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Penicillium expansum versus Antagonist Yeasts and Patulin Degradation *in vitro*

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ABSTRACT

Taking into account the preliminary antagonistic/biodegradation property showed by Pichia membranifaciens and Sporobolomyces roseus, which decreased the initial patulin concentration of 588.4 to 290.0 μg/mL, ability of P. ohmeri 158 in biocontrol against Penicillium expansum and patulin decrease in vitro was performed. The culture supernatant of P. ohmeri 158 was effective against 66.17% micelial growth, indicating antibiosis related with the killer phenomenon. The initial patulin concentration of 223 μg in the presence of P. ohmeri 158 cells was decreased over 83% of the original concentration, when incubated at 25ºC/2 days and > 99% after 5 days incubation time, with undetectable patulin level after 15 days. The initial pH 4.0 decreased to pH 3.3 along 15 days experiment, suggesting that patulin decrease was an active process and a consequence of yeast metabolism. The results suggested that P. ohmeri 158 could be a promising alternative for the inhibition of P. expansum growth and patulin degradation.

Key words: *Penicillium expansum*, antagonist yeasts, biocontrol, patulin, degradation

INTRODUCTION

The perishable post-harvest fruits should be free of fungal deterioration trigged by the environmental factors as mechanical damages caused by the harvest and storage (Arras et al., 1998; Hussein and Brasel, 2001). Phytosanity can be the critical point in the food safety in the globalized agribusiness, where the Brazilian fresh fruits have been introduced as promising natural food (World Health Organization-International Agency for Research on Cancer-WHO-IARC, 1996; Food and Drug Administration-FDA, 2002). The Food and Agricultural Organization (FAO) estimated contamination with mycotoxins in 25% of the world's crop, and the main fungi involved belonged to the *Aspergillus*, *Penicillium* and *Fusarium* genera (Pitt et al., 2000; Ono et al., 2006). However, the predominant post-harvest fungus in apple is patulin producing *Penicillium expansum*. This mycotoxin causes gastrointestinal distress and neurotoxic effect in the rodents, immunotoxicity in mice and rabbit, and genotoxicity in the mammalian cells (Hopkins,

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1993; Sharma, 1993; Wouters and Speijers, 1996; Moss and Long, 2002).

The control of diseases in the post-harvest fruit is based on the chemical fungicides, but progressive loss of the effectiveness and the emergence of resistant pathogens are the topic of concern, due to the increasing level of agrotoxic residues (Sugar and Spotts, 1999; Castoria et al., 2001). Biological control using antagonist microorganism has been a promising alternative, which provides safe application in both human health and ecosystem (Janisiewicz et al., 2000; Usall et al., 2001). Emphasized is the biological control using yeast, i.e. safe antagonist without health hazard coupled with low ecological impact, reliable in refrigerated storage of fruit (Usall et al., 2001). Patulin degradation to (E)-ascladiol involved fermentative process by *Saccharomyces cerevisiae* (Sumbu et al., 1983; Moss and Long, 2002).

In our previous study, screening of 44 yeasts isolated from the different ecosystem source showed fifteen isolates with inhibitory activity against *P. expansum* strain #2 based on the nutrient and space competition, and five strains with antagonism based on antibiosis, with the culture supernatant of *C. guilliermondii* P3 and *P. ohmeri* 158 showing the higher antagonism (Levy et al., 2002; Levy, 2003; Coelho, 2005). Levy *et al* (2002) demonstrated the decrease of patulin *in vitro* using *Pichia membranifaciens* and *Sporobolomyces roseus* strains. However, the use of antagonist yeasts with wide activity and patulin decrease could be promising, and additional studies about the mode of action would be necessary before *in vivo* application. Taking into account the prominent activity of *P. ohmeri* 158 against mycotoxin producing *P. expansum* in solid culture, the antifungal activity in broth culture and patulin decrease by *Pichia ohmeri* was evaluated *in vitro* in this work.

MATERIALS AND METHODS

Microorganisms

Single spored *P. expansum* strain #2 isolated from the apple was used as test microorganism (107 μg/mL patulin production). *P. expansum* was subcultured each six months on Potato Dextrose agar (PDA, 21ºC/120 h), and stored at 4ºC. The spore inoculum was prepared suspending reactivated culture into 3 mL sterile distilled water with 0.1% (v/v) Tween 80, and it was adjusted to 1 x 10^5 spores/mL (Newbauer chamber). *C. guilliermondii* P3 and *P. ohmeri* 158 were isolated from the papaya and anthill, respectivelly, and biochemically characterized by the conventional method (Kreeger Van Rij, 1984; Barnett et al., 1990) and API 20 C AUX test (Biomérieux Vitek, Marcy-l'Etoile, France), followed by identification according to Kurtzman and Fell (1998). *C. guilliermondii* P3 and *P. ohmeri* 158 grown on the surface of GYMP agar slant (2.0% glucose, 1.0% malt extract, 0.5% yeast extract, 0.2% $NaHPO_4$ and 1.8% agar) were maintained at 4ºC, carrying out each six months reactivation in the same culture medium. For assay, the strains were activated on Yeast medium (YM agar-2.0% glucose, 0.5% yeast extract, 1.0% NaCl, 0.23% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, 1.8% agar) at 25ºC/48 h (Levy, 2003).

Antagonism in broth culture

C. guilliermondii P3 and *P. ohmeri* 158 were activated in YM broth culture at 25ºC/24 h (150 rpm), and 100 μL of cell suspension (3.0 x 10^6 cells) was inoculated in five Erlenmeyer flasks (25 mL of YM broth) and incubated at 25ºC in static condition. Each 24, 48, 72, 96 and 120 h, one of flask was centrifuged (6500 x g/15 min) and filter sterilized (Millipore 0.20 μm). An aliquot (1 mL) of sterilized supernatant was added into 1 mL of YM medium (tube 120 x 13 mm), and inoculated with *P. expansum* strain #2 (10^5 spores). A control without supernatant (10^5 spores of *P. expansum* strain #2 in 1 mL sterile water plus 1 mL YM medium) was used for the comparison. The tubes were incubated at 25ºC/12 h, and microscopic analysis of conidia germination percentage and fungal growth determination by measuring the hyphal length. One inhibition unit (UI) was defined as 50% conidia inhibition or 50% hyphal growth inhibition (Chen et al., 1999). The experiment in three repetitions was carried along four months. For each repetition, the hyphal lengths were measured for 40 randomly selected hyphae and the mean hypha length was used for the comparison. Conidia germination was based on four counts of 100 conidia per repetition (Chen et al., 1999). The data of three repetitions were analysed by Tukey test (ANOVA/MANOVA) comparing the mean values obtained from 120 data for hyphal length and 12 data for propagules percentage.

Toxigenicity of *P. expansum*

P. expansum strain #2 toxigenicity was determined by the high-performance liquid chromatography (HPLC, Shimadzu, Japan). Ten mg of working standard of patulin (99.0% purity-ACROS, New Jersey, USES) was dissolved in 1 mL chloroform, subdivided in five flasks (200 μL), dried under N_2 gas and stored at -20ºC. Patulin was suspended in ethanol and quantified at 275 nm (Cintra 20), according to the "Official Methods of Analysis"-AOAC (2000), procedure n. 974.18.

P. expansum was grown in 10 Erlenmeyer flasks with 25 mL of malt extract (pH 4.4) (malt extract 0.6%, dextrose 0.6%, maltose 0.18%, yeast extract 0.14%) at 25ºC/15 days and the filtrate was submitted to three partitions with 25 mL of ethyl acetate. Pooled organic phases (750 mL) were dried with 100 g anhydrous sodium sulfate (30 min) and evaporated at 40ºC until aproximately 25 mL. The solution was cleaned up using silica gel column (500 x 10 mm, with 8.5 g of silica gel 60G, 70-230 mesh, MERCK). Briefly, the solution was introduced into the column and the elution was carried out with 100 mL benzene/ethyl acetate (75:25). The eluate was collected, evaporated at 40ºC until aproximately 5 mL, and dried under N_2 gas (AOAC, 2000).

Residue was dissolved in 100 μL of acetonitrile/water (1:9), and analysed by the reversed-phase HPLC (Shimadzu, Japan), equipped with a CTO-10A VP column oven, a LC10AD pump, and a SPD-10 UV detector. Separations were performed on a 250 x 4.6 mm I.D. C_{18} column of 5 μm diameter (Trennsaule). The mobile phase was acetonitrile/water (5:95), and the flow rate was 1.5 mL/min; the elution was monitored by UV absorption at 275 nm (Kawashima et al., 2002). Under these conditions, patulin has a retention time of 7.4 min. Patulin was quantified by comparing the peak areas in the sample with those of an authentic reference standard (ACROS, New Jersey, USA) at 7 concentration levels: 0.020; 0.035; 0.050; 0.100; 0.200; 0.250; and 0.350 μg/mL (R^2 = 0.999). The detection limit of the toxin was measured as three times the baseline standard deviation of blank (0.020 μg/mL), and the quantification limit was measured as five times the detection limit (Codex Alimentarius Commission, 1998; Valente Soares, 2001).

Patulin degradation by *P. ohmeri*

The assay of patulin degradation was carried out adding 223 μg of patulin (previously produced by *P. expansum*, as described on item 2.4.) in four Erlenmeyer flasks with 25 mL of Yeast Medium (YM) pH 4.0, and inoculated with *P. ohmeri* (3.0 x 10^6 cells). Four more Erlenmeyer flasks (25 mL of YM pH 4.0) were added with patulin alone (without yeast) as control, in order to check patulin stability during assay. After 2, 5, 10 and 15 days incubation at 25ºC in a Biochemical Oxygen Demand (B.O.D., MERCK), remained patulin was quantified as described above (AOAC, 2000) and pH value was determined.

Statistical analysis

The mean values of hyphal length measurement and propagules percentage from the antifungal assay analysed for the antagonistic activity were compared by Tukey test using ANOVA/MANOVA program (STATISTICA version 5.0, 1995).

RESULTS AND DISCUSSION

In the previous studies, screening of 44 yeasts isolated from the different ecosystem source resulted 20 antagonists with any anti-*Penicillium expansum* strain #2 activity (Levy et al., 2002; Levy, 2003; Coelho, 2005). Eight antagonist yeasts were isolated from the apple (*P. membranifaciens* strains A1 to A7; *Sporobolomyces roseus* strain A8), three from the corn silage (*Debaryomyces hansenii* strains C1; C5; C7), 6 from anthill (*P. ohmeri* 158; *Aureobasidium* sp. 102; *Candida homilentoma* 69; *D. hansenii* 31; *P. anomala* 142; *P. guilliermondii* 42), 1 from papaya (*C. guilliermondii* P3) and 2 from grape fruits (*Rhodotorula mucilaginosa* strains G3 and G5). Fifteen antagonists showed inhibitory activity against *P. expansum* strain #2 based mainly on nutrient and space competition. The assay in solid medium (25ºC/120hrs) pointed the strongest nutrient competition antagonism by *D. hansenii* strain C1 (31 mm inhibition diameter), while *D. hansenii* strain C7 (15 mm) showed higher antibiosis and parasitism pattern (Levy et al., 2002; Coelho, 2005).

Taking into account the approach concerning the antagonism based on antibiosis, the extracellular activity was tested performing the assay with

culture supernatant in YM agar, against *P. expansum* strain #2 (10^5 spores). This assay reduced the number of active isolates into five (*D. hansenii* strain C7; *P. membranifaciens* strains A4 and A8; *C. guilliermondii* strain P3; and *P. ohmeri* strain 158), with the culture supernatant of *C. guilliermondii* P3 and *P. ohmeri* 158 showing higher antagonism, indicating probable production of effective extracellular compounds (data not shown, Coelho, 2005).

In this work, the antagonism of *P. ohmeri* 158 against *P. expansum* was evaluated in broth culture due to the sensibility of the method, with microscopic analysis that allowed the measurement of hyphal length and conidia germination percentage. Testing the incubation-time range from 24 to 120 h of *C. guilliermondii* P3 and *P. ohmeri* 158 in YM broth, both yeasts culture supernatants showed higher inhibitory activity at 48 and 72 h incubation time. *C. guilliermondii* P3 was more effective against conidia germination (inhibition rate of 58.15%) while *P. ohmeri* showed better inhibition on micelial growth (66.17%, Figure 1). The exogenous compound activity associated with antibiosis could probably be related with the killer phenomenon, once both strains were killer positive against *S. cerevisiae* NCYC 1006 (Coelho, 2005). Among the 20 yeasts showing anti-*Penicillium* activity, 9 were identified as killer positive, suggesting involvement of such factor as promising agent in the control of patulin producing *P. expansum*. Walker et al. (1995) reported susceptibility of yeast and filamentous fungi caused by killer positive strains of *S. cerevisiae*, *P. anomala* and *Williopsis mrakii*.

The killer phenotype is based on the secretion of a low molecular mass protein or glycoprotein toxin able to kill sensitive cells belonging to the same or related yeast genera, but without direct cell-cell contact (Bevan and Makower, 1963). A *Pichia* strain (CCA 510) showed the best killer activity against 92% of isolated fermentative yeasts of the process (Ceccato-Antonini et al., 2004). De La Pena et al. (1981) demonstrated that *S. cerevisiae* toxin was a protein which bond to a receptor on cell wall of sensitive yeasts, disrupting the electrochemical gradient across the cell membrane and hence the intracellular ionic balance. The zigocin secreted by *Zygosaccharomyces bailii* was a rapid process mostly related with the disruption of cytoplasmic membrane function *in vivo* (Weiler and Schmitt, 2003). Wicaltin, a chromosomal killer toxin in *Williopsis californica*, as the HK toxin of *Hansenula mrakii* were targeted to β-1,3-D-glucan skeleton in the cell wall (Yamamoto et al., 1986; Theisen et al., 2000).

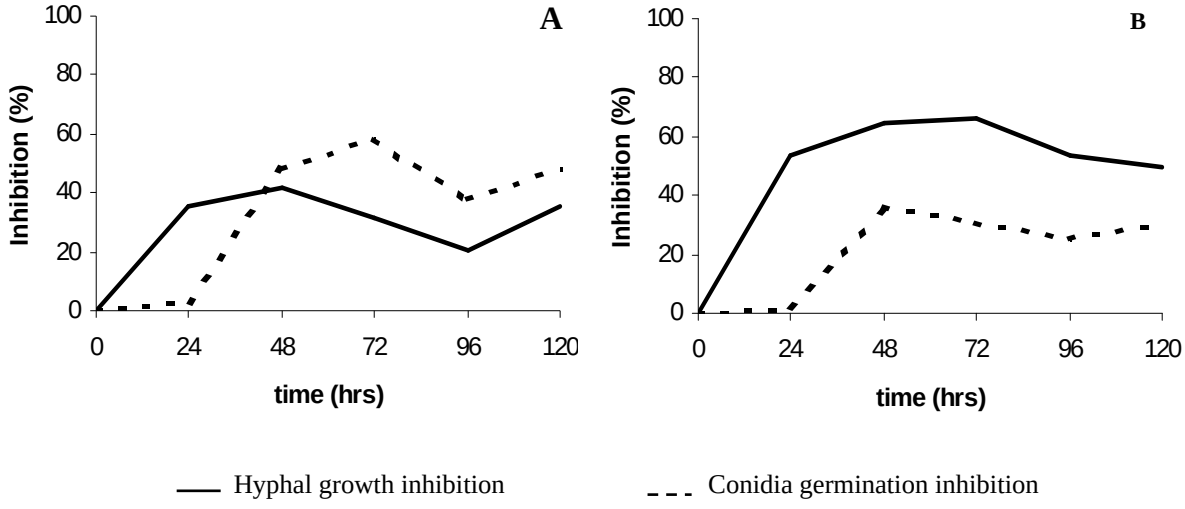

Figure 1 - Inhibitory activity of *C. guilliermondii* P3 (A) and *P. ohmeri* 158 (B) culture supernatant against germination and hyphal growth of *P. expansum* #2 (10^5 spores) after 12 hrs at 25ºC in static condition.

Previous microscopic analysis data showed consistent attachment of *D. hansenii* C7 onto cell wall of *P. expansum* hyphae (Levy, 2003). Attachment of yeast cells on mycelium surface of the pathogen and subsequent changes in hyphae viability suggested the antagonistic activity based on hyperparasitism. Arras et al. (1998) also reported attachment of *P. guilliermondii* onto *P. italicum* mycelium. Wisniewski et al. (1991) reported the attachment of *P. guilliermondii* onto *Botrytis cinerea* mycelium. *C. oleophila* I-182 in Aspire® by Ecogen, Langhorne, PA (Sugar and Spotts, 1999; El Ghaouth et al., 2000) and *Cryptococcus albidus* in Yield Plus® (Ancor Yeast, Cape Town, South Africa), both with antagonistic activiy based on hyperparasitism, which have already been commercialized in the United States (El Ghaouth et al., 2000; Droby et al., 2003).

Considering that such a biological control property would be enhanced if the bioactive yeasts also could degrade the produced mycotoxin, the preliminary study with *P. membranifaciens* and *S. roseus* decreased the initial patulin level from 588.4 to 290.0 μg after incubation at 25ºC/15 days (Levy et al., 2002). Taking this into account, and the best effectivness of *P. ohmeri* 158 against *P. expansum* hyphal growth in the present work, ability of *P. ohmeri* 158 in patulin degradation was performed. Added patulin was decreased over 83% of the original content, when incubated with *P. ohmeri* at 25ºC/48 h in the static condition and completely destroyed after 15 days with undetectable patulin level (Figure 2).

The patulin content in the controls remained between 140 μg/25 mL (or 5.6 μg/mL) and 180 μg/25 mL (or 7.2 μg/mL) along 15 days experiment and the different values observed suggested problems during the mycotoxin extraction procedure (Fig. 2). The detection limit by HPLC-UV detector for the pure standard was 6.7 ng/mL. The limit of the quantification was determined as the smallest quantity of the toxin that allowed confirmation by the spectrum obtained. In this study the quantification limit was 0.03 μg/mL.

The pH values were accomplished in order to determine patulin stability during the assay, as this mycotoxin was stable in solutions at pH ranging from pH 3.0 to 5.5 (Engel and Teuber, 1984), i.e., compatible with pH values (pH 2.9-3.3) in natural apple fruits (Jay et al., 2005). The initial pH of 4.0 decreased to pH 3.3 along 15 days of *P. ohmeri* cultivation in YM broth (Figure 2), suggesting that patulin decrease was an active process and a consequence of yeast metabolism, i.e., this mycotoxin was unstable at alkali solutions (Engel and Teuber, 1984). The patulin decrease using yeasts could be explained by two possible hypotheses. The first one suggested patulin degradation by an enzymic action during the fermentative condition associated with an inducible process. Sumbu et al. (1983) demonstrated that yeast cells preconditioned to grow in the presence of 50 μg/mL of patulin for 3 h and submitted to apple juice spiked with higher patulin concentrations, they became resistant to the doses of > 200 μg/mL, indicating an inducible process. This phenomenon was confirmed when the toxin was added into juice simultaneously with cycloheximide, an antifungal agent which blocked the peptide bond formation, resulting in repression of protein synthesis without toxin decrease (Sumbu et al., 1983). Stinson et al. (1978) reported complete degradation of patulin during fermentation of apple juice to wine by *S. cerevisiae*. Moss and Long (2002) showed decreasing patulin level by *S. cerevisiae* fermentative process, reducing this toxin to (E)-ascladiol, in contrast with lower biodegradation effectiveness in shaken flask culture.

The second hypothesis suggested that the toxin decrease could be associated with the adsorption ability of yeast cell wall components, such as glucan/mannan. For example, Yiannikouris et al. (2004) showed that *S. cerevisiae* strains with high cell wall glucan content exhibited higher affinity rates for zearalenone, when their total cell wall fraction (TCW) and its alkali-insoluble glucan fraction (AIG) were submitted to the toxin. Furthermore, the best adsorption efficacy of AIG was evidenced by the chitin content decreasing compared to the TCW fraction, thus increasing glucan flexibility and toxin accessibility to the glucan network (Yiannikouris et al., 2004).

Although any toxicological or epidemiological data in humans was not reported, the presence of patulin could be indicative of the quality of fruit used in the processed apple products (Moss, 1996; Leggott et al., 2001). Recently, "The Guidance Document of FDA Components and Industry on Apple Juice and Apple Juice Products"

recommended that level of patulin in fruit juice and fruit products should be lower than 50 μg/L (Richard et al., 2003), and the European Commission established 25 μg/kg as maximum level for patulin in solid apple products, including apple compote and puree and 10 μg/kg in apple products intended for the infants and young children (European Commission, 2003; Boonzaaijer et al., 2005). The World Health Organization (WHO) altered the limit of patulin ingestion from 7.0 to 0.4 μg/kg bw/day (Bolger, 2002; Baert et al., 2004).

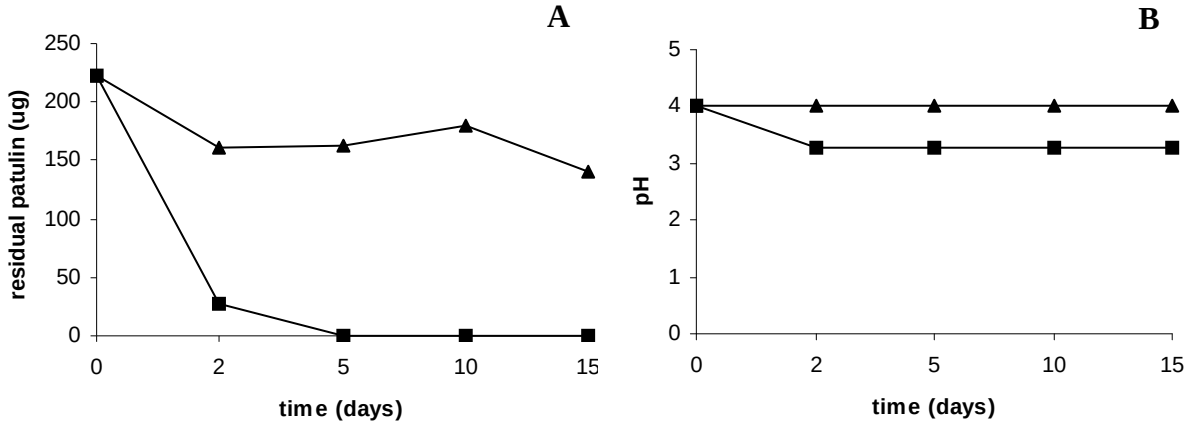

Figure 2 - Decrease of patulin (A) and pH determination (B) along 15 days by *P. ohmeri* 158 cultivation in Yeast Medium. ▲, Yeast Medium added with patulin alone (control); ■, Yeast Medium added with patulin and *P. ohmeri* 158.

Burda (1992) found patulin levels between 51 and 1130 μg/L and Gokmen and Acar (1998) reported the presence of patulin in 215 samples ranging from 7 to 376 μg/L. Concentrations between 15 and 285.3 μg/L of patulin in apple juice produced in Iran were found by Cheraghali et al. (2005). However, this toxin could reach higher levels when juices were made from the visibly rotten apples, ranging from 500 to 2500 μg/L (Steiner et al., 1999), or when fruits artificially contaminated with *P. expansum* NRRL 1172 or toxigenic *P. variabile* were stored at 0 and 4ºC during 90 days, with patulin levels between 1066 and 6400 μg/L (Ross-Urbano et al., 1998).

It should be noted that the patulin level used in the present study (223 μg/25 mL; or 8.92 μg/mL) and the patulin content in the controls along 15 days experiment (140-180 μg/mL; or 5.6-7.2 μg/mL) were higher than that recommended by the European Commission (2003) and those normally found in the apple and apple juices, and consequently not safe for human consumption, indicating that it would be possible to reduce significantly high doses of patulin to an acceptable or undetectable level using yeasts as biodegradation agents, once *P. ohmeri* was commonly used in fermentative process in the food industry (Kurtzman and Fell, 1998). The effective antagonism of *P. ohmeri* versus *P. expansum* hyphal growth showed in this work suggested that the inhibition of fungal development was a promising alternative for the biologial control, and consequently should guarantee a low or undetectable patulin levels. However, further studies would be necessary to determine the likely mode of action of *P. ohmeri* against patulin decrease.

To concludes, the frequent occurrence of *P. expansum* in apple fruits associated with patulin hazard in quality control at the harvesting and storage stages, suggested that the use of non-deleterious antagonist yeasts screened from the natural ecosystem, with additional biodegradation /adsorption properties should be an effective and profitable choice of biological control to avoid the fruit spoilage and mycotoxin production by the post-harvest fungi, to the increased trading of fruits.

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RESUMO

Considerando o antagonismo e degradação de patulina detectados em *Pichia membranifaciens* e *Sporobolomyces roseus* no estudo preliminar, este trabalho avaliou o efeito antagônico de *Pichia ohmeri* 158 no desenvolvimento de *Penicillium expansum* e a degradação de patulina "in vitro". O sobrenadante do cultivo de *P. ohmeri* 158 inibiu 66,17% do desenvolvimento micelial, indicando antibiose relacionada ao fator *killer*. A concentração inicial de patulina (223 μg) na presença de células íntegras de *P. ohmeri* foi reduzida em mais de 83% após dois dias de incubação a 25ºC e superior a 99% após 5 dias, com níveis indetectáveis no 15º dia. O decréscimo do pH 4,0 inicial para pH 3,3 sugeriu que a eliminação de patulina é um processo ativo e uma conseqüência do metabolismo da levedura. Os resultados obtidos concluem que *P. ohmeri* 158 é uma alternativa promissora na inibição do desenvolvimento de *P. expansum* e na degradação de patulina.

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