

Microbial volatile organic compound dynamics in solid-state fermentation: evaluation of antifungal activity

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Abstract: Volatile organic compounds (VOCs) are low-molecular-weight compounds biosynthetically produced by plants, animals, humans, fungi, and bacteria, and involved in inter- and intraspecific signalling. Microbial VOCs are metabolic molecules that play a crucial role in microbial communication, including the regulation of *quorum* sensing. In recent years, VOCs have gained interest in research focused on new environmentally friendly molecules active against phytopathogens. This interest aligns with the European Farm to Fork and Biodiversity Strategy under the European Green Deal, which aims to reduce the use of chemical and hazardous pesticides by half by 2030. Numerous VOCs produced by different bacterial genera, including *Bacillus*, *Pseudomonas*, and *Streptomyces* are known for their activity against phytopathogenic bacteria and fungi. Generally, microbial VOCs activity is exerted through growth inhibition of other microorganisms present in the same environment, affecting hyphae morphology or conidiation. Even if the production of VOCs occurs naturally in microbial metabolism, the volatilome of each species can be influenced by the microbial community composition, nutrient availability, and temperature

Streptomyces, gram-positive and filamentous bacteria belonging to the phylum Actinobacteria, are among the highest producers of natural secondary metabolites with antimicrobial and antibiotic activities. They are widespread in soil, particularly in the rhizosphere. They are also found in symbiosis with plants, mainly in roots. Interest in *Streptomyces* spp. has increased in recent years thanks to genomic and metabolomic analyses, which have revealed a high number of potential secondary metabolites present in cryptic Biosynthetic Gene Clusters (BGCs), i.e. clusters of genes that are expressed under specific conditions, such as the presence of stress or the substrate in which they grow on. For these properties, diverse *Streptomyces* strains are being studied as possible biocontrol agents (BCAs) or as sources of specific bioactive molecules that can serve as alternatives to conventional chemicals. Moreover, *Streptomyces* produce a wide range of volatiles. The best-known VOC produced by the *Streptomyces* genus is geosmin, a compound with the earthy odour associated with petrichor. Various volatiles produced by *Streptomyces* spp., like 3-methyl-butanol, 2-pentanone, and 2-hexenal, have antimicrobial activity against several phytopathogens, including *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium culmorum*, and *Botrytis cinerea*. Growing *Streptomyces* spp. on unconventional substrates, such as agrifood waste, through solid-state fermentation could valorise agricultural wastes and trigger cryptic BGCs with antimicrobial activity that can be used in phytopathogen control. Solid-State Fermentation (SSF) is an alternative cultivation process in which microorganisms are grown on a solid substrate without the presence of free water. This method is used for the industrial applications to produce secondary metabolites, spores, and biotransformations.

Streptomyces sp. DEF603 is a strain isolated from *Vitis vinifera*, whose antimicrobial activity has been tested in vitro. SSF of *Streptomyces* sp. DEF603 on defatted wheat bran was previously optimised in our research group. Genomic analysis confirmed the presence of genes encoding for molecules with antimicrobial activity in *Streptomyces* sp. DEF603 genome.

In this work, the volatilome released by *Streptomyces* sp. DEF603 during SSF was analysed together with the evaluation of the antimicrobial activity to find potential active molecules against phytopathogens. An untargeted HS-SPME-GC-MS analysis was carried out at four sampling times (4, 7, 10, and 14 days after inoculation) during SSF to follow the shifts and changes in VOCs composition in both inoculated and non-inoculated (control) wheat bran. The analysis allowed us to putatively annotate 132 compounds, mainly belonging to the classes of terpenoids, alkanes, aldehydes, ketones and alcohols among others. Data were successively analysed through Principal Component Analyses (PCAs) highlighting a clear separation (i) between inoculated and non-inoculated samples and (ii) among samples collected at different time points. Subsequently, the data were analysed using ANOVA, and significantly altered metabolites were visualized with a clustered heat map. This analysis revealed that the volatilomes segregated into two distinct clusters: non-inoculated and inoculated substrates. Subclusters were also observed in both groups, whose disposition in the graph is related to chemical variations induced by the time (days of fermentation). In the volatilome of *Streptomyces*-fermented substrate, common VOCs produced by *Streptomyces*, such as geosmin and 2-methyl-pentanone, were also found.

The antimicrobial activity of VOCs produced by *Streptomyces* strain DEF603 during SSF was tested against four phytopathogens: *Fusarium culmorum*, *Fusarium oxysporum* f.sp. *basilici*, *Botrytis cinerea*, and *Alternaria alternata*. We observed that the antimicrobial activity increased with time in SSF, reaching a peak at 14 days with growth inhibition of almost 70% in all fungi, except for *F. oxysporum* f.sp. *basilici*, which was less sensitive to microbial VOCs. At the same time, VOCs produced by non-inoculated defatted wheat bran appeared to both increase the growth and the development of aerial mycelium of the four fungal pathogens. In addition to these results, VOCs produced on SSF caused a morphological variation in *B. cinerea* aerial mycelium and spore production.

Synthesis of bioactive VOCs by *Streptomyces* sp. strain DEF603 active against phytopathogens is a further confirmation of the potential of this strain to act as a putative BCA to be applied in the field as a complement to chemicals, in line with principles of the Integrated Pest Management (IPM). Further analyses are required to confirm the efficacy of the strain in systems that try to mimic open field conditions.

Key words: Solid-State Fermentation, *Streptomyces*, Volatile Organic Compounds, biocontrol agent, phytopathogens control