Design circular economy bioprocesses for winemaking waste conversion into biostimulants: Functional evaluation.

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Abstract

A circular economy process has been developed to convert the lees, a major waste product in winemaking, into new agronomic biostimulants through enzymatic bioprocessing. This biostimulant was chemically characterized by analyzing antioxidant activity, as well as the composition of phenolic compounds, peptides, and amino acid content. The potency of the biostimulant in soils was also evaluated by measuring the activity of soil enzymes (dehydrogenases, glucosidases, phosphatases, and ureases) and its impact on bacterial biodiversity through metabarcoding. The biostimulant potency in plants was assessed in grapevines, analyzing functional parameters such as net photosynthetic rate and delayed fluorescence. This study was completed with an analysis of gene expression in plants using the RNAseq technique. In conclusion, the new bioprocessing approach for valorizing winemaking lees allows the production of a biostimulant that has a very positive effect on plants, enhancing photosynthetic efficiency and the expression of crucial genes in grapevine metabolism, as well as inducing an increase in soil fertility and promoting the induction of bacteria that enhance plant growth.

Keywords: biostimulants, grapes, lees, enzymatic hydrolysis.

Introduction

Typical wastes and by-products from wineries include grape pomace (skins and seeds, representing on average about 60% of the total winemaking by-products and stems about 14%), grape solids and fermentation (yeast) lees (25%), wastewaters rich in organic compounds (up to about 15 L/L of wine) (De Iseppi et al., 2020), carbon dioxide from the fermentation process, exhausted filtration materials and fining agents. Derived from wine industries, approximately seven million tons of grape pomace and lees are originated annually worldwide (Bordiga et al., 2019). It is estimated that from 100 kg of grapes 20-25 kg of by-products are obtained and that the annual quantity produced in Spain is approximately 1200 million kg (assuming an annual grape production of 6500 million kg).

In recent years, there is a growing interest in reusing these food wastes not only to reduce their environmental impact by the circular production of by-products but also to entail an economic benefit derived from the reuse of products with added value (Dwyer et al., 2014)· Given a circular economy approach, some of these wastes can be successfully “recycled”, reused, or recovered, improving both the economic and environmental. These by-products are typically used for animal feeding, composting, industrial biomass, or distillate production (Bordiga et al., 2015). However, grape pomace and lees are high added-value by-products due to its wide variety of compounds. The wine lees are a combination of yeasts, organic acids, insoluble carbohydrates, inorganic salts, lignin, proteins, phenolic compounds, and ethanol (Jara-Palacios, 2019). These compounds are susceptible of extraction or transformation and exploitation with the consequent economic benefit.

The biostimulant capacity implies, among other properties, the capacity to protect against abiotic stress situations (du Jardin, 2015). Among the abiotic stressors, those involving oxidative stress, such as high levels of ozone, are of special relevance. Considering the relevance of the wine industry in mediterranean countries such as Spain, Greece, and Italy, with high level of tropospheric ozone, there is an emerging interest in search for protection strategies to avoid the damages described above, being of special interest those that do not generate environmental toxicity such as extracts of plant origin. In this context the enzymatic technology developed by our group can be a good option to convert viticulture wastes into biostimulants, providing an interesting option for circular economy.

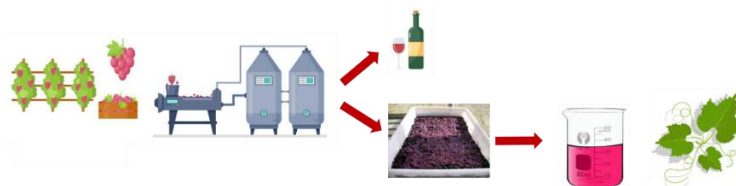

Fig 1. Diagram of extraction or transformation and explotation of wine lees.

Material and Methods:

Biostimulant preparation:

Agro-industrial waste from the wine industry wastes was obtained from the companies "Alvinesa Natural Ingredients, S.A" (Daimiel, Ciudad Realo) and from the wineries "Cooperativa Nuestra Señora del Socorro" (Rociana del condado, Huelva). The lees were processed by enzymatic hydrolysis using subtilisin (EC 3.4.21.62), a protease from *Bacillus licheniformis* (Biocom, Spain) as hydrolytic agents in a bioreactor with controlled temperature (55°C) and pH (pH 9), using the pH-stat method (Parrado et al., 2006).
In addition, enzymatic biostimulant production was monitored by studying the performance of the process as a function of the evolution of the extraction of soluble hydrolyzed biomolecules as peptides, carbohydrates and polyphenols.

Chemical characterization

The evaluation of the antioxidant capacity was evaluated using the PPPH, ABTS assays. The determination of total phenols was determined by the Folin-Ciocalteu methods by reacting the extracts with the homonymous reagent (absorbance at 765 nm after 2 hours).

Physiological Status in Plants

Analyses of photosynthetic parameters

At the end of the experiment, photosynthetic parameters in plants were measured by an IRGA (LI-6400XT, LI-COR Inc., Nev., EEUU) with a light chamber for the leaf (Li-6400-02B, Li-Cor Inc.). Measurements were recorded between 10 am and 2 pm from random leaf in each plant (n=20) and the parameters used were described in Macias-Benitez et al. (2021). Data were recorded and the net photosynthetic rate (AN), the electron transport rate (ETR), and the effective quantum yield of PSII (PhiPSII) was determined.

Delayed Fluorescence Measurements

Delayed fluorescence (DF) was detected using a plant imaging system (NightShade LB 985, Berthold Technologies, Germany) equipped with a deeply cooled CCD camera according to López-Jurado et al. (2020). From plants of each treatment, 2–3 intact leaves of approximately the same size were separated and placed in the plant imaging system. The leaves were illuminated for 20 s with light supplied from far red (730 nm), red (660 nm), green (565 nm), and blue (470 nm) LED panels at 2, 105, 40, and 110 µmol.m–2.s–1, respectively. Immediately after the LEDs were turned off, DF was measured, and the recorded intensities of light were converted to counts per second (cps). Data were then normalized to each leaf area to obtain comparable cps values across treatments.

RNAseq:

Sample Collection:

The sample collection was carried out following the protocol provided by the company Corning.

Extraction, purification of Samples and library Preparation:

The extraction and purification of the input RNA was performed by GENEWIZ Multiomics & Synthesis Solutions from Azenta Life Sciences.

Mapping sequence reads to the reference genome

Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Vitis_vinifera_GCA_030704535.1 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step.

Extracting gene hit counts

Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2. The hit counts were summarized and reported using the gene_id feature in the annotation file. Only unique reads that fell within exon regions were counted.

Differential Gene Expression Analysis

After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the customer-defined groups of samples was performed. The Wald test was used to generate p-values and log2 fold changes. Genes with an adjusted p-value < 0.05 and absolute log2 fold change > 1 were called as differentially expressed genes for each comparison.

Bioinformatics tools for functional analysis:

To verify the annotation, and thus the function of the overexpressed genes and proteins, the gene ontology provided by UniprotKB, annotations from NCBI, PATRIC, and Ecogene were consulted, as well as the gene ontology assigned by the JCVI Microbial Resource Center. Additionally, these genes and proteins were sorted according to the orthologous classification provided by KEGG (Kanehisa et al., 2008), incorporating into this classification those genes and proteins reviewed by the various annotations and ontologies mentioned earlier. As the first functional analysis, the different functional categories described in the clusters of orthologous groups (COG) associated with each overexpressed gene or protein were consulted.

Studies on soil:

Metabarcoding

Soil DNA Extraction and Illumina MiSeq Sequencing

Soil samples underwent genomic DNA extraction utilizing the DNeasy Power-Soil DNA isolation kit (Qiagen) in accordance with the provided instructions. The extracted DNA, amounting to a final volume of 100 µl, underwent scrutiny alongside a DNA extraction blank in each round to assess potential cross-contamination.

Library preparation ensued by targeting the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. This was achieved through amplification utilizing the primer pair Bakt 341F/Bakt 805R, with Illumina-specific sequencing sequences appended to their 5' ends (Herlemann et al., 2011).

The PCR was conducted in a 25 µl volume, comprising 12.5 µl of Supreme NZYTaq 2x Green Master Mix (NZYTech), 0.5 µM of each primer, 2.5 µl of template DNA, and ultrapure water. Cycling parameters included an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min.

Subsequently, barcoding sequences necessary for multiplexing different libraries during sequencing were appended in a second PCR round under identical conditions, albeit with only five cycles and an annealing temperature of 60°C. A negative control devoid of DNA served to monitor potential contamination during library preparation.

Validation of library size occurred through gel electrophoresis, followed by purification of the libraries using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). Equimolar pooling was conducted based on quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific). Ultimately, the pooled library underwent paired-end sequencing on an Illumina MiSeq PE300 platform.

Analysis of Microbial Community Composition

Sequencing data underwent thorough processing utilizing Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0) following the methodology outlined by Caporaso et al. (2010). Initially, raw FASTQ files were subjected to demultiplexing and trimmed using CUTADAPT 1.3 (Martin, 2011), followed by merging via FLASH (Magoč and Salzberg, 2011). Quality filtering and labeling were performed by QIIME 1.9.0 with stringent criteria, including merging sequences with an overlap exceeding 30 bp, allowing for two nucleotide mismatches in primer matching, discarding reads shorter than 300 nucleotides, and quality filtering merged reads with a minimum Phred quality score of 20.

Subsequently, all chimeric sequences were identified and excised utilizing the UCHIME algorithm (Edgar et al., 2011) integrated into VSEARCH, utilizing the Greengenes reference database (DeSantis et al., 2006). The remaining sequences were clustered into operational taxonomic units (OTUs) employing a de novo approach at a threshold of ≥ 100% identity, with singleton OTUs being filtered out. Each OTU was taxonomically assigned a representative sequence using the RDP classifier (Wang et al., 2007) with a confidence threshold of 97%.

Various alpha diversity indices, including Chao, Good's coverage, Simpson, Shannon, and phylogenetic diversity, were computed to assess species diversity within each sample.

The OTU data files generated by QIIME were imported into R version 3.5.1 (R Core Team, 2018) for further analysis and visualization. OTU counts and taxonomic assignments were amalgamated into a phyloseq object using the phyloseq R package (McMurdie and Holmes, 2013).

Subsequently, rarefaction, relative abundance, and heatmap plots were constructed utilizing a combination of Vegan (Oksanen et al., 2018) and ggplot2 (Wickham, 2016) R packages. Principal coordinate analysis (PCoA) utilizing the Weighted-Unifrac distance metric was employed to visualize the microbial community structure relative to each treatment and time point. Additionally, Venn diagrams depicting shared, common, and/or unique OTUs among samples were generated using the Venn Diagram R package (Chen, 2018).

The original sequence data have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB35168.

Enzymatic activities in soil:

For each experimental treatment, the activity levels of four soil enzymes were measured in triplicate at days 2,5,7,12,20 and 30 during the incubation period. Dehydrogenase activity was measured as the reduction of 2-p-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride to iodonitrophenyl formazan (García et al. Citation1993). Urease activity was determined using urea as substrate (Kandeler and Gerber 1988). β-glucosidase activity was determined using p-nitrophenyl-β-D-glucopyranoside as substrate (Eivazi and Zakaria Citation1993). Phosphatase activity was measured using p-nitrophenyl phosphate as substrate (Tabatabai and Bremner Citation1969).

Statistical Analysis

To discern disparities in soil chemical and biochemical attributes across various treatments, we employed one-way analysis of variance (ANOVA), supplemented by the least significant difference (LSD) test. These analyses were conducted using the agricolae R package (Mendiburu, 2019), with all statistical significance levels set at $p < 0.05$.

Results and discussion:

Extraction and characterization process of the biostimulant:

The composition of the lees makes it an interesting product to be used as a biostimulant. To fully exploit its properties, it is necessary to degrade the insoluble fraction and make it more available. To achieve this goal, enzymatic hydrolysis has been proposed using the enzyme subtilisin. This protease extracts, solubilizes, and hydrolyzes the initial proteins into soluble peptides, and also leads to the solubilization of hydrophobic compounds such as lipids and bioactive metabolites. The enzymatic hydrolysate showed an approximate increase of 13% compared to the control without enzymes, reaching up to a 38% total solubilization The biostimulant´s characterization is shown in Table 1.

	Lyophilized (% humidity)		FOLIN (mg EG/L)		DPPH (µmoles TE/L)		ABTS (mM)		KJELDHL (% N)	
Samples	MEDIA	SD	MEDIA	SD	MEDIA	SD	MEDIA	SD	MEDIA	SD
Lees	94,05	0,03	2133,84	233,82	8377,91	157,54	34,16	1,47	4,80	0,16
BSs	90,58	1,08	19328,89	2402,99	60008,52	2778,85	214,66	2,90	6,91	0,27

Table1. Characterization of the biostimulant obtained from the enzymatic hydrolysis of grape residues.

As shown in the table, the biostimulant showed a higher percentage of nitrogen with respect to the lees, and also obtained approximately 10 times more antioxidant activity compared to the control.

Evaluation of a biostimulant activity:

Application in plants:

Biostimulant capacity of products obtained after enzymatic hydrolysis of lees were assayed in grape plants grown inside the University of Seville Glasshouse General Services on a phytoclimatic chamber. Plants were foliar sprayed with an aqueous solution grape less-derived products. Control plants were spayed with water.

Imaging techniques were applied to evaluate the leaves on which biostimulant treatments were directly applied. Color and general appearance were evaluated, as well as the spectral profile in both the visible and near infrared. Multivariate statistical techniques were used to analyze colorimetric and spectral differences between leaves on which biostimulants were applied and leaves of control vines. In this way, the effect of these biostimulants on the plant was studied.

Delayed fluorescence, is the extremely weak light emitted by pre-illuminated intact plants, it provides powerful tool to study stress reactions in plants. The biostimulants produced from winemaking residues induce an increase in induced fluorescence (Figure 1), which indicates that they stimulate the plant by improving the photosynthetic system.

Additionally, to evaluate physiological status in plants, Net photosynthetic rate, ETR, PhiPS2, Fv'/Fm' and delayed fluorescence were analyzed. After exposure to the biostimulant, the net photosynthetic rate, ETR, and PhiPS2 were significantly affected (Table 2), showing an increase in all parameters except for the Fv'/Fm' ratio, which remained practically the same as that of the control. Both the electron transport rate (ETR) and the efficiency of photosystem II (PhiPS2) showed an increase of 5%; however, the net photosynthetic rate increased by 28%, and delayed fluorescence increased by around 36% compared to the control plants.

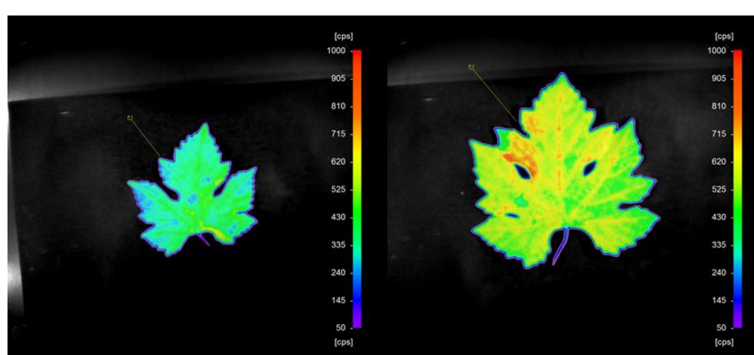

Figure 1. Delayed fluorescence of grape plant after biostimulant applied.

	Net photosynthetic rate		ETR		PhiPS2		Fv'/Fm'		Fluorescense	
	MEDIA	SD	MEDIA	SD	MEDIA	SD	MEDIA	SD	MEDIA	SD
BSs	9,00178	1,105024	27,68898	3,407564	0,131489	0,016182	0,914489	0,025827	44,99814	6,474643
Control	7,221032	0,5061	26,47586	3,004994	0,124123	0,038577	0,925686	0,016208	33,00665	2,56511

Table 2. Physiological parameters. The parameters that have been measured are Net photosynthetic rate, ETR (Electron Transport Rate), PhiPS2 (Photosystem II Efficiency), Fv'/Fm' (Photosystem II Fluorescence), and delayed fluorescence.

Analyze gene expression in plants:

Next, RNA-seq has been carried out, RNA-seq is a powerful technique used to globally analyze gene expression in plants. In this case, it has been used to study how biostimulants affect gene expression in plants, providing valuable information about the underlying mechanisms of their action and their effects on plant growth and development.

The results obtained showed that when the biostimulant was applied to the plants, it caused 64% of the differentially expressed genes to be inhibited, while 36% of the genes were overexpressed compared to the control plants.

As observed in Figure 2, three cellular behaviors stood out for concentrating the highest number of differentially expressed genes, namely the plasma membrane with around 20% of the genes, the cytoplasm with 6%, and the chloroplast with 4%.

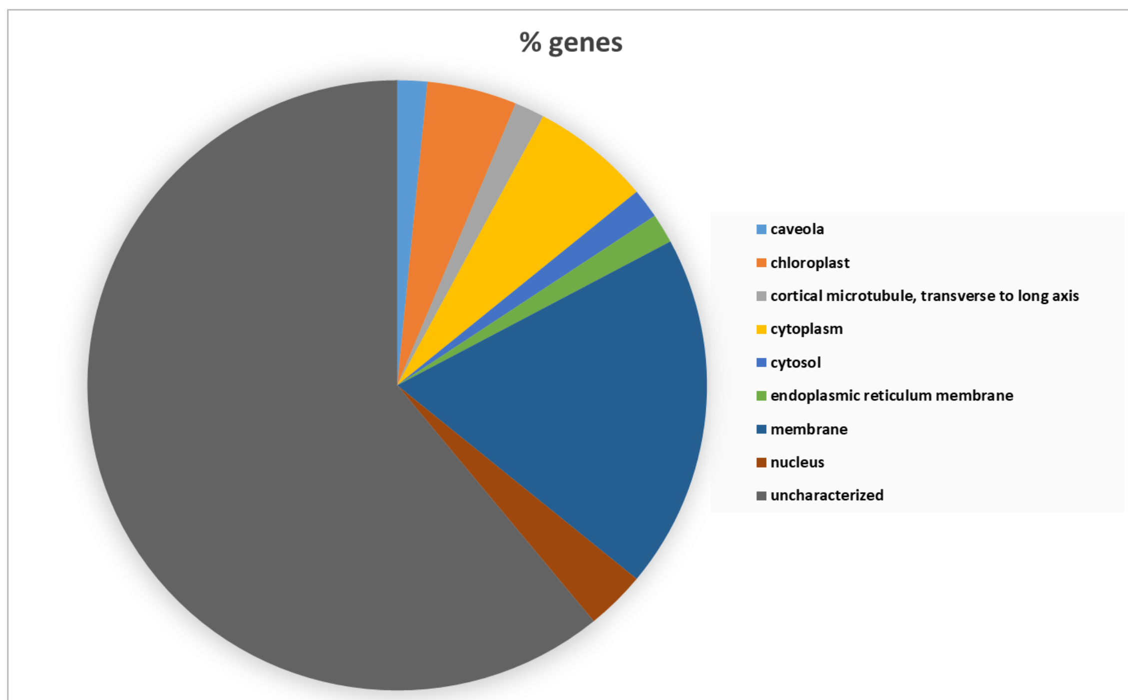

Figure 2. Distribution of differentially expressed genes across various cellular components of the eukaryotic plant cell according to the Uniprot database.

Next, the percentage of differentially expressed genes located within each category defined in the clusters of orthologous groups was globally studied. As observed in Figure 3, around 40% of the genes were annotated as 'Uncharacterized protein.' The most representative categories by the number of genes are Secondary metabolites biosynthesis, transport, and catabolism with 10%, and the groups Amino acid transport and metabolism and Transcription with approximately 6% each.

It's worth noting that the application of the biostimulant has led to the overexpression of certain proteins important for plant metabolism, such as, for example, glutathione transferase (VIT_04s0079g00690), transferring alkyl or aryl groups, other than methyl groups. This enzyme is relevant for its involvement in glutathione metabolism, which is an organic molecule with critical functions in meristem development, senescence, intracellular redox reaction homeostasis, and plant defense against xenobiotics, among others.

Another group of important proteins are Fe2OG dioxygenase domain-containing proteins (VIT_18s0001g03430 and VIT_18s0001g14310), which are involved in the biosynthesis of flavonoids, a major class of plant secondary metabolites that serve a multitude of functions including pigmentation and antioxidant activity.

Finally, highlight the Phenylalanine ammonia-lyase (VIT_06s0004g02620). These proteins are involved in the biosynthesis of phenylpropanoids. Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine and have a wide variety of functions as both structural and signaling molecules.

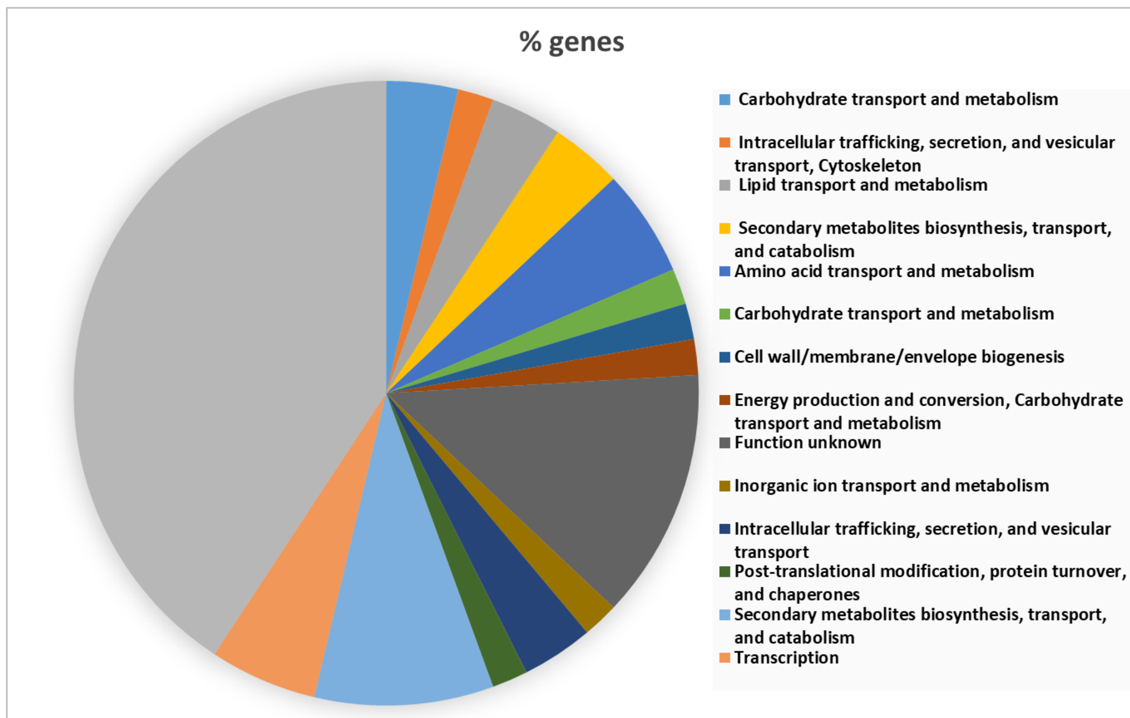

Figure 3. Distribution of the percentage of differentially expressed genes across various functional categories assigned by COG ("Cluster of orthologous groups") in plants subjected to biostimulant application.

Application in soils:

Effects on Soil Bacterial Community Composition:

The control soil samples exhibited 15 genera, with the main genera being Nocardioides, Sphingomonas, Rubrobacter, and Blastococcus, but their abundances varied according to the incubation time (Figure 4).

Surprisingly, in soils treated with the biostimulant, the microbiota changed drastically, with 18 genera of bacteria observed, with the most abundant groups being Bacillus, Lysinibacillus, Desemzia, Clostridium, Solibacillus, Flavisolibacter, Nocardioides, and Georgenia. The abundance of bacteria varied according to the incubation time, with both the populations of Bacillus and Lysinibacillus decreasing by approximately 50%, while Clostridium and Solibacillus decreased by around 80% after 30 days of incubation. Conversely, other genera increased in abundance, for instance, Desemzia increased by around 25%, Georgenia by 67%, and Nocardiales by approximately 90% compared to the data obtained after 5 days.

These genera that have emerged thanks to the action of the biostimulant become relevant because most of them belong to a group of bacteria called psychrophilic and psychrotolerant phosphate-solubilizing bacteria (PSB). These bacteria have the ability to improve low-temperature conditions while maintaining their physiological activities; therefore, they represent a solution to the negative effects caused by low temperatures, which disrupt physiological and metabolic processes, resulting in significant crop losses worldwide (Rizvi et al., 2021).

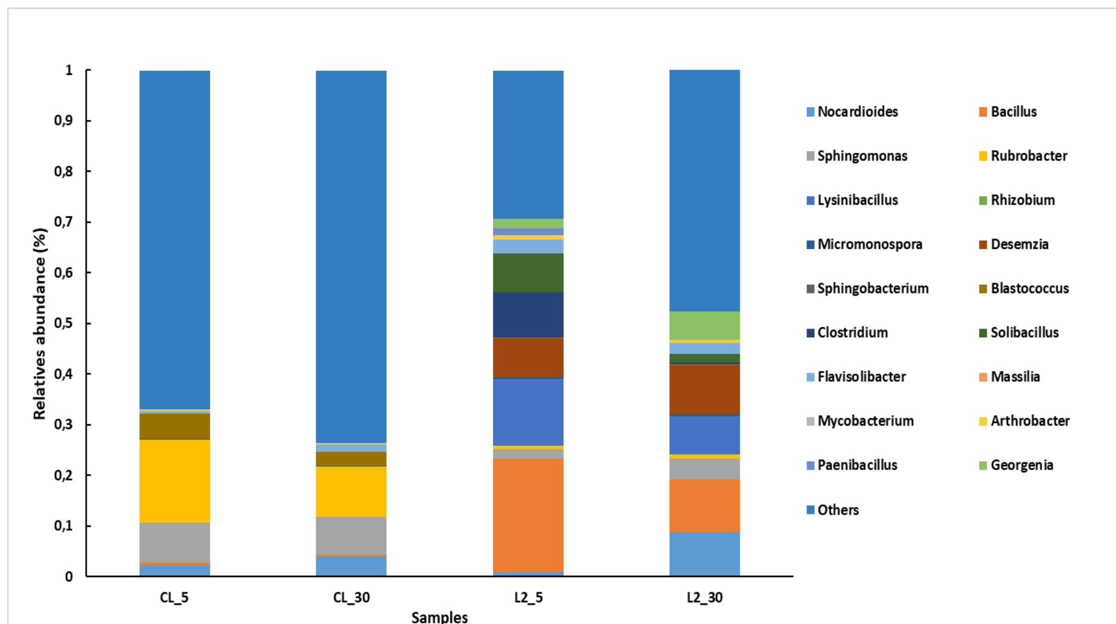

Figure 4. Bacterial community composition at genus level. C, control sample; L, lees treated sample. T5: day 5; t30: day 30.

Influence of Biostimulant on Soil Enzyme Activities

Soil enzymes play a pivotal role as mediators and catalysts in numerous biological processes within the soil, offering a comprehensive biological evaluation of soil functions (Nannipieri et al., 2002).

Dehydrogenase, Glucosidase, Phosphatase, and Urease enzymes have served as indicators to gauge the impact of agronomic practices on soil quality or health (Gajda and Mortyniuk 2005, Baležentienė and Klimas 2009). These enzymes play a crucial role in releasing essential elements such as C, N, and P, which are vital for plant nutrition. Dehydrogenase activity offers insights into soil microbial processes, serving as a marker for organic matter oxidation rates, as it is exclusively found in living systems. Among these enzymes, ß-glucosidase is frequently utilized to assess soil quality under various management regimes, while urease catalyzes the conversion of urea into ammonium and carbon dioxide, and phosphatase is integral to the phosphorus cycle (Gajda and Martyniuk 2005, Gil-Sostres et al. 2005).

The soils treated with the biostimulant showed a significant stimulation of dehydrogenase, glucosidase, and phosphatase activities on days 2 and 5, progressively decreasing in the following days, with a more pronounced decline observed in the dehydrogenase and phosphatase enzymes (Figures A-C). Regarding urease enzyme, no significant differences were observed among all measurements (Figure 5D).

These results are consistent with those obtained in Metabarcoding, as it appears that this increased enzymatic activity could be due to the higher number of bacteria found when the biostimulant is added to the soil. Furthermore, as explained in the previous section, these bacteria are PSB (phosphate-solubilizing bacteria); therefore, it is logical to think that this increased phosphatase enzyme activity, for example, is partly due to the phosphate solubilization generated by these bacteria. Additionally, they are PGPR (plant growth-promoting rhizobacteria), so they provide various benefits to the soil, which can be observed in the measured enzymatic activities.

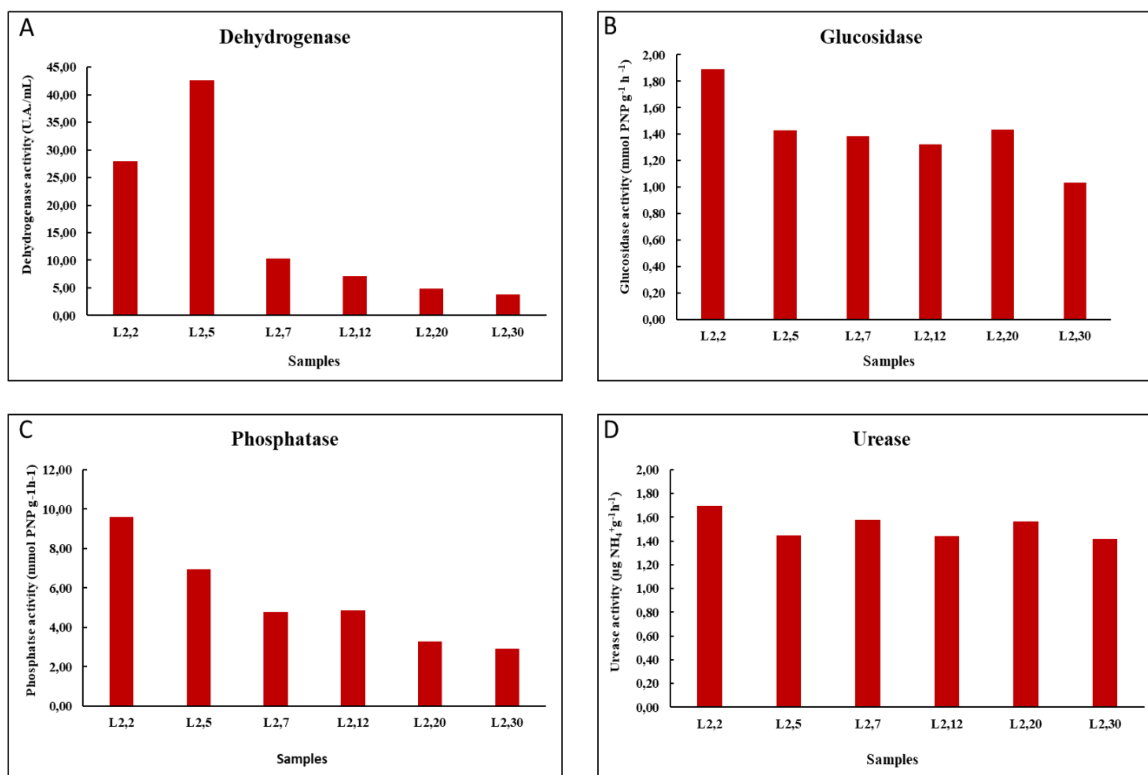

Figure 5. Evolution during the experimental period dehydrogenase, glucosidase, phosphatase and urease activities in soils with BS**.**

Conclusion

A new circular economy process for converting winemaking waste into new agronomic bio-stimulants has been developed, and its potency in plants has been verified through its ability to induce the photosynthetic efficiency and the expression of crucial genes in grapevine metabolism, as well as inducing an increase in soil fertility and promoting the induction of bacteria with biofertilizer activity.

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