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EVALUATION OF ANTIFUNGAL ACTIVITY OF SELECTED LACTIC ACID BACTERIA STRAINS AGAINST SPOILAGE MOULD *PENICILLIUM EXPANSUM*

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Abstract

Various spoilage fungi that contaminate fruits, vegetables and other food commodities produce economic losses as well as harmful effects on human and animal health caused by ingestion of mycotoxins The public concern about the use of chemical fungicides during cultivation and storage of fruits and vegetable determined the search for new strains of lactic acid bacteria (LAB) able to control the fungal growth of mycotoxigenic species. The effect of 8 LAB strains have been assayed on the growth of Penicillium expansum isolated from infected apples. Interaction between LAB strains and selected spoilage fungi was tested by overlay assay method. Discrete spots of liquid cultures of tested LAB strains were placed on MRS agar and after incubation, overlaid with soft PDA containing fungal propagules. Data concerning the evolution of the diameters of clear visible inhibition zones of fungal growth around the LAB strain spots were monitored for 10 days. A number of 5 strains have shown antifungal activity with clear inhibition zone developed at 5 days, the effect of 2 strains being sustained until the 10th day of monitoring. P. expansum spores were cultivated with 3 efficient LAB strains on PD liquid medium and monitored for 17 days. The inhibition of spore forming lasted for 2 strains until the end of monitoring period and for the other strain until the end of the first week. Completely sporulated mycelium with powdery aspect was evidenced in control flask with pure culture of P. expansum after 5 days. Probably chemical interactions appeared between fungus and LAB strains that changed the color of culture media towards reddish-brown comparatively to control. The results could be used in biotechnological methods for the control of spoilage fungi with application in fruits preservation.

Key words: *antifungal activity, inhibition zone, lactic acid bacteria, Penicillium expansum.*

INTRODUCTION

A wide spectrum of filamentous fungi can contaminate various food commodities, with important economic losses around the world.

Fungal infection causes food spoilage with high impact on organoleptic properties. The harmful effects on human and animal health is determined by the contamination with mycotoxins produced by fungi belonging mainly to genera *Aspergillus, Fusarium* and *Penicillium* (Gerez et al., 2009; Bryden, 2012).

Penicillium expansum, the blue mold of apples is a mycotoxigenic species recognized for the capacity to produce patulin and citrinin as secondary metabolites with toxic effect when ingested (Watanabe, 2008).

The effects of highly toxic metabolites synthesized by fungal species on human health include carcinogenic, teratogenic, immunotoxic, neurotoxic, hepatotoxic and nephrotoxic (Bryden, 2007; Wild and Gong, 2009).

Even though several physical and chemical methods have been developed to control fungal growth, some moulds acquired resistance to chemical treatment and preservatives.

In the last decades, biopreservation, the control of one organism by another, has become an alternative to conventional methods motivated by general public demands for preservatives-free food and feed (Schnurer and Magnusson, 2005).

Lactic acid bacteria have been considered natural biological antagonists for mycotoxigenic fungi that contaminate various fruits and vegetables (Trias et al., 2008). Blagojev et al. (2012) reviewed the possibility of using various species of lactic acid bacteria as biopreservatives for the control of mycotoxigenic fungi and the main mechanisms involved in antimicrobial efficiency of lactic acid bacteria such as: the yield of organic acids, competition for nutrients and production of antagonistic compounds. Species belonging to genera *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* are recognized for their ability to synthesize bacteriocins (microbial proteins) with antifungal effect against various mycotoxigenic fungi (Erginkaya et al., 2011). Muhialdin et al. (2011) listed a series of antifungal compounds produced by lactic acid bacteria and their spectrum range of inhibitory activity against mycotoxigenic species. Cyclic dipeptide released by *Pediococcus pentosaceous* inhibited *Penicillium expansum*, the bacteriocins produce holes in the membrane of the cell causing content leakage and alteration of the trans-membrane potential. Recent studies showed that lactic acid bacterial biofilms rich in exopolysccharides could be used as biocontrol agents (Ünal et al., 2011).

The aim of this work was to assess the effect of different lactic acid bacterial strains on growth of mycotoxigenic fungal species *Penicillium expansum* and to select performant strains as biopreservatives for fruits.

MATERIALS AND METHODS

The 8 strains of lactic acid bacteria were obtained from dr.Medana Zamfir, Institute for Biology Bucharest and have been assayed on the growth of possible mycotoxigenic fungus *Penicillium expansum* isolated from infected apples. The fungus was isolated by plating decimal dilutions of heavily infected apple fragments on PDA (produced by Merck KGaA Germany), after incubation at 25 ^{o}C for 5 days. Pure cultures were streaked on PDA in test tubes. Microscopic observation and photographs have been carried out for morphological characterization. Taxonomic identification was done according to determinative manual for food-borne fungi (Samson and van Reenen-Hoekstra, 1988). Interaction between lactic acid bacteria and selected fungi was tested by overlay assay method (Magnusson et al., 2003). Discrete spots of 48 hours liquid cultures of the 8 lactic acid bacterial strains were placed on MRS agar (Liofilchem Italy) and after 24 h incubation at 30^{o}C, overlaid with soft PDA containing propagules of test fungi. Data were collected after 72 hours incubation at 25^{o}C concerning the diameters of clear visible inhibition zones of fungal growth around the lactic acid bacterial strain spots. Values obtained represented the mean of three replicates per assay. The Petri dish cultures were monitored for the persistence of the inhibition zones over a period of 10 days. A reversion of the zone of inhibition after 96 h was recorded as a fungistatic action while those with inhibition zone for at least 7 days were recorded as being fungicidal in action (Adebayo and Aderiye, 2010). Fungal spores of *Penicillium expansum* were cultivated with 3 efficient strains of lactic acid bacteria on PD liquid medium and monitored for 17 days. Fifty milliliters of sterile PD broth containing 10^5 spores of *Penicillium expansum* mL^{-1} and 2 mL each of the LAB isolates were added into triplicate 100 mL Erlenmeyer flasks. In the control experiment, only 2 mL of sterile MRS broth was added to the PD broth containing 10^5 spores of *Penicillium expansum*. The flasks were incubated at 25°C for 5 days.

The percentage inhibition was determined by comparing the growth of the control with the treated fungi. The dry weight was determined by drying the mycelia in an oven at 70°C for 48 hours. The data obtained represent the mean of 3 replicates per variant. The percent inhibition was calculated using the formula:

$$GI\ (\%) = C_o - C_F/C_o \times 100$$

where C_o was the dry weight of the control and C_F was the dry weight of fungal mycelium after inhibition by lactic acid bacteria (Hamed et al., 2011).

RESULTS AND DISCUSSIONS

Penicillium expansum isolated in pure culture from heavily infected apples (Figure 1) presented blue-green colonies with yellowish reverse and fruity odour. Microscopical aspects from Figure 2 reveal the specific conidiophores branching, the cylindrical metulae bearing 5-8 phialides and greenish subglobose conidia, smooth-walled.

The effect of 8 lactic acid bacterial strains on *Penicillium expansum* assayed by overlay method ranged from no visible inhibition to clear visible inhibition zone as shown in the aspect of petri plates. The assay results (Figure 3) showed that after 3 days 5 lactic acid bacterial strains (13, 15, 43, Lpa and Lpl) had inhibition zone diameter over 25 mm. Two strains (53, LCM5) had no inhibitory effect on mycotoxigenic fungus *Penicillium expansum* and one strain (64) failed to grow. The persistence of clear inhibition zones around 4 strains (13, 15, 43 and Lpa) until the 10th day suggest the fungicide effect against test fungus. The strain Lpl presented reversion of the inhibition zone after 6 days showing a potential fungistatic effect. The results are in accordance with data obtained in other studies: 9 of the 17 lactic acid bacterial strains isolated from indigenous Nigerian fermented foods exhibited a high fungicidal activity against spoilage fungi *Penicillium citrinum*, *Aspergillus niger*, *Aspergillus flavus*, as there was no reversion of the delay in growth caused by these strains before and after 7 days incubation (Adebayo and Aderiye, 2010).

Figure 1. *Penicillium expansum* - pure culture from infected apple

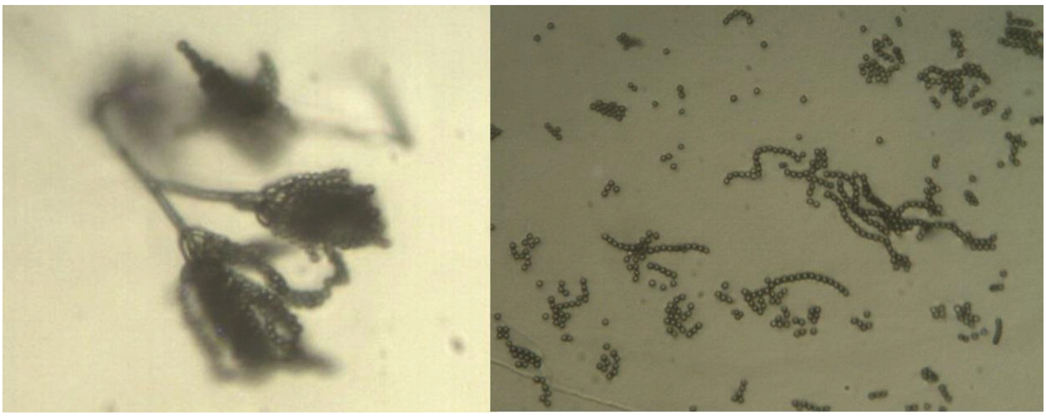

Figure 2. Microscopical aspect of conidiophores ramification (left) and chains of greenish conidia (right)

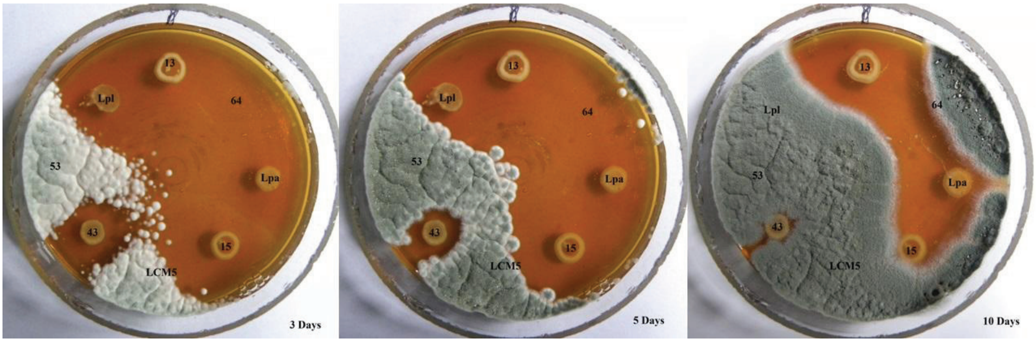

Figure 3. Evolution of inhibitory effect of lactic acid bacteria against *Penicillium expansum*

Our research by optic microscopy evidenced hyphal alterations of *Penicillium expansum* (Figure 4) with frequent loss of cellular content, fragmentation and delays in sporulation process as a consequence of the antifungal activity in the inhibition zone around lactic acid bacteria spots.

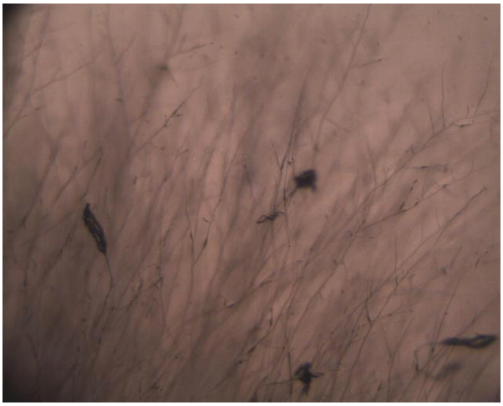

Figure 4. Microscopic aspect with altered fungal hyphae from inhibition zone

Results are in concordance with co-cultivation assay carried out by Ström (2005) when mycelial growth of mycotoxin-producing *Aspergillus nidulans* was 36% inhibited by *Lactobacillus plantarum* and increased vacuolization, disturbed branching and swollen hyphal have been described.

Trias et al. (2008) showed that 4 lactic acid bacterial strains isolated from fresh fruits and vegetables inhibited the development of infection produced by *Penicillium expansum* on apples by 20%, due to high capacity to produce organic acids. Concentrated culture filtrate of *Lactobacillus plantarum* 21B isolated from sourdough, with increased production of phenyllactic and 4-hydoxy-phenyllactic acid, presented efficient antifungal activity against 7 mycotoxigenic fungi including *Penicillium expansum* (Lavermicocca et al., 2000).

Antifungal activity of 3 lactic acid bacteria (strains 15, 43, Lpa) on fungal mycelia growth and sporulation was assessed in liquid media (Figure 5). All strains inhibited the sporulation of test fungus until the 5th day as compared with the control represented by pure culture of *Penicillium expansum* completely sporulated. Perhaps biochemical interactions appeared consequently to the beginning of fungal sporulation when co-cultivated with lactic acid bacterial strain Lpa, determining the color change of the medium towards reddish-brown compared with the control. The inhibition of spore forming lasted for the strains 15 and 43 until the end of monitoring period.

The growth inhibition percent of *Penicillium expansum* by the three lactic acid bacterial strains ranged from 3.40 % for Lpa to 21.96 % for strain 15 (Table 1). Literature also cites data from *in vitro* assay where various isolates of *Lactobacillus* showed a broad spectrum of antifungal activity ranging from no inhibition against *Rhizoctonia solani* to 75 % against *Fusarium oxysporum* (Hamed et al., 2011).

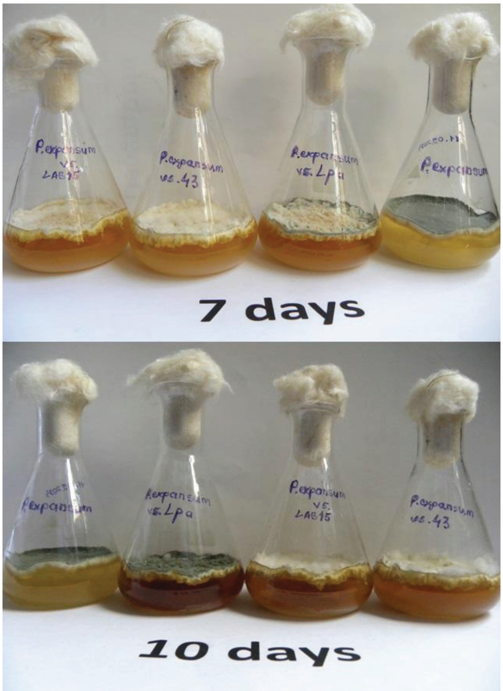

Figure 5. Evolution of antifungal effect of lactic acid bacteria against *Penicillium expansum*

Table 1.The dry weight and percentage growth inhibition of *P. expansum* by 3 strains of lactic acid bacteria

Variant	Dry weight (mg)	Growth inhibition (%)
P. expansum	2.64	Control
P.expansum - Strain 43	2.18	17.42
P.expansum - Strain 15	2.06	21.96
P.expansum- Strain Lpa	2.35	3.40

Other research reported partial (inhibition of conidia formation) or total (inhibition of conidial and aerial hyphae formation) inhibition of *Penicillium* expansum in the presence of yeasts isolated from apple or other fruits, as well as a ten times reduction of quantity of patulin (Taczman-Bruckner, 2005).

Our results obtained on liquid media confirmed the prolonged antifungal effect from overlay assay on solid media in concordance with data reported by Adebayo and Aderiye (2010) in experiments with lactic acid bacteria assayed for antifungal effect of cell free supernatants by agar well diffusion method and co-cultivation in liquid media.

CONCLUSIONS

A number of 5 lactic acid bacterial strains have shown antifungal activity on *Penicillium expansum.*

The lactic acid bacterial strains 13, 15, 43 and Lpa presented fungicidal activity against potential mycotoxigenic fungus.

The strain Lpl of lactic acid bacteria presented potential high fungistatic activity.

The effect of lactic acid bacteria in liquid media consisted in delaying the sporulation process and reduced dry weight of mycelium.

The results could be used in biotechnological methods for the control of spoilage fungi with application in fruits preservation.

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