APPLICATION OF CHITOSAN AND YEAST AS GROWTH INHIBITORS OF PENICILLUM DIGITATUM

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Abstract

The effect of degree of acetylation (DA) and molecular weight (MW) of chitosan on growth of *Penicillum digitatum* alone or in combination with the biocontrol yeast *Pichia guillermondii* was studied. Several chitosan concentrations were added to plates with Nutrient Yeast Dextrose Agar (NYDA) for determination of minimum inhibitory concentration (MIC) of *P. digitatum and P. guillermondii*. Radial growth (RG) as percentage inhibition (% RI), germination rates (k), percentage of spore germination (% SG) and percentage of germination inhibition (% GI) were also determined. Chitosans were obtained using chitin purified by lactic acid fermentation, Chitosan was prepared by homogenous deacetylacion with alkali (65%) at room temperature 25ºC, HDRT, by Freeze-Pump out- Thaw cycles, FPT and commercial chitosans (Kitomer and Fluka). The MIC showed that the FPT and HDRT chitosans allowed yeast growth thus in competition with the *P. digitatum* growth. The radial growths of fungi in media supplemented with chitosans (FPT and HDRT) were 100% inhibited at 2.5g/L for the first 48h. After 120h the inhibition decreased until 15% and 53%, respectively, yeast inoculation increased the %RI until 40% for 100 kDa and 10% for 400 kDa at 1.2g/L of chitosan. All treatments reduced the k values. The maximum germination percentages of spores (SG_{max}) decreased for both chitosans either in the presence or in absence of yeast, chitosan alone showed a better antimicrobial effect during spore germination (% SG), whereas low MW chitosan allowed the use of yeast as a biocontrol agent since this was more effective in the apical growth of fungi.

Introduction

Chitosan, a deacetylated form of chitin, has been reported as potential food preservative. It was suggested that the antifungal activities are due to the amino groups that give a polycationic character in the polysaccharide chain.[1] The use of chitosan in food applications has been encouraged for its biocompatibility, no toxicity, and biodegradability. The variations in preparation methods of chitosan is an important factor to be considered for its application, since it produces differences in the degree of deacetylation, as well as the distribution of acetyl groups, chain length and conformational structure. Those characteristics have an influence on the antimicrobial activity and other properties[2,3].

Fungal diseases are the most common causes of commercial loses in citrus fruits, such as those provoked by *Penicillum digitatum*, named green mould. These post-harvest diseases are often controlled by the application of chemical fungicides,[4,5] however, there are several problems

associated with the use of such chemicals as the fungicide toxicity, the development of pathogens resistance, as well as the risk to human health and environment pollution. The biological control as well as the application of natural antimicrobial compounds, i.e. chitosan, has been proposed as effective alternatives to reduce the use of chemical fungicides.[6]

Recently, yeast has been used as antagonist of post-harvest pathogens on a variety of vegetables and fruits. The mechanisms of yeast against microbial phytopathogens are the capability of rapidly growth on organic surfaces and in wounds, and the colonization and competition against the pathogens for nutrients and space.[4,7] *Candida guillermondii* and its anamorphous *Pichia guillermondii* are natural antagonists of phytopathogen fungi, these yeasts have been targeted as potential antagonists against *P. digitatum*.[4,8,9]

There are very scarce reports on the evaluation of combined applications of natural antimicrobial compounds and microbial antagonist to control the fungal decay caused by *Penicillium digitatum*. The aim of this work was to study the effect of degree of acetylation (DA) and molecular weight (MW) of chitosans on the growth of *Penicillum digitatum* alone or in combination with the biocontrol yeast *Pichia guillermondii*.

Material and Methods

Materials

Chitin was purified by a modified process reported by Cira *et al*[10], which lactic acid fermentations were carried out at 25ºC and 35 °C temperatures (Table 1). These chitins were used for the preparation of chitosans by homogenous deacetylation with alkali (65% w/v) at 25ºC (HDRT)[11] (LB-I) and by Freeze-Pump out-Thaw cycles with alkali (50% w/v) at 100°C (FPT)[12] (LB-II). Commercial chitosans, Fluka and Kitomer, were also utilized in this study. Commercial chitosan (Kitomer) was treated with FTP three deacetylation cycles (LMPB). Chitosans were characterized for determination of DA by nuclear magnetic resonance spectroscopy (H^1NMR) and Molecular Weight (MW) by viscosity and gel permeation chromatography (GPC).

Table 1. Chitins obtained by biological method used for chitosan preparation and their molecular weights.

Chitins	Temperature during lactic acid fermentation (°C)	kDa	Deacetylation processes	Chitosans code
Chitin-I	25	360	HRDT	LB-I
Chitin-II	35	930	FPT	LB-II

Microorganisms

Pichia guillermondii was isolated from the surface of Valencia orange *(Citrus sinensis*) and Italian lemon (*Citrus limon var. Eureka*) of Nuevo Padilla Tamaulipas. The microorganisms were cultivated in dextrose Sabouraud broth at 30° C. The inoculums for the determination of minimum inhibitory concentration (MIC) were established at concentrations of 10^8 cells/mL and 10^3 cells/mL for interactions with *P. digitatum*. The mould was cultivated in potato dextrose agar (PDA) at 30° C for 5 days. The spores were harvested in a sterile solution of 0.1 vol % Tween 80 by magnetic stirring. Spore suspension was counted in a Neubauer chamber until concentrations of 10^6 spores/mL for MIC and 10^3 spores /mL for determinations of percentage of spore germination (% SG) and radial growth (RG).

MIC, RG and SG assays

Several chitosan concentrations (0.625, 1.2, 2,5 and 3 g/L) were added to Nutrient Yeast Dextrose Agar (NYDA) in plates for determination of MIC of *P. digitatum* and *P. guillermondii*. RG was measured daily in agar plates, in presence and in absence of yeast. The results were compared with the control and expressed as a radial growth percentage inhibition (% RI). Germination rates (k) were also determined in liquid culture media Nutrient Yeast Dextrose (NYD) with or without

suspension of yeast at 30° C and 48h. Percentage of germination inhibition (% GI) and germination spores percentage (SG %) were determined every hour and adjusted to the logistic model (equation 1).

$$SG = SG_{max}/[1+((SG_{max}-S_0)/S_0)exp^{-kt}] \quad (1)$$

where:

S_0 is the initial percentage of germination spores, k is spore germination rate, and SG_{max} maximal percentage of germination spores.

Results and discussions

Chitosans preparation and characterization

The M_W of the chitins employed were an important factor for further preparation of chitosans. Therefore the characterization of chitosans showed that the highest DA (40%) and M_W of 400kDa for the chitosan LB-II was obtained by FPT method using Chitin II (M_W 930 kDa). Whereas LB-I chitosan obtained by HDRT method showed a DA of 33.75% and the lowest M_W (100kDa) starting from Chitin-I with M_W of 360kDa (Table 1). The DA decreased until 0.68% using a commercial chitosan (Kitomer) with 10% DA after treatment of three FPT deacetylations. The low DA obtained did not present any remarkable decrease in M_W (319 kDa) (Table 2).[12]

On the other hand the yeast was more sensitive to the biopolymer than the mould. Low DA significantly increased the yeast inhibition hence the MIC decreased. This may be due to the amino groups that interact with cells explaining the increment of the antimicrobial activity of the biopolymer.[3] The M_W chitosans with 400 and 550 kDa showed higher MIC (3.5g/L) of mould than those with relatively low M_W (100 and 300kDa). Based on the antimicrobial activity, chitosans of 100kDa (33.75 %DA) and 400 kDa (40.7 %DA) were the most suitable to interact with the mould. The determination of fungal radial growth was carried out selecting chitosans that allowed the growth of yeast (Table 2).

Table 2. Degree of acetylation (DA) and molecular weight (M_W) of chitosans and their minimum inhibitory concentration (MICs) determined for yeast and mould at 48 h 30ºC in nutrient yeast dextrose agar.

Sample	(%) DA	kDa	MIC for yeast (g/L)	MIC for mould (g/L)
LMPB[a]	0.68	319	> 0.625	> 2.5
Commercial[1]	< 10*	550	> 0.625	> 3.5
Commercial[2]	< 10*	400	> 1.25	> 3.5
LB-I	33.75	100	> 2.5	> 2.5
LB-II	40.7	400	> 3	> 3.5

[a]FPT and three cycles of deacetylation, [1]Fluka Biochemika high molecular weight, [2]Fluka Biochemika medium molecular weight. *values reported by the manufacturer

Determination of Radial growth and %RI of Penicillum digitatum

Radial Growth was 100% inhibited for all concentrations of LB-I chitosan during the first 48h with or without yeast. The fungi were less sensitive with LB-II chitosan due that required higher concentrations than 2.5g/L. The retardation of lag phase can be explained to the higher fungistatic activity of chitosan during the spore germination.[1] The addition of yeast showed the highest %RI at 120h. The LB-I chitosan displayed lower %RI than LB-II chitosan, whereas the addition of yeast increased the % RI with LB-I chitosan. This maybe due to the competition between yeast and the mould for nutrients [4,5,7] and besides the retard of spore germination by chitosan. For the LB-II chitosan, the addition of yeast does not affect the inhibition because the biopolymer has an

antimicrobial effect against the yeast and therefore it prevents its action as an antagonist, (Table 3) (Figure 1).

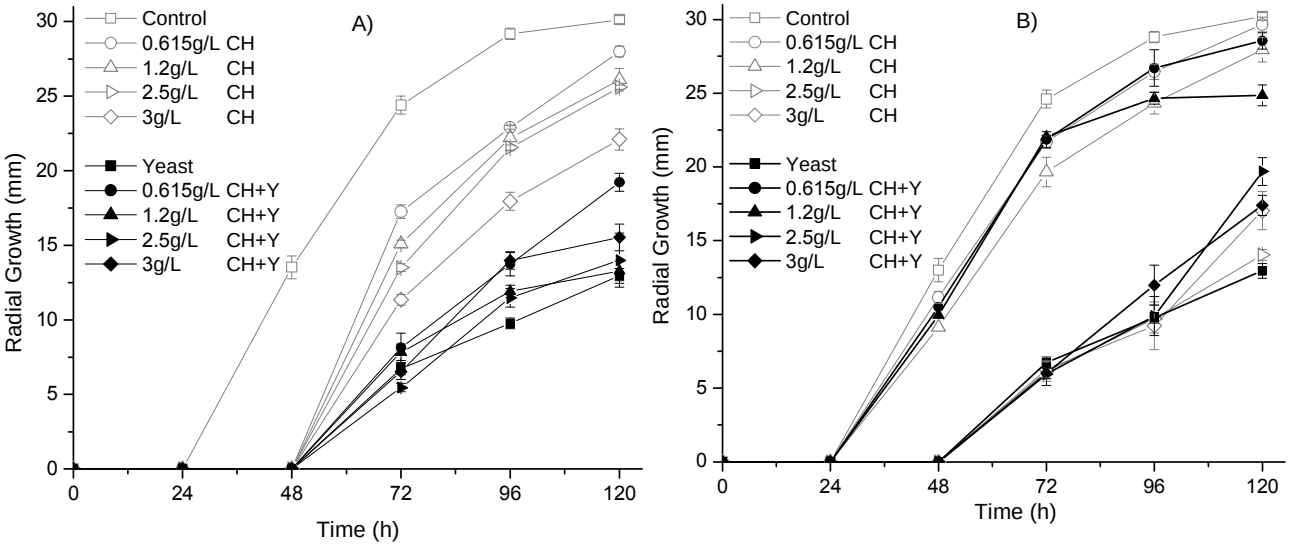

Figure 1. The effect of different concentrations of chitosans and *Pichia guillermondii* on RG of *Penicillium digitatum* : A) LB-I (100kDa) and B)LB-II (400kDa)

Table 3. %RI of *P. digitatum* at different concentrations of chitosans and *P. guillermondii* at 48 and 120h of incubation.

LB-I Chitosan (100 kDa)					LB-II Chitosan (400 kDa)			
Without Yeast			With Yeast		Without Yeast		With Yeast	
Chitosan g/L	%RI		%RI		%RI		%RI	
	48 h	120 h	48 h	120 h	48 h	120 h	48 h	120 h
0	-	-	100	**57.02**	-	-	100	**57.02**
0.615	100	7.05	100	36.16	14	1.82	22	5.23
1.2	100	13.36	100	55.93	30	7.45	26	17.51
2.5	100	15.02	100	53.53	100	53.56	100	34.65
3	100	26.64	100	48.46	100	43.63	100	42.28

Inhibition of spores germination of Penicillum digitatum

The presence of yeast in media without chitosan decreased the germination rate of *P. digitatum* from 0.372 to 0.224 1/h, and both chitosans in presence or absence of yeast also diminished the germination rates. The lowest spore germination rates were estimated for 1.2 and 2.5 g/L of LB-II chitosans without yeast with values of 0.153 and 0.156 1/h, respectively. Although the presence of yeast mixed with chitosan in the media increased the germination rates, the maximum germination SG_{max} did not change significantly, for instance, at concentration of 2.5 g/L without yeast it was 44% GS, with yeast it was determined as 42 %GS. In the case of LB-I chitosan, the concentration that reduced the SG_{max} was 2.5g/L of chitosan without yeast (57%).

It is remarkable that chitosan presence not only reduces the germination rates but also decreases the SG_{max} at concentrations of 2.5 g/L. It has been reported that the chitosan was more available in the liquid media so the interactions between the charges of NH_2 groups with the negatives charges in the cell membrane altered cell permeability, playing an important role to delay spore germination.[13] LB-II chitosan at concentration of 2.5g/L produced more than 95 % GI at 12h for all treatments, 82 %GI at 24 h and 59.5%GI at 48h. The presence of yeast did not change those values. Results indicate that chitosan has a strongly effect during the spore germination stage as it was reported for *Aspergillus niger*[1] (Figure 2).

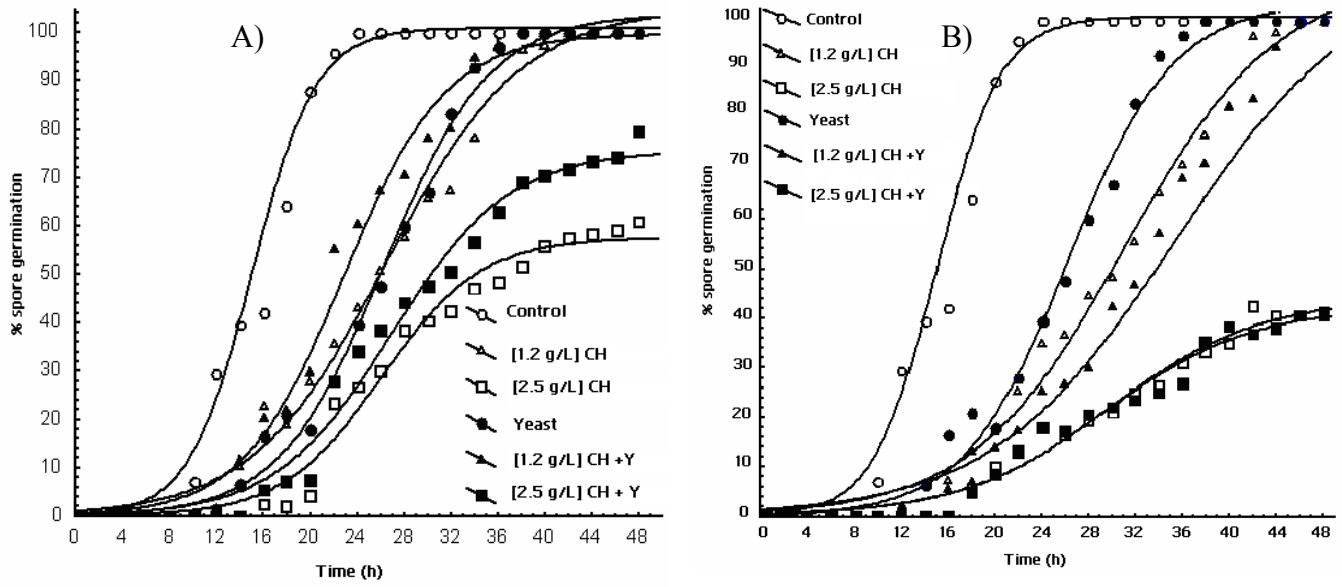

Figure 2. Spore germination of *P. digitatum* in media with *P. guillermondii* at 1.2 and 2.5 g/L and of A) LB-I, B) LB-II Chitosans

SEM microscopy showed the growth of yeast on the mycelium with LB-I chitosan at concentration of 1.2 g/L, as well as the production of polysaccharide excreted by the mould due to the chitosan cleavage (Figure 3).

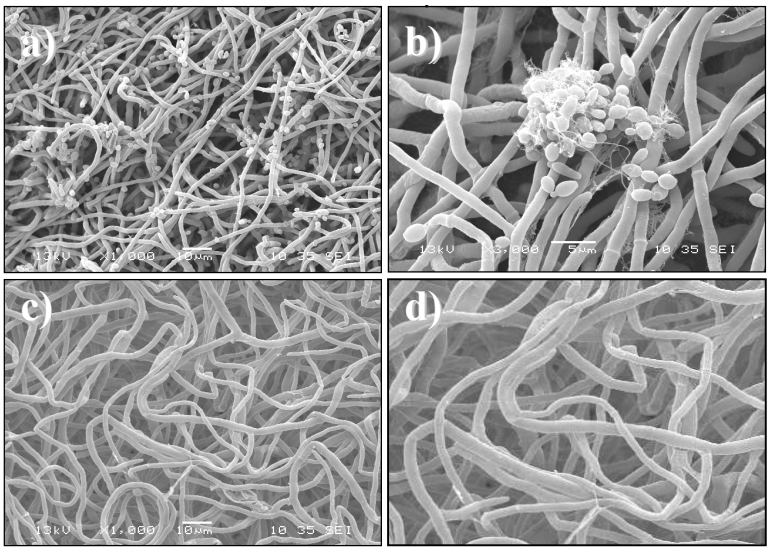

Figure 3. Scanning electron micrographs of P. digitatum in 1.2 g/L of chitosan medium and *P. guillermondii* at 96h, a) 1,000x y b) 3,000x and control media (non added yeast): c) 1,000x y d) 3,000x.

The results showed that the different methods of chitosan preparation affect the DA and M_W thereby the antimicrobial activities of the biopolymers. The yeast was more suppressed with chitosans with low DA. The chitosans of low and medium M_W presented the main effect during the spore germination stage at 2.5g/L. The use of yeast in combination with relatively low M_W chitosan (100 kDa) improved the fungal growth inhibition therefore this chitosan can be formulated with yeast as biocontrol agents.

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