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Biocatalysts in Control of Phytopatogenic Fungi and Methods for Antifungal Effect Detection

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1. Introduction

The diseases in plants can be reduced with chemical control, in the case of plant fungi illness can be controlled using fungicides. Chemical fungicides are toxic substances that are used to prevent growth or kill fungi harmful to plants, animals or humans. Their use presents many environmental problems such as: the development of resistance to insecticides in pest populations, the resurgence of the populations treated, chemical waste accumulation, risks and legal complications, destruction of beneficial species. Moreover, it is need to be taking in consideration high cost of fumigants, equipment, labor work and material. Thus, to minimize an environmental damage it is very important to substitute chemical control by the biological control.

Biocontrol means to use natural enemies or their metabolites against pathogens causing diseases. This method provides a decrease of pathogenic microorganisms' population. The main advantages are: little or no adverse side effects to the other biological systems different from pathogenic microorganisms; rare resistance of pathogenic forms to biological control; not harmful to environment; the favorable relation cost *vs* benefit; prevention of food contamination by chemicals. Biocontrol methods apply to regulate phytopathogenic fungi growth by using an antagonistic microorganism (fungi or bacteria) and / or its derivatives as an active ingredient. These biofungicides, called like that due to its biological origin, are easily assimilated into the environment and they are alternative techniques to replace the use of chemicals in agriculture. Development of new biocontrol approaches and biofungicides, as well as methodologies for detection of antifungal activity are the one of the important goals of biotechnology.

A fungal disease can be described as polycyclic whether the causative agent is capable of producing spores and re-infect plants during a growing season or monocyclic when the causative agent must wait for a new season. This classification applies to regions with four seasons where pathogens must produce specific structures to survive the winter. Phytopathogenic fungi can also be distinguished by the types of produced spores and the method by which they penetrate into the plant. Once the pathogen has penetrated, it

produces a haustorium and grows inside the plant (biotrophic power), or kills target cells and feeds on dead tissue (necrotrophic power). The identification of pathogens is performed based on the signs and symptoms of the disease. Signs refer to the observation of some of the structures of the pathogen (such as sporulation). Symptoms are secondary evidence produced by the plant where a pathogen is present (such as wilting leaves).

The fungal cell wall contains different chemical constituents such as polysaccharides, proteins, chitin and other substances. The cell wall formation varies among species, also varies with age of the fungus, since substances may be present in young hyphae, disappear in the older or deposit other materials to mask the presence of initial constituents. Also the composition of the medium, the pH and temperature influence composition of fungi walls.

Enzymes such exo-1,3- β -D-glucosidase (laminarinase; EC 3.2.1.6) and β -N-acetyl-D-glucosaminidase (chitinase; EC 3.2.1.14) are hydrolytic enzymes produced by *Trichoderma spp* wich are strong inhibitors of many important plant pathogens, mainly of the genus *Phytophthora*, *Rhizoctonia*, *Sclerotium*, *Pythium* and *Fusarium* among others. *Trichoderma* species are most commonly used as antagonists to control plant diseases caused by fungi and they do not attack plants. There are essentially three types of mechanisms by which *Trichoderma* strains influence the plant pathogen: direct competition for space or nutrients, production of antibiotic metabolites, volatile or nonvolatile nature, and direct parasitism of certain species of *Trichoderma* on plant pathogenic fungi. *Trichoderma* species that act as competitive hyperparasites, produce antifungal metabolites and hydrolytic enzymes. Their activity is related to structural changes at the cellular level, such as vacuolation, granulation, disintegration of cytoplasm and cell lysis, which found in organisms they interact with. The chitinases and laminarinases hydrolyze the fungi cell wall and are able to degrade chlamydospores, conidia and polysaccharides of the mature hyphae.

Trichoderma enzymes (chitinase and laminarinase) are substantially more antifungal against wider range of pathogens (e.g. *Fusarium oxysporum*). They are effective and non-toxic in comparison to other purified enzymes from any other source when assayed under the same conditions (Lorito et al., 1998).

F. oxysporum is a pathogenic plant fungus. It causes severe chlorosis, defoliation, desiccation, and wilt in leaves of plants which can lead to plant death. This generates considerable economic loss in many important crops (Jimenez-Gasco et al., 2004).

The use of mycolytic enzymes as antifungal treatments in the protection of some commercially important crops is promising. Nevertheless, the application of enzyme *in situ* needs to increase enzyme stability and protection against environmental factors (Wang & Chio, 1998) that may be achieved by means of immobilization. The immobilization on solid supports can affect the enzyme mobility to fungi in soil contained system and increases its heterogeneity. Therefore, the immobilization in liposomes possibly can help to avoid the problems related to union of enzyme on solid support. However, gradual release of enzymes is desirable in the hydroponic systems with frequent fluid current. The immobilization in solid support may be useful. Recently, in our laboratory a research focused on the immobilization of chitinase and laminarinase on liposomes and brown seaweed bagasse was performed in order to analyze their stability and activity against *Fusarium oxysporum* during tomato growth under greenhouse conditions and hydroponic green fodder (HGF) production, respectively.

The objectives of this study are: to describe the bioluminescence assay approach for detection of mycolytic activity of biocatalysts (chitinase and laminarinase) using *Fusarium*

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oxysporun as model of phytopathogenic fungus; to demonstrate the effect of chitinase and laminarinase immobilized on brown seaweed bagasse on *Fusarium oxysporum* growth in HGF system; to compare the partitioning behavior of chitinase and laminarinase in soya lecithin liposomes, using a thermodynamic approach based on the variation of partitioning with temperature as well as to define the synergetic activity of microencapsulated enzymes against *Fusarium oxysporum in vitro* and *in vivo* testing in the presence and absence of chemical fungicide thiabendazole.

2. Methods for antifungal effect detection

2.1 Common methods

Fungal growth inhibition could be measured by different methods, which are affected by several *in vitro* factors (Table 1). These variables should be considered when a susceptibility test is designed or interpreted.

Organism specific factors	Drug specific factor	Variables that influence results
Variable growth characteristics Pleomorphism (in yeasts) Type of Metabolism (aerobic or anaerobic) Medium, pH and incubation temperature can affect growth and pleomorphism	Limited aqueous solubility of some agents Partial inhibition of growth over a wide concentration range giving trailing end points Buffer and pH effects on activity Interaction with media components and buffer	Inoculum Medium formulation and pH Agar versus broth Type of buffer Temperature and duration of incubation Minimal inhibitory concentration (MIC) end point criteria

Table 1. Variables related to the drug, organism and technique used in antifungal testing.

Broth dilution test was established as the standard reference for antifungal susceptibility testing, serving as the basis for comparison, for the development of novel tools for antifungal susceptibility testing (Rex *et al.*, 2008a; Rex *et al.*, 2008b). These alternative methods require correlation with MIC results in broth dilution one. At first, National Committee for Clinical Laboratory Standards (NCCLS) recommended broth macrodilution methods, but broth microdilution tests were later determined, having the same effect (Espinel-Ingroff et al., 1992). The methodology is useful for testing common filamentous fungi or yeasts, including the dermatophytes. The fungi encompass *Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., *P. boydii*, *S. prolificans*, *S. chenckii* (Espinel-Ingroff et al, 1995), *Trichophyton*, *Microsporum*, *Epidermophyton* spp. (Ghannoum *et al.*, 2004), *Candida* spp. and *C. neoformans* (Rex *et al.*, 2008b). Difference between methodologies is test volume: for macrodilution is 1 ml and test is done in test tubes, while microdilution is 200 µl and the test is performed in a 96-multiwell microdilution plate. Medium, antifungal substance and inoculum are added to the test tubes or wells and are incubated at 35°C from 21 to 74 hours depending of the fungus. Results are expressed as MIC or MEC (minimal effective concentration). The MIC is the lowest concentration of an antifungal agent that inhibits organism's growth and the MEC is the lowest concentration of drug that leads to the growth

of compact hyphal forms as compared to the hyphal growth seen in the growth control assay. MEC evaluation is more appropriated than MIC reading for testing echinocandin antifungal agents (Espinel-Ingroff et al., 2003).

NCCLS, now Clinical and Laboratory Standard Institute (CLSI), recommended some alternative methodologies to the conventional broth dilution tests to probe yeasts and molds susceptibility, which provide reproducible results: YeastOne, Alamar Blue, MTT–test, E-test and Disc diffusion.

Disk diffusion testing is a simple and economic alternative to broth dilution tests. It has been probed with yeasts inhibition. Furthermore there have been identified parameters for testing the antifungal effect over filamentous fungi to five agents (amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole) by this method (Diekema et al., 2003). Results are provided between 8 to 24 hours, quicker than broth dilution test and the use of nonsupplemented Mueller-Hinton agar instead supplemented one should make this method more available to conventional laboratories at a less cost. There is a good correlation between minimal inhibitory concentration (MIC) and diameter of inhibition in disk diffusion testing.

The E-test (stable agar gradient method) is an alternative procedure to test antifungal susceptibility of yeast (Espinel-Ingroff *et al.*, 1996) and molds (Espinel-Ingroff *et al.*, 2001; Szekely *et al.*, 1999). The method is based on a combination of the concepts of dilution and diffusion tests. It quantifies antifungal susceptibility directly as MIC values, like dilution methods. E-test also consists of a predefined and continuous concentration gradient, making this methodology more precise than conventional procedures based on discontinuous two-fold serial dilutions, and it is not affected by antifungal agent properties (such as molecular weight, diffusion characteristics and aqueous solubility) or by different growth rates of fungus as disk diffusion testing (AB BIODIS, 2000). This method involves placing a plastic strip containing a gradient of an antifungal agent on the surface of an inoculated agar plate (plates are inoculated with a suspension of yeast or mold, turbidity equal to 0.5 McFarland standard (1 McFarland standard for *C. neoformans*)), across the entire surface of agar in three directions. The drug diffuses into the agar and establishes a stable concentration gradient. Inhibition of fungal growth produces an ellipse, and the MIC is read where the ellipse intersects the test strip. Plates are incubated at 35 °C between 24 to 72 h.

MICs determination can be facilitated for a method which quantifies the hyphal growth of filamentous fungi and overcomes observer bias, which can be getting by colorimetric methods based on the measurement of metabolic activity. Alternative methods use different colorimetric growth indicators and they take at least 24 h before reading. The commercially available YeastOne (Trek Diagnostics Systems) consists of a microtiter plate with dried antifungal drugs (Table 2). Every well includes an oxidation-reduction indicator (Alamar Blue) that changes from blue to pink in the presence of microbial growth. The first well to show a change from pink (growth) to purple or blue (growth inhibition) is recorded as the MIC. Easy set-up procedures eliminate time-consuming broth dilution alternative and results are ready after 24 hours of incubation. Several multicenter studies found good correlation between microbroth dilution and Alamar Blue colorimetric susceptibility tests among *Candida sp.* and *C. neoformans* (Eraso et al., 2008).

Another preliminary colorimetric test used for filamentous fungi and yeast isolates utilized the yellow tetrazolium salt dye 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). This salt is cut by dehydrogenases to form its purple formazan derivative, which can be measured spectrophotometrically at 550 nm (Levitz & Diamond, 1985). All

living and metabolically active fungi can cleave MTT. This method has demonstrated a good agreement with MICs of standard broth dilution tests for the fungal inhibition test of yeasts (Clancy & Nguyen, 1997) and some molds (Meletiadis *et al.*, 2000). Initial inoculum and the dye MTT are incubated for 48 h or more to get results.

The methods described above require a long time to perform. Thus, the development of the faster methods for antifungal activity detection is obvious.

Antifungal Agent	Dilution Range µg/ml
Amphotericin B	0.12 - 8
5-Flucytosine	0.060 - 64
Anidulafungin	0.015 - 8
Caspofungin	0.008 - 8
Micafungin	0.008 - 8
Fluconazole	0.120 - 256
Itraconazole	0.015 - 16
Posaconazole	0.008 - 8
Voriconazole	0.008 - 8

Table 2. Antifungal agents and its dilution range on a YeastOne plate.

2.2 Bioluminescence ATP assay as antifungal susceptibility testing

Due to conventional antifungal methods need long periods of time to evaluate inhibitory effect of the antifungal compounds, a research focused in the development of a faster and reliable technique using luciferase catalyzed bioluminescent reaction for detection of ATP released from the cells destroyed by mycolytic compounds was carried out in our laboratory.

The luciferase catalyzed reaction allows to selective ATP detection (Ansehn & Nilsson, 1984)). However, ATP of dead cells is very unstable metabolite due to ATPases activity, which in this case catalyzes its hydrolysis. To improve detection of ATP released from dead fungal cells, there were applied vanadate ions as inhibitor of a wide spectrum of ATP-ases (Angelis & Gobbetti, 2004). Assay was carried out testing the susceptibility of *Fusarium oxysporum* to such micolytic agents as chitinase (C), laminarinase (L) and syringomycin-E (SR-E) (Cano *et al.*, 2008; De Lucca *et al.*, 1999).

Firstly, vanadate concentration that doesn't affect fungi's viability has been determined by evaluation of the *F. oxysporum* radial growth in agar poisoned with different concentration of ammonium vanadate. Results led to choose 0.75 mM as right vanadate concentration for next assays.

The effect of the mycolitic agents on extracellular and intracellular ATP levels in the presence and absence of vanadate (applied at 0.75 mM) was evaluated (Fig. 1). These assays were performed using enzymes at 20 µg/ml (Cano *et al.*, 2008) and syringomycin E at 2 µg/ml (Espinel-Ingroff et al., 1995) as final concentrations. Results confirm the hypothesis that vanadate addition leads to keep ATP of dead cells due to ATPases inhibition. The increase of extracellular ATP could be considered as the measurable parameter that demonstrates the effect of mycolytic antifungal compounds because it has linear correlation with the CFU (colony forming unit) change rate (Fig. 2).
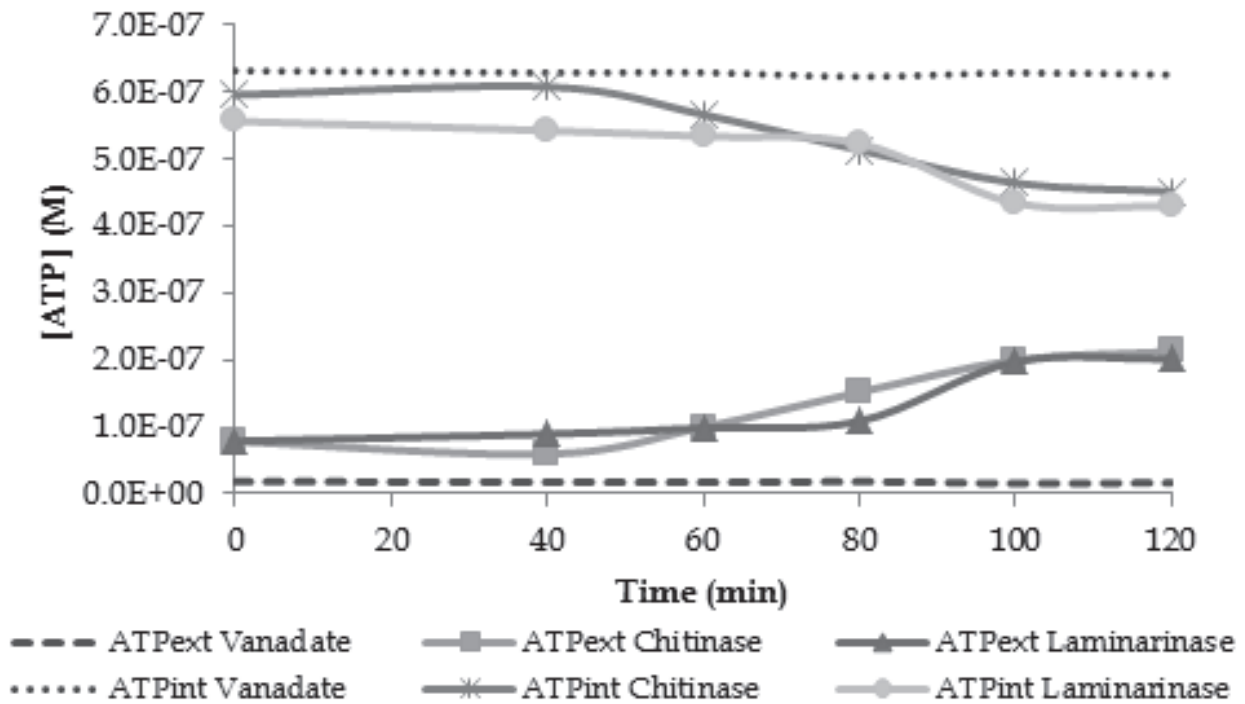
a)
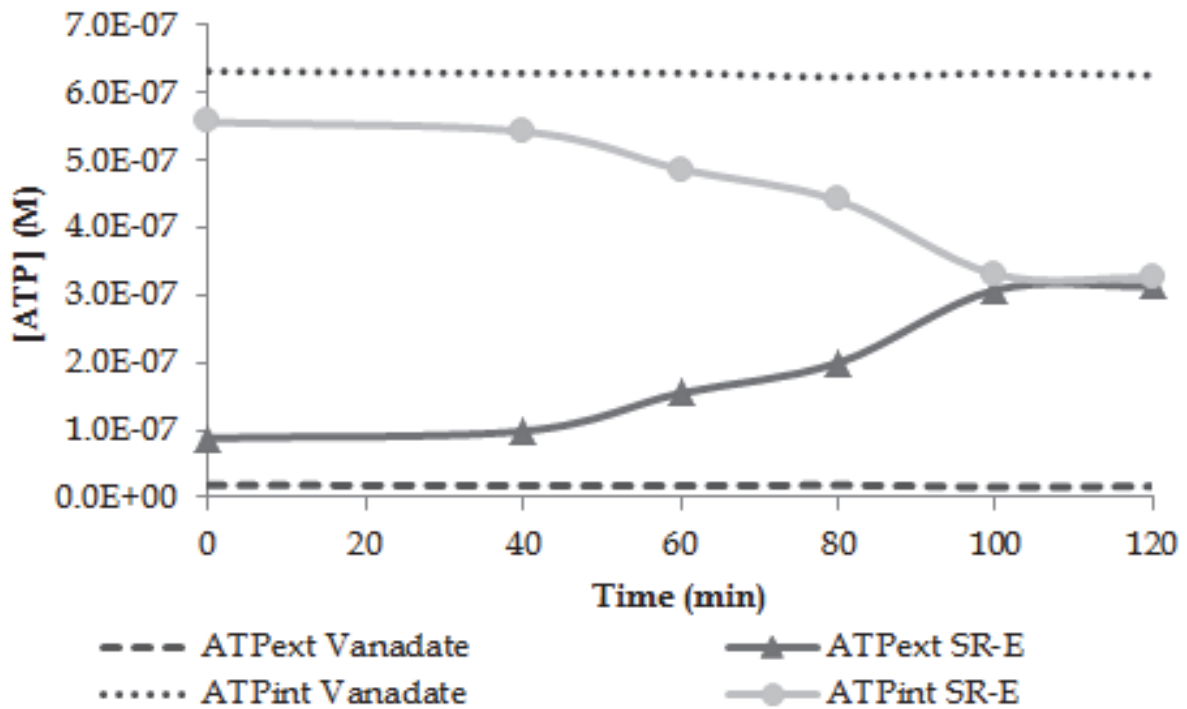
b)

Fig. 1. Intracellular ATP (ATPint) and extracellular ATP (ATPext) detected after 120 min of incubation with antifungal biochemical compounds applied to *Fusarium oxysporum*: a) Chitinase and laminarinase; b) Syringomycin E. The dotted lines indicates the extracellular ATP y intracellular ATP levels in control without antifungal agents.

The decline of intracellular ATP occurs simultaneously with the decrease of fungus viability: the CFU concentration remained constant in the absence of biofungicides, and decreased in their presence. This parameter also may be indicative to measure the inhibitory effect of antifungal compounds. According to the relationship between the CFU/ml and ATP concentration (Fig. 2), is defined that each CFU of *Fusarium oxysporum* affected by mycolitic activity of SR-E corresponds to 10^{-11} mol of ATP. However, it should be reported that ATP level in cells is affected by multiple factors such as crop growth stage, culture media and the presence of metabolic regulators, which may influence the relationship between the number of CFU and the amount of ATP per cell (Stanley, 1986).

Selection of change in intracellular ATP level as indicative parameter of antifungal effect is confirmed by results of kinetic study performed with different concentrations of the tested substances (Fig. 3). The increase of biofungicides concentration leads to greater decrease of intracellular ATP level as well as fungus viability, i.e. a good correlation is observed.

The obtained results led to conclusion that the bioluminescent assay may be considered as the fast and reliable method to antifungal activity evaluation.
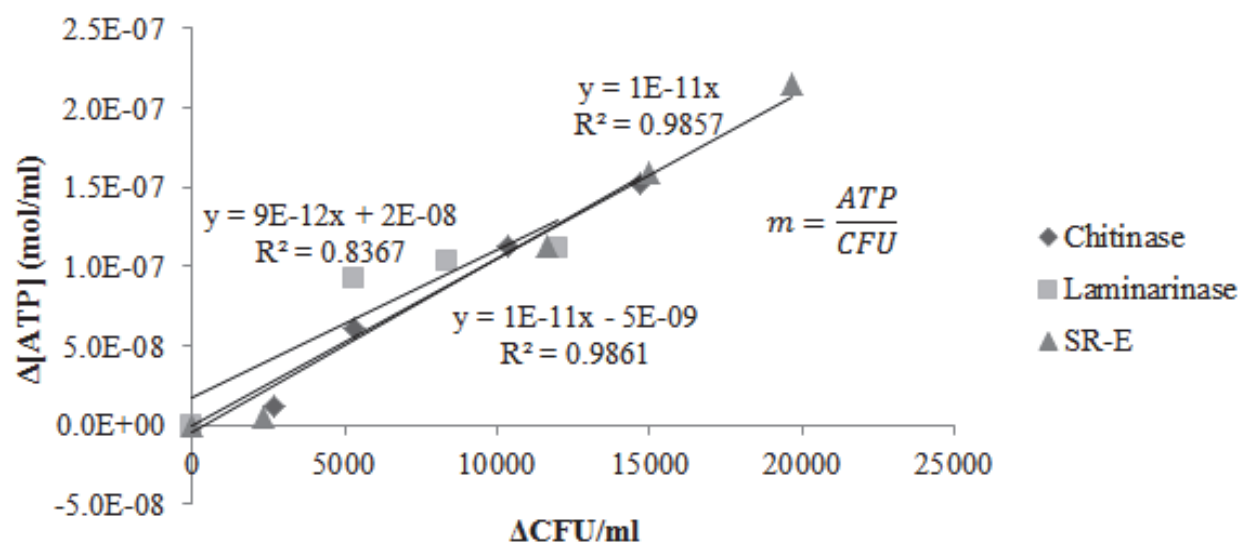

Fig. 2. Correlation between ΔCFU/ml and extracellular ΔATP to estimate the ATP quantity on a CFU of *Fusarium oxysporum*.

3. Immobilization of chitinase and laminarinase in seaweed bagasse as an alternative for fungal control in HGF system

The effect of chitnase and laminarinase (Sigma, USA) on *Fusarium oxysporum* viability was evaluated in the HGF production system. Free and immobilized on seaweed bagasse enzymes were applied. Enzymes were immobilized by adsorption on bagasse at 4°C and 250 rpm. The protein adsorption kinetics and their activity were evaluated at different contact times of immobilization. Immobilized protein was calculated by measuring initial and final protein concentration in the medium by Bradford method (1976). Chitinase and laminarinase activities were determined spectrophotometrically according to the methods described by Pantoom (2008) and Lethbridge (1978), respectively. The results are presented in Table 3. The greater enzymatic activity of chitinase and laminarinase immobilized on seaweed bagasse was detected at 120 min.
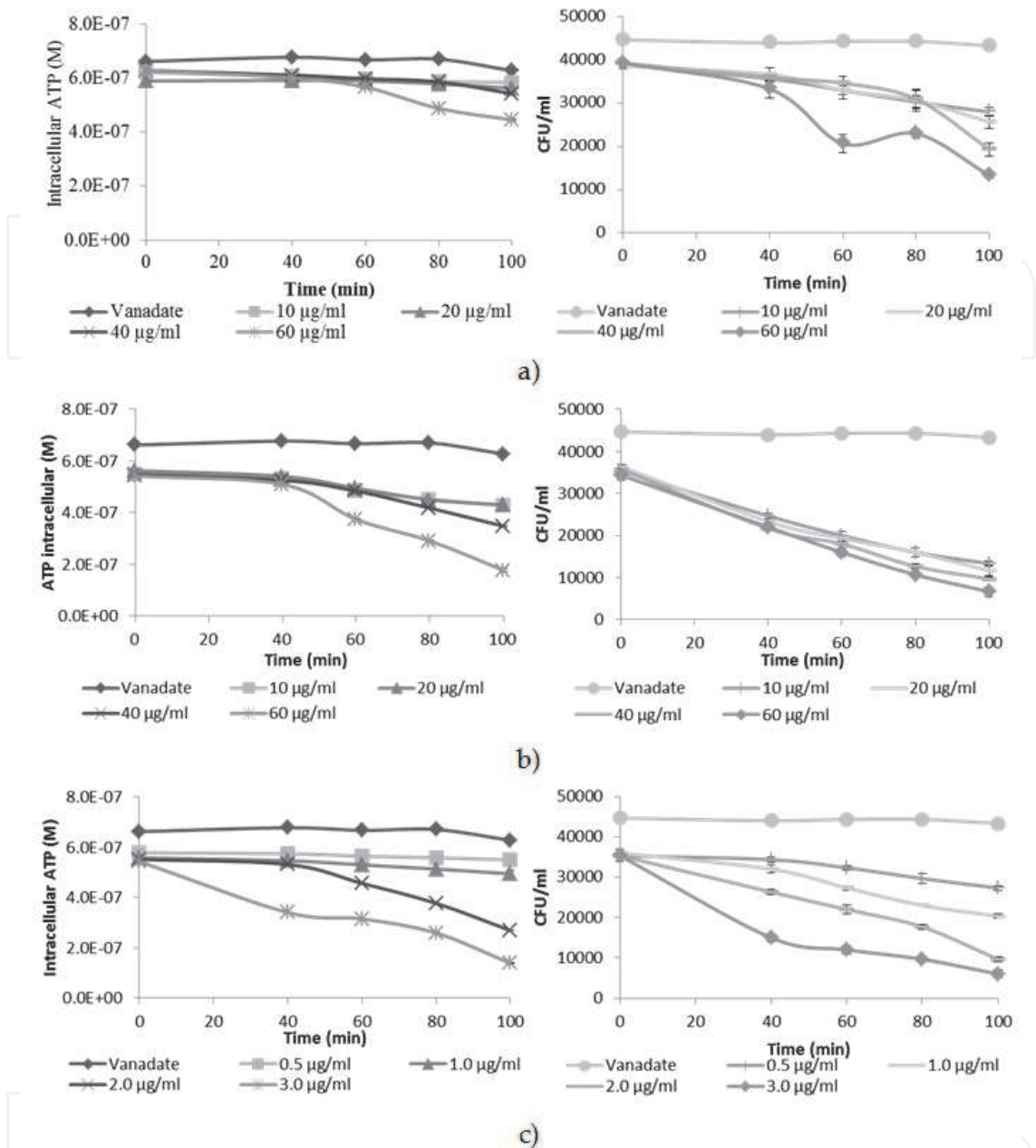
Fig. 3. Kinetics of intracellular ATP (left) and *F. oxysporum* viability (CFU/ml) (right) in the presence of vanadate and different biofungicides concentrations: a) Chitinase; b) Laminarinase; c) Syringomicin E.

After immobilization, antifungal properties of these preparations were compared with free enzymes on wheat HGF. This experiment was carried out under controlled conditions (on an environmental chamber at 21ºC). Five grams of wheat seeds were used for each test. Seeds were irrigated with 20 ml of nutritious solution per day and inoculated with 10 4 *F. oxysporum* spores/seed gram. The first day of assay was added 2.5 ml of chitinase or laminarinase (at 20 µg/ml) or 2.5 g of immobilized enzymes. At the harvest day (10th day) fungal viability expressed as CFU/ HGF g as well as height, wet and dry mass were evaluated. Table 4 summarizes the effect of free and immobilized on seaweed bagasse chitinase and laminarinase on HGF production and fungus viability.

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Time (min)	Chitinase		Laminarinase	
	Immobilized protein (%)	IU/g of bagasse	Immobilized protein (%)	IU/g of bagasse
0	0	0	0	0
60	38.87	0.017	31.5	0.13
90	62.71	0.018	35.26	0.14
120	65.7	0.02	82.76	0.17
180	66.48	0.019	83.75	0.14

Table 3. Kinetics of chitinase and laminarinase immobilization.

Treatment	Wet mass (g)	Dry mass (g)	Height (cm)	Fungal viability (CFU/g of HGF)
Control without F. oxysporum	38.23	5.74	19.33	5.5×10^5
Control	39.63	6.07	19.66	2.8×10^6
Chitinase	49.86	5.85	20.33	8×10^4
Laminarinase	47.33	5.7	20.16	1.5×10^6
Immobilized chitinase	62.5	5.5	24.5	6×10^4
Immobilized laminarinase	50.06	5.2	24.3	1×10^4

Table 4. Effect of chitinase and laminarinase free and immobilized on seaweed bagasse in HGF characteristics.

Table 4 shows that treatments with chitinase and laminarinase immobilized were effective to inhibit *F. oxysporum* viability compared with the controls. Moreover it was demonstrated that the addition on seaweed bagasse led to increase HGF wet mass and height. Also, the addition of seaweed bagasse on HGF system led to increase plants height and yield.

Thus, with immobilized chitinase and laminarinase on seaweed bagasse led to better control of phytopathogenic fungi under HGF system.

4. Liposomes as chitinase and laminarinase carriers

4.1 Properties of liposomes

Liposomes are vesicles of colloidal dimensions and spherical shape, with a membrane composed of a lipid bilayer in which (phospho) lipid bilayer sequesters part of the solvent (Lasic, 1995). Liposomes were discovered in 1961 by Alec D. Bangham who was studying phospholipids and blood clotting, and since then they became very versatile tools in biology, biochemistry and medicine.

Due to their chemical composition, structure and small size, liposomes exhibit several properties which may be useful in various applications (Table 5). The most important properties include bilayer phase behavior, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands. Additionally liposomes exhibit many special biological characteristics, including specific interactions with biological membranes and various cells (Lasic, 1995).

Liposomes are broadly classified by their structure, composition and size (Table 6). The size, lamellarity (unilamellar or multilamellar) and lipid composition of the bilayers influence

Use	Application area	Reference
Containing drugs or markers. As a model, tool, or reagent in the basic studies of cell interactions. Recognition processes.	Pharmacology and medicine	Lasic, 1995
Used to improve the therapeutic efficacy of the encapsulated drug molecules. Site specific targeting.	Pharmacokinetics and biodistribution	Lasic, 1995
As carriers of nystatin and amphotericin *B*, particularly as a means of overcoming toxic side effects and low aqueous solubility of these antibiotics.	Pharmacology	Yamskov *et al.*, 2008
Antimicrobial effect of liposome-encapsulated polymyxin B. formulations against a *P. aeruginosa.*	Microbiology	McAllister *et al.*, 1999
To yield better correlations with partitioning and solvation of ketoprofen.	Pharmacology	Lozano & Martínez, 2006
To deliver exogenous genetic material intra cellularly via fusion with the cell	Pharmacology	Ravichandiran *et al.*, 2011
The antimicrobial and antiviral activity of liposomes as carriers of essential oils.	Microbiology	Martin *et al.*, 2010
Drug delivery systems such antifungal, local anesthetics and Retinoids.	Pharmacology	Granda & Diduk, 1996
Encapsulation of chitinase and laminarinase on soya lecithin liposomes against *Fusarium oxysporum.*	Biotechnology	Joublanc *et al.*, 2010

Table 5. Applications of liposomes in different science areas.

many of the important properties like the fluidity, permeability, stability and structure, these can be controlled and customized to serve specific needs. The properties are also

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influenced by external parameters like the temperature, ionic strength and the presence of certain molecules nearby.

Liposomes are under investigation both as models for biological membranes and as carriers for various bioactive agents such as drugs, diagnostic and genetic materials, and vaccines. The thermodynamics of molecules transfer can be studied by measuring the partition coefficient as a function of temperature. Such data were used for the prediction of absorption, membrane permeability, and *in vivo* distribution in the case of various drugs (Bangham, 1993; Ávila *et al.*, 2003).

To obtain liposomes, different phospholipids may be applied such soya lecithin, which is the popular and commercial name for a naturally occurring mixture of phospholipids (also called phosphatides or phosphoglycerides). Different soya lecithin samples vary in color from light tan to dark reddish brown. Lecithin is the gummy material contained in crude vegetable oils and removed by degumming. Soybeans are by far the most important source of commercial lecithin and lecithin is the most important by-product of the soy oil processing industry because of its many applications in foods and industrial products. Three main phospholipids in this mixture called "commercial soy lecithin" are 33.0% of phosphatidylcholine (also called "pure" or "chemical" lecithin to distinguish it from the natural mixture), 14.1% of phosphatidylethanolamine (popularly called "cephalin"), and 16.8% of phosphatidylinositols (also called inositol phosphatides) as well as 0.4% of phosphatidylserine. Commercial soy lecithin also typically contains unrefined soy oil as well as additives insoluble in organic solvents (Beare- Rogers *et al.* 1992).

Structure	* Multilamellar: Spherically concentric multilamellar (many bilayers) structures. * Unilamellar: Spherical concentric unilamellar (one bilayer) structures.
Composition	* Phospholipids, cholesterol, phosphatidylethanolamine, free fatty acids, divalent cations. * Conventional, pH-sensitive, cationic, immune and long-circulating.
Size (nm)	* Small unilamellar 20-50 * Large unilamellar, 200-1000 * Multilamellar, 400-3500

Table 6. Classification of liposomas according to structure, composition and size.

Liposomes are widely used to deliver drugs for cancer and other diseases, as well as physiologically active substances in cosmetic products. Their application for immobilization of mycolytic enzymes such as chitinase and laminarinase, and the effect of microencapsulation on their antifungal properties are studied by our scientific group.

4.2 Comparison of the partitioning behavior of chitinase and laminarinase in soya lecithin liposomes, using a thermodynamic approach based on the variation of partitioning with temperature

Liposomes were prepared similar to Bangham method (1993). This resulted in the formation of multilamellar vesicles (MLVs), which was verified by microscopy according to Ávila *et al.*

2003. The molal partition coefficients ($K_{o/w}$), were calculated by Lozano & Martínez (2006) reported method. The standard free energy of transfer ($\Delta G_{w \to o}$), from aqueous media to organic system was calculated in agreement Ávila & Martínez (2003) approach. The temperature dependence of partitioning (van't Hoff method) was employed to obtain data on the enthalpy of transfer ($\Delta H_{w \to o}$). The entropy of transfer ($\Delta S_{w \to o}$) was quantified by means equation $\Delta S_{w \to o} = (\Delta H_{w \to o} - \Delta G_{w \to o})/ T$, and van't Hoff linearization.

The 'partition' means, in this case, that the enzyme is distributed between two phases in a dynamic equilibrium. It is a heterogeneous equilibrium since the 'solute' is distributed between two distinct phases: water and liposomes lipids. As the evidence confirming the distribution process of the enzyme might be considered the decrease of its concentration in aqueous phase after liposome formation related to the partition process between these two phases. Fig. 4 shows the temperature dependence of the partition coefficients for laminarinase and chitinase in studied systems. The $K_{o/w}$ values diminish with rising temperature in chitinase contained systems and increase for laminarinase microencapsulation (Fig. 4). The partition coefficients of enzymes laminarinase and chitinase ($K_{o/w}$) are greater than 1 indicating affinity of enzymes for microencapsulation in liposomes. However, the mechanisms of microencapsulation are different for each enzyme that may be related with differences of their primary structure and amount of lipophilic nature aminoacids (Nobe *et al.*, 2004).

The thermodynamic functions related to the transfer of laminarinase and chitinase from aqueous media to soya lecithin liposomes are summarize in Table 7. In both cases values of $\Delta G_{w \to o}$ at 25 °C are similar and negative. This indicates the preference of each enzyme for the organic phase confirming that enzyme transfer from aqueous media to organic system is spontaneous.

The enthalpic changes imply to energetic requirements and the entropic changes the molecular randomness (increase or decrease in the molecular disorder), resulting in the net transfer of enzyme from water to organic phase. The $\Delta S_{w \to o}$ values defined for chitinase and laminarinase microencapsulation in soy lecithin liposomes are differed to the sign: positive for laminarinase immobilization and negative for chitinase microencapsulation (Table 7). The enthalpy of chitinase transfer ($\Delta H_{w \to o}$) is negative and that of laminarinase is positive. Therefore, the process is exothermic and endothermic, respectively. Negative enthalpy indicates the presence of significant interaction between molecules of chitinase and soya lecithin phospholipids. Phospholipids can establish hydrogen bonding as donor or acceptor of hydrogen (Ávila & Martínez, 2003). On the other hand, after a certain number of enzyme molecules have migrated from the aqueous to the liposome organic phase, the original cavities occupied by the protein in the aqueous phase now are occupied by water molecules. This event is accompanied by release of energy due to water-water interactions. However, depending on enzyme's molecular structure, it is also necessary to keep in mind that the water molecules can organize around the enzyme hydrophobic aminoacids (hydrophobic hydration). This event is accompanied by an intake of energy in addition to a local entropy increase which is related to the separation of some water molecules.

Table 7 shows that for the laminarinase, transfer processes from water to lecithin liposomes were endothermic, and imply high increments in the system net entropy. The entropies of transfer ($\Delta S_{w \to o}$) are positive only for laminarinase contained system. The increase in entropy at the transfer of laminarinase to lecithin liposomes is possibly due to the disorder produced in the hydrophobic core of the lipid layers during separating the phospholipids hydrophobic tails to accommodate the protein molecules in liposomes. The obtained results

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indicate that the transfer of laminarinase is entropy driven due to positive value of entropy, while chitinase transfer is enthalpy driven due to its negative value.

Thus, laminarinase and chitinase microencapsulation performed by means of thermodynamically different mechanism that might be taken in account for process optimization.
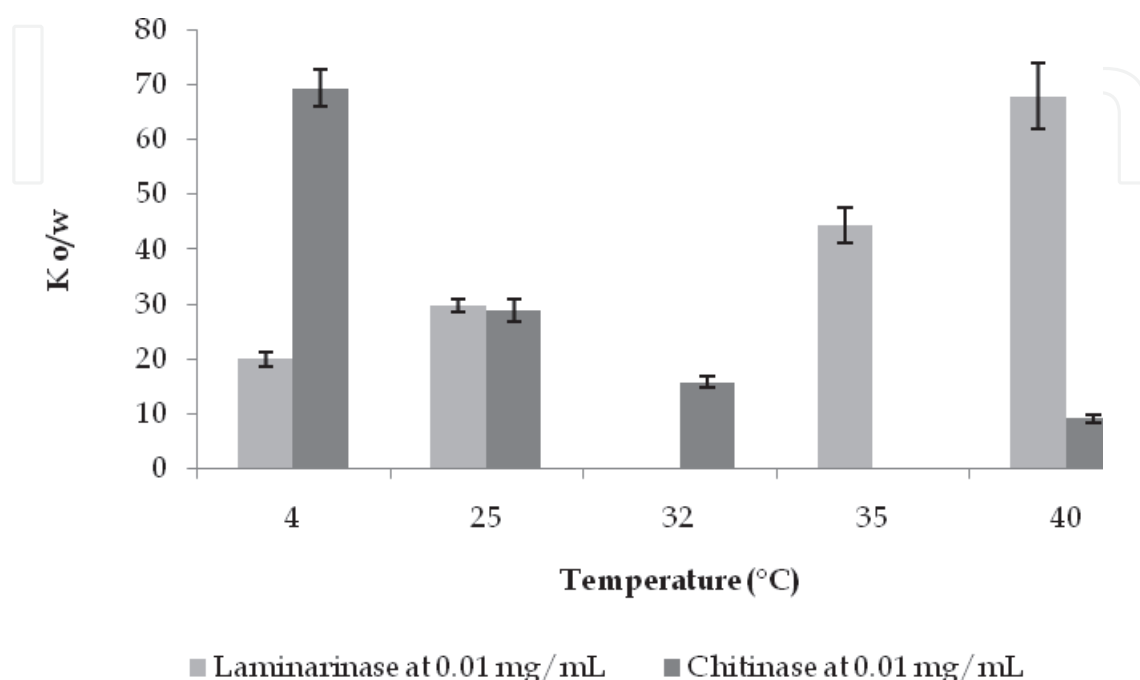

Fig. 4. Partition coefficients of enzymes (laminarinase and chitinase) in soya lecithin liposomes system as a function of temperature (±0.1 °C), in molality (± standard deviation).

Enzyme, mg/Ml	Laminarinase at 0.01 mg/mL	Chitinase at 0.01 mg/Ml
$\Delta G_{w \to o}$, kJ/mol	-8.4	-8.3
$\Delta H_{w \to o}$, kJ/mol	19.4	-39.3
$\Delta S_{w \to o}$, J/(mol x K) from equation	93.4	-103.9

Table 7. Free energy, enthalpy and entropy for the transfer of enzymes (laminarinase and chitinase) from aqueous media to soya lecithin liposomes.

4.3 Liposome storage stability

The liposomes number were measure by optical light microscopy (40X) immediately after their preparation and each tenth day during their storage at 4° and 25°C (Ávila *et al.*, 2003).

The enzyme presence and rising temperature for liposome formation led to decrease in their number and storage stability over long period of time (Table 8). In the presence of chitinase significant decreasing was observed in twentieth day under both storage temperatures and disappearance at 40th and 30th days, respectively for 4° and 25°C. The number chitinase contained liposomes obtained at 25°C (Table 8) was significantly lower than number of liposomes at the same temperature without enzymes or in the presence of laminarinase (Table 8) that seems to relate to a different interaction mechanisms. In the presence of

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laminarinase the concentration was similar to quantified in the system without enzymes at 4°C and it was greater than detected at 25°C. Significant decreasing was observed in 10th and 20th day and disappearance after 50 and 30 days, respectively, for 4° and 25°C. In this case, at 25°C liposomes number did not decrease as drastically as in liposomes without enzymes. Effects of enzymes on the stability of liposomes could be related to the interaction of proteins and lipids, which in the case of chitinase destabilized the liposomes, while laminarinase stabilized them for short periods of interaction followed by destabilization.

Enzyme concentration (mg/mL)	Temperature (°C)	Time (days)							
		0	10	20	30	40	50	60	70
		Liposomes/Ml							
Without enzyme	4	9 E+09	9 E+09	9 E+09	9 E+09	6 E+09	5 E+09	5 E+09	5 E+09
	25	6 E+09	8 E+08	6 E+08	9 E+07	7 E+06	5 E+06	5 E+06	4 E+06
Chitinase 0.005	4	4.63 E+09	4.81 E+09	2.76 E+09	2.78 E+09	n.d.	n.d.	n.d.	n.d.
	25	6.83 E+06	6.08 E+06	2 E+06	n.d.	n.d.	n.d.	n.d.	n.d.
Chitinase 0.01	4	2.88 E+09	2.43 E+09	2.12 E+09	1.68 E+09	n.d.	n.d.	n.d.	n.d.
	25	3 E+06	3.17 E+06	1.25 E+06	n.d.	n.d.	n.d.	n.d.	n.d.
Laminarinase 0.005	4	8.65 E+09	8.68 E+09	7.75 E+09	2.22 E+09	2.17 E+07	n.d.	n.d.	n.d.
	25	7.1 E+09	5.53 E+09	5.83 E+08	n.d.	n.d.	n.d.	n.d.	n.d.
Laminarinase 0.01	4	8.44 E+09	8.68 E+09	9.02 E+09	1.67 E+09	3.17 E+07	n.d.	n.d.	n.d.
	25	6.3 E+09	5.4 E+09	4.9 E+08	n.d.	n.d.	n.d.	n.d.	n.d.

(n.d.- undetectable by means of optical microscopy)

Table 8. Liposome storage stability at 4°C and 25°C

There are two aspects that affect stability of liposome systems: 1) the liposome component may degrade by hydrolysis and oxidation; chemical changes in the layer-forming molecules may affect physical stability; e.g., if phospholipids lose one of their acyl chains (turn into their lysoforms), the liposome structure is affected; and 2) the physical structure of the liposomes may be affected by changes within the lipid-layer, aggregation, or fusion. The storage stability may be increased by the use of purified phospholipids (Bangham, 1993; Ávila *et al.*, 2003). Thus, the soya lecithin liposomes are sensible to the enzyme presence and are better stored at low temperature.

4.4 Enzyme activity and storage stability

Chitinase activity was quantitatively determined by colorimetric measuring the nitrophenyl group of p-nitrophenyl-β-D-N-acetyl-glucosamide served as substrate, as described previously by Li *et al.* (2004). The laminarinase (β-1, 3-glucanase) activity was measured according to the method of Singh (1999) using laminarine from *Laminaria digitata* as substrate. The activity of laminarinase was determined spectrophotometrically by measuring the release of reducing sugar by the method of Somogyi-Nelson (Nelson, 1944).
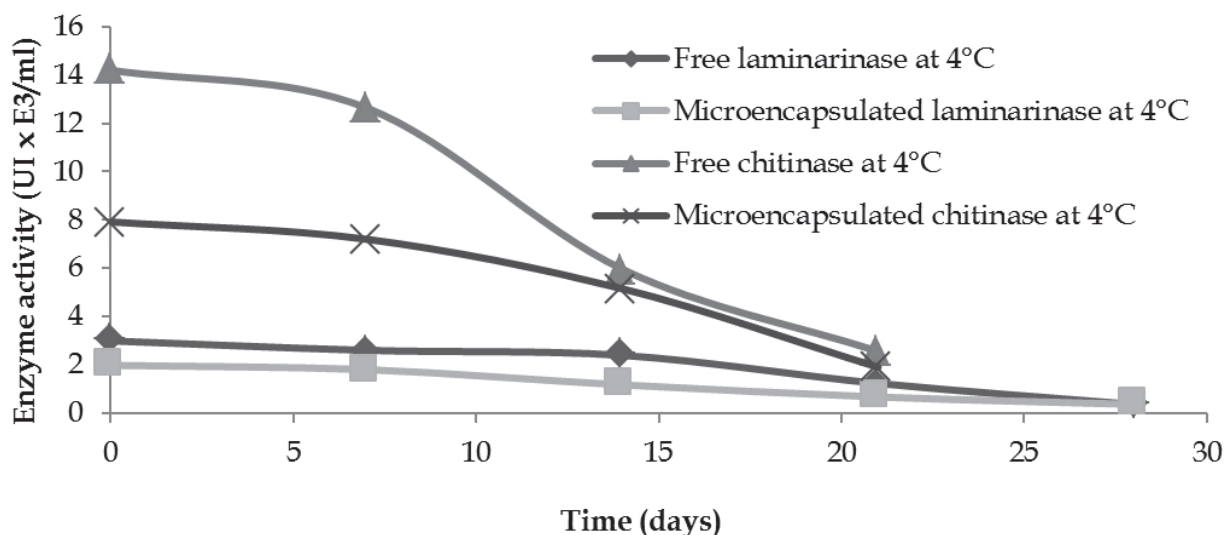
Fig. 5. Chitinase and laminarinase activity during storage at 4°C for microencapsulated in liposomes and free enzyme using at 0.01 mg/mL.

Results described activity of free and encapsulated chitinase and laminarinase during their storage at 4°C are shown in Fig. 5. Microencapsulated enzymes are less active than free enzymes, possibly due to encapsulation effect (Fig. 5). In the presence of liposome the chitinase activity was twice less than free enzyme, while laminarinase was significantly less active after encapsulation. The lower activity is related to partly enzyme encapsulation. The enzyme concentration is less in microencapsulated form than in free form.

Moreover, Chaize (2004) reports that once encapsulated, the enzymes have encountered another problem. The permeability barrier of the lipid membrane drastically diminishes the activity of the enzyme trapped in the liposome by reducing the entrance rate of the substrate molecules and then reducing the substrate concentration inside the liposome. It could be the reason for decreasing of chitinase and laminarinase activity after their microencapsulation.

Microencapsulated enzymes lost activity slower than free enzymes. Thus, immobilization of laminarinase and chitinase led to increase the enzymes stability.

4.5 Definition of the activity of microencapsulated enzymes against *Fusarium oxysporum in vitro* and *in vivo* testing

The inhibitory effects of free and microencapsulated chitinase and laminarinase were estimated by using the radial growth inhibition assay as described previously by Prapagdee *et al*. (2007). Fungal growth inhibition was expressed as the percentage of radial growth inhibition relative to the control assay. The means and standard deviations of all obtained results were calculated. Data were analyzed by one-way analysis of variance (ANOVA). Significant differences (p ≤ 0.05) between the means were determined by the Duncan multiple range tests (Bewick *et al*., 2004).

4.5.1 *In vitro* antifungal activity of free and immobilized enzymes, chemical fungicide and their mixtures

Previously we reported that chitinase and laminarinase from *Trichaderma spp*. applied at 0.02 mg/ml in solid agar media totally inhibited the growth of *Fusrium oxysporum* (Joublanc *et al*., 2010). Free enzymes (laminarinase and chitinase) applied at 10 µg/ml each partially

inhibited the *F. oxysporum* growth and spore production. In these cases the inhibition increased with increasing fungus growth time. In contrast, separately applied microencapsulated enzymes inhibit the fungus growth only during the first four days. The difference may be explained by significantly lesser activity of enzymes encapsulated in liposomes. Moreover, the inhibition of *F. oxysporum* mycelia growth in the presence of liposomes without enzymes is very similar to the one observed in the presence of separately applied microencapsulated enzymes. This leads to conclusion, that the inhibition of *F. oxysporum* growth in these cases is also related to lipid presence. This result is consistent with other reports (Ment *et al.*, 2010) that discussed similar effect observed with other fungi types and other lipid extracts.

The concentration (spores/ml) was decreased in comparison with one detected in the presence of liposomes without enzyme. Conidia concentration decrease was greater in the presence of free enzymes that related to their higher activity and greater growth inhibition.

However, the growth of *Fusarium oxysporum* was totally inhibited in the presence of free and microencapsulated chitinase and laminarinase mixtures applied at 0.005 and 0.01 mg/ml, concentrations lower than 0.02 mg/ml. Our results also are in agreement with the findings of Lorito *et al.* (1993), who indicated that mixtures of hydrolytic enzymes with a complimentary mode of action may benefit an organism by improving its antifungal activity through mycoparasitism or survival in an antagonistic environment.

Use of free or microencapsulated laminarinase and chitinase at 0.01 mg/mL allows at least double decrease of chemical fungicide concentration to obtaining complete inhibition of fungal growth. It can be useful in agriculture practice to reduce levels of chemical fungicides. Thus, the results demonstrate a synergistic effect on the *Fusarium oxysporum* growth: the effect of two treatments (laminarinase/chitinase, or enzyme/thiabendazole) is greater than the effect of each treatment applied individually. Considering the synergistic effects of microencapsulated enzymes demonstrated in the present study, it may be supposed an advantage of its potential application in agro-industry.

In vitro testing performed using soil contained system was also carried out. One milliliter of free or microencapsulated enzymes , as well as their micture at 0.005 and 0.01 mg/mL was added at each tube contained 1 g of soil. Next assay was performed using chemical fungicide (thiabendazole) solution added to soil at final concentration 0.006 mg/g of soil followed by addition of 1 ml of free or microencapsulated enzymes solution at 5 or 10 µg/mL. Control assays were carried out using soil without treatments, adding 1 mL of acetate buffer at pH 5.4, as well as using 1 mL of liposomes suspension prepared without enzymes or with thiabendazole at 6 or 12.5 µg/g of soil without enzyme addition.

All tubes were inoculated with *F. oxysporum* using 1.3 E+05 conidia/mL suspension obtained from a 12 day old culture. Immediately after tube inoculation fungus viability (CFU/g of soil) was evaluated (Madigan *et al.*, 2003). The same measurements were carried out after tube incubation at 25°C at 20th, 30th and 40th day.

It has been demonstrated that the enzymes addition led to decrease of the fungus proliferation that was expressed in lower values of CFU/(g of soil). Free and immobilized laminarinase significantly controlled the fungus viability only at 10 or 20 µg/mL, while chitinase applied at 5 µg/mL also provoked the inhibitory effect. The effect was greater with microencapsulated chitinase than free enzyme, while it was not observed in the case of laminarinase. The results obtained in assays carried out in soil demonstrate fungistatic effect (an inhibiting effect upon the growth and reproduction of fungi without its total destruction) which is more pronounced in the presence of higher enzyme concentrations.

The low fungistatic effect also was demonstrated in assay carried out with liposomes without enzymes that demonstrated the lecithin capacity to inhibit conidia germination. The difference between free and microencapsulated enzyme activities decreased during the assay, possibly due to greater storage stability of enzymes immobilized in soya lecithin liposomes. The relative activity of microencapsulated enzymes was superior to free enzymes. It may be considered as higher enzymatic stability under storage in the soil. Microencapsulation of chitinase and laminarinase protects the protein active structure from these inactivation factors.

The synergic effect to control fungus viability was demonstrated. A mixture of chitinases and β- 1,3-glucanase was significantly more effective against phytopathogenic fungus than either of these enzymes used individually.

With thiabendazole applied at 12.5 μg/mL the fungicide effect was observed, while at 6 μg/mL the effect was lower. The enzyme addition led to greater inhibition of *F. oxysporum* viability. The greater inhibition was observed in the presence of 10 μg/mL of microencapsulated chitinase, as well as laminarinase. Each mixture provoked a fungicide effect detected at 30th day of assay. The effect of free enzyme applied in the presence of thiadendazole was slightly lower than of microencapsulated enzyme. This difference may be related to an inactivation of enzymes in the presence of chemical fungicide. Free enzymes were inactivated after 40 days. Due to inhibitory effect of chemical fungicide, the initial activity of immobilized chitinase was greater than of free enzyme activity in soil. Initially, free laminarinase was more active than microencapsulated preparation but drastically lost its activity in the next days of assay. Thus, the use of microencapsulated enzymes has advantages in comparison to free enzyme application due to their greater enzyme stability as well as higher inhibitory effect on *F. oxysporum* viability with and without chemical fungicide.

4.5.2 *In vivo* testing under greenhouse conditions

Microencapsulated laminarinase and chitinase were applied in the soil under greenhouse condition against *Fusarium oxysporum* to the tomato crops (*L. esculentum Mill*). *Fusarium oxysporum* is one of the most important pathogens of the tomato plants and the other vegetable crops in the greenhouse and field conditions (Larkin *et al*, 1996). Tomato diseases are normally controlled by means of the selected fungicides, e.g. thiabendazole. Due to fungi adaptability and population diversity, the pathogen frequently overcomes all of these currently used disease control strategies. The use of broad-spectrum fungicides results in imbalances within the microbial community creating unfavorable conditions for the activity of beneficial organisms, contaminates the environment, affects ozone layer, and must be applied every season due to its null residual activity and the rapid re-colonization of soils by the phytopathogens. The enzymes of *Trichoderma* are strong inhibitors of many important plant pathogens. Chitinases and laminarinases are able to lyse polysaccharides of mature hyphae, conidia, chlamydospores, and sclerotia.

Three different treatments included encapsulated enzymes were applied: the mixture of laminarinase and chtinase at 10 μg/g of soil and 5 μg/ g of soil, respectively; immobilized laminarinase at 10 μg/g of soil with thiabendazole at 6 μg/g of soil, and immobilized chitinase at 10 μg/g of soil in the presence of thiabendazole at the same concentration. The assays with thiabendazole at 12.5 μg/g of soil and with liposomes without enzyme were carried out. Two controls inoculated and not inoculated with *F. oxysporum* and treated with

the buffer without additional substances were performed. Thus, 7 different groups of plants were employed with 10 plants in each group.

Tomato seedlings (*L. esculentum* Mill) were grown according to conventional techniques (Montealegre *et al.*, 2010) during 14 days. Then, they were transplanted into previously sterilized 30 g of the soil / peatmoss mixture (weight ratio 1:1) using as substrate for plant growth. The corresponding treatments were added at total volume of 15 mL to reach the mentioned final concentration. The pots were inoculated with 15 mL of *F. oxysporum* conidias at 1E+05 conidias/mL. The pots were kept at random complete block under controlled greenhouse conditions (controlled range of temperature: 20ºC (minimum) and 30ºC (maximum); 12 h natural light; irrigation was applied once at three days).

The group of plants grown on the substrate infected with *F. oxysporum* lost 90% of its population due to fungus attack. In all cases the damage at the root of the plant caused by fungus was observed. In other blocks corresponding to other treatments the dead plants were not observed.

Obtained results demonstrated that application of all treatments contained enzymes or chemical fungicide led to control of CFU/(g of soil). The best results were obtained with thiabendazole at 12.5 µg/(g of soil) and at 6 µg/(g of soil) in the presence of chitinase microencapsulated in liposomes at 10 µg/mL. With three other treatments, a similar effect to fungus viability was observed. The CFU/(g of soil) measurements were significantly greater in the untreated infected pots than in the pots with applied treatments, although the increase of CFU/(g of soil) was observed in all cases. The activity maintained at the initial level for the first 14 days followed by its decrease for the next two weeks.

The growth of plants under treatments contained enzymes and/or liposomes with and without thiabendazole was greater than in the case of non-treated and non-infected control. The lowest growth was detected on the infected group without treatments that indicated positive effect of microencapsulated enzymes applied in different treatments to control of phytopathogenic fungus *F. oxysporum* and protection of tomato plants (*L. esculentum* Mill).

5. Conclusion

To determine antifungal activity of biochemical micolytic compounds, the bioluminescent assay may be considered as the fast and reliable method of its evaluation.

It can be concluded that immobilization of chitinase and laminarinase can be a good alternative for enzyme stabilization. Mycolitic enzymes immobilized on seaweed bagasse can be used for the control of phytopathogenic fungi in HGF system.

Chitinase and laminarinase have affinity to soya lecithin liposomes. The findings on the thermodynamic properties of enzymes microencapsulation on liposomes can be considered for process optimization in future studies and applications. Stability of enzyme preparations was increased. Finally, the possibility of using of mycolytic enzymes immobilized in liposomes for the control of some pathogens was confirmed. This finding may provide the alternative means of reducing the dependency on synthetic chemical fungicides. The synergistic effect on viability of *F. oxysporum* was demonstrated in the presence of mixture of encapsulated enzymes and enzymes with thiabendazole. Thus, it confirmed the original idea regarding of use the mycolytic enzyme immobilized in soya lecithin liposome for control of some pathogens.

6. Acknowledgements

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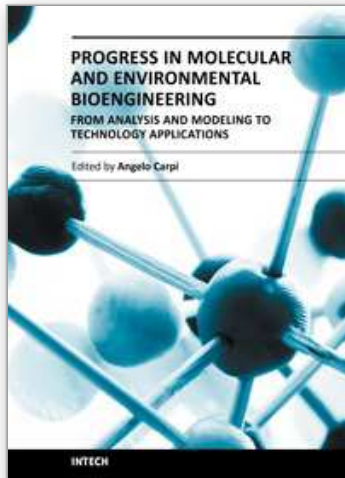
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