IMPROVING SOIL FERTILITY THROUGH THE USE OF NOVEL OXIDIC MATERIALS FUNCTIONALIZED WITH HALOPHILIC BACTERIAL ENZYMES

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Abstract: This study investigates the role of halophilic bacterial enzymes, specifically proteolytic enzymes synthesized by a strain from Movila Miresei Salt Lake, Romania, in enhancing anthropized soil fertility through hybrid structures. Utilizing a moderately halophilic (MH) medium with 10% NaCl and 1% casein, the enzymes were biosynthesized, purified via acetone precipitation and gel filtration, and immobilized on titanium dioxide (TiO_2) and silica (SiO_2) nanomaterials through direct adsorption. Titanium dioxide demonstrated superior immobilization effectiveness. These enzyme-nanomaterial hybrids were mixed into anthropized soil at a 1:1 ratio. The improvement in soil biodegradation was assessed after 72 hours by measuring activities of alkaline phosphatase, urease, and dehydrogenase in soil samples containing immobilized enzymes and in those with only nanomaterials.

Keywords: proteases, halophiles, hybrid systems, nanomaterials.

INTRODUCTION

Soil is an essential part of the terrestrial ecosystem, providing fundamental support for all forms of terrestrial life. To prevent erosion, infertility, pollution with hard-to-degrade materials, groundwater contamination, and loss of biodiversity, adequate soil protection programs are necessary. The quality of the soil is linked to its biological properties, which can be influenced by changes in the environment. Micro-populations within the soil are sensitive and respond rapidly to any environmental shifts. Profiles of soil microbiota and enzymatic systems are closely correlated, serving as indicators of health and quality (Pajares *et al*., 2011; Joshi *et al.*, 2018). Enzymes, primarily synthesized by microorganisms in the soil, play a

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key role in nutrient cycling and are reflective of microbiological activity and fertility (Benítez *et al*., 2000). Assessing enzymatic activity at this level can reveal information about changes occurring within the soil's structure, even long before alterations in its chemical composition or physical properties become apparent (Nannipieri *et al*., 2018). Biochemical processes in soil are catalyzed by a series of enzymes, including glucosidases, xylosidases, amylase, dehydrogenase, chitinase, urease, proteases, phenol oxidases, aminopeptidases, and phosphatases. Recognized as reliable indicators of soil quality, soil enzymes reflect the underlying biology of the soil and are closely correlated with its health and functionality (Alkorta *et al*., 2003; Karaca *et al*., 2010). These enzymes, synthesized by microorganisms within the soil, play vital roles in nutrient cycling and fertility, and their assessment through enzymatic activity assays provides insights into the ecosystem health status of soils. Building on this understanding of soil enzymes and their role in ecosystem health, there has been a growing interest in exploring innovative approaches to enhance soil quality and functionality. One such approach involves the integration of natural enzymatic mechanisms with advanced materials, such as oxidic nanostructures.

Combining one of the most efficient and fragile natural mechanisms (the enzymatic one) with a well-defined and promising oxidic (tubular/1D) nanostructure represents a valuable direction for obtaining hybrid systems. Many attempts have been made to immobilize and encapsulate biologically active species, especially enzymes, in suitable inorganic matrices, usually mesoporous silica with pore diameters ranging from 15–300 Å, compatible with enzyme molecular diameters (Takahashi *et al*., 2000).

The aim of this research was to develop a hybrid system composed of synthesized titanium oxide and silica nanostructures onto which halophilic bacterial proteases were immobilized. This hybrid system was evaluated to ascertain its effect on soil fertility, particularly to determine its capability to influence the biodegradation rate in anthropized soils. To evaluate the biodegradation rate, activities of alkaline phosphatase, urease, and dehydrogenase in anthropized soil samples were monitored. Produced by halophilic bacteria, these enzymes are relevant for studying in situ processes and act as indicators of the anthropized soil condition, offering insights into their utility in potential biotechnological processes aimed at improving soil fertility.

MATERIALS AND METHODS

SAMPLING AND SITE DESCRIPTION

For the functionalization of oxide nanostructures with bacterial enzymes, nanomaterials based on titanium dioxide and silica were utilized. The synthesis of SiO_2 nanotubes was conducted using the sol-gel method, as detailed in our

previously published results (Anastasescu, 2009; Anastasescu, 2010). Similarly, the synthesis of TiO_2 powders was achieved through the sol-gel method followed by hydrothermal treatment to obtain nanotubes (Preda *et al*., 2013). The tested samples consisted of ten different synthesized nanomaterials, represented by titanium dioxide and silica materials, labeled as follows: S1 – sample of titanate nanotubes (NaTNT/135°C/72h AIE), S2 – sample of titanate nanotubes (NaTNT 135°C/72h BIE), S3 – sample of copper-doped titanate nanotubes (Cu-TiNT), S4 – sample of titanate nanorods (NaTNR 175°C/72h HT), S5 – sample of titanate nanorods (NaTINR/H/175°C, 72h), S6 – sample of silica nanotubes (SiO_2-t), S7 – sample of chemically functionalized silica nanotubes (SiO_2tF), S8 – sample of large silica nanotubes (SiO_2-T), S9 – sample of silica nanotubes modified with direct Au ($AuSiO_2$t), S10 – sample of chemically functionalized silica nanotubes modified with Au ($AuSiO_2$tF). The source of the proteolytic enzymes was derived from the halophilic strain IBB37, isolated from a mud sample collected from Lake Movila Miresei, the saltiest lake in Romania, located in Braila county. The soil used for experimental tests, collected from the Paltinu area in Prahova county, Romania, was identified as eubasic brown mountain forest soil. The A horizon (uppermost layer) of this soil was characterized by brown humus, weak-gray, well-structured, glomerular-granular in nature. Soil samples were collected from a depth of 10 cm, chosen for its representativeness of biological activity and chemical composition. The samples were stored at 4°C in a refrigerator. The methodology for determining the soil's pH was applied according to ISO guideline 17152-1 (ISO, 2008), involving suspending the soil in a 1 M KCl solution (1:5 w/v) and using a pre-calibrated HANNA HI 4522 pH meter. The analyzed soil had a pH of 8.

BACTERIAL STRAINS, MEDIA, AND GROWTH CONDITIONS

For the experimental procedures, the halophilic bacterial strain IBB37 was selected based on its ability to produce proteases. This strain was cultivated on MH medium, composed of the following constituents (g/L): yeast extract, 10; proteose peptone, 5; glucose, 1; $MgCl_2 \cdot 6H_2O$, 7; $MgSO_4 \cdot 7H_2O$, 9.6; $CaCl_2 \cdot 2H_2O$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.026; and NaCl, 100 (Ventosa *et al*., 1972). The strain IBB37 was isolated through the pour plate method (Leboffe & Pierce, 2012) from a sediment sample collected from Movila Miresii Salt Lake, located approximately 170 kilometers east of Bucharest, in Eastern Romania.

MOLECULAR IDENTIFICATION OF THE WILD BACTERIAL STRAIN

Genomic DNA was extracted using a Nucleospin Tissue kit (Macherey-Nagel) following the manufacturer's instructions. The 16S rDNA gene sequence was amplified by PCR with universal primers: 27F (AGAGTTTGATCACTGGCTCAG) and 1492R (ACGGCTTACCTTGTTACGACTT) (Stancu, 2015; Marchesi *et al.*,

1998) which allowed the amplification of a 1500 bp DNA fragment. 1 µL of genomic DNA was used for PCR amplification in a total volume of 25 µL reaction mixture, containing a mixture of dNTP, MgCl2, universal bacterial primers 27F and 1492R, and Taq polymerase (Promega), dissolved in suitable buffer. For the amplification reactions, the Mastercycler pro S (Eppendorf) equipment was used. The PCR reaction steps were as follows: 10 minutes of initial denaturation at 94°C; 30 cycles of denaturation at 94°C for 1 minute; 30 seconds of hybridization at 55°C; 2 minutes of extension at 72°C; and 10 minutes of final extension at 72°C. Amplicons stained with Red Safe were electrophoresed on a 1% (wt/vol) agarose gel and documented under UV-transillumination, in the presence of a molecular weight-size marker from New England Biolabs. The obtained PCR products were purified with a PureLink® PCR Purification kit (Invitrogen) and then sequenced with an ABI 3730XL sequencer by CeMIA SA (Larissa, Greece).

BIOSYNTHESIS OF EXOENZYMES

The capacity of the bacterial strain IBB37 to produce proteolytic enzymes was tested by spot seeding on MH medium agarized with a concentration of 10% NaCl and 1% casein (Rohban *et al*., 2009). The enzymatic activity level (LEA) of the bacterial sample was determined based on the ratio of the hydrolysis zone diameter to the bacterial colony diameter in millimeters, following the approach of Menasria (Menasria *et al*., 2018). The categorization of enzymatic activity levels was defined as low for an LEA less than 1.3, medium for an LEA between 1.31 and 1.99, and high for an LEA exceeding 2.

PURIFICATION OF EXTRACELLULAR PROTEOLYTIC ENZYMES

Proteases biosynthesis was performed in MH liquid medium supplemented with 10% NaCl and 1% casein (Cojoc *et al.*, 2009). The medium was seeded with an inoculum from a liquid bacterial culture in the exponential growth phase at a ratio of 9:1. Following the growth of the bacterial culture and the biosynthesis of the enzyme, the culture liquid was subjected to centrifugation at a speed of 9500 rpm, at a temperature of 4°C, for a duration of 20 minutes. Separation and purification of the exoenzymes were accomplished by precipitation with acetone followed by gel filtration. The protease activity was determined by the modified Anson method for casein, dosing the hydrolysis products released from casein under the action of the enzyme with the Folin-Ciocâlteu color reagent. Enzyme activity was expressed in units/mg protein (U/mg). One unit of proteolytic activity is defined as that amount of enzyme that releases 1 µmol of tyrosine per minute. The reaction mixture, consisting of 400 µL of 0.5% casein in 50 mM Tris-HCl, pH 8 and 10% NaCl, was incubated with 100 µL of enzyme extract, at 30°C, for 30 minutes. The enzymatic reaction was stopped by the addition of 500 µL of

10% trichloroacetic acid. After 10 minutes of incubation at room temperature, the samples were filtered through Whatman 1 paper. The obtained filtrate was used for the color reaction. The samples, containing 1 mL filtrate, 1 mL 0.2 N HCl, 4 mL 0.5N NaOH solution and 1.2 mL Folin Ciocâlteu reagent diluted 1:2, were vortexed and incubated for 30 minutes at room temperature to perfect the color, then the extinction was read at 578 nm versus distilled water. The µmoles of tyrosine corresponding to the measured extinction were determined from the calibration curve. Protein concentration was determined by the method of Lowry (Lowry *et al*., 1951) and expressed as mg protein/mL liquid.

THE IMMOBILIZATION CAPACITY OF PROTEOLYTIC ENZYMES ON INORGANIC SUPPORT

The immobilization of proteolytic enzymes onto an inorganic support was achieved through the direct absorption method, resulting in a hybrid system. This immobilization process involved contacting 0.05g of the nanomaterial with 5 mL of partially purified enzyme extract. The coupling was conducted at a temperature of 4°C over a period of 24 hours. Following decantation, the preparation was washed with 10 mL of a 0.05M Tris-HCl buffer solution at pH 8. The immobilized product was subsequently stored in the refrigerator in 50 mL of the same buffer solution. To determine the immobilization capacity, the protein was dosed by the method of Lowry in the influent (initial enzyme solution), effluent (decanted solution after coupling), and wash buffer solutions. The difference between the initial amount of enzyme and the excess amount was then calculated, and by reporting the amount of enzyme fixed to 1g of support, the immobilization capacity was determined (mg enzyme/gram of support).

HYBRID SYSTEM FUNCTIONALITY TESTING

The hybrid system, created by immobilizing the target enzymes on various inorganic supports composed of titanium dioxide and silica, was evaluated by coupling it in a 1:1 ratio with soil, utilized as an experimental model. The assessment of the biodegradation rate involved monitoring the activities of alkaline phosphatase, urease, and dehydrogenase within the soil sample. This was done both in conjunction with the hybrid system and in soil treated solely with the oxidic matrix, following a 72-hour coupling period. Additionally, enzymatic activities were determined in an untreated soil sample.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

The activity of alkaline phosphatase was assessed in both treated and untreated soil samples, utilizing p-nitrophenyl phosphate as a substrate. This

measurement was conducted using the spectrophotometric method described by Tabatabai (Tabatabai *et al.*, 1969). The amount of p-nitrophenol released per unit time, determined in the alkaline medium at 400 nm, is considered a measure of phosphatase activity. Thus, 1g of soil sample was incubated in a water bath at 37°C/1h together with 4mL Tris-HCl 0.05 M pH 11 and 1 mL p-nitrophenyl phosphate 0.115 M. After the incubation step, 1 mL of 0.5M $CaCl_2$ and 4 mL of 0.5M NaOH were added in each tube with reaction mixture. The final reaction mixture was filtered and the concentration of p-nitrophenol in the analyzed samples was determined measuring the absorbance values at 400 nm. Enzyme activity was expressed as µmole/h/mL.

UREASE ACTIVITY ASSAY

Urease activity was evaluated using the Nessler reagent method, a process that relies on the colorimetric quantification of ammonia produced as a result of the enzyme's action on urea. Within an alkaline environment, the Nessler reagent reacts with ammonia to form a yellow-orange complex. The intensity of this coloration is directly proportional to the enzyme activity, providing a measurable indication of the urease function. The reaction mixture contained: 4 mL Tris-HCl buffer solution 0.05M, pH 8, 0.5 mL urea solution 0.06M in Tris-HCl buffer solution, 0.5 g of the sample to be analyzed. The mixture was homogenized and incubated 20 min/37°C. The reaction was stopped by adding 1 mL of 10% trichloroacetic acid solution. Denatured proteins were removed by filtration, and ammonia was measured by mixing 3 mL of the filtrate with 0.5 mL of Nessler's reagent. The sample was stirred and the optical density was determined at 436 nm against distilled water. In parallel, a control sample was also made which was treated under the same conditions, but in which the enzyme reaction was stopped before adding the enzyme source. The unit of urease activity was defined as the amount of enzyme that forms 1 µmol of ammonia within 1 minute at 37°C.

DEHYDROGENASE ACTIVITY ASSAY

Soil dehydrogenase activity was determined using the Casida (1977) method. The reaction mixture consisted of 3 g of soil, 1 mL of 3% glucose, 0.5 mL of 3% triphenyl tetrazolium chloride, and 1 mL of distilled water. In a different test tube, the same mixture was used, but glucose was omitted. The obtained preparations were incubated for 24 hours at 37°C, after which the formazane was extracted with ethanol and the mixture was filtered. The optical density of the filtrate was measured at 490 nm using ethanol as a blank. The formazan concentration was determined based on a standard curve of triphenyl formazan chloride. Enzymatic activity was expressed as µg formazan/g sol.

RESULTS AND DISCUSSIONS

PHYLOGENETIC EXAMINATION OF THE BACTERIAL ISOLATE IBB37

The purified strain isolated from the hypersaline lake Movila Miresii showed Gram-negative rods with beige colonies, glossy, flat, and straight edges and was selected for subsequent experiments based on the high proteolytic activity, a fast growth rate, the capacity to grow over a wide range of salinities (0–4M NaCl), and the ability to synthesize various exoenzymes. The organism showed a similarity of 96.48% with that of *Salinivibrio costicola* subsp. *alkaliphilus.*

BIOSYNTHESIS AND PURIFICATION OF EXTRACELLULAR PROTEOLYTIC ENZYMES

Experimental results demonstrated the ability of the bacterial strain IBB37 to produce proteolytic enzymes, evidenced by the clarification of the culture medium and the formation of a precipitation ring around the culture spot, suggesting significant proteolytic activity. According to the methods described by Rohban (2009), the hydrolysis is depicted in Figure 1, where the precipitation ring serves as a direct indicator of the enzymatic activity of the strain IBB37. Additionally, the calculation of the hydrolysis index, following the formula, yielded a value of 3.3, thereby classifying the proteolytic activity of strain IBB37 as high. The purified protease exhibits enzymatic activity across a pH range of 6 to 8, with an optimum activity at a pH value of 8, indicating its classification as an alkaline protease. It demonstrates enzymatic activity within a temperature range of 15 to 60°C, achieving optimal activity at 30°C. Furthermore, the enzyme retains its activity in the presence of NaCl, across a concentration range of 0 to 3M, with optimal activity observed within a concentration range of 0 to 2M.

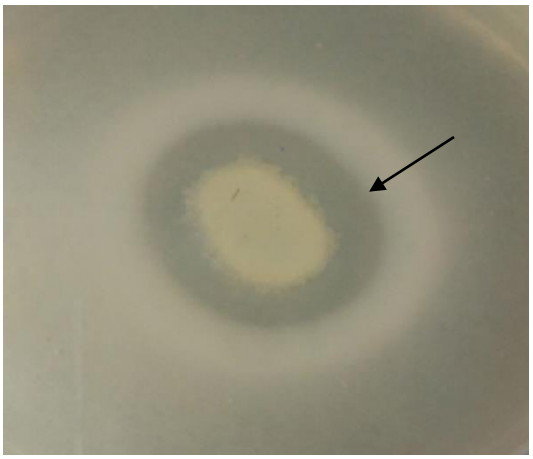

Figure 1. The ability of the bacterial strain IBB37 to produce proteolytic enzymes.

The stages of isolating extracellular protease from the *Salinivibrio* sp. IBB37 strain are detailed in Table 1. Initially, the crude enzyme, with a specific activity of 0.8 U/mg, underwent a first purification step using acetone extraction, which led to a 2.5-fold improvement in specific activity. Subsequently, through gel filtration, there was a significant increase in specific activity up to 8.9 U/mg, marking a cumulative purification of 11.2-fold compared to the initial extract.

Table 1

Purification steps of extracellular protease from Salinivibrio sp. IBB37 bacterial strain

Purification steps	Total protein (mg)	Enzymatic activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	1042.5	834	0.8	1	100
Acetone precipitation	364.4	720.7	2	2.5	86.4
BioGel S100	57.1	510.5	8.9	11.2	61.2

THE IMMOBILIZATION CAPACITY OF PROTEOLYTIC ENZYMES ON INORGANIC SUPPORT

The investigation into the immobilization capabilities of titanium dioxide (TiO_2) and silica (SiO_2) as inorganic supports for proteolytic enzymes reveals distinct differences in efficiency and capacity, as illustrated in Figure 2.

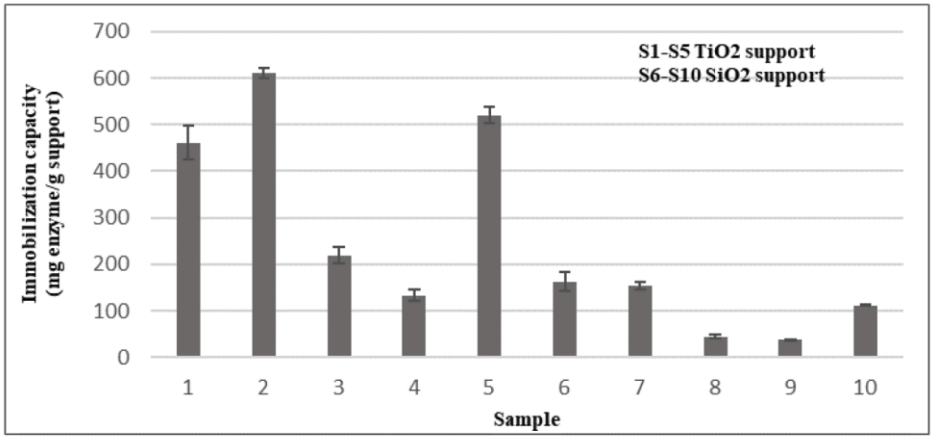

Figure 2. The capacity of different inorganic support to immobilize proteolytic enzymes. The bars in the histogram indicate the average value with its corresponding standard deviation derived from two repeated measurements.

In our study's exploration of enzyme immobilization on TiO_2 and SiO_2 supports, we observed a broad range of capacities, particularly for TiO_2, which

varied from 144.64 mg enzyme/g support in sample S4 to 621.3 mg enzyme/g support in sample S2. This variation not only highlights the significant potential of TiO_2 materials in enzyme immobilization but also suggests underlying mechanisms at play, which might be attributed to the specific structural and porosity characteristics of TiO_2. These properties likely facilitate more effective interactions with proteolytic enzymes, enhancing their immobilization capacity. In contrast, SiO_2 supports demonstrated lower and more uniform capacities, with the highest at 182.14 mg enzyme/g support (sample S6) and the lowest at 39.28 mg enzyme/g support (sample S9). This indicates that while SiO_2 supports are consistent, they may offer less optimal conditions for enzyme interaction compared to TiO_2, potentially due to differences in surface chemistry and porosity that impact the enzyme's ability to bind effectively. The superior performance of TiO_2 supports can be linked to their ability to offer a more conducive environment for enzyme immobilization. This is in line with the findings of HU *et al*. (2015), who emphasized the critical role of nanosupport characteristics in improving enzyme stability and activity. The high immobilization capacities we observed for TiO_2 supports resonate with these studies, suggesting that the specific interaction between the enzyme molecules and the oxidic surfaces of TiO_2 plays a pivotal role in the observed efficiency. Moreover, the variation in immobilization capacities among the TiO_2 samples and the more uniform performance of SiO_2 supports underscore the importance of material selection and functionalization in enzyme immobilization strategies. Our findings extend the conversation about the applicability of such technologies in agriculture, as indicated by SINHA & KHARE (2015), by demonstrating the enhanced potential of TiO_2 in supporting enzyme activity and stability under variable environmental conditions.

TESTING THE FUNCTIONALITY OF THE OBTAINED HYBRID SYSTEM

In this investigation, we assessed the activity of three key soil enzymes – alkaline phosphatase, urease, and dehydrogenase – in anthropized soils treated with titanium dioxide, silica nanostructures, and hybrid systems. These enzymes are crucial in soil metabolism and nutrient cycling, thereby acting as indicators of soil health and biodegradation efficacy. Alkaline phosphatase, essential for breaking down phosphate esters to facilitate nutrient recycling, showed varying levels of activity in soils treated with titanium dioxide and silica, from 0.267 µmole/h/mL in Soil-S9 to 0.542 µmole/h/mL in Soil-S4, as Table 2 illustrates. In soils incorporating the hybrid system, alkaline phosphatase's activity was more consistent, ranging between 0.634 and 0.781 µmole/h/mL. This uniformity suggests that the hybrid systems may facilitate more stable enzyme-support interactions, potentially making them more suitable for applications that rely on steady biodegradation processes (Table 3).

Table 2

Enzyme activities in the soil sample coupled with the titanium dioxide and silica nanostructures

Sample	Alkaline phosphatase activity (µmole/h/mL)	Urease activity (µmole/mL·min)	Dehydrogenase activity (µg formazan/g soil)
Soil	0.514	0.057	3.065
Soil-S1	0.428	0.172	3.466
Soil-S2	0.381	0.095	5.737
Soil-S3	0.424	0.060	4.273
Soil-S4	0.542	0.131	1.866
Soil-S5	0.505	0.115	5.089
Soil-S6	0.363	0.159	4.052
Soil-S7	0.374	0.183	1.885
Soil-S8	0.390	0.189	5.798
Soil-S9	0.267	0.241	2.611
Soil-S10	0.298	0.086	6.411

Table 3

Enzymatic activities in the soil sample coupled with the hybrid system formed based on the immobilization of proteases on the inorganic support

Sample	Alkaline phosphatase activity (µmole/h/mL)	Urease activity (µmole/mL·min)	Dehydrogenase activity (µg formazan/g soil)
S1	0.751	0.192	2.481
S2	0.781	0.204	3.679
S3	0.719	0.125	2.989
S4	0.741	0.137	2.795
S5	0.692	0.118	3.226
S6	0.683	0.173	2.843
S7	0.702	0.278	2.756
S8	0.713	0.295	3.015
S9	0.7	0.315	3.054
S10	0.634	0.142	3.1

Urease, which catalyzes the conversion of urea into ammonia and carbon dioxide, exhibited activity levels from 0.057 to 0.241 µmole/mL·min in soils coupled with nanostructures, as shown in Table 2. This variation underscores the diverse ways in which urease interacts with different nanostructures, with samples like Soil-S9 displaying increased activity, possibly due to more effective enzyme-nanostructure engagement. These observations underscore the critical nature of enzyme-nanostructure interactions for achieving optimal biodegradation outcomes. In the hybrid system, detailed in Table 3, urease activity was observed to range from 0.125 to 0.315 µmole/mL·min, with samples S8 and S9 exhibiting the highest activities. This suggests that certain hybrid configurations may optimize enzyme performance. However, the reduced activity in sample S3 hints at less synergistic enzyme-support interactions, which could be influenced by the specific characteristics of the inorganic support or the immobilization approach.

Dehydrogenase activity, a marker of microbial life in soil, was recorded between 1.866 and 5.798 µg formazan/g soil in samples with nanostructures (Table 2), pointing to significant variations in enzyme interaction with different supports. Samples S2 and S8 showed superior activities, suggesting optimal enzyme-support engagements. Meanwhile, within the hybrid system, as Table 3 reveals, dehydrogenase activity demonstrated a tighter range from 2.481 to 3.679 µg formazan/g soil, indicating a more regulated interaction landscape that could benefit consistent biodegradation applications.

The data presented in this study shine a light on the selective sensitivity of enzymes to various nanostructured supports and illustrate the hybrid systems' potential as effective platforms for facilitating controlled biodegradation.

CONCLUSIONS

This study has provided a comprehensive evaluation of the enzymatic activities of alkaline phosphatase, urease, and dehydrogenase in saline soils, coupled with titanium dioxide, silica nanostructures, and hybrid systems. The findings reveal the complex and nuanced interactions between these enzymes and various nanostructures, shedding light on their roles in soil metabolism, nutrient cycling, and biodegradation processes. The variation in alkaline phosphatase activity across different nanostructures emphasizes the enzyme's sensitivity and potential for controlled biodegradation. Urease activity, with its diverse range, underscores the importance of understanding enzyme-nanostructure interactions for achieving desired outcomes in saline soils. The dehydrogenase activity results highlight the need for strategic selection of nanostructures to optimize biodegradation rates. The hybrid system, in particular, demonstrated a more uniform enzymatic activity, suggesting a stable and controlled environment that may be advantageous for applications requiring consistent biodegradation processes. This uniformity aligns with the study's goal of controlled biodegradation and may contribute to efficiency in soils. In conclusion, the insights gained from this study contribute to the broader understanding of enzyme-nanostructure interactions and their implications for soil quality enhancement and biodegradation processes.

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