SCAP
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Biochemical and microbiological soil effects of a biostimulant based on *Bacillus licheniformis*-fermented sludge

Efectos bioquímicos y microbiológicos en el suelo de un bioestimulante a base de lodos fermentados de *Bacillus licheniformis*

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ABSTRACT

A fermentative process has been applied to carry out the conversion of sludge from slaughterhouse wastewater treatment into a biostimulant complex. The three biostimulant components are: a) The *Bacillus licheniformis* biomass, which is a plant growth promoter rhizobacteria (PGPR), and the organism in charge of the fermentative process, b) the enzymatic secretion of said microorganism, mainly peptidases and amidases (50%), involved in N metabolism, and glucanases (33.3%), involved in carbohydrate metabolism, and c) the hydrolyzed sludge organic matter, largely composed of peptides and free aminoacids. The biostimulant has been evaluated in soil at the biochemical and microbiological level. It induced a strong microbial stimulation of the soil, and produced the specific stimulation of PGPR soil bacteria, which was studied through a metabarcoding. Moreover, mainly when applying the soluble fraction of the biostimulant complex, it was found that the Bacillus licheniformis inoculum remains active in the environment at the end of the experiment.

Keywords: Protein hydrolysate, metabarcoding, *PGPR*, enzymes, biodiversity

RESUMEN

Se ha aplicado un proceso fermentativo para llevar a cabo la conversión de lodos procedentes de la depuración de aguas residuales de matadero en un complejo bioestimulante. Los componentes bioestimulantes son: a) la biomasa de *Bacillus licheniformis* que es una rizobacteria promotora del crecimiento vegetal (PGPR), y organismo encargado del proceso fermentativo, b) la secreción enzimática de dicho microorganismo, principalmente peptidasas y amidasas (50%), implicadas en el metabolismo del N, y las glucanasas (33,3%), implicadas en el metabolismo de los hidratos de carbono, y c) la materia orgánica del lodo hidrolizado, compuesta en gran parte por péptidos y aminoácidos libres. El bioestimulante ha sido evaluado en suelo a nivel bioquímico y microbiológico. Indujo una fuerte estimulación microbiana del suelo y produjo la estimulación específica de las bacterias del suelo PGPR, que fue estudiada a través de un metabarcode. Además, principalmente al aplicar la fracción soluble del complejo bioestimulante, se encontró que el inóculo de Bacillus licheniformis permanece activo en el ambiente al final del experimento.

Palabras-clave: Hidrolizado protéico, metabarcoding, *PRPR*, enzimas, biodiversidad

INTRODUCTION

Biostimulants are applied to plants or to the rhizosphere in order to enhance the natural process, improving the absorption of nutrients and the efficiency, quality and tolerance of crops to abiotic stresses (du Jardin, 2015). In soil they have shown a positive effect over the soil biological fraction, having a direct implication over the soil fertility (Caballero *et al*., 2020). Changes produced by biostimulants can be monitorized studing the nutrient-recycling enzymes expressed by the soil microbiota, that act as indicators of the quality and state of fertility of soil, and at microbiological level, thanks to metabarcoding techniques using 16S rRNA sequencing (Parlapani *et al*., 2018).

Sewage sludge, the organic by-product resulted from the treatment of wastewater, supposes a serious environmental issue. Scientific community is constantly searching for new alternatives uses. Considering sluge as raw material for the formulation of biostimulants is gaining interest as long as it does not exceed limit values for organic pollutants, nor heavy metals and it is sanitized in order to eliminate its pathogenic microorganisms.

Biostimulants, obtained by enzymatic or fermentative technology from different substrates, and composed by low molecular weight peptides and free amino acids, microbial metabolites, such as phytohormone analogues, polysaccharides, humic substances, beneficial microorganisms, etc., have shown positive effects on soil biostimulation and bioremediation of soil contaminants (Tejada *et al*., 2013, 2014).

Recent results in our group revealed that exogenous aplication of subtilisine from *Bacillus*, a naturally-produced-in-soil enzyme, produced stimulation of soil enzymes and changes in microbial biodiversity, favouring specific PGPR species (Caballero *et al*., 2020). Thus, a *Bacillus*-fermented sludge, composed by such enzyme and by a broad spectrum of other enzymes, would lead to interesting changes.

Our purpose is to evaluate the soil biostimulating capacicity, both at biochemical and microbiological level, of a *Bacillus*-fermented sludge obtained by fermentative technology.

MATERIAL AND METHODS

Biostimulant obtention and experimental design

Biostimulant products were obtained through a physical-fermentative process as described by Rodriguez-Morgado *et al*. (2019) (Figure 1).

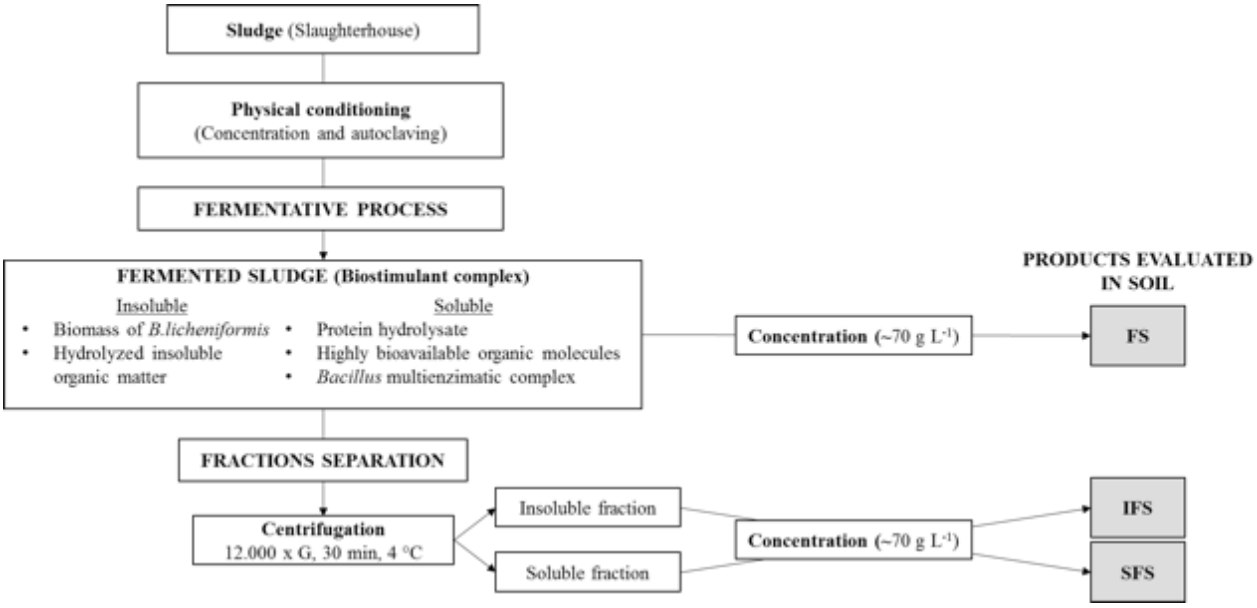

Figure 1 - Diagram of the process for obtaining experimental products.

FS product contains a total dry matter of 63.1±0.17 g L^{-1}, being 14.6% soluble matter. Bacterial concentration (*B. licheniformis*) in FS is 2.01 x 10^8 ± 1.11 x 10^8 CFU g^{-1}. IFS (insoluble fraction) is composed by the *Bacillus* biomass and by the hydrolyzed insoluble organic matter; SFS (soluble fraction) is composed by the enzymatic secretion of *Bacillus* and by highly bioavailable soluble hydrolyzed organic matter, mainly composed by peptides and free aminoacids.

Experimental design was stablished according to previous studies (Caballero *et al*., 2020). Thus, microcosms of 250 g of soil were preincubated at 30–40 % at water holding capacity for 7 days. After this phase each product were added (0.1 and 0.5 % w/w) to soil under the following experimental conditions:

- *C:* Control
- *SFS:* Addition of FS
- *SIFS:* Addition of IFS
- *SSFS:* Addition of SFS

Analytical techniques

Proteomic study: Sample preparation and LC-MS analysis were carried out following the procedure described by Parrado *et al*. (2014).

Metabarcoding analysis: Soil DNA extraction, Illumina MiSeq sequencing and the analysis of the microbial community composition was performed as described in a previous work (Macias-Benitez *et al*., 2020).

Determination of soil enzymatic activities

Dehydrogenase activity was measured as described by García *et al*.; phosphatase activity was determined as described by Tabatabai and Bremner.

Statistical analysis

Soil enzymatic activities were compared using a one-way analysis of variance (ANOVA), followed by a Tukey test. Level of significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Enzimatic characterization of the biostimulant

LC-MS analysis reported that the enzimatic component present in the soluble fraction of the biostimulant is comprised by proteins with hydrolitic and transport functions (Information not shown). Secreted hydrolases were 50% peptidases and amidases, related to N metabolism, and 33.3% glucanases, related to carbohydrate metabolism.

Evaluation of the biostimulant capacity in soil

<u>Changes at biochemical level</u>

It was observed that the soluble fracion of fermented sludge (SFS), and to a lesser extent the complete product (FS), produced the greatest stimulation on the soil biological activity (dehydrogenase activity SFS 0.5 % w/w: 2,89 ± 0,10 mmol INTF g^{-1} h^{-1}; FS 0.5 % w/w: 1,66 ± 0,23 mmol INTF g^{-1} h^{-1}, Figure 2).

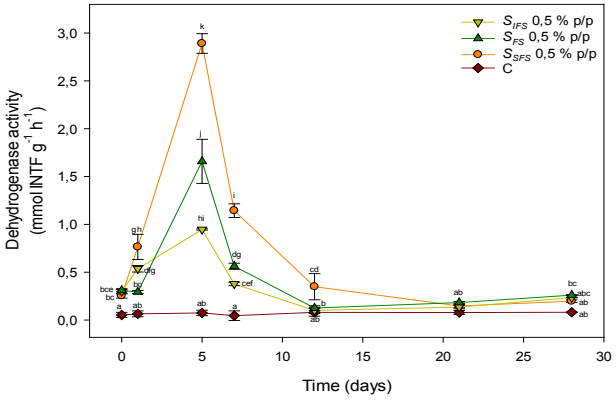

Figure 2 - Dehydrogenase activity in controls and soils treated with biostimulants. Points (mean ± SD) with the same letter(s) are not significantly different from each other ($p > 0.05$).

In all cases, maximum peaks of stimulation were reached on day 5. The main reason to such stimulation is the molecular size profile of the soluble organic component of fermented sludge, constituted 65.95 ± 0.09% by organic molecules of molecular size under 1 KDa, mainly peptides, free amino acids and other highly bioavailable organic molecules, easily assimilated by the soil microbiota (Estrada *et al*., 2013).

Also notable was the stimulation produced by SFS on phosphatase activity coinciding with the peak of dehydrogenase activity (SFS 0.5 % w/w: 0.46 ± 0,02 mmol PNF g^{-1} h^{-1}, Figure 3), explained by the depletion of phosphorus as a consequence of the stimulation of the soil microbiota, by synthesing microbial phosphatases to make it more bioavailable.

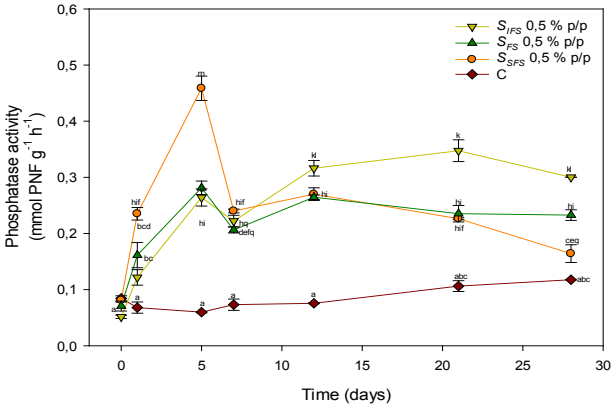

Figure 3 - Phosphatase activity in controls and soils treated with the biostimulants. Points (mean ± SD) with the same letter(s) are not significantly different from each other (p>0.05).

Changes at microbiological level

Biostimulants produced changes within the soil microbial structure compared to the control, causing the stimulation of some bacterial genera bibliographically classified as beneficial microbes.

Showing a low presence in control samples, the relative abundance (RA) of Oxalobacteriaceae family, which encompasses several endophytic bacteria classified as plant growth promoters (PGPB) (Lambrecht *et al*., 2000), was induced at 5 days by the three treatments (increase in RA of 10.1%, 7.9% and 8.9% for TFS, SFS and IFS respectively). Within this family, the endophytic genus Herbaspirillum, includes nitrogen-fixing species, producers of phytohormones, such as gibberellin and auxin and siderophores, and have the ability to solubilize inorganic phosphorus among other PGPB capabilities (Rosconi *et al*., 2013).

Comamonadaceae family also was clearly induced (increase in RA of more than 50 %) by TFS and SFS treatments.

Maybe the most drastic change was found by SFS in the Moraxellaceae family coming to suppose 18.3% of total RA at five days. This change is entirely due to the increase of genus *Acinetobacter* that includes certain PGPB involved on the production of hormones, solubilization of phosphate, and production of siderophores. In addition, other strains of *Acinetobacter* exhibit indirect PGPR activity via the growth suppression of phytopathogenic fungi, and potential biocontrol properties against pathogenic bacteria (Xue *et al*., 2009).

Regarding to Bacillaceae family, it is represented by the genus *Bacillus* known to exert PGPR activity, protecting plants from phytopathogen and simultaneously increase the yield in different crops (Elanchezhiyan *et al*., 2018). As expected, it shows the highest RA at the beginning in TFS and IFS treated soils, decreasing along the time, while oppositely, in SFS treatment, it increases reaching similar values to TFS and IFS soils at five days (10.84% of total relative abundance).

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