

Abstract

Fruits are particularly vulnerable to fungal infestations or other physiological disorders during storage thus diminishing their market value. Post-harvest fruit treatments with fungicides like thiabendazole (TBZ), imazalil (IMZ), and *ortho*-phenyl-phenol (OPP), and antioxidants like diphenylamine (DPA) are the most effective means to minimize fruit spoilage in storage. The latter is no more registered for use in the EU, although exemption authorizations for 120 days have been given considering that there are no equally effective alternatives in the market for the control of apple scald.

Pesticide application methods in fruit-packaging plants (dipping, drenching, spraying, or waxing) are water-based generating at the end of the process large volumes of wastewaters containing high concentrations of pesticide (0.2 to 2 g/l depending on the molecule). Direct discharge of these wastewaters into the environment without prior treatment constitutes a serious point-source of contamination. This problem becomes more acute when the high persistence (TBZ, IMZ) and toxicity (all) of the pesticides used is taken in account. Therefore, a wastewater treatment system able to efficiently depurate the wastewaters derived from the post-harvest handling of fruits is required, considering also that authorization for the use of those pesticides TBZ, IMZ, OPP, and DPA was granted under the clause that an effective treatment of the wastewaters produced is operative. However, the only system actually patented (CONTROL TEC-ECO® system) is highly costly precluding its market uptake. Thus, a sustainable, cheap, effective, and environmentally friendly method for the depuration of the fruit packaging wastewater is needed. Microbial degradation of pesticide is considered the most important process for their environmental loss. The development of biological treatment facilities based on the degrading ability of microorganisms seems promising. The implementation of such systems will require inoculation with start-up inocula possessing high degradation capacity against the target compounds. However, very little (OPP, DPA) or nothing (TBZ, IMZ) is known regarding the microbial degradation of those chemicals.

The current doctoral thesis aimed to fulfill this gap and provide novel insights into the microbial degradation of those pesticides with future practical implications for environmental protection. Thus the main research aim of this thesis was to isolate and

characterize bacteria able to degrade the major pesticides used in the fruit packaging industry (TBZ, IMZ, OPP, and DPA). Enrichment cultures from soils collected from wastewater disposal sites led to the isolation of TBZ, OPP, and DPA-degrading bacteria, while, despite different attempts, it was not possible to isolate any bacterium able to degrade IMZ.

Three TBZ-degrading consortia able to rapidly degrade the fungicide and use it as a C and N source were obtained. DGGE analysis and cloning showed that all TBZ-degrading consortia were composed of two to eight main members of proteobacteria. Different microbiological (antibiotics treatment) and molecular (DNA/RNA-DGGE, cloning, q-PCR) approaches, showed that a *Sphingomonas* sp. strain was responsible for the degradation of TBZ, while the role of other bacterial members was probably supportive regarding nutrition or degradation of metabolites produced (C1-compounds). However, the bacterium was not obtained in pure culture due to its limited capacity to grow on the agar media tested. The TBZ-degrading consortium was able to degrade 750 mg/l TBZ in liquid culture and 500 mg/kg TBZ in artificially contaminated soil. Moreover, it was able to degrade TBZ in a wide range of pH (4.5-7.5) and temperatures (15-37°C) and in the presence of OPP and DPA and their respective degrading bacteria.

An OPP-degrading bacterium identified as *S. haloaromaticamans* was isolated in pure culture. The bacterium was able to rapidly degrade OPP only when supplemented with casamino acids or in the co-presence of at least another bacterial strain (e.g. *Pseudomonas stutzeri*), an observation suggesting the incapacity of the degrading isolate to synthesize some essential amino acids like the ones included in casamino acids. The isolated *S. haloaromaticamans* strain was able to metabolize up to 150 mg/l OPP within 7 days and to use it as an energy source for growth. OPP was metabolized by the isolate in a wide range of pH (4.5-9) and temperatures (4-37°C) and in the presence of different pesticides (TBZ and DPA) used in the fruit packaging industry and of their pesticide-degrading microbes.

Regarding DPA, a *P. monteilii* was isolated which was able to degrade rapidly DPA and use it as a carbon and nitrogen source. Molecular analysis showed that the specific strain had polymorphisms in the different copies of its 16S rRNA gene, a common feature of many Pseudomonads. The isolated bacterium was able to rapidly degrade spillage level concentrations of DPA in liquid media (2 g/l) and in artificially contaminated soil (1 g/kg). Its degrading activity was not impaired in a wide range of

pH (4.5-9) and temperatures (4-37°C) or by the co-presence of other pesticides possibly contained in wastewaters (TBZ and OPP) and their respective degrading bacteria.

Overall, the results of this thesis provide novel insights into the largely unknown area of microbial degradation of the pesticides contained in wastewaters from the fruit-packaging industry. The pesticide-degrading bacteria isolated showed promising properties which could make them valuable tools in future biotechnological applications including their use a) as tailor-made inocula in biological wastewater treatment facilities, b) in *in situ* bioaugmentation of pesticide-polluted wastewater disposal sites adjacent to fruit-packaging plants and c) for bioaugmentation of modified biobed systems receiving wastewaters from the fruit packaging industry. Follow up studies will focus on both basic and practical challenges that have arisen including a) the elucidation of the metabolic pathway *via* advanced analytical tools and of the genetic mechanisms involved in the degradation of the pesticides studied *via* comparative genomic – proteomic analysis and b) the full-scale application of the isolated bacteria in biological wastewater treatment facilities.