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Effect of Marine Microorganisms on Limestone as an Approach for Calcareous Soil

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Received: 5 April 2018; Accepted: 15 June 2018; Published: 19 June 2018

Abstract: Calcareous soils generally have low levels of organic matter and nitrogen; they require modification to promote their support for agriculture production. Calcareous soils are commonly found in important agricultural areas throughout the world, mainly around the Mediterranean, America and Australia. In this study, we the isolated and identified different groups of microorganisms, from a product made from seaweed, in relation to their soil improvement properties. The objective was to use these microorganisms for the solubilization of specific soil elements and reduce their accumulation as a result of overfertilization. The isolated microorganisms were grown in specific culture media and were applied on limestone to determine their effect on mobility of Ca, Mg and K. Also, changes in soil properties such as pH, texture and density were evaluated. This study demonstrated that the treatments applied were able to modify the solubility of Ca, Mg and K, increasing it, in some cases, up to 3500%. In addition, an increase of organic matter close to 200% was observed. Both the group of molds and yeasts, and the group of nitrogen-fixing microorganisms, modified the proportion of sand, silt and clay in the treated limestone. These results open possibilities for the widespread use of marine microorganisms on a large scale in the agricultural sector, since they improve the nutrient availability present in the soil.

Keywords: cation mobility; edaphic properties; isolation microorganisms; Algaenzims®

1. Introduction

Soil degradation, by acidification/basification and salinization, is the main consequence of intensive agricultural techniques [1]. This is even more significant in calcareous soils, which are distributed all around the world. For example, they are present near the Mediterranean in North Africa, North America (USA, Mexico), South America (Argentina, Chile) and Australia [2]. Nowadays, many efforts to recover the productivity and fertility of soils are being undertaken. Technologies that facilitate the absorption of some nutrients and reduce the effects of soil degradation are the most recurring [3–5].

From an environmental point of view, mineral sources allow the solving of serious problems in agricultural production, of which reduction of acidity, erosion, soil salinity and loss of nutrients are the most representative. In recent years, the role of microorganisms in the physicochemical modification of soil has been investigated. This modification is affected by the translocation of the metal cations in the soil [6,7].

On the other hand, organic sources are the core of organic fertilizers due to their capacity to supply nutrients to crops and improve the properties of agricultural land [8,9]. However, when the crops suffer a lack of nutrients from the organic sources, it is necessary to incorporate chemical fertilizers.

Nowadays, the proper handling of the relationship between microorganisms, plants and soil has become a promising biotechnology alternative for generating cleaner and more sustainable production systems [10,11]. Therefore, the knowledge of microbial diversity can help restoration systems to develop themselves. Microorganisms promote the growth of plants by their nutritional intake or by improving soil features through a better aggregation of particles, increasing soil retention, porosity, water retention and erosion control [12].

In this sense, biofertilizers have attracted attention due to their organic properties and efficient nutrient liberation. These characteristics make them a promising option for the substitution of synthetic fertilizers.

Algaenzims® is a commercial biofertilizer made from marine algae of the *Sargassum* genus, which has many microorganisms that remain viable after the manufacturing process of the product. Algaenzims® has shown remarkable improvement in the growth of different crops: at least 65% increase in peanut production, 100% in sweet potato and up to 50% in lucerne and serrano chili [13]. This effect is attributed to the high microbial load, which has an active role in the results and benefits of the product. Thus, it is important to identify the effect of such microorganisms on soil characteristics, either by modifying its texture or structure.

Furthermore, in Mexico there are large surfaces of calcareous soils that represent important challenges. To attend to these problems, in this study, the isolation and identification of different groups of microorganisms from a product made from seaweed are presented. In addition, the effect of added microorganisms on soils was determined. Since high heterogeneity of natural soils induces interference in and low reproducibility of results, it is common to use model soils, as done by Celis et al. [14], Kuyukina et al. [15] and Hwang et al. [16]. For this reason, in this study, it was decided to use limestone as a model of an extremely calcareous soil.

2. Materials and Methods

2.1. Isolation of Microbial Groups from Algaenzims®

The microorganism isolation from Algaenzims® was performed by selecting the proper growth medium in order to obtain the nutritional and environmental conditions to promote the growth of the different kinds of microorganisms. Nutritive agar for Mesophilic Aerobic Bacteria (MAB), Sabouraud agar for Yeasts and Molds (YM), agar for Nitrogen-Fixing microorganisms (NF) and halophilic agar for Halophilic bacteria (HALO) were used.

Standard methods for agar formulations were followed. For nitrogen fixing microorganisms, the culture medium was prepared using dibasic potassium phosphate (2 g L^{-1}), D-mannitol (20 g L^{-1}) and bacterial agar (20 g L^{-1}). The culture medium for halophilic microorganisms was formulated using sodium citrate (10 g L^{-1}), sodium thiosulfate (10 g L^{-1}), sucrose (20 g L^{-1}), sodium chloride (25 g L^{-1}), ferric chloride (1 g L^{-1}), dibasic potassium phosphate (2 g L^{-1}), magnesium sulfate (5 g L^{-1}) and agar (20 g L^{-1}).

Applying the serial dilution in peptone water method (10^{-1} to 10^{-10}), the isolation of different groups of microbes from Algaenzims® was carried out. A volume of 1 mL of each dilution was transferred to Petri dishes which contained the corresponding culture medium. The dishes were incubated at the following temperatures: nutritive agar at 37 °C for 24 h, Sabouraud agar at 25 °C for 48 h and HALO and the nitrogen fixing agar at room temperature for 1 week. After incubation, quantitative measurements of microbial colonies were performed. This procedure made it possible to detect the limit dilution at which the microbial growth was shown; it also made it possible to determine the microbial load in the initial sample [17,18].

2.2. Strain Isolation, Propagation and First Characterization

Using selective agars inoculated with the strains coming from the microbial count, a qualitative study for morphological characterization of the different colonies was made. Microorganisms that showed different morphology were isolated and incubated in Petri dishes at the temperatures and times indicated above. After the incubation, the strains were reseeded in culture tubes. This step was carried out in order to obtain pure strains for preservation, morphology analysis (color, elevation and shape) and inoculation of the medium applied to the limestone. Strain typification was carried out by biochemical tests and Gram-stain using Phenotype MicroArray™ of BIOLOG system. The propagation of the microbial groups isolated from Algaenzims® was made using a nutritive broth for YM and MAB; and halophilic and nitrogen fixers broths, for HALO and FN microbial groups, respectively, for which the same formulations were used as for solid culture media without adding agar.

The isolated strains were kept on Difco brand Brain Heart Infusion agar, which was prepared according to the manufacturer's instructions, incubated at 35 °C until visible growth was observed, and then kept in refrigeration at 4 °C.

2.3. Limestone Pretreatment

The limestone was dried and sterilized at 120 °C for 2 h. After that, the limestone was stored in plastic bags.

2.4. Microbial Treatments

The first four treatments (T1, T2, T3 and T4) consisted of the addition of 300 mL of the different microbial group inoculums (MAB, YM, NF and HALO, respectively) into the 1 L capacity plastic pots with limestone (1 kg per pot). The concentration of the inoculums added was 1% of the initial field capacity (36 mL inoculum in 3.6 L distilled water). T5 treatment consisted of the addition of 300 mL of Algaenzims® at the same concentration. The Control treatment consisted of placing 1 kg of the material in identical pots, to which distilled water was added at the same time and the same volume as the rest of the treatments. A plastic container was placed at the base of each pot to recover the leachates; the assays were replicated 4 times, placing the pots in a completely random arrangement. This study did not consider a treatment using only the culture medium. The reason for this was to avoid the possibility that some adventitious microorganisms, present in the study area, had been incubated and grown in the system. On the other hand, it is unlikely that the influence of the composition of the culture medium was significant in the results, since the microbial culture was applied in a dilution of 1% in water.

Each month, reinforcements of all treatments in the same amount and concentration as the first application were applied. The pots were kept in treatment for four months, covered in plastic simulating a greenhouse.

Limestone samples were taken every month. Quantification of microbial groups was carried out using the technique previously described for the isolation and quantification of microorganisms from Algaenzims®. Leachates were recovered at the final of the assays to quantify K, Ca and Mg by atomic absorption (Varian AA240FS) using an acetylene flow of 7.40 L min^{-1} and a flow of 10.0 L min^{-1} of nitrous oxide and calibrating the optical parameters according to the manufacturer's recommendations.

2.5. Limestone Soil Assessments

Texture is one of the most basic physical properties of soils; the different contents of specific particles have very different hydraulic characteristics of the soil, such as water retention and hydraulic conductivity [19]. At the end of the assays, samples (50 g) of limestone from each treatment were collected. The soil texture was determined by sieving with mesh: 20, 50, 60, 80, 100, 140, 200 and >200 (RO-TAP Ws-Tyler Rx 29), shaking for 5 min [20,21]. The 20–100 sieved fraction (>0.15 mm) was considered as sand, 140–200 (<0.15 and >0.075 mm) as silt and <200 (<0.075 mm) as clay.

The pH of the limestone was determined following the protocol described by Motsara and Roy [21] using a pH-meter Orion 420A.

The bulk density (Db) of limestone was determined by a standard method as follows: first an empty 100 mL beaker was weighed, afterwards, a beaker with limestone (gently compacted) was weighed, finally the mass of the limestone was calculated by difference. The corresponding density was obtained, dividing the mass by 100 mL.

Organic matter was determined by placing 4 g of limestone in an Erlenmeyer flask which contained 10 mL of $K_2Cr_2O_7$ 1N. After that, the mixture was softly stirred, and 20 mL of concentrated H_2SO_4 was added, and the stirring continued for 10 min. Then, 100 mL of distilled water was added, and the suspension was filtered. The filtrate was analyzed by spectrophotometry at 610 nm [22,23].

The collected data were processed with Microsoft Excel 2013 for graphing and obtaining measures of central tendency and dispersion.

3. Results

3.1. Isolation of Microbial Groups from Algaenzims®

As observed in Table 1, the aforementioned groups of microorganisms were found. The low growth shown for some microorganisms (i.e., mold and yeast) may be due to inhibition by lack of optimal conditions for its proper adaptation in this stage. The presence of these different microbial groups is due to the raw material used for producing Algaenzims®, and they can play an important role as plant growth stimulators.

Table 1. Microbial counts in Algaenzims®.

Microbial Group	CFU mL^{-1}
Mesophilic aerobes	4.90×10^3
Yeast and Molds	5.00×10^1
Nitrogen fixers	2.50×10^4
Halophilous	1.00×10^3

Table 2 shows the identification of the 22 microorganisms isolated from Algaenzims®. As observed, the diversity of viable microorganisms found in the product is wide, which may explain part of its fertilizing activity.

Table 2. Characterization of strains isolated from the Algaenzims® product by biochemical tests.

Key	Microorganism or Assigned Key
MAB-4	*Escherichia vulneris*
MAB-5	*Enterobacter nimipressuratis*
MAB-6	*Corynebacterium nitrilophilus*
MAB-7	*Xenorhabdus nematophilus*
MAB-8	*Serratia marsecens*
MAB-9	*Hafnia alvel BioGroup 1*
MAB-10	*Bacillus thermoglucosidasius*
NF-2	*Bacillus thermoglucosidasius*
NF-3	*Acinetobacter calcoaceticus*
NF-4	NF-4
NF-5	FN-5
YM-1	*Aquaspirillum dispar*
YM-2	*Brevundimimonas vesicularis*
YM-3	YM-3
YM-4	YM-4
HALO-1	*Bacillus thermoglucosidasius*
HALO-2	*Vibrio* spp.
HALO-3	HALO-3
HALO-4	HALO-4
HALO-5	HALO-5
HALO-6	HALO-6
HALO-7	HALO-7

3.2. Microbial Counts

Initial concentration of microorganisms inoculated in each treatment to limestone and the microbial counts determined each month for four months is presented in Table 3. The mesophilic aerobic bacteria (T1) showed an oscillation in the CFU g^{-1} of up to 4 orders of magnitude between months 2 to 3 and 3 to 4. This could be due to the main feature of the mesophilic microorganisms of developing optimally in temperatures near to 37 °C, being very susceptible to sudden changes in temperature. Regarding the group of yeasts and molds (T2), a stable growth could be observed during the four months, which suggests a good adaptation in the limestone. In the case of nitrogen-fixing microorganisms (T3), a good adaptation to the medium was observed, finding monthly microbial counts higher than those quantified at the start of the assay. Finally, in the treatment with halophilic bacteria (T4), the microorganism achieved a good adaptation, showing only a slight variation in the count of the second month.

Table 3. Counts of different microbial groups from limestone samples (CFU g^{-1}).

Treatment	Initial Count	Month 1	Month 2	Month 3	Month 4
T1	2.46×10^3	5.00×10^5	5.00×10^3	5.00×10^7	5.00×10^3
T2	3.00×10^3	3.70×10^5	1.80×10^5	2.50×10^5	5.50×10^5
T3	1.10×10^4	2.50×10^4	2.20×10^5	2.70×10^7	4.20×10^5
T4	1.26×10^4	2.50×10^4	2.50×10^3	2.40×10^5	3.90×10^5

T1: treatment with MAB; T2: treatment with YM, T3: treatment with NF, T4: treatment with HALO.

With respect to the treatment in which Algaenzims® was added to the limestone (T5), Table 4 shows the microbial counts determined throughout the trial. First, it was observed that both YM and NF have a good adaptation to the limestone; this was reflected in the microbial growth in the first month and the almost constant count during the following three months. On the other hand, the MAB presented a sustained growth during the first two months, after which the growth stopped, and the counts fell in the fourth month. The group of halophilic microorganisms (HALO) showed an increase in the first month followed by a significant decrease in the second month; however, from the third month, a progressive increase was observed, indicating a moderate adaptation of the microorganism. As could be observed, the different groups of microorganisms in Algaenzims® were better adapted together than individually.

Table 4. Counts of the different microbial groups in limestone treated with Algaenzims® (T5).

Microorganism in Algaenzims	Initial Count	Month 1	Month 2	Month 3	Month 4
MAB	5.00×10^1	5.00×10^4	5.00×10^5	5.00×10^5	5.00×10^3
YM	4.90×10^3	4.50×10^5	2.70×10^5	7.10×10^5	6.10×10^5
NF	2.50×10^4	5.00×10^5	3.30×10^5	2.27×10^6	8.00×10^5
HALO	1.00×10^3	2.50×10^8	1.00×10^4	8.50×10^5	7.60×10^5

3.3. Quantification of Ca, Mg and K in Leachates

The concentration of calcium, potassium and magnesium was quantified in the leachates at the end of the experiments. As can be seen in Figure 1, in all treatments there were a marked increase in the availability of Ca, Mg and K compared to the Control. In the case of calcium, its mobility was strongly enhanced when the limestone was treated with both MAB and Algaenzims®. Improvements were also observed both with the use of HALO and YM, while treatment with NF was not effective. In the case of magnesium, the increase in mobility was more notable when the treatment with YM was applied. The mobility of potassium was improved with all of the treatments applied. It is noted that there are microorganisms in the Algaenzims® able to solubilize calcium, magnesium and potassium in the soil, making them available for plants.

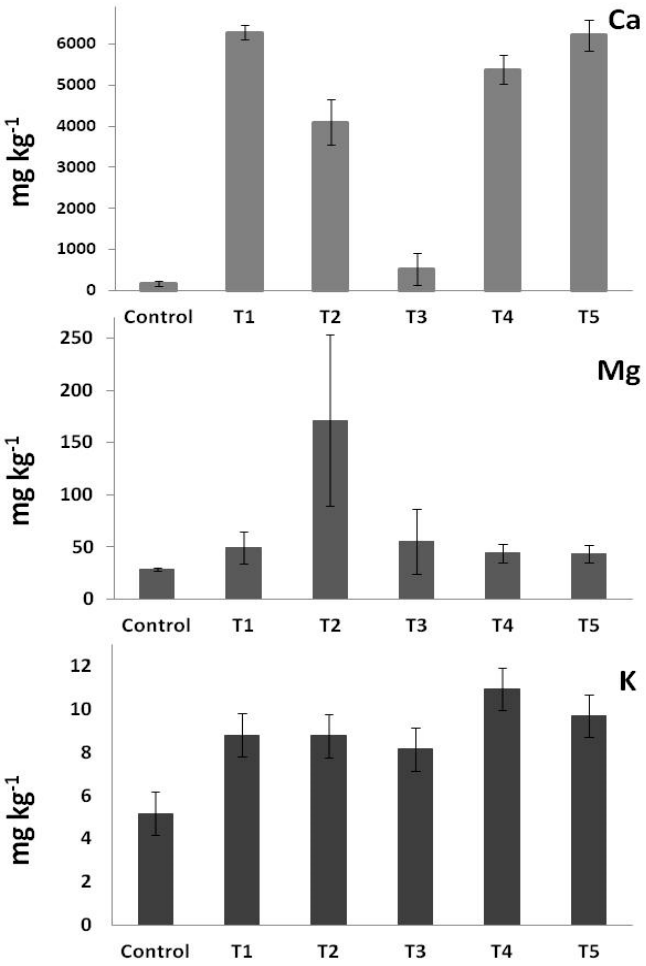

Figure 1. Concentration of Ca, Mg and K in the leachates of limestone treated (T1: mesophilic aerobic bacteria, T2: yeast and molds, T3: nitrogen fixer microorganisms, T4: halophilic bacteria and T5: Algaenzims®).

3.4. Limestone Soil Assessments

The texture of the limestone samples collected after four months for the different treatments presented the behavior shown in Table 5. It was observed that all treatments promoted the change of texture in soils compared with the Control. The most notable change was obtained with the YM (T2) treatment, exhibiting a 7.65% decrease in sand content and an increase in clay and silt fractions in all treatments.

Table 5. Textural composition of limestone treated after four months.

Treatment	Sand %	Clay %	Silt %
Control	88.33 ± 4.0	9.26 ± 3.0	2.41 ± 0.7
T1	83.31 ± 1.5	13.09 ± 1.0	3.60 ± 0.4
T2	81.57 ± 1.5	15.00 ± 1.0	3.44 ± 0.8
T3	81.83 ± 1.5	14.32 ± 0.5	3.85 ± 0.4
T4	83.30 ± 1.5	13.30 ± 0.5	3.40 ± 0.3
T5	86.22 ± 3.0	10.80 ± 2.0	2.98 ± 0.1

The final pH values of the limestone samples with the different treatments after four months are shown in Figure 2. It could be observed that there was a decrease in pH in the limestone treated with

FN and HALO compared to the Control, while the pH increased when the limestone was treated with MAB, AE and YM.

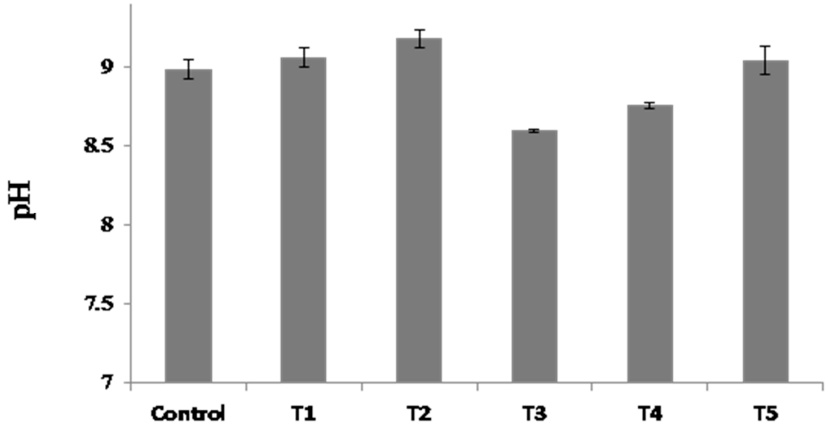

Figure 2. Values of pH of the treated limestone after four months (T1: mesophilic aerobic bacteria; T2: yeast and molds; T3: nitrogen fixer microorganisms; T4: halophilic bacteria and T5: Algaenzims®).

The values of bulk density determined for the Control and the treated limestone are shown in Table 6. It was observed that there were no significant changes in the values of the treated limestone compared to the Control after four months.

Table 6. Bulk density of control and limestone-treated samples.

Treatment	Bulk Density (g cm^{-3})
Control	1.711 ± 0.04
T1	1.651 ± 0.05
T2	1.683 ± 0.08
T3	1.754 ± 0.06
T4	1.716 ± 0.07
T5	1.683 ± 0.05

The amount of organic matter in the limestone after four months of having applied the different treatments is shown in Figure 3. A higher OM content could be observed in all the treated samples compared to the Control, especially for the T3 and T4 treatments, which showed 100% increases with respect to the Control.

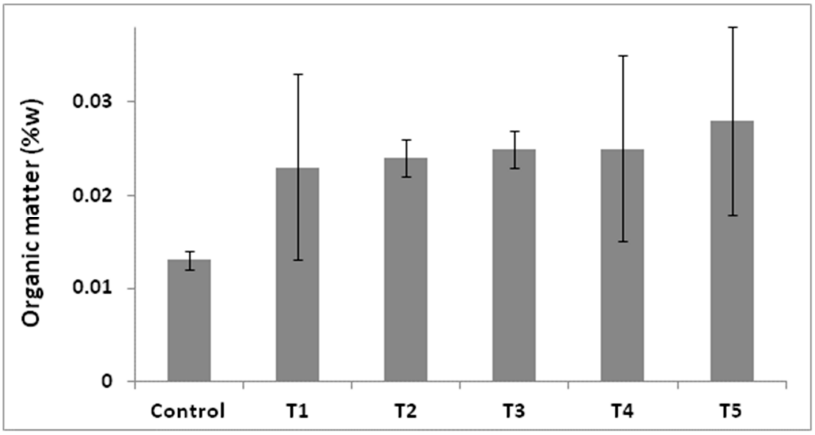

Figure 3. Amount of organic matter in the treated limestone (T1: mesophilic aerobic bacteria; T2: yeast and molds; T3: nitrogen fixer microorganisms; T4: halophilic bacteria and T5: Algaenzims®).

4. Discussion

4.1. Isolation of Microbial Groups from Algaenzims®

In recent years, there has been an increase in scientific attention on marine biodiversity, in which microorganisms are of great interest due to their metabolism and unique physiological capacities [24]. It is estimated that the epipelagic zone contains around 4.4×10^{28} microorganisms and more than 99% of the species have not been able to be cultivated yet [25]. This explains the large number and variety of microorganisms isolated from the studied product and the impossibility of characterizing some of them. Marine microorganisms have great potential for industrial applications; such is the case of the work developed by Pucci et al. [26], who isolated 403 strains from the Patagonian Sea with the capacity to metabolize hydrocarbons. On the other hand, Muñoz [27] isolated 17 strains with autotrophic capabilities, which implies the possibility of metabolizing inorganic molecules by synthesis or hydrolysis. Likewise, Jussie et al. [28] isolated 161 strains from the South Atlantic Ocean, of which 58 had cellulolytic and/or proteolytic properties. There are reports of microbial concentrations reaching up to 3.63×10^4 microbial cells per milliliter in the Colombian marine muddy snows [29]. León et al. [30] isolated 62 actinomycetes with antimicrobial activity in Ancon Bay, Peru. It is well known that many microorganisms are symbiotic with specific systems and contribute to the hydrolysis of organic and inorganic macromolecules [31].

4.2. Microbial Counts in the Treatments

The small variations observed in the counts could be due to changes in the environmental temperature that affected the humidity. However, the variation in the microbial counts was lower than that observed for T1, since the replication processes of the yeasts and molds are much slower than those of the bacteria [32].

As in T1, it was possible to notice an increase in microbial counts in the third month, although the evolution was slower because of the process of N_2 fixation of the atmosphere is slower than the assimilation of N_2 available for heterotrophy microorganisms such as those present in T1.

The small variations shown by T4, which could be due to environmental stress [32,33], coincide with the decreases in the microbial populations of T1 and T2 for the same period.

This may be due to Algaenzims® providing the nutrients required for the growth of each group of microorganisms through a synergistic association that allows them to achieve greater adaptation to adverse nutritional conditions.

4.3. Quantification of Ca, Mg and K in Leachates

The microorganisms present in Algaenzims® are able to solubilize calcium, magnesium and potassium in the soil, promoting their availability to the plants. This is important because the mobility of the cations (Mg, K and Ca) is of vital importance for the soil-plant interaction [6]. Of the studied elements, calcium is the most influential in the soils found in the semi-arid zones of northern Mexico, since it is present in high concentrations [34].

4.4. Limestone Soil Assessments

Texture is one of the parameters that influence the physiology of plants, since organic matter has a direct influence on the dynamics of carbon and nitrogen during water/drought cycles. In this test, the decrease in the percentage of sand and the consequent increase in the proportion of clay and silt in the treated limestone was observed. This effect may be due to the formation of aggregates, as well as the quantity and activity of colloids, type of dominant cations, presence of inorganic cements, quantity and type of organic matter, activity of microorganisms, wetting and drying of the soil. [35,36]. The ability to modify the texture of a soil is of great importance, since the texture affects the interaction of the soil

with water and with the salts present, as indicated by Fernández et al. [37,38]. Texture also affects the soil's ability to retain organic matter [39] and the biological activity of the microbial flora [4,40].

The decrease in pH may be due to the activity of soil microorganisms producing CO_2, to the humidification of organic matter due to the biological activity involved in the transformation of organic compounds and to the presence of basic cations produced by leaching. The increase in pH could be due to the formation of compounds between acidic materials with carbonates, displacing Ca and Mg, which, when free, form oxides and hydroxides of calcium and magnesium, respectively [35].

The treatments did not modify Bulk density. This may be due to the fact that limestone has low binder capacity and plasticity, as well as low water retention capacity and low chemical activity [41].

Organic matter plays an important role on fertility, structure and physical-chemical soil properties. The main factors that influence the amount of organic matter in the soil are humidity, temperature, pH, the availability of nutrients and the mineral nature of the soil. The notable increase of organic matter in the treated clay may be due to the fact that the microorganisms achieved a good adaptation in this substrate, which allowed their growth and proliferation causing an increase in the amount of organic matter. The results obtained agree with the results reported by Matus and Maire [36], who observed that soils with clay textures retain and contain a greater amount of organic matter than sandy soils. In the present study, the treatments used caused the change of texture towards a composition with a higher concentration of clay and less sand, which is consistent with that observed by Howard et al. [23].

Future research should be performed to study the possibility that isolated microorganisms have the capacity to mobilize other chemical elements and substances for agricultural use. It is also convenient to review the effects of microorganisms on model plants to rule out negative effects. Additionally, testing of a Control considering only the culture medium should be developed to ensure the effects of different treatments.

5. Conclusions

The isolated organisms modify several properties of the soil employed in the experiments. The most relevant correspond to the solubility of K, Mg and Ca in the limestone. Specifically, solubility of Ca increases more than 3500% for treatments T1 and T5. Besides, the concentration of organic matter is also improved in all treatments. These properties provide the soil with better features to be more productive.

The microorganisms present in the Algaenzims® act in a synergic mechanism; this implies that they are better together than alone. For this reason, the effect of isolated microorganisms is lesser than that of the studied product.

Author Contributions: J.A.V.S. designed the experiments, collected and analyzed the data and prepared the draft; L.D.J. and J.E.B. advised on the assays design, on the interpretation and analysis of data, and reviewed and corrected the manuscript. J.O.C.P. and N.E.G.E. supported in the soil treatments; and J.S.L.A. helped in the statistical analysis of data.

Funding: This research received no external funding.

Acknowledgments: The authors acknowledge the economic support provided by the National Council of Science and Technology (CONACyT-Mexico) for the financial support and the scholarship for doctoral studies for Villarreal J.A.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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