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# Effect of chitin on the antagonistic activity of *Cryptococcus laurentii* against *Penicillium expansum* in pear fruit

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**Abstract**

This study was designed to evaluate the impact of chitin on the antagonistic activity of *Cryptococcus laurentii* against the postharvest blue mold rot caused by *Penicillium expansum* in pear fruit. The results showed that the antagonistic activity of *C. laurentii* obtained from the culture media of nutrient yeast dextrose broth (NYDB) amended with chitin at 0.5–1.0% was improved greatly compared with the case that without chitin. The addition of chitin to NYDB did not influence the growth of *C. laurentii,* however, its population was found to increase rapidly thereafter in pear fruit wounds compared to that harvested from NYDB without chitin. Moreover, the cell-free filtrate of the chitin-supplement culture media in which the yeast was incubated for 24 h emerged a direct antifungal activity against *P. expansum* in pear fruit wounds, with the associated high level of chitinase activity. These results suggested that the use of chitin may be an effective method to induce the antagonistic activity of *C. laurentii*. To our knowledge, this is the first report regarding the chitin could enhance the efficacy of postharvest biocontrol yeasts.


*Keywords:* Biocontrol; Blue mold; Chitin; *Cryptococcus laurentii*; Pear; *Penicillium expansum*; Postharvest

## 1. Introduction

The biological control of postharvest diseases by using antagonistic yeasts is one of the most promising non-fungicidal means (Janisiewicz and Korsten, 2002). Recently two commercial products, Aspire (based on *Candida oleophila*) and Yield Plus (based on *Cryptococcus albidus*) have been registered in the United States or South Africa (Janisiewicz and Korsten, 2002; Fravel, 2005). Strains of *Cryptococcus laurentii* are also widely known postharvest biocontrol yeasts, which have been shown with high antagonistic activity in pear fruit (Sugar and Spotts, 1999; Zhang et al., 2005) and other fruit (Zhang et al., 2007)). However, like other non-fungicides means, presently all the biocontrol yeasts can not reduce postharvest diseases as effective as synthetic fungicides (Janisiewicz and Korsten, 2002; Droby et al., 2003).

Several strategies have been proposed to enhance the efficacy of postharvest biocontrol yeasts. Besides the substantial studied integration strategy (Janisiewicz and Korsten, 2002; Droby et al., 2003; Droby, 2006), the physiological manipulation may also be a useful method (Janisiewicz and Korsten, 2002). However, the physiology and genetic characteristics of most postharvest biocontrol yeasts are poorly known and the antagonistic mechanism by which the yeast antagonists inhibit the fungal diseases is not fully understood yet (Janisiewicz and Korsten, 2002). Therefore, there are limited documents available evaluating potential for enhancement of efficacy of biocontrol yeasts through the physiological manipulation. For instance, Teixidó et al. (1998) found that the activity of *Candida sake* could be enhanced by cultivation of the yeast in a low water activity modified liquid media. Recently, Li and Tian (2006) reported that the efficacy of *C. laurentii* is increased by accumulation of a high level of internal trehalose in the yeast.

Chitin is the second most abundant biopolymer on Earth (about $1 \times 10^{13}$ kg), which has omnifarious application potential in agriculture-food industry (Synowiecki and Al-Khateeb, 2003;

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Prashanth and Tharanathan, 2007), such as a biopesticide which has been approved by U.S. Environmental Protection Agency (EPA) and as a food additive which has been approved by U.S. Food and Drug Administration (FDA). Recently, Vivekananthan et al. (2004a,b) reported that the antagonistic efficacy of biocontrol bacteria *Pseudomonas fluorescens* and *Bacillus subtilis* increased after the cultivation in a growth media added with chitin. However, the results from these authors also showed there was no beneficial effect on reduction of diseases when the yeast *Saccharomyces cerevisiae* was grown in a media plus chitin (Vivekananthan et al., 2004a,b).

The objective of this study was to determine the influence of chitin on the efficacy of the biocontrol yeast *C. laurentii* in controlling the blue mold rot in pear fruit and to evaluate the effect of chitin on the population growth of *C. laurentii* and the chitinase activity excreted by *C. laurentii*.

## 2. Materials and methods

### 2.1. Fruit material

Pear fruit (*Pyrus pyrifolia* Nakai.) cultivar "Shuijing" were harvested at commercial maturity and selected for uniformity of size and ripeness and removed any fruit with apparent injuries or infections. Fruit were surface-disinfected with sodium hypochlorite at 0.1% (vol/vol) for 1 min, rinsed with tap water and allowed to air dry at the room temperature (20 °C).

### 2.2. Antagonist

The yeast antagonist *C. laurentii* (Kufferath) Skinner was originally isolated from the surfaces of pear fruit and identified as described previously (Yu et al., 2007). The yeast was streaked on nutrient yeast dextrose agar (NYDA) media (containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar in 1 l of distilled water) and incubated at 28 °C for 48 h. The yeast cells were cultivated in nutrient yeast dextrose broth (NYDB) or NYDB amended with chitin powder (NYDB + chitin) on a gyratory shaker at 200 rpm at 28 °C for 24 h. Then the yeast cells were harvested by centrifuging at 7000 ×*g* for 10 min and were washed twice with sterile distilled water to remove the growth media. The yeast cell was counted using a hemocytometer. The growth media was filtered through a Millipore membrane (0.45 μm) and the supernatant was used for biocontrol and chitinase activity assay.

### 2.3. Fungal pathogen

The pathogen *P. expansum* was originally isolated from a pear fruit showing the typical blue rots and its hyphal growth and morphology was examined with an Olympus BH2 microscope. The fungus was cultured on the potato-dextrose agar (PDA) media (containing the extract from 200 g boiled potato, 20 g glucose and 20 g agar in 1 l of distilled water) at 25 °C in the dark. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of *P. expansum* with sterile distilled water. The spore concentration was counted using a hemocytometer.

### 2.4. Biocontrol of blue mold rot in pear wounds

The surface of pears were wounded with a sterile cork borer (approximately 5 mm-diameter and 3 mm deep) and treated with 30 μl (1) *C. laurentii* cells which were harvested either from the media of NYDB or NYDB + chitin in which the yeast was incubated for 24 h at $10^6$, $10^7$ and $10^8$ cells/ml; (2) a solution of cell-free filtered supernatant (through a Millipore membrane (0.45 μm)) from the media of NYDB or NYDB + chitin in which the yeast was incubated for 24 h. Two hours later, 30 μl of *P. expansum* suspensions ($1 \times 10^4$ spores/ml) were inoculated onto each wound. The treatment with sterile distilled water then inoculated with the pathogens was used as the positive control. In each experiment, wounded fruit treated with sterile distilled water, any suspensions and the supernatant but without pathogen inoculums was used as the negative controls. Because none of the negative controls exhibited disease symptoms, they were not mentioned in the results.

After air-drying, the samples were stored in trays covered with plastic film to maintain a high relative humidity at 25 °C. The number of the infected fruit and their lesion diameters were examined daily. There were four replicates per treatment and 24 fruit each replicate. All treatments were arranged in a randomized complete block design and conducted at least twice. The data are from one individual experiment and are representative of two independent experiments with similar results.

### 2.5. Population growth of antagonist in wounds

The fruit samples were wounded as described above. Then, the wounds were treated with 30 μl of *C. laurentii* cells which were harvested either from NYDB or NYDB + chitin at $1 \times 10^7$ cells/ml. The samples were taken at different times at 25 °C after the treatment. The tissue was removed with a sterile cork borer (1 cm diameter by 1 cm deep) and ground with a mortar and pestle in 10 ml of sterile water. The yeast cells were counted using a haemacytometer. There were three replicates per treatment and 6 fruit per replicate. All treatments were arranged in a randomized complete block design and conducted at least twice. The data are from one individual experiment and are representative of two independent experiments with similar results.

### 2.6. Assay for chitinase activity

Aliquots of 10 ml of growth media were centrifuged at 3000 rpm for 10 min to remove yeast cells, filtered through a Millipore membrane (0.45 μm) and the supernatant was used for assay of the enzymes activity. The reaction mixture was made up by adding 1 ml of a solution of colloid chitin (0.4%, wt/vol), 1 ml of 50 mmol/l acetate buffer (Ph 5.0), and 1 ml of the yeast culture filtrate. The mixture was incubated for 1 h at 50 °C. Non-degraded substrate was removed by centrifugation at 3000 rpm for 5 min and the amount of degraded substrate was measured in the supernatant at 550 nm. One enzyme unit (U) of the chitinase activity is defined as the amount of the increase of

$A_{550}$ by 0.01 in 1 h and expressed as U per ml (culture filtrate). For each treatment, there were three replicates and the data are from one individual experiment and are representative of two independent experiments with similar results. All treatments were arranged in a randomized complete block design and conducted at least twice. The data are from one individual experiment and are representative of two independent experiments with similar results.

### 2.7. Statistical analyses

The data were analyzed on analysis of the variance (ANOVA) in the Statistical Program (SPSS/PC ver. II.x, SPSS Inc. Chicago, Illinois, USA) and the Duncan's multiple range test was applied for the means separation. The data are from one individual experiment and are representative of two independent experiments.

## 3. Results

### 3.1. Biocontrol of blue mold rot

Application of *C. laurentii* at $1\times10^8$ cells/ml reduced the incidence of blue mold rot in pear fruit by approximately 60%, compared with the water control after 6 days of incubation at 25 °C (Fig. 1, $P<0.05$). The antagonistic activity of *C. laurentii* was shown to be greatly enhanced through cultivation in the NYDB media amended with chitin, especially at the optimal concentration (1.0%), compared with that cultivated in NYDB without chitin (Figs. 1 and 2, $P<0.05$). However, there was no difference of the biocontrol efficacy of the yeast cultivated in NYDB and in NYDB containing chitin at 2.0% ($P>0.05$).

The cell-free culture filtrates of chitin-supplement media (0.5% and 1.0%) in which *C. laurentii* was incubated for 24 h emerged a strong inhibition of the lesion diameter of blue mold

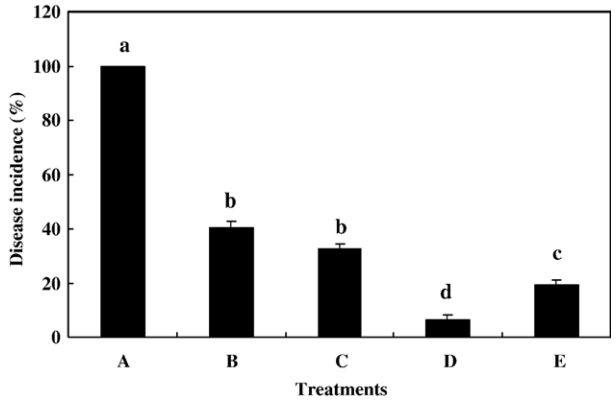


Fig. 1. Efficacy of *Cryptococcus laurentii* ($1\times10^8$ cells/ml) in inhibiting the disease incidence of blue mold caused by *Penicillium expansum* in pear fruit wounds after 6 days of incubation at 25 °C after incubation in nutrient yeast dextrose broth (NYDB) **(B)**, NYDB amended with chitin at 2.0% **(C)**, 1.0% **(D)**, 0.5% **(E)**. The treatment with water and inoculated with *P. expansum* was served as the positive control **(A)**. Bars represent standard errors. Different letter indicates significant differences ($P=0.01$) according to the Duncan's multiple range test.

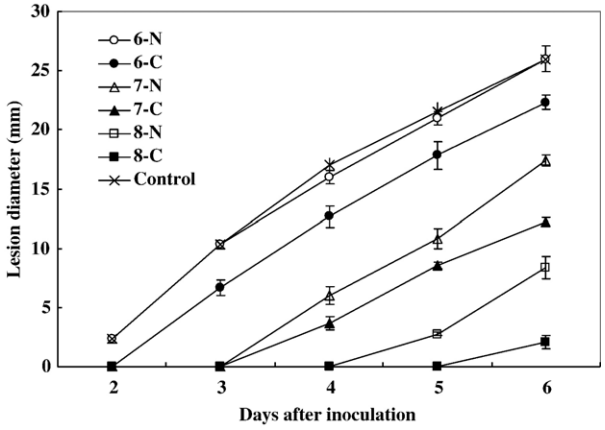


Fig. 2. Efficacy of *Cryptococcus laurentii* in inhibiting the lesion diameter of blue mold caused by *Penicillium expansum* in pear fruit wounds at 25 °C. The treatment with water and inoculated with *P. expansum* was served as the control. Bars represent standard errors. (○) *C. laurentii* grown in NYDB at $1\times10^6$ cells/ml (●) *C. laurentii* grown in NYDB with chitin at 1.0% at $1\times10^6$ cells/ml (△) *C. laurentii* grown in NYDB at $1\times10^7$ cells/ml (▲) *C. laurentii* grown in NYDB with chitin at 1.0% at $1\times10^7$ cells/ml (□) *C. laurentii* grown in NYDB at $1\times10^8$ cells/ml (■)*C. laurentii* grown in NYDB with chitin at 1.0% at $1\times10^8$ cells/ml.

in pear fruit wounds after 4, 5 and 7 days of incubation at 25 °C compared to the case that incubated in NYDB without chitin (Fig. 3, $P<0.05$). However, there was not any inhibition of the blue mold rot in wounds by use of either a solution of chitin at various concentrations or a solution of NYDB or their combination compared with the water control (data not shown).

### 3.2. Population growth of antagonist in pear wounds

The yeast *C. laurentii* proliferated rapidly in pear wounds, especially during the first 48 hand stabilized relatively thereafter (Fig. 4). Addition of chitin to NYDB did not influence the population of *C. laurentii* in liquid medium (data not shown). However, after chitin treatment, the growth of yeast in pear fruit

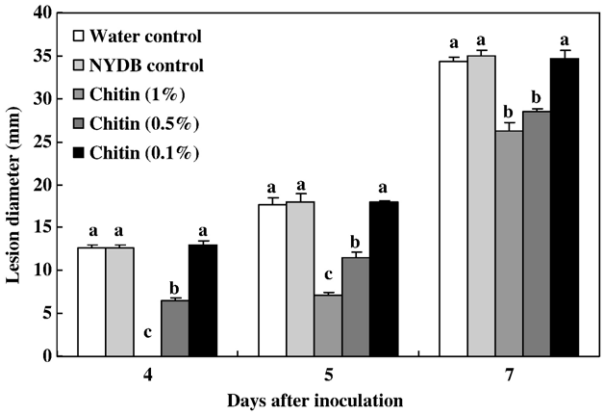


Fig. 3. Effect of cell-free culture filtrate of *Cryptococcus laurentii* on reduction of the lesion diameter of blue mold caused by *Penicillium expansum* in pear fruit wounds at 25 °C. Each value is the mean of three separate determinations. Bars represent standard errors. Different letter indicates significant differences ($P=0.05$) according to the Duncan's multiple range test and the data from each time point are separated.

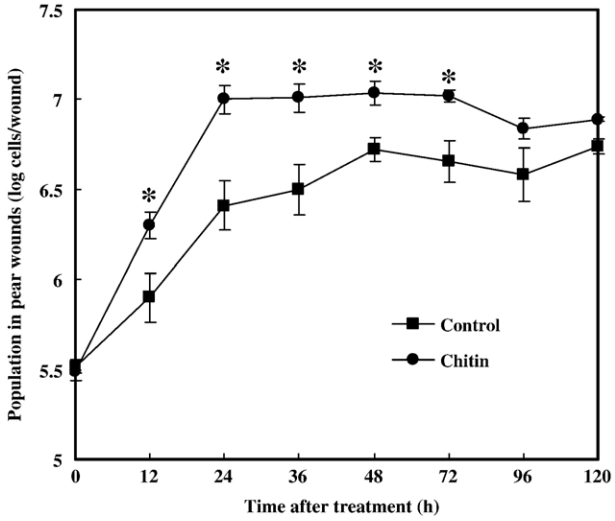


Fig. 4. Population of *Cryptococcus laurentii* after cultivation in nutrient yeast dextrose broth (NYDB) or NYDB amended with chitin at 1.0% in pear fruit wounds at 25 °C. Bars represent standard errors. An asterisk (*) indicates significant differences ($P=0.05$) according to the Duncan's multiple range test.

wounds increased greatly during 72 h of incubation compared with the nonchitin treatment control ($P<0.05$). After 24 h of incubation in the wounds, the amount of the yeast grown in NYDB plus chitin was more than three fold higher than that grown in NYDB without chitin ($P<0.05$).

### 3.3. Chitinase activity

After 24 h of incubation in culture media amended with chitin at 0.5–2%, the chitinase activity of the cell-free culture filtrates increased compared with that grown in NYDB, especially at the optimal chitin concentration, 1% (Fig. 5). There was no difference of the chitinase activity between the cell-free culture filtrates of NYDB and NYDB plus chitin at 0.1%.

## 4. Discussion

The results from this study showed that the biological activity of *C. laurentii* against the blue mold rot on pear fruit was greatly enhanced when the yeast was harvested from NYDB with chitin, especially at the optimal concentration 1%, compared with the case that *C. laurentii* grown in NYDB without chitin. Since that chitin has no risks to humans and to the environment because it has been approved as a biopesticide by EPA and a food additive by FDA, and that chitin is a cheap and widely available natural resource, the utilization of chitin might be an effective, safe and economic approach to enhance the biocontrol efficacy of *C. laurentii*.

The results from the present study also showed that *C. laurentii* obtained from the chitin-supplement media proliferated more rapidly in pear fruit wounds than that grown in NYDB without chitin (Fig. 4). Previous studies have shown that chitin can increase the population of several biocontrol bacteria, which was closely associated with the high level of decay control (Kokalis-Burelle et al., 1992; Bell et al., 1998). Considering that the competition for nutrients and space is proposed to be the principal modes of action of *C. laurentii* in antagonism with pathogens (Roberts, 1990; Filonow, 1998; Janisiewicz and Korsten, 2002) and that the results from this study that the efficacy of *C. laurentii* in reducing the mold infections was depended on the yeast inoculum density: the higher the inoculum concentration, the more inhibition of the blue mold was obtained (Fig. 2), we assume that the enhanced population of the yeast antagonist might be a major reasons leading to the increased biocontrol efficacy against the blue mold rot in pear fruit. However, the mechanism for the enhancement of the yeast growth in fruit wounds after chitin treatment is not clear, which deserves to be elucidated further.

On the other hand, our results showed that the cell-free filtrate of the chitin-supplement culture media of *C. laurentii* exhibited a direct antifungal activity against *P. expansum* in pear fruit wounds. Previously, McCormack et al. (1994) found that some strains of *C. laurentii* could produce antibacterial compound, however, it is not clear whether this strain of *C. laurentii* can produce antifungal compounds. While it is known that some strains of *C. laurentii* were found to excrete chitinase although the relationship between the chitinase and biocontrol had not been evaluated (Buzzini and Martini, 2002). And the chitinase activity has been reported in other postharvest biocontrol yeasts such as *Candida guilliermondii*, *Candida oleophila*, *Cryptococcus albidus* and *Pichia membranefaciens* (Fan et al., 2002; Saligkarias et al., 2002; Bar-Shimon et al., 2004; Chan and Tian, 2005). The chitinase activity is usually induced in the presence of the chitin, which may have diverse biological roles including the antifungal activity (Dahiya et al., 2006; Gohel et al., 2006; Li, 2006). Specifically for the yeast chitinase, Carstens et al. (2003a,b) demonstrated that the *S. cerevisiae* chitinase could inhibit spore germination and hyphal growth of *Botrytis cinerea in vitro* and constitutive overexpression of this chitinase in tobacco plants resulted in increased disease tolerance with the associated higher chitinase activities. Thus, we proposed that the enhanced chitinase

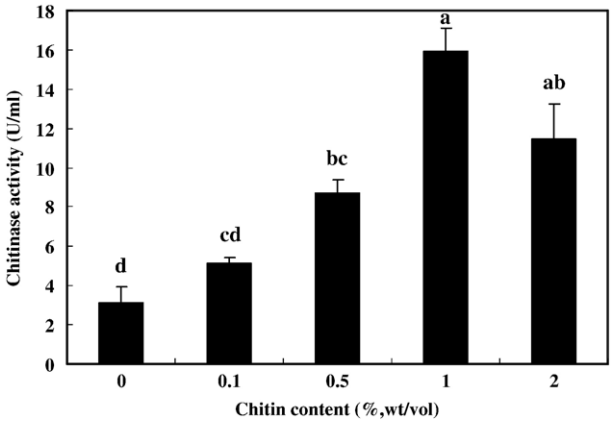


Fig. 5. Chitinase activity of *Cryptococcus laurentii* after cultivation in nutrient yeast dextrose broth (NYDB) or NYDB amended with chitin at 0, 0.1, 0.5, 1.0 and 2.0%. Bars represent standard errors. Different letter indicates significant differences ($P=0.05$) according to the Duncan's multiple range test.

activity after chitin treatment as shown in this study (Fig. 5) might be another reason for the enhanced antagonistic activity of *C. laurentii* in addition to the increased population levels of *C. laurentii* by the use of chitin.

In conclusion, our results showed that the antagonistic activity of *C. laurentii* could be enhanced by chitin treatment, which may offer great practical potential in reducing the postharvest diseases of pear fruit. To our knowledge, this is the first report regarding the chitin could enhance the efficacy of postharvest biocontrol yeasts.

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## References

Bar-Shimon, M., Yehuda, H., Cohen, L., Weiss, B., Kobeshnikov, A., Daus, A., Goldway, M., Wisniewski, M., Droby, S., 2004. Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*. Current Genetics 45, 140–148.

Bell, A.A., Hubbard, J.C., Liu, L., Davis, R.M., Subbarao, K.V., 1998. Effects of chitin and chitosan on the incidence and severity of *Fusarium* yellows in celery. Plant Disease 82, 322–328.

Buzzini, P., Martini, A., 2002. Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. Journal of Applied Microbiology 93, 1020–1025.

Carstens, M., Vivier, M.A., Van Rensburg, P., Pretorius, I.S., 2003a. Overexpression, secretion and antifungal activity of the *Saccharomyces cerevisiae* chitinase. Annals of Microbiology 53, 15–28.

Carstens, M., Vivier, M.A., Pretorius, I.S., 2003b. The *Saccharomyces cerevisiae* chitinase, encoded by the CTS1-2 gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. Transgenic Research 12, 497–508.

Chan, Z.L., Tian, S.P., 2005. Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. Postharvest Biology and Technology 36, 215–223.

Dahiya, N., Tewari, R., Hoondal, G.S., 2006. Biotechnological aspects of chitinolytic enzymes: a review. Applied Microbiology and Biotechnology 71, 773–782.

Droby, S., 2006. Improving quality and safety of fresh fruits and vegetables after harvest by the use of biocontrol agents and natural materials. Acta Horticulturae 709, 45–51.

Droby, S., Wsiniewski, M., Ei-Ghaouth, A., Wilson, C., 2003. Biological control of postharvest diseases of fruit and vegetables: current achievements and future challenges. Acta Horticulturae 628, 703–713.

Fan, Q., Tian, S.P., Liu, H.B., Xu, Y., 2002. Production of beta-1, 3-glucanase and chitinase of two biocontrol agents and their possible modes of action. Chinese Science Bulletin 47, 292–296.

Fravel, D.R., 2005. Commercialization and implementation of biocontrol. Annual Review of Plant Biology 43, 337–359.

Filonow, A.B., 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. Biocontrol Science and Technology 8, 243–256.

Gohel, V., Singh, A., Vimal, M., Ashwini, P., Chhatpar, H.S., 2006. Bioprospecting and antifungal potential of chitinolytic microorganisms. African Journal of Biotechnology 5, 54–72.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40, 411–441.

Kokalis-Burelle, N., Backman, P.A., Rodriguez-Kabana, R., Ploper, L.O., 1992. Potential for biological control of early leaf spot of peanut using *Bacillus cereus* and chitin foliar amendments. Biological Control 2, 321–328.

Li, D., 2006. Review of fungal chitinases. Mycopathologia 161, 345–360.

Li, B.Q., Tian, S.P., 2006. Effects of trehalose on stress tolerance and biocontrol efficacy of *Cryptococcus laurentii*. Journal of Applied Microbiology 100, 854–861.

McCormack, P.J., Wildman, H.G., Jeffries, P., 1994. Production of antibacterial compounds by phylloplane-inhabiting yeasts and yeastlike fungi. Applied and Environmental Microbiology 60, 927–931.

Prashanth, K.V.H., Tharanathan, R.N., 2007. Chitin/chitosan: modifications and their unlimited application potential – an overview. Trends in Food Science and Technology 18, 117–131.

Roberts, R.G., 1990. Postharvest biological control of gray mold of apples by *Cryptococcus laurentii*. Phytopathology 80, 526–530.

Saligkarias, I.D., Gravanis, F.T., Epton, H.A.S., 2002. Biological control of Botrytis cinerea on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: II. A study on mode of action. Biological Control 25, 151–161.

Sugar, D., Spotts, R.A., 1999. Control of postharvest decay in pear by four laboratory-grown yeasts and two registered biocontrol products. Plant Disease 83, 155–158.

Synowiecki, J., Al-Khateeb, N.A., 2003. Production, properties, and some new applications of chitin and its derivatives. Critical Reviews in Food Science and Nutrition 43, 145–171.

Teixidó, N., Viñas, I., Usall, J., Magan, N., 1998. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. Phytopathology 88, 960–964.

Vivekananthan, R., Ravi, M., Ramanathan, A., Samiyappan, R., 2004a. Lytic enzymes induced by *Pseudomonas fluorescens* and other biocontrol organisms mediate defence against the anthracnose pathogen in mango. World Journal of Microbiology and Biotechnology 20, 235–244.

Vivekananthana, R., Ravia, M., Saravanakumara, D., Kumarb, N., Prakasama, V., Samiyappan, R., 2004b. Microbially induced defense related proteins against postharvest anthracnose infection in mango. Crop Protection 23, 1061–1067.

Yu, T., Li, H., Zheng, X., 2007. Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. International Journal of Food Microbiology 114, 261–266.

Zhang, H., Zheng, X., Fu, C., Xi, Y., 2005. Postharvest biological control of gray mold rot of pear with *Cryptococcus laurentii*. Postharvest Biology and Technology 35, 79–86.

Zhang, H., Zheng, X., Yu, T, 2007. Biological control of postharvest diseases of peach with *Cryptococcus laurentii*. Food Control 18, 287–291.