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# Use of Biostimulants Obtained from Sewage Sludge for the Restoration of Soils Polluted by Diuron: Effect on Soil Biochemical Properties

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**Abstract:** The use of biostimulants in the bioremediation of polluted soils in order to eliminate/reduce the toxic effects of pesticides on the soil is currently a very common environmental practice. In this study, we studied the bioremediation effect of three biostimulants obtained from sewage sludge by way of an enzymatic hydrolysis and fermentation process in a diuron-contaminated soil for 55 days under laboratory conditions. During this period of time, the enzymatic activities, bacterial community and the evolution of diuron in the soil were analyzed. Compared with the unpolluted soil, the application of diuron decreased the dehydrogenase, β-glucosidase and phosphatase activities by 60%, 40.7%, and 60.6%, respectively. The Gram-positive bacterial population was decreased by 48.5%, while the Gram-negative population was decreased by 57.7% and the fungal population was decreased by 54.3%. The application of the three biostimulants to the soil decreased the diuron concentration. However, this decrease was higher when the biostimulant obtained by enzymatic hydrolysis was applied. This may be due to the fact that this biostimulant contains a higher quality of low molecular weight proteins than the other two biostimulants obtained by fermentation processes.

**Keywords:** diuron; biostimulant; sewage sludge; enzymatic hydrolysis; fermentation processes; bioremediation of contaminated soils



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## 1. Introduction

The implementation of EU directives 91/271/EEC and 98/15/EEC on wastewater treatment has led to an increase in the number of wastewater treatment plants, thus resulting in large amounts of wastewater sludge [1].

One of the alternative uses for these sewage sludge is as an organic amendment to improve the physical, chemical and biological properties of agricultural soils and to improve crop growth and nutrition [2–6]. Similarly, sewage sludge has been used in the bioremediation of soils polluted by organic pesticides [7,8]. In this regard, the role of sewage sludge is the same as for other sources of organic matter applied to soils contaminated by pesticides. Thus, they play a role in stimulating pesticide-tolerant soil microorganisms, which leads to an acceleration in the degradation of this organic xenobiotic and mayo also allow the sorption of such pesticides; thus, reducing their concentration in the soil solution and, consequently, reducing their toxicity [9,10].

However, despite having a high content of organic matter, these organic wastes are also usually characterized by a high content of heavy metals, organic pollutants and pathogenic organisms, which could be a source of contamination for the environment and human health [11–14].

It has been known for many years that composting this organic waste minimizes and eliminates the undesirable effects of the heavy metals, organic pollutants and pathogenic microorganisms present in said sewage sludge [15]. However, the composting of sewage sludge presents several problems, including the emission of unwanted gases, such as ammonia and greenhouse gases, that can affect local residents, thereby reducing the quality of the final product [16]. In addition to the low quality of the final product, the presence of large solids that hinder some agricultural applications and slow down assimilation by microorganisms and soil plants limits its use further [12].

In recent years, various authors have proposed an alternative to composting sewage sludge. Thus, Rodríguez-Morgado et al. [12] obtained a biostimulant from sewage sludge using enzymatic hydrolysis processes with an endoprotease enzyme obtained from *Bacillus licheniformis*. The effectiveness of this biostimulant in the bioremediation of soils contaminated by pesticides was verified by Rodríguez-Morgado et al. [12] and Tejada et al. [17], who found that these organic compounds stimulated the biochemical activity of the soil, as well as its microbial biomass, thereby accelerating the degradation of oxyfluorfen and chlorpyrifos in the soil.

In addition to these enzymatic biostimulants, the use of various microorganisms, such as *Bacillus licheniformis* to obtain biostimulants by way of fermentation processes, has also received attention recently given that this bacterium is capable of producing and secreting numerous hydrolytic enzymes that are capable of degrading different organic substrates into amino acids and low molecular weight peptides (<300 daltons), which can also be easily assimilated by microorganisms (Rodríguez-Morgado et al. [12].

Rodríguez-Morgado et al. [18] obtained four new biostimulants from fermentation processes using the bacterium *Bacillus licheniformis* ATCC21415. When these biostimulants were applied to the soil, they caused a stimulation of the soil microorganisms. Consequently, these new products, which also act as biostimulants for the soil microbial community, could be very useful in bioremediation of soils contaminated with organic xenobiotics.

Diuron [3-(3,4-dichlorophenyl) -1,1-dimethylurea] is a herbicide from the phenyl amide family and a subclass of phenyl-urea used to control pre-emergent weeds in a wide variety of crops [19]. However, it is very persistent in the soil, with a half-life of around 330 days [20]. Diuron is classified by the European Commission (Directive 2000/60/EC) as a highly toxic herbicide that negatively affects both terrestrial and aquatic biota and human health [21,22]. As such, its use in Europe is prohibited, although it is still used in other parts of the world [22]. The continuous use of this herbicide, together with its high persistence, means that diuron is a highly polluting chemical compound in the soil [23].

It has been known for many years that the study of biological parameters in soil, such as enzymatic activities or microbial biodiversity, is of great importance due to their rapid response after the addition of various chemical compounds to the soil [24,25].

Enzymatic activities are important biological indicators of the soil because they are very sensitive to changes related to the biogeochemical cycle and the dynamics of soil organic matter [26]. In this regard, dehydrogenase activity is an intracellular enzyme related to the oxidative phosphorylation, making it a good indication of microbial activity [27]. Soil alkaline phosphatase activity plays an important role in organic P mineralization [28], and β-glucosidase activity provides information on cellulose degradation [26].

On the other hand, understanding the structuring of the microbial community and the patterns of those microbes that are sensitive to changes in the soil ecosystem can be of great importance in order to understand the dynamics of any soil-applied compound in the soil [12].

Consequently, the study of these biological properties could be very useful for understanding the effect of various biostimulants obtained from sewage sludge on the bioremediation of soils contaminated with xenobiotics.

There are currently no studies on the use of such soil biostimulants obtained either by enzymatic hydrolysis processes or by bacterial fermentation in the bioremediation of soils polluted by the diuron herbicide. As such, the objective of this work was to study and

compare the bioremediation effect of three biostimulants obtained from sewage sludge in a soil contaminated with the herbicide diuron and its influence on the biological properties of that soil. Two of these biostimulants were obtained using bacterial fermentation processes, while the third was obtained using an enzymatic hydrolysis process.

## 2. Materials and Methods

### *2.1. Characteristics of Experimental Biostimulants, Soil and Diuron*

The sewage sludge used to obtain the experimental biostimulants had a maturity of four months and was supplied by the Experimental Water Treatment Plant at the Center for New Water Technologies (CENTA) located in Carrión de los Céspedes (Seville, Spain).

Two different methodologies were used to obtain the experimental biostimulants. To obtain the biostimulants by enzymatic hydrolysis, the procedure described by Rodríguez-Morgado et al. [12] was used, while to obtain the biostimulants obtained by fermentation with *Bacillus licheniformis*, the methodology described by Rodríguez-Morgado et al. [18] was used.

Figure 1 shows the production of these experimental biostimulants schematically. Table 1 shows the chemical characteristics of the sewage sludge and the three experimental biostimulants. The analytical methods used to determine these chemical parameters are detailed in Rodríguez-Morgado et al. [12].

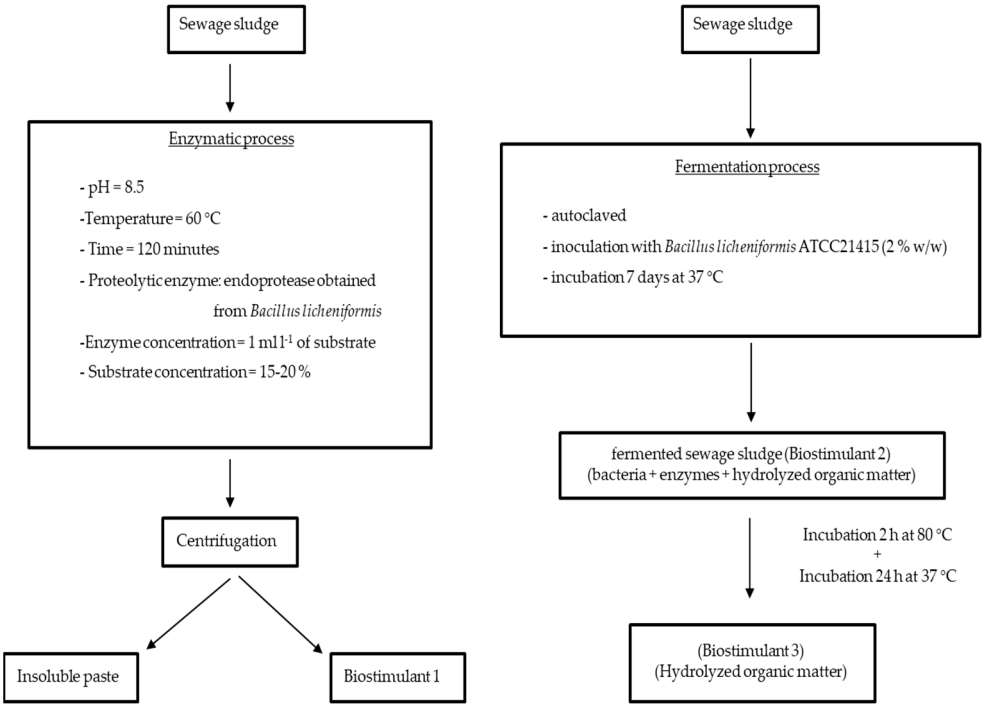


**Figure 1.** Scheme of the enzymatic hydrolysis and fermentation process in sewage sludge to obtain the experimental biostimulants.

**Table 1.** Incubation treatments.

| |
|---|
| 1. C: Control soil. Without BS and without diuron |
| 2. C+BS1: Soil amended with BS1 |
| 3. C+BS2: Soil amended with BS2 |
| 4. C+BS3: Soil amended with BS3 |
| 5. C+D: Soil contaminated with diuron |
| 6. C+D+BS1: Soil contaminated with diuron and amended with BS1 |
| 7. C+D+BS2: Soil contaminated with diuron and amended with BS2 |
| 8. C+D+BS3: Soil contaminated with diuron and amended with BS3 |

A sandy clay loam agricultural soil from southern Spain with a particle size distribution of 48.6 ± 4.9 % coarse sand, 13 ± 2.5 % fine sand, 12.3 ± 2.9 % silt and 26 ± 3.5 % clay, a pH of 7.9 ± 0.2, and 1.7 ± 0.3 % of organic matter was selected for this study. The soil texture was determined using Robinson's pipette method [29]. Soil pH was determined for the 1:2.5 soil/water extract and organic matter was determined by $K_2Cr_2O_7$ oxidation [30]. According to the WRB [31] classification, this soil was an Arenic Calcaric Regosol.

The herbicide used in this experiment was diuron, as found in the commercial formulation Diruokey 80% (W/P) from Industrial Química Key, S.A. (Spain). The dose used was 2 kg $ha^{-1}$, as described by Tejada et al. [25].

### *2.2. Incubation Layout and Soil Analysis*

A 9.3 kg sample of dry soil was mixed with 9.23 mg of herbicide to obtain an application dose of 2 kg $ha^{-1}$, as described by Tejada et al. [25]. The mixture was vigorously stirred for 24 h. The soil was mixed with three biostimulants in 15 kg pots. The experimental biostimulants were applied to the soil at a dose of 1% of soil organic matter, such that 1.49 kg, 1.48 kg and 1.68 kg of biostimulant 1 (BS1), biostimulant 2 (BS2) and biostimulant 3 (BS3) were applied to 300 g of soil, respectively. In the diuron-contaminated soil, biostimulants were added three days after application of the herbicide. Unamended soil and non-polluted soil were used as the control.

The soils were incubated in microcosm at 25 ± 1 °C for 55 days and the moisture content was maintained at 60% of the water-holding capacity. All treatments were performed in triplicate and are described in Table 1.

For each experimental treatment, a sufficient aliquot of soil was taken to determine the dehydrogenase, β-glucosidase and alkaline phosphatase activities and diuron content at 2, 6, 10, 20, 35 and 55 days of incubation; samples were taken at 2, 10 and 55 days to determine microbial fatty acids. All samples were kept at −20 °C until analysis.

Dehydrogenase activity was analyzed according to García et al.'s [32] methodology and using 1 g of sample as the reduction of 0.2 mL of 4% INT (2-p-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride). The p-iodonitrotetrazolium chloride to iodonitrotetrazolium formazan (INTF) formed was extracted with a mixture of ethylene chloride and acetone. INTF was measured in a spectrophotometer at 490 nm. Controls were prepared without substrate.

Alkaline phosphatase activity was measured by the method of Tabatabai and Bremner [33] except that incubation was at 30 °C in maleate buffer (2 mL, pH 6.5) for 90 min and 0.5 mL of substrate (p-nitrophenyl phosphate) added to 0.5 g to soil. Controls were prepared without substrate.

β-Glucosidase activity was determined using 2 mL of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of substrate (p-nitrophenyl phosphate as the substrate) [34].

Ester-linked microbial fatty acids were extracted and quantified according to the methodology described by Montes de Oca-Vásques et al. [35].

The extraction of diuron from the soils was performed according to Tejada et al. [25] using methanol (2 g soil/10 mL metanol). The recovery percentage was 96.9 ± 2.4%. Soil herbicide concentration was determined by high-performance liquid chromatography (HPLC). The analytical conditions are described in Tejada et al. [25].

### *2.3. Statistical Analysis*

To verify whether the use of the biostimulants obtained by fermentation and hydrolysis significantly improved the aforementioned biological properties and the biodegradation of diuron in soil, an analysis of variance (ANOVA) was performed using two factors (treatments and sampling time).

In the event of statistically significant differences in this first analysis, a multiple comparison test was performed by applying the Tukey HSD. Statistical analyses were performed using the Statgraphics Plus 2.1 software package.

## 3. Results

### *3.1. Characteristics of the New Biostimulants*

Table 2 shows the chemical properties and molecular weight distribution of the proteins from the experimental sewage sludge (SS) and of the three BSs obtained from said sludge.

**Table 2.** Chemical properties (mean ± standard error, $n$ = 3) of sewage sludge and biostimulants obtained by enzymatic hydrolysis and fermentation processes.

| | SS | BS1 | BS2 | BS3 |
|---|---|---|---|---|
| Dry matter (%) | 5.3a ± 0.7 | 5.6a ± 0.3 | 5.6a ± 0.2 | 5.4a ± 0.2 |
| pH | 6.4a ± 0.3 | 8.0b ± 0.2 | 8.0b ± 0.3 | 8.3b ± 0.2 |
| Organic matter (g $kg^{-1}$) | 477a ± 17 | 478a ± 12 | 475a ± 11 | 468a ± 19 |
| N (g $kg^{-1}$) | 29.2a ± 6.3 | 34.3a ± 4.7 | 31.4a ± 4.7 | 29.6a ± 5.2 |
| P (g $kg^{-1}$) | 10.9a ± 1.8 | 3.1b ± 0.8 | 11.7a ± 1.9 | 12.5a ± 1.9 |
| K (g $kg^{-1}$) | 5.8a ± 1.3 | 19.7b ± 4.1 | 6.0a ± 1.0 | 6.3a ± 1.5 |
| S (g $kg^{-1}$) | 14.9a ± 2.0 | 6.8b ± 1.5 | 18.4a ± 2.0 | 15.7a ± 1.8 |
| Ca (g $kg^{-1}$) | 41.0a ± 3.6 | 47.3a ± 4.4 | 45.9a ± 3.8 | 42.8a ± 2.7 |
| Mg (g $kg^{-1}$) | 6.6a ± 1.3 | 8.2a ± 1.1 | 6.9a ± 1.7 | 7.5a ± 1.2 |
| Fe (mg $kg^{-1}$) | 16.3a ± 1.9 | 15.5a ± 1.3 | 16.8a ± 1.1 | 18.1a ± 1.6 |
| Cu (mg $kg^{-1}$) | 322a ± 11 | 314b ± 2.1 | 318a ± 15 | 312a ± 10 |
| Mn (mg $kg^{-1}$) | 150a ± 6 | 91.4b ± 4.8 | 138a ± 4.6 | 131a ± 8 |
| Zn (mg $kg^{-1}$) | 79.5a ± 11.6 | 2.3b ± 0.7 | 71.7a ± 10.1 | 75.4a ± 9.8 |
| Pb (mg $kg^{-1}$) | 39.8a ± 7.9 | 0.88b ± 0.45 | 33.5a ± 5.7 | 36.5a ± 5.6 |
| As (mg $kg^{-1}$) | 4.1a ± 1.3 | 0.13b ± 0.06 | 3.8a ± 0.7 | 3.7a ± 0.5 |
| Cd (mg $kg^{-1}$) | 1.3a ± 0.6 | 0.17b ± 0.08 | 1.1a ± 0.3 | 1.2a ± 0.2 |
| Protein molecular-weight distribution (Da) | | | | |
| >10,000 | 98.8a ± 1.3 | 22.8c ± 2.2 | 40.0b ± 2.1 | 42.8b ± 2.7 |
| 10,000–5000 | 0.0a ± 0.0 | 9.7b ± 1.1 | 15.6c ± 2.1 | 13.8c ± 1.6 |
| 5000–1000 | 1.2a ± 0.5 | 6.2b ± 1.2 | 11.8c ± 1.9 | 11.7c ± 1.3 |
| 1000–300 | 0.0a ± 0.0 | 2.0b ± 0.4 | 1.6b ± 0.4 | 2.0b ± 0.5 |
| <300 | 0.0a ± 0.0 | 59.3c ± 4.7 | 31.0b ± 2.5 | 29.7b ± 3.2 |

[a] Files followed by the same letter(s) are not significantly different according to the Tukey test ($p \leq 0.05$). BS1: biostimulant obtained by enzymatic hydrolysis processes. BS2: biostimulant obtained by fermentation processes consisting of bacteria, enzymes and hydrolyzed organic matter. BS3: biostimulant obtained by fermentation processes constituted by hydrolyzed organic matter.

Firstly, the results indicate that there was a significant increase in pH for the new BSs obtained with respect to that obtained for SS. The organic matter content in the new BSs obtained was very similar to that for SS.

With respect to the macronutrients analyzed, the results indicate that there are no significant differences between the N, Ca and Mg contents in SS and the BSs obtained. The P, K and S contents were only significantly ($p < 0.05$) higher in BS1 than in SS, while these macronutrients were very similar in the latter and in BS2 and BS3.

As regards the micronutrients analyzed, the results indicate that there were no significant differences between SS and BS2 and BS3. Except for Fe, the contents of these micronutrients decreased significantly ($p < 0.05$) in BS1 compared to SS.

The enzymatic hydrolysis and fermentation process resulted in a higher quantity of proteins of smaller molecular size. A statistical analysis indicated that this increase in lower molecular weight proteins was higher in BS1 than in BS2 and BS3.

### *3.2. Soil Biochemical Properties and Microbial Community*

During the first days of the experiment, the application of the three BSs to soil significantly ($p \leq 0.05$) stimulated the dehydrogenase activity (Table 3). Compared with the control treatment and for treatments BS1, BS2 and BS3, dehydrogenase activity increased significantly ($p \leq 0.05$) by 89.5%, 70.4% and 56.8%, respectively, 6 days after the start of the

experiment. During the induction period, this enzymatic activity decreased until reaching values similar to the control treatment at the end of the experimental period.

**Table 3.** Evolution of dehydrogenase and β-glucosidase activities (mean ± standard error, $n$ = 3) in soils amended with the three experimental biostimulants and polluted with diuron during the experimental period.

| | **Dehydrogenase Activity (μg INTF $g^{-1}$ $h^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **2** | **6** | **10** | **20** | **35** | **55** |
| C | 3.4b ± 0.5 | 3.2b ± 0.7 | 3.0b ± 0.4 | 3.2b ± 0.3 | 3.4b ± 0.5 | 3.5b ± 0.4 |
| C+BS1 | 23.5d ± 2.4 | 30.4d ± 3.1 | 21.3c ± 2.6 | 5.3b ± 1.1 | 4.2b ± 0.8 | 3.6b ± 0.2 |
| C+BS2 | 11.3c ± 1.50 | 10.8c ± 1.9 | 4.9b ± 1.7 | 3.6b ± 0.4 | 3.6b ± 0.3 | 3.4b ± 0.5 |
| C+BS3 | 9.30c ± 1.10 | 7.4bc ± 1.7 | 4.0b ± 1.2 | 3.4b ± 0.5 | 3.5b ± 0.5 | 3.5b ± 0.3 |
| C+D | 1.9a ± 0.3 | 1.6a ± 0.2 | 1.3a ± 0.3 | 1.6a ± 0.4 | 1.7a ± 0.2 | 1.4a ± 0.2 |
| C+D+BS1 | 17.2c ± 2.2 | 11.4c ± 1.6 | 8.31c ± 1.9 | 4.4b ± 1.8 | 3.3b ± 1,2 | 2.8ab ± 0.9 |
| C+D+BS2 | 9.7c ± 1.5 | 5.2b ± 1.2 | 3.9b ± 1.1 | 2.6ab ± 0.7 | 2.4a ± 0.9 | 2.3a ± 0.3 |
| C+D+BS3 | 7.3bc ± 1.9 | 3.1b ± 1.3 | 2.7ab ± 0.9 | 2.1a ± 0.5 | 2.2a ± 0.6 | 2.1a ± 0.6 |
| | β-glucosidase Activity (μmol PNP $g^{-1}$ $h^{-1}$) | | | | | |
| C | 0.57b ± 0.08 | 0.55b ± 0.05 | 0.58b ± 0.06 | 0.59b ± 0.05 | 0.55b ± 0.08 | 0.54b ± 0.07 |
| C+BS1 | 1.70c± 0.3 | 2.6c ± 0.5 | 2.0c ± 0.3 | 0.98c ± 0.09 | 0.58b ± 0.09 | 0.59b ± 0.07 |
| C+BS2 | 0.57b ± 0.05 | 0.55b ± 0.08 | 0.58b ± 0.09 | 0.56b ± 0.08 | 0.54b ± 0.07 | 0.55b ± 0.06 |
| C+BS3 | 0.56b ± 0.05 | 0.54b ± 0.07 | 0.57b ± 0.08 | 0.57b ± 0.05 | 0.56b ± 0.06 | 0.57b ± 0.05 |
| C+D | 0.34a ± 0.07 | 0.32a ± 0.10 | 0.34a ± 0.09 | 0.35a ± 0.08 | 0.32a ± 0.10 | 0.27a ± 0.09 |
| C+D+BS1 | 1.1c ± 0.3 | 1.4c ± 0.06 | 0.98c ± 0.07 | 0.60b ± 0.09 | 0.50b ± 0.06 | 0.47b ± 0.04 |
| C+D+BS2 | 0.45a ± 0.05 | 0.40a ± 0.04 | 0.40a ± 0.05 | 0.38a ± 0.04 | 0.31a ± 0.06 | 0.33a ± 0.05 |
| C+D+BS3 | 0.43a ± 0.03 | 0.38a ± 0.05 | 0.41a ± 0.04 | 0.37a ± 0.04 | 0.37a ± 0.10 | 0.35a ± 0.06 |

Columns (mean ± standard error) followed by the same letter(s) are not significantly different ($p$ < 0.05). INTF 2-p-iodo-3-nitrophenyl formazan; PNP p-nitrophenol.

The behavior of the β-glucosidase and alkaline phosphatase activities differed markedly depending on the type of BS applied to the soil (Tables 3 and 4). Thus, when BS1 was applied to the non-polluted soil, the behavior of these extracellular activities was similar to that for dehydrogenase activity. Compared with the control treatment, a 78.8% increase in β-glucosidase activity and a 79.5% increase in phosphatase activity were observed 6 days after the start of the experiment, when BS1 was applied to uncontaminated soil. When BS2 and BS3 were applied to the non-polluted soil, urease, β-glucosidase and phosphatase activities remained unchanged.

**Table 4.** Evolution of alkaline phosphatase activity (mean ± standard error, n = 3) in soils amended with the three experimental biostimulants and polluted with diuron during the experimental period.

| | **Alkaline Phosphatase Activity (μmol PNP $g^{-1}$ $h^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **2** | **6** | **10** | **20** | **35** | **55** |
| C | 3.3b ± 1.0 | 3.5b ± 1.2 | 3.4b ± 0.97 | 3.3b ± 1.1 | 3.1b ± 1.0 | 3.3b ± 1.1 |
| C+BS1 | 7.3c ± 2.3 | 17.1d ± 3.5 | 10.6c ± 2.8 | 5.2 b± 2.0 | 3.8b ± 1.1 | 3.2b ± 1.0 |
| C+BS2 | 3.1b ± 0.9 | 3.4b ± 1.0 | 3.4b ± 1.3 | 3.3b ± 1.0 | 3.2b ± 1.4 | 3.4b ± 0.9 |
| C+BS3 | 3.2b ± 0.7 | 3.1b ± 0.8 | 3.5b ± 0.7 | 3.4b ± 1.3 | 3.4b ± 0.9 | 3.3b ± 1.3 |
| C+D | 1.5a ± 0.7 | 1.8a ± 0.9 | 1.4a ± 0.5 | 1.4a ± 0.8 | 1.5a ± 0.7 | 1.3a ± 0.6 |
| C+D+BS1 | 4.8b ± 1.8 | 8.6c ± 2.9 | 5.3b ± 1.7 | 4.2b ± 1.0 | 3.0b ± 0.6 | 2.4b ± 0.5 |
| C+D+BS2 | 1.4a ± 0.3 | 1.6a ± 0.3 | 1.7a ± 0.4 | 1.4a ± 0.3 | 1.3a ± 0.2 | 1.4a ± 0.2 |
| C+D+BS3 | 1.6a ± 0.4 | 1.5a ± 0.4 | 1.6a ± 0.3 | 1.3a ± 0.2 | 1.5a ± 0.4 | 1.5a ± 0.3 |

Columns (mean ± standard error) followed by the same letter(s) are not significantly different ($p$ < 0.05). PNP p-nitrophenol.

The application of diuron to the soil significantly ($p \leq 0.05$) decreased the enzymatic activities during the experimental period (Tables 2 and 3). Thus, at the end of the incubation period, and compared to treatment C, the dehydrogenase activity in C+D treatment

decreased significantly (60%, $p < 0.05$), the β-glucosidase activity decreased significantly (40.7%, $p \leq 0.05$) and the alkaline phosphatase activity also decreased significantly (60.6%, $p \leq 0.05$).

The application of the three BSs to polluted soil decreased the inhibition of dehydrogenase activity (Table 3). However, this decrease depended on the type of BS applied. Thus, at the end of the experimental period and compared with treatment C, the dehydrogenase activity decreased by 20% for treatment C+D+BS1, 34.3% for treatment C+D+BS1, and 40% for treatment C+D+BS3. On the other hand, the application of BS1 to the polluted soil also decreased the inhibition of β-glucosidase and phosphatase activities. Thus, at the end of the incubation period and compared to control treatment, β-glucosidase and alkaline phosphatase activities decreased significantly ($p \leq 0.05$) by 13% and 27.36%, respectively (Tables 3 and 4). Compared with treatment C+D, the application of BS2 and BS3 to the contaminated soil did not produce changes in terms of β-glucosidase and phosphatase activities (Tables 3 and 4).

During the first days of the experiment, the application of the three BSs to the non-polluted soil increased the population of bacteria and fungi (Table 5). This increase depended on the type of BS applied to the soil, with higher values being observed for treatment C+BS1, followed by treatments C+BS2 and C+BS3. At the end of the experiment, the bacteria population was similar to that for the control treatment.

**Table 5.** Evolution of bacterial Gram$^+$, bacterial Gram$^-$, total bacterial and fungal PLFAs (nmol g$^{-1}$) during the experimental period. Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p > 0.05$).

| | bacGram$^+$ | bacGram$^-$ | Total Bacterial PLFA | Fungal PLFA |
|---|---|---|---|---|
| C (2d) | 13.8b ± 3.5 | 2.5b ± 1.0 | 16.3b ± 4.8 | 1.5b ± 0.3 |
| C (10d) | 13.4b ± 2.8 | 2.3b ± 0.94 | 15.7b ± 3.6 | 1.2b ± 0.2 |
| C (55d) | 13.0b ± 2.9 | 2.6b ± 1.1 | 15.6b ± 4.1 | 1.4b ± 0.2 |
| C+BS1 (2d) | 32.5c ± 3.3 | 5.2c ±1.9 | 37.5c ± 4.6 | 2.6c ± 0.3 |
| C+BS1 (10d) | 27.3c ± 2.0 | 3.0b ± 1.6 | 30.3c ± 3.4 | 1.9b ± 0.3 |
| C+BS1 (55d) | 13.8b ± 2.4 | 2.7b ± 1.2 | 16.5b ± 3.5 | 1.5b ± 0.4 |
| C+BS2 (2d) | 21.3c ± 3.2 | 3.5b ± 1.3 | 24.8bc ± 4.2 | 2.0b ± 0.3 |
| C+BS2 (10d) | 17.4b ± 2.6 | 2.9b ± 0.95 | 20.3b ± 3.5 | 1.8b ± 0.2 |
| C+BS2 (55d) | 13.2b ± 2.1 | 2.2b ± 1.0 | 15.4b ± 3.3 | 1.5b ± 0.3 |
| C+BS3 (2d) | 19.0b ± 2.3 | 3.1b ± 1.1 | 22.1b ± 3.5 | 2.0b ± 0.2 |
| C+BS3 (10d) | 15.3b ± 2.0 | 2.6b ± 0.84 | 17.9b ± 2.1 | 1.7b ± 0.1 |
| C+BS3 (55d) | 13.0b ± 2.3 | 2.2b ± 0.90 | 15.2b ± 2.5 | 1.4b ± 0.2 |
| C+D (2d) | 6.5a ± 1.0 | 1.1a ± 0.1 | 7.6a ± 1.2 | 0.63a ± 0.12 |
| C+D (10d) | 6.0a ± 1.7 | 1.3a ± 0.2 | 7.3a ± 1.8 | 0.61a ± 0.11 |
| C+D (55d) | 6.7a ± 1.3 | 1.1a ± 0.1 | 7.8a ± 1.6 | 0.64a ± 0.15 |
| C+D+BS1 (2d) | 23.8c ± 3.0 | 3.8b ± 1.1 | 27.6bc ± 4.0 | 0.89ab ± 0.13 |
| C+D+BS1 (10d) | 19.6b ± 2.8 | 2.1b ± 0.93 | 21.7b ± 3.7 | 0.80a ± 0.08 |
| C+D+BS1 (55d) | 9.3ab ± 1.5 | 1.7ab ± 0.2 | 11.0ab ± 1.8 | 0.76a ± 0.11 |
| C+D+BS2 (2d) | 17.4b ± 2.9 | 3.0b ±1.2 | 20.4b ± 4.0 | 0.77a ± 0.08 |
| C+D+BS2 (10d) | 12.4b ± 2.2 | 1.9ab ± 0.56 | 14.6b ± 2.8 | 0.77a ± 0.14 |
| C+D+BS2 (55d) | 7.6a ± 1.1 | 1.3a ± 0.2 | 8.9a ± 1.5 | 0.74a ± 0.12 |
| C+D+BS3 (2d) | 15.3b ± 2.4 | 2.8b ± 0.4 | 18.1b ± 3.0 | 0.75a ± 0.15 |
| C+D+BS3 (10d) | 10.8ab ± 2.0 | 1.6ab ± 0.4 | 12.4b ± 2.6 | 0.72a ± 0.11 |
| C+D+BS3 (55d) | 7.1a ± 1.8 | 1.3a ± 0.1 | 8.4a ± 2.0 | 0.70a ± 0.09 |

Columns (mean ± standard error) followed by the same letter(s) are not significantly different ($p < 0.05$).

When diuron was applied to the unamended soil, the total population of bacteria and fungi decreased significantly ($p \leq 0.05$; Table 5). At the end of the experiment, and compared with the control soil, the Gram-positive bacterial population in soils contaminated with diuron decreased by 48.5%, the Gram-negative population decreased by 57.7% and the fungal population decreased by 54.3% (Table 5).

The application of BSs to soils contaminated with soils decreased the inhibition of the bacterial and fungal population. At the end of the experiment, and in comparison with

treatment C+D, the inhibition of total bacterial PLFA and fungal PLFA decreased by 29.0% and 15.8% for treatment C+D+BS1, 12.3% and 13.5% for treatment C+D+BS2, and 7.1% and 8.6% for treatment C+D+BS3, respectively (Table 4).

### *3.3. Persistence of Diuron in Soil*

The diuron concentration in the soil decreased until the end of the incubation period (Table 6). The application of the three BS significantly ($p \leq 0.05$) decreased the herbicide concentration. At the end of the experiment, and compared with treatment C+D, the diuron concentration decreased by 73.4% for treatment C+D+BS1, 30.3% for treatment C+D+BS2, and 24.8% for treatment C+D+BS3.

**Table 6.** Evolution of diuron (mean ± standard error, n = 3) in soils during the incubation period.

| | **Diuron (mg kg$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **2** | **6** | **10** | **20** | **35** | **55** |
| C+D | 24.5a ± 0.4 | 23.3a ± 0.8 | 20.3a ± 0.5 | 14.5b ± 1.0 | 13.6b ± 1.2 | 10.9b ± 1.2 |
| C+D+BS1 | 19.3a ± 1.1 | 13.4b ± 1.4 | 8.7c ± 1.1 | 7.0b ± 0.8 | 5.7c ± 0.5 | 2.9bd± 0.4 |
| C+D+BS2 | 21,94a ± 1.5 | 16.7b ± 1.7 | 15.0b ± 1.6 | 11.8b ± 1.2 | 9.9b ± 1.1 | 7.6c ± 0.9 |
| C+D+BS3 | 22.9a ± 1.7 | 20.8a ± 1.2 | 18.6b ± 1.3 | 13.7b ± 1.5 | 12.0b ± 1.5 | 8.2c ± 0.7 |

Columns followed by the same letter(s) are not significantly different ($p \geq 0.05$).

## 4. Discussion

The results obtained suggest that application of the different biostimulants obtained from sewage sludge increased the enzymatic activities determined during the first days of incubation. These results are in line with those obtained by Rodríguez-Morgado et al. [12,18] who observed an increase in soil biochemical activity after the application of different biostimulants obtained from sewage sludge by both enzymatic hydrolysis processes, as well as by fermentation processes using the bacterium *Bacillus licheniformis*.

However, this stimulation of the soil biochemical activity was different depending on the type of biostimulant applied to the soil. The application of the biostimulant obtained by enzymatic hydrolysis stimulated the intracellular and extracellular enzymatic activities, while the application of the biostimulants obtained by fermentation processes stimulated only the intracellular activity analyzed.

Tejada et al. [10] have suggested that the production process of these biostimulants is the cause of the differences in the stimulation of soil enzymatic activities. Thus, during the fermentation process, the bacterium Bacillus licheniformis excretes a large amount of enzymes that break down organic compounds into inorganic ones. For this reason, when biostimulants of this type are applied, soil microorganisms easily absorb these inorganic compounds, without needing to excrete any enzymes. Additionally, Rodríguez-Morgado et al. [18] suggest that in the biostimulants obtained by fermentation processes the presence of live bacteria and enzymes can stimulate the dehydrogenase activity of the soil more than these biostimulants without live bacteria and enzymes.

The application of biostimulants obtained by enzymatic hydrolysis processes stimulated the dehydrogenase, glucosidase and alkaline phosharase activities. Tejada et al. [10] suggest that in the process of enzymatic hydrolysis, a large part of the organic substrates are not degraded by the action of proteolytic enzymes. Therefore, when these biostimulants are applied to the soil, soil microorganisms must excrete different enzymes to obtain energy and nutrients. Therefore, the biochemical activity of the soil when applying this type of biostimulants is higher.

Rodríguez-Morgado et al. [12,18] and Tejada et al. [17] suggest that the protein size of the biostimulant applied to the soil plays a fundamental role in this enzymatic stimulation. Thus, these authors suggest that increasing the percentage of lower molecular weight proteins in the organic fraction increases the microbial stimulation of the soil. The smaller size of these proteins suggests that N is more readily available to soil microorganisms.

According to Rodríguez-Morgado et al. [12,18], microorganisms are also better able to directly absorb these low molecular weight peptides compared with other peptides of higher molecular weight. For this reason, in our experiment, the highest enzymatic activity was observed when BS1 was applied.

Unlike other sources of conventional organic matter (compost, manure and vermicomposts), the residual effect of the experimental biostimulants was null. According to Tejada and Benitez [36], these sources of conventional organic matter are usually characterized by having a higher content of high molecular weight proteins, which are more difficult to degrade, which may explain why after the application of the experimental biostimulants, microbial stimulation was observed for a short period of time.

The microbial biomass presented a very similar behavior to the soil biochemical activity, with a significant increase in the bacterial and fungal population during the first few days of incubation when BS1 was applied compared with BS2 and BS3. Possibly the differences described in the biostimulants related to the manufacturing process and chemical composition are responsible for the differences found in the stimulation of the soil microbial community.

The application of diuron produced a negative effect on the biochemical activity and microbial community in the soil. These results are in agreement with those obtained by Tejada et al. [25], who observed that this herbicide significantly inhibited the soil biochemical activity. These authors suggest that the decrease in the soil biochemical activity by herbicides could be due to the fact that these compounds suppress some microbial populations involved in the nutrient cycle, hindering the interaction between the enzymatic active sites and soluble substrates. These authors also suggested that only microorganisms that are tolerant to this pollutant will degrade it over time. Our findings also are in agreement with those obtained by Romero et al. [37], who highlighted that after the pollution of soil with diuron, there are active microbial communities capable of degrading this molecule, thus obtaining energy and a carbon source.

However, this degradation is usually very slow, which is why several authors have considered the use of different sources of organic matter to accelerate said degradation, and thus, eliminate or reduce diuron in the soil more rapidly [38].

Rubio-Bellido et al. [38] have suggested the use of composts obtained from different organic wastes for the remediation of soils polluted by diuron. However, they highlight that these composts may simply reduce the bioaccessibility of diuron as it is adsorbed to said organic matter.

Different biostimulants mainly obtained from different organic wastes by enzymatic hydrolysis processes have been used for the bioremediation of soils polluted by various herbicides in recent years [9,10,12]. The results obtained in these studies suggest that the application of these biostimulants to contaminated soils significantly decreases the herbicide concentration. These authors have also suggested that the high content of low molecular weight proteins, which are easily absorbed by toxic-tolerant microorganisms, facilitates their proliferation in their soil and, consequently, the degradation process.

The results obtained in our experiment are in line with those obtained by these authors. However, the different chemical composition, mainly in terms of the content of low molecular weight proteins, makes the effect of each biostimulant in diuron-contaminated soil different. In this sense, the biostimulant obtained by enzymatic hydrolysis is better than those obtained by fermentation processes.

During the fermentation process, the bacterium *Bacillus licheniformis* excretes a large number of enzymes to degrade organic compounds and obtain carbon and energy. This causes said organic compounds to break down into less complex forms that are easily assimilated by the bacteria. Consequently, when we apply these biostimulants with a low molecular weight high-protein content to the soil, they are easily absorbed by soil microorganisms without the need to excrete enzymes to degrade said organic compounds.

During the enzymatic hydrolysis process, the subtilisin enzyme added to the reactor will only degrade high molecular weight proteins to low molecular weight proteins, without

altering or modifying the chemical structure of other organic forms. As such, when a biostimulant with these characteristics is applied to the soil, the soil microorganisms must degrade these unaltered organic remnants, and consequently, excrete different types of enzymes depending on the type of organic compound to be degraded.

This causes the microbial stimulation in the soil to increase when the biostimulant obtained by enzymatic hydrolysis is applied, and consequently, results in a higher degradation of diuron in the soil.

## 5. Conclusions

Biostimulants obtained from sewage sludge by enzymatic hydrolysis and fermentation processes using *Bacillus licheniformis* are very useful in the bioremediation of soils polluted with diuron. This is due to the high content of low molecular weight proteins, which are easily assimilated by microorganisms.

The higher quantity of low molecular weight proteins in the biostimulants obtained by enzymatic hydrolysis processes compared to the biostimulants obtained by fermentation processes is responsible for the higher microbial activity, and consequently, the sharp reduction in diuron concentration. In summary, the use of biostimulants obtained by enzymatic hydrolysis in the bioremediation of soils contaminated with the herbicide diuron is recommended.

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