Investigation of pseudomonads, pathogenic to oyster mushroom and the possibilities of their biological control

Ph.D. THESIS

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1. INTRODUCTION

The mushroom cultivation is increasingly important today, both in our country and worldwide. Many species of edible mushrooms can be grown easily; furthermore, they are delicious and nutritious, therefore becoming increasingly popular for food. The oyster mushroom (*Pleurotus ostreatus*) is one of the most extensively cultivated mushrooms in the world, closely behind the bottom mushroom (*Agaricus bisporus*). However, the success of cultivation depends on the proliferation of different bacterial and fungal pathogens. These cause problems both in the cultivation processes and during the storage.

In the case of Hungarian mushroom growers, the most harmful microorganisms are the members of the fungal genus *Trichoderma* causing green mold disease and members of the genus *Pseudomonas* causing bacterial browning and yellowing of mushroom caps. This study investigates the significance of the pseudomonads, mostly *Ps. tolaasii* in causing diseases and the possibilities to control the pathogenic strains. It is difficult to achieve an efficient control because of the bans of chemical usage during edible mushroom production. In our case the situation is even more complicated, as not all members of the *Pseudomonas* genus are harmful, some of them play essential roles in healthy cap formation, therefore they can not be completely excluded from cultivation. During the past years, numerous investigations have been focused on the reduction of pathogenic forms, but none of them proved to be perfect.

The aims of this study were to evaluate the diversity of the *Pseudomonas* genus at a Hungarian oyster mushroom farm; to prove the presence of *Ps. tolaasii* and investigate its significance in the pathogenic processes; to investigate the antagonistic and necrotic abilities of the isolated *Pseudomonas* strains against oyster mushroom; to select microorganisms from the genera

Pseudomonas and *Bacillus* and from the group of bacteriophages that may be suitable for biocontrol purposes.

2. AIMS

- to investigate of the *Pseudomonas* genus at a Hungarian mushroom farm, to assess the diversity, examine the presence of *Pseudomonas tolaasii* and its significance in the mushroom deterioration,
- to determine the antagonistic and necrotic characters of the isolated strains, to develop a method for the examination of necrotic phenomenon, to investigate the relationship between the enzyme production and necrotic effect,
- to select microorganism-groups for effective biocontrol against pathogenic pseudomonads,
- to study the competition properties of previously isolated non-pathogenic, fluorescent *Pseudomonas* strains against oyster mushroom pathogenic strains in artificially mixed cultures; molecular monitoring of the competition process,
- to isolate *Bacillus* strains, apathogenic to oyster mushroom, to test their biocontol abilities against pathogen pseudomonads,
- to isolate and investigate the effectivity of bacteriophages, to determine their host spectra,
- to establish integrated protection and its investigation in production of oyster mushroom.

3. METHODS

- ♦Selective isolation of *Pseudomonas* genus
- ♦Characterization of the isolates with classical biochemical methods
    - ●Gram-staining
    - ●Fluorescence on King's B medium
    - ●Growth ability at lower and higher temperatures

- ●Sensitivity to copper-ions
- ●Assimilation of different carbon sources
- ●Nitrate reduction
- ●Oxidase reaction

♦Investigation of enzyme activities

- ●Lipase, caseinase, gelatinase enzymes on solid medium
- ●Palmitoyl-esterase, β-glucosidase, N-acetyl-glucosaminidase (NAG-ase), trypsin-like protease, cellobiohydrolase, chymotrypsin-like protease, chitobiosidase using chromogenic substrates

♦Investigation of lipopeptide producing abilities

- ●WLA-test, reverse WLA-test, thin layer chromatography (TLC)

♦Molecular characterization

- ●Specific PCR for detection of *Ps. tolaasii*
- ●BOX, ERIC and REP PCR
- ●ARDRA and restriction enzyme digestion of the *rpoB* gene
- ●Investigation of the hypervariable region of the *rpoB* gene

♦Isolation of effective biocontrol strains

- ●Fluorescent members of *Pseudomonas* genus
- ●*Bacillus* genus
- ●Bacteriophages
- ●Integrated protection

♦Bacteriophages, and further investigation of Bf7

- ●Determination of host spectra with spot-lysis assays
- ●RAPD analysis and restriction enzyme digestion
- ●One step growth experiment of Bf7 phage
- ●Bf7 genome sequence determination

4. SUMMARY OF RESULTS

Pleurotus ostreatus is one of the most extensively cultivated mushrooms in the world; however, the success of cultivation often depends on the proliferation of different bacterial pathogens. *Pseudomonas tolaasii* is thought as the major cause of brown blotch disease of *Agaricus bisporus* and yellowing of *Pleurotus ostreatus*.

Many investigations were carried out to find an appropriate method for the prevention or control of these diseases. Although several trials were attempted to use chemical wash formulations for *A. bisporus* including calcium chloride, sodium hypochlorite, hydrogen peroxide, 2-bromo- 2-nitropropane-1,3-diol (bronopol) and several antibiotics, none of them was found to be fully effective and harmless to humans The possibility of biological control by competition was also investigated. Our results are listed below:

1. We isolated 60 bacterial strains from infected caps, straw samples and from stagnant water around the spawn bags using selective medium (T-2) developed by Gould et al.

2. We investigated these strains with different biochemical and molecular biological methods in order to determine their virulence and pathogenicity towards oyster mushroom and to confirm the presence of *Ps. tolaasii*. Results of the biochemical tests revealed great diversity among the strains. Fifty isolates could produce fluorescent pigments and three had nitrate reductase activity. Twenty-eight, 80 and 60% of the strains had gelatinase, caseinase and lipase activity, respectively. Production of all of these 3 enzymes could be observed in the case of 16 strains, suggesting that these could participate in the development of disease. In case of 11 isolates none of these enzyme activities could be detected, so their presence on the mushroom caps is not necessarily a negative effect. Growth test on restricted temperatures (4 and 42°C) resulted that most of them are able to grow at 4°C and unable to grow at 42°C except from 4 isolates.

Most of them were able to grow on various carbon sources and there were no significant differences between the isolates.

3. Based on the WLA-test, reverse WLA-test and the TLC we could conclude that 12 isolates were able to produce lipopeptide like substances, 8 of these were WLIP-like, 2 were tolaasin-like and 2 were representing another type of lipopeptides.

4. The BOX, ERIC and REP repeated sequence analyses confirmed the diversity revealed in the biochemical tests. Detection of *Ps. tolaasii* using specific PCR proved that the 2 tolaasin-like lipopeptide producer strains were really belonged to this species.

5. Analysis of the partial *rpoB* gene sequences demonstrated that most of the strains belonginged to *Ps. fluorescens* bv. V (37 isolates) and the others were: *Ps. putida* (4) *Ps. fluorescens* bv. I (2), *Ps. brenneri* (2), *Ps. mucidolens* (1), *Ps. entomophila* (1), *Ps. synxantha* (1), *Ps. syringae* (1), *Ps. mandelii* (1). The presence of the 2 *Ps. tolaasii* were proved with this method also. Based on the phylogenetic analysis they are taxonomically closely related to *Ps. fluorescens* bv. V and to other WLIP producing strains. Seven isolates belonged to other genera, despite the fact that they were able to grow on *Pseudomonas*-selective medium. Three of them belonged to *Rhizobium*, 2 to *Stenotrophomonas* and 2 to *Ochrobactrum* genus. The *Rhizobium* and *Stenotrophomonas* genera are closely related to the *Pseudomonas* genus, this could explain the ability to grow on selective media. On the basis of the *rpoB* gene sequence analysis we could conclude that not the *Ps. tolaasii* caused the main problems in case of this Hungarian mushroom farm, the relevance of other fluorescent strains belonging to the *Ps. fluorescens* bv. V was also significant. It is possible that they play key roles at first steps of the rotting process, but later the last mentioned species take over their place.

6. In order to implement biological protection of oyster mushroom, we investigated firstly the previously isolated, non-pathogenic pseudomonads. Thirteen non-pathogenic, fluorescent *Pseudomonas* isolates were tested against *Ps. tolaasii*. Ten of them were effective inhibitors and the best four were selected for further investigations, in production of oyster mushroom. Strains PS-2 and 54 proved to be the best ones; they might be applied as biocontrol products in the future.

7. Another applicable group for searching biocontrol agents was the *Bacillus* genus. More than 60 strains were isolated with selective method. Among these isolates only 7 were appropriate for further study; 4 *B. subtilis*, 2 *B. pumilus* and 1 *B. licheniformis*. Their direct test in production of oyster mushroom is not yet carried out.

8. The third possible biocontrol opportunity was the highly specialized bacteriophage group. Sixteen lytic bacteriophages that infect *Pseudomonas tolaasii* LMG 2342^{T} were isolated from smashed sporocarps of oyster mushroom (*Pleurotus ostreatus*). Molecular investigations revealed that they all have dsDNA genomes about 30 kbp in size. Identical restriction patterns resulting from restriction enzyme analysis suggest that the isolates probably belong to the same phage species. However, there was a difference between these phage isolates in their infecting abilities. Phage isolate Bf7 was investigated and characterized more deeply. Morphological characterization of Bf7 by Transmission Electron Microscopy (TEM) showed that it has a short, non-contractile tail, an icosahedral phage head and the size is about 62-68 nm in diameter, suggesting that it belongs to the *Podoviridae* family. Complete genome sequence analysis of the Bf7 phage isolate revealed a 30580 bp genome, 58.4% G+C content, 35 open reading frames encoding different proteins showing homology to proteins of the bacteriophage *Caulobacter crescentus* ΦCd1 from the *Podoviridae* family. Phage Bf7 forms clear plaques

(1-3 mm in diameter) after 18 h incubation at 20°C on *P. tolaasii* 2342^T. This property depends mostly on the temperature. Clear plaques formed at 5, 10, 20 and 25°C after 18-48 hours incubation on *Ps. tolaasii* 2342^T, but no plaques generated at 30 and 40°C. The single-step growth experiments revealed that the Bf7 bacteriophage has a latent period of about 140 min. The calculated burst size was 237 PFU per infected cell at 20°C, MOI of 0.06, taken into account the latent period, the eclipse and the rise periods. Moreover, the phage was resistant to chloroform treatment for at least one month, which is an essential parameter of the long-time storage.

9. Development of integrated control is still in progress. First of all the selected phages should not inhibit the growth of pseudomonads used as biocontrol agents. PS-2 and 54 are resistant to the isolated bacteriophages, so they could be applied simultaneously. Further investigations are necessary. The implementation of integrated control is not yet realized, but there are many interested mushroom growers, so rapid development is expected.

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