Production of biostimulants from okara through enzymatic hydrolisis and fermentation with *Bacillus licheniformis*. Comparative effect on soil biological properties.

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Abstrac

In this work okara (OK), a by-product of soy milk manufacturing, is submitted to an enzymatic hydrolysis and a fermentative process to produce different soil biostimulants (BS): EH, hydrolysate obtained by the enzymatic process; FHEB, fermentation broth with Bacillus licheniformis and the enzymes secreted during the fermentation; FHE, fermentation broth without bacteria and FH, the FHE hydrolysate in which enzymes were denatured. Enzymatic hydrolysates showed a different chemical composition compared with fermented hydrolysates and OK. It had a higher protein concentration as well as C, P and K. The proteins of OK were converted into peptides with a lower molecular weight, the fermented hydrolysates being those with the lowest molecular weight profile. The influences of hydrolysates and OK were tested in soil, finding that β-glucosidase, phosphatase and dehydrogenase activities were stimulated by every treatment. However, it was observed that EH produced a greater stimulation of dehydrogenase and phosphatase than both OK and fermented BS. The bacterial and fungal phospholipid fatty acids were also higher in soils amended with BS than those of the control and soils with OK. It has also been found that β-glucosidase, phosphatase and microbial biomass were dose-dependent in every treatment, but dehydrogenase only was dose-dependent in EH and OK treatments.

1. Introduction

The addition of organic matter to soils is a common agricultural and environmental practice used to supply nutrients and improve the soil properties with the aim of enhancing either the productivity of the agroecosystem or reclaiming degraded soils [1–3]. In order to achieve these objectives, different sources rich in organic matter such as crop residues, animal manures, sewage sludge, by-products of food processing industry, etc. have been used [4–7]. However, it is necessary that these residues break down to provide the nutrients needed to stimulate the soil microbiology but, as found by several authors, this breakdown takes some time which depends on both the soil properties and the nature of the added organic wastes [1,8,9]. Therefore, recently, new products, called biostimulants (BS), have been used. These products are obtained by hydrolytic processes, enzymatic hydrolysis being the most used one. In order to produce these BS, different industrial by-products have been used, such as carob germ [10], sewage sludge [11], wheat-condensed distiller [12], defatted sunflower seed meal [13], chicken feathers [3], rice bran [3] and by-product of the dry-mill bioethanol process [14].

Enzymatic hydrolysis of these organic wastes yields a product rich in peptides, amino acids, polysaccharides, humic acids, etc., which provide a higher concentration of organic matter, macro and micro nutrients than those of the raw materials. Therefore, they provoke an enhancement of the microbial soil activity, improving its capability to reclaim contaminated soils; thus several researchers have studied the effect of BS on the degradation of different xenobiotics in soils, such as policyclic aromatic hydrocarbon (PAH) [15] and pesticides [16]. Likewise, the use of BS as biofertilisers exerts a positive influence on plant growth and fruit quality and production [10,13,17,18]

Taking into account what has been found so far, any organic waste rich in organic matter can be used to obtain BS. In this sense, okara (OK) is an interesting raw material because of its composition. OK is a by-product of soy milk and tofu production rich in dietary fibre and proteins which contains essential amino acids [19]. Besides, it has a little quantity of fats, other carbohydrates and isoflavones. This composition makes OK an exceptional source of functional compounds in the food industry [20–23]. Nevertheless, due to the fact that OK ferments rapidly and has a high content of water, its conservation, although possible, is unprofitable. Consequently, OK has to be processed quickly and so, currently in the Western World, this by-product is mainly used as feed or fertiliser [19]. To overcome this aspect, OK could be submitted to a hydrolytic process to obtain a product with a high concentration of peptides and other functional compounds which can be used not only as a source of organic nitrogen and phosphorus but also as a sustainable tool to enhance the soil quality, contributing to making OK profitable and to reduce chemical fertilisers [24,25].
In addition to enzymatic hydrolysis to valorise a wide variety of agricultural and agro-industrial by-products, several authors have carried out a fermentative process using different microorganisms to recycle these residues, most of which focus on the production of bioethanol [26–30] and enzymes [31–37]. On the other hand, there are some research works that study the application to soils of the bio-based products obtained by fermentation. Among them, it is worth highlighting those which focus on the production of bioherbicides [38], bio-pesticides [39,40] and biosurfactants [41,42]. Many research works have brought to light the importance of biosurfactants in the restoration of contaminated soils. In this sense Magthalin et al. [43] found that cationic biosurfactants obtained from chicken tallow were very efficient in the bioremediation of chromium-contaminated soils. On the other hand, other authors have highlighted the

importance of these substances in reclamation of organic xenobiotics-contaminated soils. In this sense, Bezza et al. [44] and Mnif et al. [45] concluded that bio- surfactants enhanced the bioavailability and hence the biodegradation of PAHs and diesel oil. Finally, Wang et al. [46] observed that the phytoremediation of DDT- contaminated soil was improved when biosurfactants were used. However, there are few articles in which the production of BS by fermentative processes is studied, such as Chintagunta et al. [30] who, in fact, obtained a biomanure as a co-product in the bioethanol manufacturing process, Oliveira Mendes et al. [47] and Aceves-Díez et al. [48] who used the broth obtained after the fermentation of a culture medium with Aspergillus niger and Bacillus thuringiensis as biofertiliser, respectively.

Hence, the present paper aimed to produce BS from OK through two different processes, namely an enzymatic hydrolytic process, using an endoprotease, and a fermentative process, using Bacillus licheniformis. After obtaining the BS, its effect on soil biological properties and soil microbial community was assessed.

2. Materials and methods

2.1. Substrate, enzymatic hydrolysis and fermentative processes

The substrate used in the fermentative as well as in the enzymatic hydrolysis process was OK, a by-product of the soy milk production provided by the company Soria Natural located in Garray (Soria, Spain).

The enzymatic hydrolysis process was carried out according to the pH-stat method [49] using as hydrolytic agent the enzyme bioprotease LA-450 (Biocon Española S.A.) which is subtilisin. The process took place in a bio- reactor operating under controlled temperature without either stirring or pH control and with the following conditions: (a) temperature: 55°C; (b) OK concentration: 10% (w/v); (c) subtilisin concentration: 0.05% and (d) operating time: 3 h. Finally, the hydrolysed product was centrifuged and

the hydrolysed liquid (EH) was concentrated in a rotary evaporator until obtaining 58% of dry matter (Table 1).

Table 1. Chemical analyses and protein molecular weight distribution of OK, water-soluble fraction from OK, enzymatic hydrolysates (EH) and fermented hydrolysates (FH).

	OK	EH	FHE	FHEB	FH
Chemical analyses					
Dry matter (%)	23.3	58.0	31.5	77.4	38.9
Water solubility (%)	17	32	65	-	-
Fats (%)	23.6	-	-	-	-
Ashes (%)	4.05	-	-	-	-
Carbohydrates (%)	29.5	-	-	-	-
Soluble fibre (%)	2.8	-	-	-	-
Insoluble fibre (%)	26	-	-	-	-
Protein (%)	41.28	66.54	42.25	-	-
Soluble proteins (%)	4.4	-	-	-	-
Insoluble proteins (%)	36.88	-	-	-	-
C (%)	53.7	44.81	33.6	-	-
N (%)	6.60	10.64	6.76	-	-
C/N	8.13	4.21	5.01	-	-
K (g kg^{-1})	9.11	17.14	11.25	-	-
P (g kg^{-1})	6.53	11.16	6.89	-	-
Protein molecular weight distribution					
Molecular weight (kD)					
>10	91.14	7.85	0	-	-
10–5	1.09	15.87	0	-	-
5–1	0.80	0	28.42	-	-
<1	6.98	75.74	71.47	-	-

Note: The results are referred to dry weight.

Regarding fermentation, this was carried out in 500 mL Erlenmeyer flasks under controlled stirring and temperature by B. licheniformis ATCC 21415, which was stored under freezing at −80°C and refreshed 24 h before inoculation in LB medium (10 g tryptone, 10 g NaCl, 5 g yeast extract and 1L of water). The conditions of the fermentation were the followings: (a) OK concentration: 1% (w/v); (b) B. licheniformis concentration: 2% (w/v); (c) temperature: 37°C; (d) initial pH: 9 and (e) operating time: 312 h. Then the insoluble fibre was separated by centrifugation, obtaining a supernatant with hydrolysed proteins, bioactive products, enzymes and bacteria (FHEB). Secondly, a fraction of this supernatant was centrifuged to eliminate the bacteria, and thus a

soluble fraction with hydrolysed proteins, bioactive compounds and enzymes was obtained (FHE). Thirdly, a portion of this last fraction was heated at 80°C for 20 min to denature the enzymes. Therefore, the resultant product was composed of hydrolysed proteins, bioactive compounds and denatured enzymes (FH). Finally, all these fractions were concentrated in a rotary evaporator obtaining the following dry matter content for each BS: FHEB 77.4%, FHE 31.5% and FH 38.9% (Table 1).

2.2. Chemical characterisation of OK, enzymatic and fermented hydrolysates.

Macro elements in OK (previously burned) and in enzymatic and fermented hydrolysates were analysed by inductively coupled plasma atomic emission spectrometry. The equipment used standard operational conditions, which are detailed in Parrado et al. [37].

Ash was analysed according to standard AOAC methods [50]. Total soluble carbohydrates were determined after extraction with a mixture of ethanol/water (2/3) for 2 h. After centrifugation at 4000×g, the supernatant was filtered through a No. 1 Whatman paper, and total soluble sugars were estimated colorimetrically using the phenol–sulphuric acid method with a standard curve of glucose [51]. Crude fat content was determined gravimetrically after OK extraction with hexane for 12 h in a Soxhlet extractor [52]. Organic matter was analysed by dry combustion at 550°C for 6 h [53]. Total nitrogen was determined by the Kjeldahl method [50]. The protein content was determined by multiplying the total nitrogen content in a protein by the conversion factor 5.71, which is applicable to soy protein. Fibre content was determined gravimetrically according to standard AOAC methods [50]. OK solubility was determined in 200 mL of water with an OK concentration of 10% at 55°C for 24 h.

2.3. Protein molecular weight and solubility determination

The molecular mass distribution of protein in the samples was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare). The operational conditions are described in Parrado et al. [10].

Protein solubility was determined using the method described by Adler-Nissen [54]: briefly, 5 mL of 2.4 M trichloroacetic acid was added to 10 mL of the sample (1% w/v), the precipitate was removed by centrifugation (8000g, 10 min) and the nitrogen concentration of the supernatant was determined.

2.4. Biostimulation experimental design

The soil used in this experiment is a Haplic Regosol (Calcaric) [55]. Soil samples were collected from the 0–25 cm surface layer. The main soil characteristics and methodology used to determine the physical and chemical parameters in soil are presented elsewhere [11,17].

The biostimulation design was performed in microcosms consisting of a glass pot with 300 g of dried and 2 mm sieved soil. Prior to the treatments, soil was preincubated at 25°C for 7 days at 40% of its water-holding capacity according to Tejada et al. [56]. Soil was amended with OK and the four BS obtained at two concentrations so that the organic matter was 1% and 1.5%. An unamended soil was used as control. Distilled water was added to each soil to achieve 60% of its water-holding capacity.

The BS were liquid, so they were dissolved in distilled water before the application. On the other hand, OK, having been dried, was a powdery solid and so was dry mixed with soil. Therefore, there were five treatments per each concentration plus the control soil, with the following incubation conditions: (a) C: unamended soil; (b) OK1.5: amended soil with OK so that organic matter in soil was 1.5%; (c) OK1: amended soil with OK so that organic matter in soil was 1%; (d) EH1.5: amended soil with EH so that organic matter in soil was 1.5%; (e) EH1: amended soil with EH so that organic matter in soil

was 1%; (f) FHEB1.5: amended soil with FHEB so that organic matter in soil was 1.5%; (g) FHEB1: amended soil with FHEB so that organic matter in soil was 1%; (h) FHE1.5: amended soil with FHE so that organic matter in soil was 1.5%; (i) FHE1: amended soil with FHE so that organic matter in soil was 1%; (j) FH1.5: amended soil with FH so that organic matter in soil was 1.5%; (k) FH1: amended soil with FH so that organic matter in soil was 1%. Triplicates of every treatment were performed and were incubated for 55 days at room temperature. For each treatment and each incubation time, a sample of 20 g of soil was taken and stored at −20°C in sealed polyethylene jars prior to enzymatic activities and phospholipid fatty acid (PLFA) analyses.

2.5. Soil enzymatic activities and PLFA analyses

After 1, 3, 7, 10, 15, 30 and 55 days of the incubation period and for each treatment four enzymatic activities and bacterial Gram+, bacterial Gram− and fungal PLFAs were measured.

The dehydrogenase, urease, phosphatase and β-glucosidase enzymatic activities were determined using the methods described by García et al. [57], Kandeler and Gerber [58], Tabatabai and Bremner [59] and Masciandaro et al. [60], respectively.

Phospholipids were analysed at the Group of Soil Enzymology and Bioremediation and Organic Wastes Lab, CEBAS-CSIC, Murcia (Spain) according to Bastida et al. [61].

The concentration of each PLFA (ng g^{-1}) was used to indicate the Gram+ and Gram− bacteria as well as fungi biomass.

2.6. Statistical analysis

In order to test the significance of differences among means of the dependent variables measured in the soil for each treatment during the incubation (repeated measurements model) a mixed ANOVA model with a between-subjects factor (incubation time) and a within-subject factor (treatment) was performed. The analysis was made with the

function aov of R statistical software [62]. When the differences were statistically significant the post hoc Tukey HSD test was applied using the R package ‘agricolae’ [63].

3. Results

3.1. BS obtaining process

As mentioned above, OK is a by-product of the soy milk manufacturing process with only 23.3% of dry matter which is composed of fat (23.6%), insoluble fibre (26%) and protein (41.28%) (Table 1). OK’s chemical composition makes it an interesting product to be used as biostimulant/biofertiliser but due to its low water solubility (17%) it has very little bioavailability.

In order to make the most of OK’s chemical composition, it is necessary to degrade the insoluble fraction and make it more bioavailable. To achieve this goal, two biorefinery processes have been proposed, namely enzymatic hydrolysis and a fermentative process. The enzymatic hydrolysate showed an increase of 25% in proteins content with respect to OK. Most of these proteins in OK (91.14%) have a molecular weight higher than 10 kD while in EH 75.74% of them have a molecular weight lower than 1 kD (Table 1).

On the other hand, the fermented hydrolysates FHEB, FHE and FH had practically the same chemical composition since the differences among them are due to physical processes; so only the FHE chemical analysis is shown. The protein content is 42.25% (24% lower than

EH), almost the same as that of original OK; nonetheless, 28.42% of these have a molecular weight between 1 and 5 kD and 71.47% are below 1 kD, that is, 99.89% have a molecular weight under 5 kD.

Table 1 also gathers the elemental analysis of every product. The concentration of phosphorus is higher in EH (11.16 mg kg^{-1}) than both in OK (6.53 mg kg^{-1}) and in

FHE (6.89 mg kg−1). Regarding the potassium values, EH has the highest one (17.14 mg kg−1), followed by FHE (11.25 mg kg−1) and OK had the lowest quantity (9.11 mg kg−1). Nitrogen behaves in a similar way as phosphorus, i.e. EH presents the greatest value (10.64%) among the three products. On the contrary, the carbon content of OK (53.7%) is larger than those of EH (44.81%) and FHE (33.6%).

3.2. Soil microbial activity

3.2.1. β-glucosidase activity

In Table 2 it can be observed that every treatment produced a significant stimulation ($p < .05$) of this activity regarding control soil from the 7th day on, showing a sharp increase until reaching the maximum value between 10th and 15th days in soils amended with either enzymatic or fermentative hydrolysates. Afterwards, an abrupt decrease was produced until the end of the incubation period, reaching values similar to those of the first day. The evolution of β- glucosidase activity in soils amended with OK followed a quite different behaviour, namely, there was an increase during the entire experimental period, reaching the maximum value at the end of the incubation.

The highest β-glucosidase activities were achieved in soils amended with FHE1.5 and FHE1 on the 15th day, being 2.16 and 1.96 μmol PNP g−1 h−1, around four-fold higher than that of the control and two-fold significantly higher than the maximum values of the other products at both concentrations. On the other hand, the maximum β-glucosidase activity in soils amended with EH, FHEB, FH and OK was twice as much that of the control but did not show significant differences among them.

According to the evolution of ß-glucosidase activity over the incubation period, the maximum values, achieved on different days depending on the treatment, were significantly higher than those of the first day. The geatest differences were observed again in soils amended with FHE1.5 and FHE1.

This enzymatic activity was dose-dependent; so the higher the dose the greater the β-glucosidase activity. The differences were significant in the period between the 7th and 30th days, except when FHEB was used, in which case, practically, there were no significant differences during the entire period.

y, it is worth highlighting that at the end of the experimental period β-glucosidase activity in soils amended with fermented BS continued being significantly higher than that of the control, the highest values being found in soils with FHE1.5 and FHE1.

3.2.2. Phosphatase activity

Soil phosphatase activity was also significantly stimulated by every treatment. As happened with β-glucosidase activity, soils amended with enzymatic as well as fermentative hydrolysates displayed a fast rise until reaching the maximum values of phosphatase activity on the 10th day. In soils treated with OK this enzymatic activity increased gradually until the end of the incubation (Table 2).

The higher stimulation was found in soils amended with EH1.5 and EH1 that achieved on the 10th day values of 10.3 and 8.8 μmol PNP g^{-1} h^{-1}, three-fold significantly higher than that of the control and twice as much as the greatest activities of soils treated with OK and fermentative hydrolysates.

As happened with β-glucosidase activity, soil phosphatase activity was dose-dependent with every treatment, although FHEB, FHE and FH BS hardly displayed significant differences among them.

As opposed to what happened with β-glucosidase activity, at the end of the experimental period only enzymatic hydrolytic BS (EH1.5 and EH1) presented phosphatase activity values significantly higher than that of the control soil.

Table 2. Evolution during the experimental period of β-glucosidase and phosphatase activities (mean±standard error) (μmol PNP g−1 h−1) in soils amended with OK and BS obtained from OK.

	1	3	7	10	15	30	55
β-Glucosidase							
C	0.5 a ± 0.05	0.52 a ± 0.07	0.47 a ± 0.02	0.47 a ± 0.1	0.48 a ± 0.03	0.46 a ± 0.08	0.46 a ± 0.03
OK1.5	0.6 bc ± 0.19	0.66 be ± 0.09	0.75 d ± 0.04	0.79 h ± 0.03	0.91 g ± 0.12	0.96 gm ± 0.12	1.07 im ± 0.07
OK1	0.55 cf ± 0.04	0.60 bc ± 0.08	0.65 be ± 0.03	0.71 de ± 0.06	0.80 h ± 0.12	0.88 g ± 0.27	0.96 gm ± 0.18
EH1.5	0.63 bc ± 0.15	0.67 be ± 0.06	0.91 g ± 0.1	1.14 ij ± 0.1	0.85 gh ± 0.08	0.57 cf ± 0.15	0.51 ac ± 0.1
EH1	0.55 cf ± 0.09	0.61 bc ± 0.1	0.68 be ± 0.15	0.89 g ± 0.13	0.71 de ± 0.06	0.49 a ± 0.13	0.45 a ± 0.06
FHEB1.5	0.64 b ± 0.15	0.72 de ± 0.18	0.85 gh ± 0.21	1.02 im ± 0.22	0.84 gh ± 0.14	0.67 be ± 0.1	0.63 bc ± 0.06
FHEB1	0.50 a ± 0.04	0.66 be ± 0.08	0.72 de ± 0.04	0.82 h ± 0.12	0.73 d ± 0.12	0.56 cf ± 0.09	0.54 ac ± 0.15
FHE1.5	0.74 d ± 0.17	0.86 gh ± 0.14	1.02 im ± 0.05	1.82 k ± 0.05	2.16 l ± 0.14	1.53 kn ± 0.1	1.28 j ± 0.1
FHE1	0.67 be ± 0.02	0.78 dh ± 0.12	0.86 gh ± 0.1	1.6k n ± 0.16	1.96 kl ± 0.32	1.46 n ± 0.02	1.10 i ± 0.04
FH1.5	0.64 b ± 0.2	0.82 h ± 0.07	0.92 g ± 0.19	1.29 j ± 0.12	1.05 im ± 0.1	0.79 h ± 0.07	0.78 dh ± 0.23
FH1	0.58 cf ± 0.19	0.69 be ± 0.18	0.81 h ± 0.09	1.11 i ± 0.45	0.91 g ± 0.13	0.58 cf ± 0.12	0.61 bc ± 0.11
Phosphatase							
C	3.28 a ± 0.39	3.28 a ± 0.14	3.25 a ± 0.12	3.25 a ± 0.02	3.29 a ± 0.13	3.27 a ± 0.2	3.24 a ± 0.11
OK1.5	3.84 abc ± 0.37	4.14 bfg ± 0.46	4.68 jl ± 0.48	4.76 jl ± 0.35	5.04 l ± 0.11	5.61 n ± 0.09	6.83 d ± 0.34
OK1	3.72 ac ± 0.1	4.02 bcg ± 0.21	4.13 bfg ± 0.16	4.16 bfg ± 0.27	4.41 fgj ± 0.4	4.95 l ± 0.13	5.49 ln ± 0.24
EH1.5	6.52 de ± 0.33	8.42 h ± 0.57	9.49 km ± 0.5	10.3 k ± 0.23	8.76 hm ± 0.42	6.67 de ± 0.42	5.59 n ± 0.1
EH1	5.94 en ± 0.17	7.89 i ± 0.36	8.00 h ± 0.22	8.80 hm ± 0.24	7.76 i ± 0.25	6.24 de ± 0.16	5.48 ln ± 0.31
FHEB1.5	3.67 ac ± 0.08	4.02 bcg ± 0.15	4.13 bfg ± 0.29	4.83 jl ± 0.08	3.96 bcg ± 0.18	3.94 bc ± 0.33	3.86 bc ± 0.17
FHEB1	3.39 a ± 0.12	3.53 ac ± 0.15	3.73 ac ± 0.26	3.98 bcg ± 0.13	3.63 ac ± 0.2	3.60 ac ± 0.28	3.46 ac ± 0.06
FHE1.5	3.44 a ± 0.16	3.75 abc ± 0.19	4.47 fgj ± 0.12	4.75 jl ± 0.16	4.07 bfg ± 0.14	3.79 abc ± 0.26	3.59 ac ± 0.33
FHE1	3.39 a ± 0.36	3.58 ac ± 0.1	4.06 bfg ± 0.13	4.32 fg ± 0.08	3.70 ac ± 0.3	3.58 ac ± 0.17	3.54 ac ± 0.17
FH1.5	3.68 ac ± 0.08	3.79 abc ± 0.17	3.96 bcg ± 0.08	4.41 fgj ± 0.18	3.91 bc ± 0.18	3.64 ac ± 0.13	3.34 a ± 0.15
FH1	3.51 ac ± 0.24	3.56 ac ± 0.09	3.71 ac ± 0.09	4.03 bcg ± 0.13	3.68 ac ± 0.22	3.45 ac ± 0.14	3.25 a ± 0.1

Notes: Data followed by the same letter are not significantly different ($p < .05$). PNP: *p*-nitrophenol.

3.2.3. Urease activity

Nothing but OK at 1.5% and 1% stimulated the urease activity, which was between 25% and 70% significantly higher than those of the control and the BS from the 7th to the 55th day (Table 3).

3.2.4. Dehydrogenase activity

The application of OK and the BS to soil caused a significant stimulation of the dehydrogenase activity with respect to the control. The significantly highest differences were produced in soils amended with EH1.5 as well as EH1 on the 10th day, which reached values of 11.89 and 9.84, around fourfold that of the control and more than two-fold the maximum activity provoked by fermented BS and OK.

As was the case with β-glucosidase and phosphatase activities, dehydrogenase activity increased sharply since the 1st day until achieving, on the 10th day, the maximum value in soils in which EH, FHEB, FHE and FH were added. Following this, a decrease was produced, taking at the end of the incubation period values akin to the initial ones and to

the control, except for EH1.5 and EH1 which presented a dehydrogenase activity 59% and 42% significantly greater than those of the initial day as well as 100% and 80% higher than that of the control. In contrast, the soils treated with OK at 1.5% and 1% experienced a gradual rise of this parameter up to reaching a maximum at the end of the experimental period, a significant increase in INTF concentration being observed, in relation to the first day of 78% and 56%, respectively.

Contrary to β-glucosidase and phosphatase activities, dehydrogenase activity was not dose-dependent in soils amended with FHEB, FHE and FH although there were significant differences among them. Nonetheless, it was dose-dependent in soils amended with EH during all the incubation period and in soils treated with OK at the end of the incubation.

Table 3. Evolution during the experimental period of urease (μmol NH+4 g−1 h−1) and dehydrogenase activities (mean ± standard error) (μmol INTF g−1 h−1) in soils amended with OK and BS obtained from OK.

	1	3	7	10	15	30	55
Urease							
C	2.29 a ± 0.2	2.24 a ± 0.1	2.30 a ± 0.12	2.22 a ± 0.12	2.28 a ± 0.25	2.27 a ± 0.06	2.29 a ± 0.17
OK1.5	2.56 a ± 0.3	2.71 ab ± 0.2	2.88 bc ± 0.4	2.95 bc ± 0.22	3.03 c ± 0.05	3.83 d ± 0.02	3.99 d ± 0.18
OK1	2.52 a ± 0.19	2.59 a ± 0.61	2.88 bc ± 0.15	2.94 bc ± 0.79	3.35 cd ± 0.25	3.48 d ± 0.11	3.60 d ± 0.16
EH1.5	2.46 a ± 0.27	2.48 a ± 0.19	2.34 a ± 0.31	2.36 a ± 0.16	2.48 a ± 0.17	2.37 a ± 0.2	2.33 a ± 0.15
EH1	2.40 a ± 0.2	2.26 a ± 0.05	2.35 a ± 0.3	2.47 a ± 0.11	2.32 a ± 0.23	2.31 a ± 0.34	2.27 a ± 0.25
FHEB1.5	2.50 a ± 0.19	2.49 a ± 0.29	2.52 a ± 0.17	2.47 a ± 0.13	2.38 a ± 0.19	2.46 a ± 0.17	2.45 a ± 0.13
FHEB1	2.38 a ± 0.18	2.39 a ± 0.09	2.44 a ± 0.36	2.44 a ± 0.17	2.51 a ± 0.15	2.50 a ± 0.21	2.42 a ± 0.15
FHE1.5	2.43 a ± 0.17	2.34 a ± 0.19	2.38 a ± 0.14	2.27 a ± 0.4	2.42 a ± 0.25	2.44 a ± 0.25	2.43 a ± 0.23
FHE1	2.43 a ± 0.19	2.57 a ± 0.09	2.47 a ± 0.15	2.29 a ± 0.17	2.42 a ± 0.09	2.42 a ± 0.07	2.40 a ± 0.06
FH1.5	2.45 a ± 0.2	2.13 a ± 0.11	2.32 a ± 0.3	2.53 a ± 0.2	2.54 a ± 0.07	2.46 a ± 0.13	2.39 a ± 0.18
FH1	2.37 a ± 0.13	2.43 a ± 0.19	2.46 a ± 0.22	2.41 a ± 0.09	2.29 a ± 0.16	2.35 a ± 0.18	2.33 a ± 0.12
Dehydrogenase							
C	2.72 a ± 0.03	2.86 a ± 0.24	2.81 a ± 0.15	2.80 a ± 0.11	2.78 a ± 0.32	2.79 a ± 0.05	2.86 a ± 0.2
OK1.5	3.46 be ± 0.22	3.54 be ± 0.2	4.02 cf ± 0.09	4.36 fi ± 0.03	4.60 im ± 0.11	5.08 lm ± 0.3	6.17 h ± 0.61
OK1	3.37 be ± 0.24	3.47 be ± 0.25	3.80 bc ± 0.17	4.18 fi ± 0.18	4.27 fi ± 0.32	4.98 lm ± 0.25	5.26 lm ± 0.34
EH1.5	3.84 bc ± 0.12	4.09 cf ± 0.17	7.36 g ± 0.43	11.89 j ± 0.63	8.57 n ± 0.48	6.56 gh ± 0.52	6.09 h ± 0.39
EH1	3.74 b ± 0.18	3.45 be ± 0.19	6.18 h ± 0.4	9.84 k ± 0.03	6.97 g ± 0.28	5.37 lm ± 0.23	5.31 lm ± 0.32
FHEB1.5	3.06 ad ± 0.06	3.14 ade ± 0.13	3.40 be ± 0.17	3.75 b ± 0.06	3.46 be ± 0.11	3.11 ade ± 0.64	2.97 ad ± 0.05
FHEB1	2.92 ad ± 0.07	2.94 ad ± 0.12	3.19 de ± 0.13	3.44 be ± 0.15	3.19 de ± 0.07	3.05 ad ± 0.07	2.93 ad ± 0.15
FHE1.5	3.46 be ± 0.2	4.09 cf ± 0.36	5.28 lm ± 0.9	6.57 gh ± 0.33	4.35 fi ± 0.17	3.69 b ± 0.19	2.95 ad ± 0.28
FHE1	3.00 ad ± 0.17	3.81 bc ± 0.31	4.27 fi ± 0.48	5.53 hl ± 0.28	3.65 b ± 0.17	3.02 ad ± 0.48	2.87 a ± 0.23
FH1.5	3.18 de ± 0.44	3.85 bc ± 0.55	4.22 fi ± 0.22	5.66 hl ± 0.37	4.73 m ± 0.72	3.95 cf ± 0.35	3.02 ad ± 0.24
FH1	2.91 ad ± 0.39	3.28 de ± 0.2	3.81 bc ± 0.5	5.34 lm ± 0.25	4.67 m ± 0.86	3.23 de ± 0.28	2.96 ad ± 0.08

Notes: Data followed by the same letter are not significantly different (*p* < .05). INTF: 2-p-iodo-3-nitrophenyl formazan.

3.3. Soil microbial community

Every treatment increased the soil bacterial and fungal populations (Tables 4 and 5). Bacterial Gram+ and Gram− PLFA showed the highest values on the 10th day when the soil was treated with both enzymatic and fermented BS and at the end of the incubation in soils amended with OK. The greatest concentrations of bacterial Gram+ PLFA corresponded to soils with EH1.5 and EH1 with values three-fold significantly higher than that of the control and between 20% and 50% significantly greater with respect to the maximum reached with OK and the fermented hydrolysates at both concentrations. With regard to Gram− PLFA, the maximum concentration was found in the soil amended with OK1.5 (359.2 ng g^{-1}). This was four-fold significantly greater than that of the control and from 25% to 70% significantly higher than fungal PLFA in soils amended with EH, FHEB, FHE and FH at both doses.

Contrary to the bacterial population, the fungal population reached the maximum concentration on the 15th day, the significantly highest values yielded when FHE and FHEB were applied to the soil, which were approximately four times as much as the fungal PLFA concentration of control soil. Likewise, depending on the treatment and on the dose, fungal population in these soils was between 10% and 55% higher than the maximum values found in soils amended with the other products.

As happened with β-glucosidase phosphatase and dehydrogenase activities, it is worth mentioning that at the end of the experimental period bacterial Gram+ and Gram− as well as fungal PLFA measured in soils amended with EH and fermented BS were significantly higher when compared to the control soil. Again, the highest differences depended on the parameter measured, so the highest bacterial Gram+ and Gram− PLFA were achieved in soils treated with EH and the greatest fungal PLFA were produced by FHE. In spite of that, the three parameters displayed little differences among BS and not always were statistically significant.

Finally, as was observed with enzymatic activities, the higher the dose applied to the soil the greater the soil bacterial Gram+, Gram− and fungal populations.

Table 4. Evolution during the experimental period of bacterial Gram+ and Gram− PLFA (ng g−1) (mean ± standard error) in soils amended with OK and BS obtained from OK.

	1	3	7	10	15	30	55
Bacterial Gram+							
C	183.3 a ± 2.2	184.6 a ± 0.9	186.0 a ± 1.2	187.5 a ± 0.8	186.1 a ± 1.8	185.5 a ± 1.4	188.2a ± 0.7
OK1.5	201.3 a ± 1.6	245.0 cd ± 2.6	245.2 cd ± 0.9	335.0 f ± 0.5	397.3 l ± 0.9	435.2 k ± 1.7	475.2j ± 2.2
OK1	198.7 a ± 1	222.7 ce ± 0.8	241.5 cd ± 1	254.7 d ± 2.2	308.6 hi ± 0.7	322.7 fh ± 2.2	395.2 l ± 2.2
EH1.5	284.3 b ± 1.2	330.8 f ± 0.9	449.3 j ± 0.7	692.4 n ± 2	560.0 m ± 1.9	323.5 fh ± 1.9	314.8hi ± 1.8
EH1	282.4 b ± 0.6	320.1fh ± 1.5	423.1 k ± 0.9	559.1 m ± 2.7	442.3 j ± 1.3	321.0 fh ± 0.9	313.5hi ± 1.2
FHEB1.5	226.7 c ± 2.3	286.8 bg ± 1.6	387.9 l ± 1.1	455.3 j ± 1.4	389.3 l ± 1.6	268.9 g ± 1	214.9e ± 1.1
FHEB1	224.7 ce ± 0.5	272.6 g ± 1.0	292.2 bi ± 1.3	374.8 o ± 0.8	342.2 q ± 1.4	265.6 g ± 1.2	213.6e ± 1
FHE1.5	245.7 cd ± 0.6	315.7 hi ± 1.2	565.5 m ± 2.2	515.4 jp ± 2.7	347.7 q ± 1.1	266.2 g ± 1.7	215e ± 1.8
FHE1	236.7 c ± 0.6	301.8 bi ± 0.8	448.3 j ± 1.5	355.7 o ± 1.2	326.0 fh ± 1.8	263.7 dg ± 0.7	213e ± 1.1
FH1.5	225.6 c ± 0.7	266.9 g ± 1.1	317.5 hi ± 1	533.4 mp ± 0.9	456.8 j ± 1.3	323.3 fh ± 1.3	213.3e ± 1.3
FH1	215.7 e ± 0.7	263.9 dg ± 1.1	311.0 hi ± 1	408.6 l ± 0.4	387.9 l ± 1.2	320.6 fh ± 1.3	211.8e ± 1.7
Bacterial Gram−							
C	79.4 a ± 0.7	80.3 a ± 0.6	79.4 a ± 0.4	79 a ± 0.3	80.5 a ± 0.4	79.6 a ± 0.6	80.0 a ± 0.2
OK1.5	92.4 bc ± 1.3	102.4 de ± 0.6	125.6 h ± 0.5	203.2 lm ± 0.7	205.3 lm ± 0.6	216.3 mp ± 0.5	359.8 r ± 1.3
OK1	89.9 c ± 0.8	98.6 be ± 0.6	104.4 de ± 0.6	115.5 dn ± 0.8	136.1 g ± 0.4	160.5 f ± 1.1	208.1 lm ± 0.7
EH1.5	111.9 d ± 0.8	154.9 f ± 1.4	264.5 i ± 0.5	286.0 o ± 0.4	277.5 io ± 1	154.2 f ± 1.2	125.6 h ± 0.8
EH1	108.8 d ± 1.5	153.1 f ± 1.1	238.1 j ± 0.5	262.8 i ± 0.8	175.5 k ± 0.4	153.2 f ± 1.2	121.2 hn ± 0.1
FHEB1.5	107.5 d ± 1.3	155.5 f ± 1.2	179.4 k ± 0.6	239.3 j ± 0.6	250.2 j ± 0.5	155.6 f ± 1.7	117.5 dn ± 0.6
FHEB1	91.3 c ± 1	154.7 f ± 1	176.6 k ± 0.9	214.7 mp ± 0.4	134.5 g ± 0.9	127.8 hq ± 0.7	116.0 n ± 1.2
FHE1.5	111.1 d ± 0.3	138.2 g ± 1.2	263.3 i ± 0.4	279.4 io ± 0.6	185.0 k ± 0.4	157.3 f ± 0.9	123.3 hn ± 1.4
FHE1	93.4 bc ± 1.2	136.3 g ± 0.8	242.3 j ± 0.7	263.3 io ± 0.4	155.0 f ± 0.4	131.7 gq ± 0.9	89.5 c ± 0.5
FH1.5	92.4 bc ± 0.6	134.1 g ± 0.6	197.7 f ± 0.8	212.2 m ± 0.5	229.1 p ± 1	152.4 f ± 0.9	126.1 h ± 0.8
FH1	86.0 c ± 0.4	132.7 gq ± 1.3	158.7 m ± 0.6	206.1 lm ± 0.7	156.3 f ± 0.5	137.7 g ± 0.6	125.9 h ± 0.5

Note: Data followed by the same letter are not significantly different ($p < .05$).

Table 5. Evolution during the experimental period of fungal PLFA (ng g−1) (mean ± standard error) in soils amended with OK and BS obtained from OK.

	1	3	7	10	15	30	55
C	17.5 a ± 0.8	18.8 a ± 0.7	18.0 a ± 0.6	17.0 a ± 0.3	18.3 a ± 0.3	18.6 a ± 0.4	17.1 a ± 0.4
OK1.5	31.0 b ± 0.5	32.7 h ± 1	39.6 jm ± 0.4	47.4 lp ± 0.5	49.1 p ± 0.3	52.0 q ± 0.8	53.3 q ± 0.6
OK1	18.2 a ± 0.4	20.8 d ± 0.6	23.2 ef ± 0.7	27.7 co ± 0.3	31.5 bh ± 0.3	34.3 i ± 0.2	39.4 jm ± 0.4
EH1.5	29.1 bo ± 0.4	29.9 bo ± 0.3	42.4 jk ± 0.3	52.2 q ± 0.2	57.6 s ± 0.3	42.6 jk ± 0.5	27.3 co ± 0.6
EH1	16.6 a ± 0.3	21.4 fd ± 0.3	44.3 l ± 0.3	47.3 lp ± 0.5	46.4 lp ± 0.3	25.4 ce ± 0.2	24.0 e ± 0.7
FHEB1.5	25.3 ce ± 0.5	31.6 bh ± 0.9	33.1 hi ± 0.5	57.7 s ± 0.6	63.7 r ± 0.4	31.7 bh ± 0.4	23.7 ef ± 0.7
FHEB1	20.5 d ± 0.5	25.8 ce ± 0.7	29.2 bo ± 0.2	38.6 mn ± 0.7	52.2 q ± 0.5	29.3 bo ± 0.9	22.8 f ± 0.5
FHE1.5	24.5 e ± 0.4	33.5 hi ± 0.4	38.9 mn ± 0.9	46.0 lp ± 0.6	69.8 t ± 0.9	36.4 in ± 0.2	36.8 in ± 0.9
FHE1	21.2 fd ± 0.4	32.9 h ± 0.8	35.4 in ± 0.4	40.7 jm ± 0.4	61.3 r ± 0.6	18.7 a ± 0.4	19.3 dg ± 0.9
FH1.5	21.9 fd ± 0.4	24.7 e ± 0.5	26.6 c ± 0.5	51.2 q ± 0.2	55.8 s ± 0.8	47.5 lp ± 0.5	25.8 ce ± 0.7
FH1	24.5 a ± 0.4	22.3 f ± 0.5	27.4 co ± 0.4	48.6 p ± 0.6	45.7 l ± 0.9	38.7 mn ± 0.9	16.8 a ± 0.7

Note: Data followed by the same letter are not significantly different ($p < .05$).

4. Discussion

The application of organic wastes to soil is a well-known practice to increase soil fertility which can affect the structure of soil microbiota and therefore the dynamic of nutrients in soils [64]. One of the most important nutrients is nitrogen since it is found among the most common limiting elements to primary production and other ecosystems

processes [65]. Considering that proteinaceous materials are one of the major nitrogen components in soils [66], the first step in the liberation of this element is the breakdown of proteins by extracellular proteases which according to Jan et al. [67] is the major bottleneck in nitrogen cycling.

Both the enzymatic and fermentative hydrolyses increased the protein concentration in BS as well as the percentage of peptides with a low molecular weight which are more soluble than proteins and can be more easily taken up by microorganisms [67] and plants [13,17]. On the other hand, these results are in agreement with those of other authors who used an enzymatic hydrolytic process to obtain BS from different agro-industrial by-products and sewage sludges and obtained enzymatic hydrolysates with a high percentage of low
molecular weight peptides [11–12]. However, the molecular weight distribution in the fermented hydrolysates shown in this article is below 5 kD which is not in accordance with other researchers like Parrado et al. [37] who used a fermentative process with B. licheniformis to hydrolysate chicken feathers and found a molecular weight profile akin to that of enzymatic hydrolysates. This can be due to the nature of the raw material. In fact, Rodríguez-Morgado et al. [16] obtained a BS from chicken feathers through enzymatic hydrolysis with a higher molecular size than that of the BS produced using vegetable wastes [10,12,14]. Another aspect to bear in mind is that EH has a higher P concentration than OK and fermented BS (FHEB, FHE and FH). This is in accordance with Parrado et al. [10] who observed an increase of P in the enzymatic hydrolysate with regard to the raw material, although there are several works where the concentration of this element decreases [11,13,56], the greatest concentration being in the insoluble phase after hydrolysis. This could be due to phytic acid being associated with proteins [68] which are broken down during the enzymatic hydrolysis and

consequently this macroelement is released. Nevertheless, fermented BS and OK present a similar P concentration perhaps owing to the inmobilisation of this element by microorganisms [69].

According to Tejada et al. [70] enzymatic activities can be used as indicators of overall microbial activity in soils, the knowledge of hydrolases activities being of great importance because they are related to the cycle of such important nutrients as N, P and C. In the present study, β-glucosidase was stimulated by every treatment and was dose-dependent, but OK behaved in a quite different way to both enzymatic and fermented BS. In soils treated with OK the maximum value of this enzyme activity was displayed at the end of the experimental period, while soils with EH, FEHB, FHE and FH achieved the highest values around the 10th day. The differences in the behaviour of raw OK and hydrolysed OK could be due to its composition. β-glucosidase plays a significant role in the degradation of organic matter, catalysing the hydrolysis of low molecular weight carbohydrates in soils [71]. Original OK has a high content in insoluble fibre which is not present in the hydrolysates. Therefore, firstly, this compound has to be broken down. However, in the hydrolysates these complex compounds do not exist and all the carbohydrates are soluble and easier to breakdown. Thus, these results are in agreement with those expressed in other articles in which a gradual increase of β-glucosidase activity was found when different organic residues were added to soil [1,4,61]. Likewise, the behaviour of hydrolysates is also in accordance to what has been observed in different works where this kind of products was obtained [10,15,16]; nonetheless these authors reported that β-glucosidase reached the maximum value around the fifth and seventh days of the incubation period. Paying attention to the β-glucosidase activities of EH and fermented hydrolysates (FHEB, FHE and FH) only there were significant differences between FHE and the other

hydrolysates. The maximum values of EH, FHEB, FH and OK were similar and did not present significant differences. As mentioned in the introduction section several studies have been performed to produce enzymes by fermentation using different agro-industrial by-products. In this sense, Shubin et al. [72] carried out fermentation with B. subtilis Pa5 obtaining a broth with a high β-glucosidase activity. This could explain why soils amended with FHE had the highest β-glucosidase activity. Regarding FH, its chemical composition is similar to FHE but it was submitted to a high-temperature treatment so that the enzymes were denatured, losing its activity.

Phosphatase showed a behaviour analogous to β-glucosidase in soils amended with OK and hydrolysates which is according to many authors [4,11,12]. Soils amended with EH displayed the highest values of this enzyme followed by OK. Phosphatase enzymes are released by microorganisms to mineralise organic phosphorus [69]; so the higher the concentration of this, the higher the phosphatase activity. This could explain the greater activity in soil amended with EH, since it has the highest concentration of P, but comparing OK with fermented BS (FHEB, FHE and FH) the former presented the greatest activity, despite P concentration being similar to FHEB, FHE and FH. A possible explanation of this behaviour is that phytate is the main form of P occurring in legumes and different species of Bacillus excrete extracellular phytase which catalyses the hydrolysis of these phytates to myo-inositol and phosphoric acid [73]. According to this, during the fermentation B. licheniformis could have excreted phytase to hydrolyse the phytate and release the inorganic phosphorus, while these enzymes exist neither in the enzymatic hydrolysis nor in raw OK. So phosphorus in fermented BS is found as phosphate and in EH and OK as organic phosphorus.

On the other hand, enzymatic and fermented hydrolysates did not stimulate urease activity, possibly because of the presence of low molecular weight peptides which are

easily transported through the cellular membrane and, therefore, microorganisms do not need to excrete any enzyme to breakdown the organic compounds to obtain easily available N. These results agree with other authors who found that urease activity was not stimulated after application of different BS [11,12,56]. On the contrary, when OK was applied to soil a stimulation of urease was observed which was increasing gradually until the end of the incubation period. The results obtained with OK are similar to those found by other authors when applied to different soil organic wastes (vermicompost, manure, compost, MSW, green manure, etc.) with high molecular weight proteins which must be hydrolysed to release N. In these cases, the urease activity increased during the experimental period [7,8].

With regard to dehydrogenase activity, the present work brought to light that soils amended with EH displayed significantly highest values compared with the other treatments. In addition, the soils treated with FHEB and FH showed values similar to OK. As happened with phosphatase and β-glucosidase, in soils where OK was added dehydrogenase increased gradually, reaching the maximum activity at the end of the incubation.

Several authors have reported that the addition of organic matter of different chemical compositions to soils produces a stimulation of dehydrogenase. Such a stimulation is gradual and achieves the highest values at the end of the incubation period when the organic wastes added to soil are composed of complex organic matter which firstly has to be mineralised to release the nutrients [18,61,74]. In the same way as other enzymatic activities, in soils amended with enzymatic as well as fermented hydrolysates, dehydrogenase activity increased sharply with a maximum on the 10th day and then decreased until the end of the experimental time. Dehydrogenase is involved in the intracellular flux of electron to O2, that is to say, it is linked with respiratory processes

so that can be considered a measurement of microbial oxidative activities in soils [57,75]. Tejada et al. [70] found that dehydrogenase activity was a good index of microbial biomass in Mediterranean soils treated with different organic wastes. The sharpest and fast increase of this enzyme in soils amended with protein hydrolysates has been reported by other authors using BS obtained from different organic wastes [3,11,12,76]. As exposed above, the lower molecular weight peptides are easily taken by microorganisms. Moreover more complex proteins are resistant to being hydrolysed because they are protected by humic material and inorganic soil components such as clays and iron and aluminium oxy-hydroxides [66]. Accordingly, the higher content in low molecular weight peptides' distribution, the easier the soil microbiota takes them and the greater the dehydrogenase activity. This explains why soils amended with EH presented the highest values of the mentioned activity. Nevertheless, soils treated with fermented hydrolysates, despite having a lower molecular weight profile, had a lower dehydrogenase activity than EH and similar to OK. Possibly this is a consequence of its chemical composition, given that FHEB, FHE and FH have a considerably lower concentration of proteins than EH and similar to OK.

The effect of the different amendments on soil microbiota was studied, analysing the PLFA profile. Organic amendments stimulated both bacterial and fungal growth as observed by Rodríguez-Morgado et al. [16,18] and Bastida et al. [61]. In this sense, bacterial and fungal PLFA in soils amended with fermented and enzymatic BS increased in the first days of incubation in consonance with dehydrogenase activity and due to the same reason, namely the presence of low molecular weight peptides. Besides, Meidute et al. [77] studied the influence of different carbon and nitrogen sources and concluded that easily available carbon sources, such as simple carbohydrates, favoured bacterial

growth, especially in nitrogen-rich media but there are opportunistic fungi which also grow in this media. In this work, in
general, both bacteria and fungi underwent a higher increase in soils amended with fermented and enzymatic BS compared with OK, probably due to what has been explained above. The highest bacterial biomass corresponded to EH possibly because it has the greatest proteins content.

The present work also points out that β-glucosidase and phosphatase activities as well as bacterial and fungal PLFA in soils amended with OK, EH and fermented hydrolysates are dose-dependent but dehydrogenase activity only is dose-dependent in soils amended with EH and OK.

Finally, the BS FHEB produced the lower enzymatic stimulation and the lower bacterial and fungal proliferation compared with the other treatments. Saison et al. [78] demonstrated that in soils amended with composts, the compost-borne microorganisms were outcompeted by the soil microorganisms, the microbial structure changes being essentially due to the use of a compost rich in organic matter. Similarly, Pimmata et al. [79] in a study about bioremediation of soil by bioaugmentation drew as conclusion that a short time after bioaugmentation there was a competition with the indigenous microorganisms and consequently a decrease in the soil activity. This could explain why FHEB presented the lower enzymatic activities.

In conclusion, an enzymatic hydrolysis and a fermentative process have been developed to manufacture different hydrolysates with BS properties. These hyrolysates induced a fast and sharp increase of enzymatic activities as well as bacterial and fungal PLFA in the first days of incubation compared with control soil. Soils amended with EH reached the highest dehydrogenase activity regarding raw OK and fermented hydrolysates. The

work also concludes that B. licheniformis is outcompeted by soil microorganisms and therefore the soils amended with this BS presented the lower soil activity.

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