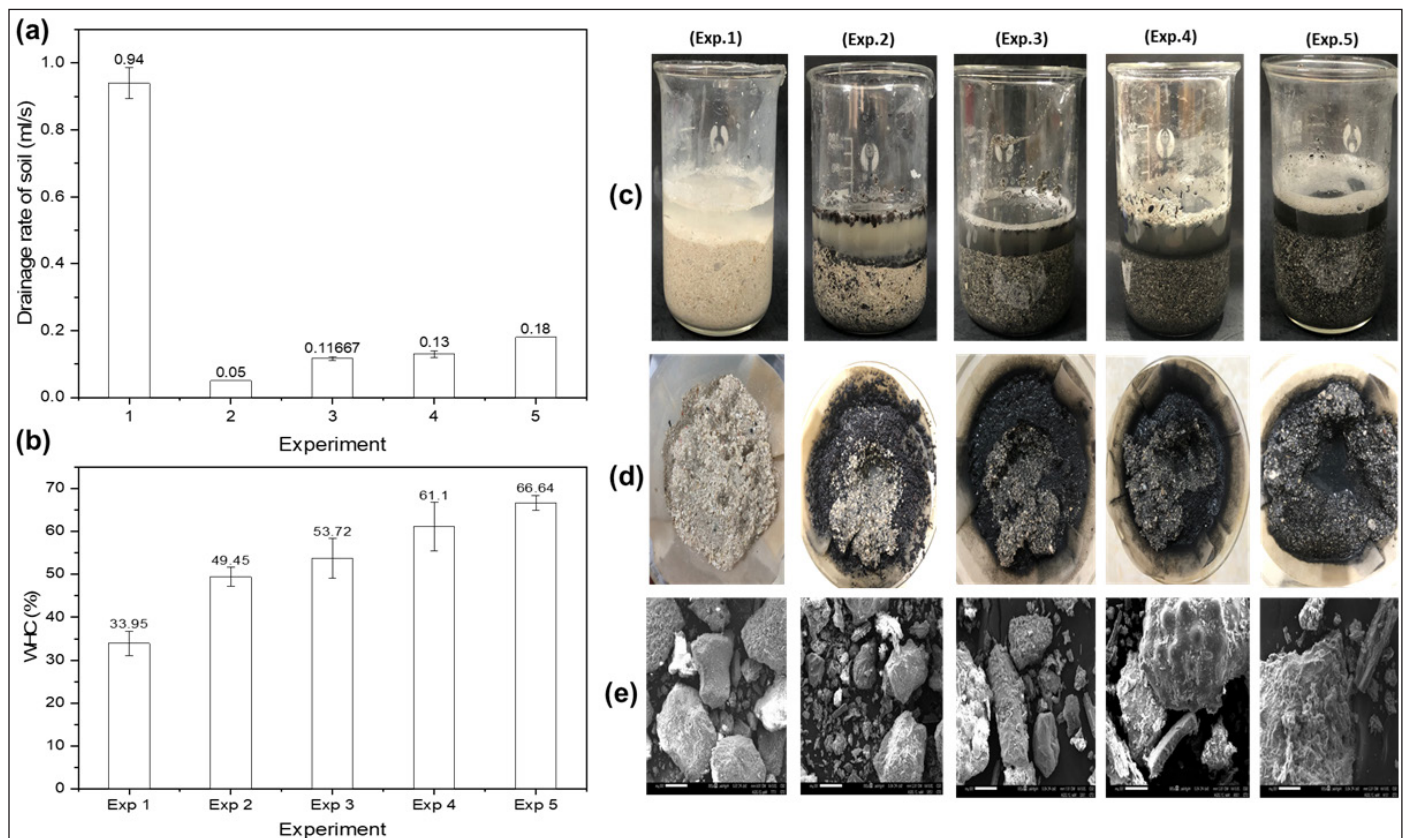


Figure 7. Drainage rate (A); WHC (B); images of the experimental samples after 2 hours of soaking the soil samples in water (C) and after filtering the water on a funnel (D); and SEM images of the surface of 5 soil samples after testing (E) by the droplet counting method. Exp.1, coral sand soil. Exp.2, mixed soil (coral sand with the addition of 13% substrate and 3% bentonite; the substrate has the following mass ratios: 3 coconut fibers: 3 smoked husks: 2 sugarcane mud: 2 sugarcane ash). Exp.3, mixed soil with TSD5 strain culture solution. Exp.4, mixed soil with 70 mg of EPS. Exp.5, mixed soil with 140 mg of EPS. Values are presented as mean $\pm$ SD; $n = 3$.

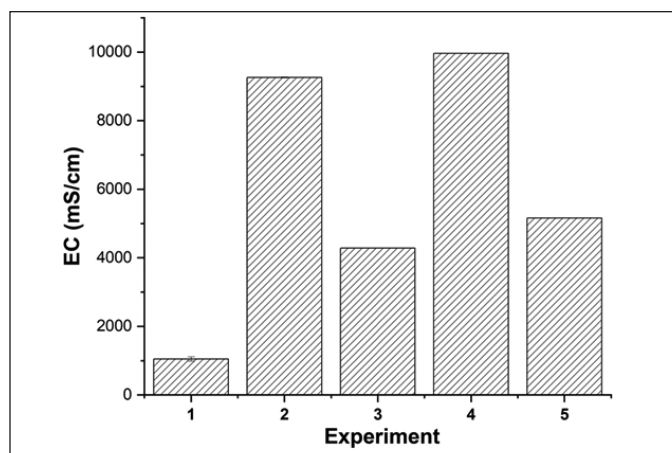


Figure 8. The EC values of water samples from five soil salinity reduction experiments. Exp.1. non-saline control; Exp.2. salt addition; Exp.3. EPS and salt addition; Exp.4. salt and LB medium addition; Exp.5. salt and culture solution of strain TSD5 addition. Values are presented as mean $\pm$ SD; $n = 3$, $p < 0.05$.

the fact that EPS has the ability to adsorb NaCl (shown in the EDX analysis results of EPS). The results contribute to confirming the salinity reduction ability of EPS as well as EPS-producing bacteria. This is very useful to improve saline soil and increase plant survival by absorbing salt from the soil, helping to reduce the salinity of free water in the soil, protecting the roots, increasing the ability of the roots to absorb water and nutrients, and promoting plant growth and development [9]. The ability to reduce salinity here is due to EPS having a very negative zeta potential and containing many negatively charged functional groups (as presented in Section 3.3), so it has the ability to adsorb cations, including Na^+ . The mechanisms of cation adsorption of EPS include: complexation, biological precipitation, ion exchange, and oxidation-reduction [13]. EPS acts as a barrier to protect bacterial cells and plant roots from high salinity due to its ability to aggregate, bind soil components and even roots, and form complexes with metals, including sodium, thereby reducing the effects of high soil salinity and reducing salt stress by maintaining the Na^+/K^+ balance that helps plants survive under adverse soil conditions [13]. EPS functional groups such as hydroxyl, carboxyl, carbonyl, amine, imidazolic, and phosphonate groups possess different metal binding affinities and can reduce the concentration of free metal ions. Therefore, increasing EPS production under metal stress helps reduce their toxicity to bacterial cells as well as plants through complexation or reduction reactions [66].

Salinity and soil structure are extremely important factors that determine soil health and plant growth. The results of the study contribute to explaining the water retention and salinity reduction mechanisms of EPS as well as EPS-producing bacteria. This is an important key to opening up directions for application in improving dry, saline soil, and enhancing soil health and sustainability. This demand is huge because the area of dry, saline soil is expanding worldwide due to the impact of climate change as well as unreasonable human farming activities (irrational use of chemical fertilizers, pesticides, saltwater irrigation, and so on). It is estimated that by 2050, up to 50% of the cultivated land area will be affected by salinity [9]. Currently, *B. velezensis* is considered a safe microorganism, being developed in microbial fertilizer preparations for soil improvement. This is a reasonable solution to replace chemical fertilizers and develop sustainable agriculture [44]. Moreover, with the properties of EPS discovered in this study, such as EPS having a large molecular size, very negative zeta potential, having many negatively charged functional groups,

and having good adhesion ability, it helps to open up further research directions on the ability to adsorb heavy metals, adsorb nutrients, and the ability to promote the development of beneficial microorganisms in the root zone for plants. In addition, many other useful properties of *B. velezensis* have been discovered in previous studies, such as the ability to dissolve inorganic phosphorus and plant growth stimulants, helping plants increase their resistance to non-metallic stress (heavy metals, pH, temperature, drought, and so on) as well as plant pathogenic microorganisms [44,20,45,46]. This study has opened up great prospects for applying the EPS-producing *B. velezensis* TSD5 strain to improve the soil as well as overcome environmental pollution problems. However, the limitation is that EPS here only has the effect of absorbing salt; the salt is still in the soil, only temporarily preventing the salt from moving with water, thereby temporarily avoiding the impact of salinity on plant roots. Therefore, in the long term, it is still necessary to have effective measures to coordinate the use of beneficial microorganisms with other field management measures to increase the effectiveness of treating saline soil. Up to now, the ecological risks of introducing EPS-producing microorganisms into the natural environment have not been discovered. However, having more assessments on the long-term safety of EPS-producing bacteria to the environment and humans is very meaningful. In addition, long-term field trials and studies on the effects of *B. velezensis* TSD5 and EPS-enriched soil on plant growth, especially coral soil-related crops, are urgently needed for sustainable agricultural development.

4. CONCLUSION

The study isolated the salt-tolerant, EPS-producing *B. velezensis* TSD5 strain from coral sand on a coral island in Khanh Hoa province, Vietnam. The strain TSD5, which exhibits salt tolerance ranging from 0% to 12.5% NaCl, produced EPS under 3% NaCl salinity stress condition at 1.25 g/l, which was better than in non-saline conditions. Strain TSD5 shares a 16S rRNA gene sequence identical to *B. velezensis* NR116240 (99.8%), a Biosafety Level 1 species. The EPS produced by strain TSD5 is characterized by a large molecular weight and consists of monosaccharides mannose, glucose, and rhamnose, containing many hydrophilic and negatively charged functional groups. It is a polyanion with significant NaCl adsorption and water-holding capacity. The application of EPS or culture liquid of the TSD5 strain to soil improved soil aggregate formation and water retention and also helped reduce soil salinity. Overall, these findings highlight the potential of EPS derived from *B. velezensis* TSD5 in the remediation of arid, saline soils, suggesting its promising applications in agriculture and environmental remediation.

5. ACKNOWLEDGMENT

This research was financially supported by the Joint Vietnam - Russia Tropical Science and Technology Research Center.

6. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. ETHICAL APPROVALS

The article does not contain any studies involving humans or animals performed by the author.

8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval

of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

9. DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing of the manuscript, and no images were manipulated using AI.

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How to cite this article:

Hue Le T, Nhan Vu D, Phuong Nguyen TH, Huong Le M, Nguyen CT, Tuyen Do T, Thang Le V, Phuong Pham M, Duy Vu D. Amending coral soil using exopolysaccharide from salt-tolerant *Bacillus velezensis* TSD5 bacteria from an atoll in Vietnam. *J Appl Biol Biotech* 2025;13(5):151–162.
DOI: 10.7324/JABB.2025.229116.