SUMMARY

Of the PhD thesis:

"Studies concerning the antifungal activity of lactic acid bacteria"

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Key words: lactic acid bacteria, antifungal activity, filamentous fungi, metabolites, silver nanoparticles, biopreservation.

The aim of the present PhD thesis is isolation and identification of some lactic acid bacteria with antifungal activity, having potential uses as biocontrol and/or biopreservation agents.

Main objectives are represented by:

1. Isolation and identification of phytopathogenic and/or contaminating filamentous fungal strains of some food products of vegetal origin, for further utilization in experiments as indicator fungi.
2. Screening for antifungal activity of some lactic acid bacteria from romanian traditional fermented products.
3. Setting the antifungal spectra of selecterd lactic acid bacteria
4. Identification and characterizatin of lactic acid bacteria strains with antifungal properties
5. Setting the action mechanism of lactic acid bacteria with antifungal properties
6. Practical appliucation of antifungal activity of lactic acid bacteria on *Penicillium expansum* from apples
7. Utilization of lactic acid bacteria in silver nanoparticles biosynthesis and antifungal activity evaluation

The doctorate thesis consists of two parts (bibliographic study and own researches) which comprise contents, the summary of the thesis, , introduction, five chapthers and references.

PART I, BIBLIOGRAPHIC STUDY with the following content:

Chapter I – CURRENT STATUS OF RESEARCH ON LACTIC ACID BACTERIA AND THEIR ROLE IN CONTROLLING FUNGAL GROWTHwhich presents the synthesized literature data concerning fungal contamination of food products, main mycotoxins, control methods of fungal contaminationgeneralities concerning morphology, physiology, metabolic particularities, lactic acid bacteria classification and antifungal activity. Factors influencing the antifungal activity, main metabolites with antifungal activity produced by different species of lactic acid bacteria, interactions between lactic acid bacteria and filamentous fungi, as well as

current applications of lactic acid bacteria in food industry for the biocontrol of fungal contamination and the involvement of lactic acid bacteria in biosynthesis of silver nanoparticles.

PART II, OWN RESEARCH comprised of four chapthers that evidence the aim and objerctives of the doctorate thesis, materials and methoids utilized, obtained results and disscusion about their siggnificance in relation with the researches in this field, general conclusions.

Chapter II – THE AIM AND OBJECTIVES OF RESEARCH CONDUCTED IN THE PHD THESIS

Chapter III – MATERIALS AND METHODS describes the characterization methods for lactic acid bacteria strains, isolation and identification of novel fungal species, screening of the methods of lactic acid bacteria, evidening the antifungal effect on solid and liquid media. Description of the utilized methods to evidence the production of antifungal agents (organic acids, biosurfactants, exopolisaccharides, laccase), specific cromatography techniques utilized, nanotechnology of silver nanoparticles biosynthesis and their parameter characterization. It is presented the *ex vivo* experiment concerning the lactic acid bacteria application as apple biopreservants, as well as the statistical methods utilized in data processing.

Chapter IV – RESULTS AND DISCUSSION it is structured in 7 subchaphers that include the research results carried out in the period 2012-2015 within the doctoral internship, in genetics and engineering laboratory, as well as in the enzymology laboratory of the Faculty of Biotechnology Bucharest. Throught this chapter are presented 113 original figures, representing graphics, photographic images and 19 tables.

During the first chapter are presented results on the isolation, identification and compiling a collection of 11 novel fungal strains belonging to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Trichotecium* and *Stachybotrys* known as pathogens of crops or associated with the contamination in food chain, having virulence properties unmitigated by cultivation in vitro. The advantage of using them as indicators gives lactic bacteria fungi which inhibit them, the possibility of effective use in countermeasures technology.

The 2-nd subchapter presents the screening results of 38 lactic acid bacteria strains tested against *Aspergillus ochraceus* and *Penicillium digitatum*. Of these, 30 strains (79%) showed antifungal activity on both species. *P.digitatum* strain was more sensitive to the action of lactic acid bacteria tested, compared to *A.ochraceus*. The strains evidenced: LAB 13, LAB 15, LAB 35, LAB 43, LAB 58, LAB 61 LAB 113, LAB 118 and LAB 122, lead to the formation of mycelium growth inhibition zones with a diameter of more than 25 mm, for at least one of the fungal species.

Next are presented the test results highlight the antifungal effect of eight selected lactic

acid bacteria against the novel isolated fungal strains and against some strains from the laboratory collection, achieving the antifungal spectrum. After testing the antifungal effect on *A.flavus* M4, *A.niger* M3, *A.ochraceus* P, *A.ochraceus* BZ, *A.ochraceus* 5S, *A.fumigatus*, *P.expansum*, *P.digitatum*, *F.verticillioides*, *T.roseum* și *S.chartarum*, found that the most effective strains on most indicator species, were LAB 13, LAB 15, LAB 43, LAB 58 și Lpl..

The 4th subchapter presents the identification and characterization of selected lactic acid bacteria strains with antifungal properties.

Molecular identification of selected lactic acid bacteria by the BOX-PCR has revealed the fact that, if the strains LAB 13, LAB 15, LAB 35 and the LAB 58 the strips profile of the resulting DNA by amplification are similar to those obtained for the strain of *Lactobacillus plantarum* Lpl, where for strain LCM5 amplicons profile is different. Also in strains LAB 64 and LAB 43 amplicons profile shows important differences, which supports the idea that they belong to different species.

Using ERIC-PCR has led to some less clearer results compared with BOX-PCR but, in general they confirm the issues highlighted in the first case.

Identification of lactic acid bacteria strains as belonging to the species *L.plantarum* create the basis of their practical use, given that bacteria belonging to this species are considered to be safe for humans and animals.

Selected lactic bacteria strains produced exopolizaharide, evidenced by optical and scanning electron microscopy (SEM) and their amount varied depending on the strain and culture conditions. There are shown the effects of the temperature, pH and carbon source on the growth of lactic acid bacteria and EPS production. Lactose was the preferd sugar by most tested strains as carbon source to produce exopolysaccharides in excess. Also, suprisingly, the amount of EPS was generally grater when the pH was incresed (pH 7,5), and optimal cultivation temperature was 38 ± 2^0C.

The results obtained for all the lactic acid bacteria strains, after 24 hours measurement of optical density (O.D.600nm), shows elevated values of it in conditions of temperature 28^0C, pH 7,5 and using glucose as a carbon source (10%).

Assaying the presence of laccase showed small halos for the strains LCM5, Lpl, Lpa, LAB 43 and LAB 13, clear halos detected for some mixed cultures (*F.verticillioides* + Lpa; *F.verticillioides* + LAB 43; *T.roseum* + Lpa; *T.roseum* + LAB 15; *T.roseum* + LAB 43; *P.digitatum* + Lpa; *A.ochraceus* + LAB 15; *S.chartarum* + LAB 43) suggests the presence of some specific interactions between the metabolites synthesized by the micro-organisms that interact and could interfere with biochemical antagonism between bacteria and fungi.

Pfeiffer chromatography confirmed the antagonism between the metabolites of lactic

bacteria strains and indicator fungal species, by changing the appearance of chromatograms at the contact zone and forming lines of precipitation.

Subchapter 5 presents the results regarding the action mechanisms of lactic acid bacteria strains with antifungal properties. In general, aspects of optical microscopy showed that the changes in the fungal mycelium by diffusible compounds synthesized from lactic acid bacteria were similar: the lysis of cell wall, vacuolation, twisting hyphae, but their intensity is different. Also, in the inhibition zones were observed changes of conidiophores and conidia.

Testing of lactic acid bacteria effect against fungal biofilm formation showed that strains LAB 13, LAB 15 and LAB 43 inhibited the growth and development of fungal mycelium to all isolates tested and blocked, through synthesized metabolites, aggregation of fungal cells, adhering to the walls of culture vessel and fungal biofilm formation in most indicator species.

Quantifying the production of organic acids by HPLC showed that most of tested strains produced lactic acid after 24 hours of incubation and this acid level has the tendency to increase in the coming days and small amounts of acetic acid, fenillactic and hidroxifenillactic acid. Bacteria effectiveness regarding production of lactic acid was in descending order: LAB 15> Lpl> LAB 35> LAB 58> LAB 43.

The strain LAB 43 presented a high antifungal activity against most indicator fungi to which was tested and a very low level of organic acids of interest, that means the possibility of intervention of other antifungal mechanisms.

The results from experiments with sterile and sterile neutralized culture filtrates confirm that the majority of lactic acid bacteria strains antifungal activity is due to the synthesis of organic acids. In th case of strains LAB 43 and LAB 58, it can be said that inhibitory activity is due to the biosynthesis of organic acids (against certain fungal targets) and production of other antifungal agents. In addition, for some bacterial strains, the antifungal activity is fungicidal type (such as strains LAB 43 and LAB 58), while for the others is fungistatic action, decreasing over a prolonged incubation.

The results indicate that lactic acid bacteria strains produce biosurfactant with anionic character.

The capacity of biosurfactants produced by lactic acid bacteria strains to adhere to solid surfaces may be another new effective mean to combat fungal contamination of food products and inhibit their development. Moreover, correlating the results relating to the biosurfactants with those concerning the inhibition of biofilm aggregates and fungal mycelia it can be appreciated that, at least for LAB 13, LAB 35 and LAB 43 strains these properties are related to the production of biosurfactants.

Practical application on the antifungal effect of lactic acid bacteria, shown in subchapter

six demonstrated that strains Lpl, LAB 43, LCM5 and Lpa had protective effect against *Penicillium expansum* on apples variety Ionatan from Voinești, having high biocontrol potential as an alternative to control chemicals.

Subchapter seven presents issues of novelty on the use of lactic acid bacteria strains isolated from romanian traditional fermented products concerning silver nanoparticles biosynthesis. Silver nanoparticles biosynthesized using lactic acid bacteria filtrates and were characterized by transmission electron microscopy (TEM) showed, generally, a good dispersion, approximately spherical shape, with parallel stripes certifying crystal structure and dimensions below 45 nm.

The strains which produced silver nanoparticles with enhanced antifungal activity against *Aspergillus flavus* M4, *Aspergillus ochraceus* BZ and *Penicillium expansum* were LAB 132, LCM5, LAB 58 and LAB 35, followed by LAB 43 and the LAB 13, on which the antifungal activity differ from fungal species, but most often superior to the reference strains (Lpa and Lpl). Antifungal activity exercised by silver nanoparticles synthesized by lactic acid bacteria strains was maintained more than 14 days, suggesting their fungicidal effect.

Chapter V – CONCLUSIONS contains general conclusions of experiments and emphasis original contributions to the study of the antifungal effect of lactic acid bacteria and their possible practical applications in nanotechnology.

The originality and innovative character of this work consist in improving methods for biopreservation and biocontrol by using new performant lactic acid bacteria strains, capable of producing antagonist compounds with impact on controlling phytopathogenic fungi and/or contaminants of food of vegetal origin.

The references include 283 titles of specialty papers published nationally and internationally.