Development of a biocontrol agent on active biomass formulation to control *Penicillium expansum* responsible for the postharvest blue mold disease of apple

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Développement d'une formulation de biomasse active d'un agent de contrôle biologique (souche de levure 1113-5) de la pourriture bleue causée par Penicillium expansum sur pomme en post-récolte.

Résumé

Une souche de levure (1113-5) isolée lors d'une étude antérieure et ayant un haut pouvoir antifongique contre les deux principaux pathrogènes responsables des maladies post-récolte des pommes au Maroc; Penicillium expansum *et Botrytis cinerea, a été utilisée dans cette étude.*

L'objectif de ce travail est d'évaluer et d'optimiser les potentialités de la production en masse de cette souche en fermenteur de laboratoire. L'effet des conditions de stockage sur la stabilité de la biomasse séchée par la technique du lit fluidisé et le maintien du pouvoir inhibiteur contre P. expansum *à l'échelle pilote ont été également évalués. Une production importante en biomasse de la levure (107 g* l^{-1} *en poids sec) a été obtenue par fermentation discontinue alimentée (i.e., fed-batch) par une solution de glucose. La biomasse produite a été séchée en lit fluidisé et a gardé une viabilité optimale de 60%. Ce travail a montré aussi que la levure séchée a maintenu son activité antifongique après 7 mois de stockage à 4 °C. Le plus fort pouvoir antagoniste contre* P. expansum *a été obtenu en appliquant la levure à une concentration de* 1.10^8 *CFU* ml^{-1} *sur les pommes blessées.*

Mots-clés : Contrôle biologique, levure, antagonisme, pomme, biofongicide, biomasse, pourriture bleue, Penicillium expansum.

إنتاج وصياغة خميرة مضادة للفطر المسبب لمرض ما بعد الجني عند التفاح : بنيسليوم إكسبانصوم *(Penicillium expansum)*

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ملـخص

في دراسة سابقة، تم عزل من على سطح فاكهة التفاح صنف (Golden Dilicious) خميرة (1113 -5) تتميز بقدرة تضادية جد عالية تجاه فطر بنيسليوم إكسبانصوم *(Penicillium expansum)* المسبب للعفونة الزرقاء في محاصيل التفاح بالمغرب.

تهدف هذه الدراسة إلى تقييم وتطوير إمكانيات الإنتاج وبوفرة لخميرة مكافحة لمرض ما بعد الجني عند التفاح، باستعمال آلة التخمير المخبرية(fermenteur) كما تمت أيضا دراسة تأثير ظروف التخزين على الاستقرار والقدرة التضادية لخلايا الخميرة المجففة ضد فطر *P. expansum* ولقد سجل انتاج وفير لهذه الخميرة باستعمال تقنية فيد- باتش «fed-batch» بالكليكوز وحصول على وزن جاف صاف للكتلة الحيوية يقدر ب 107 غرام للتر الواحد.

كما تم تجفيف الكتلة الحيوية المنتجة عن تقنية السرير السائل (fluid bed)، والتي أسفرت عن قابلية قصوى للحياة تقدر بـ60 بالمائة في ظروف رطوبة باقية تقدر ب 10 ,5 (كتلة/كتلة).

وأظهرت النتائج أيضا أن الخميرة المجففة تحتفظ بنشاطها التضادي تجاه بنيسليوم إكسبانصوم لمدة تفوق 7 أشهر عند تخزينها تحت 4 درجات حرارية، وأن أفضل نشاط تضادي على التفاح المجروح اصطناعيا يحقق بإضافة الخميرة بتركيز مائة مليون خلية في المليليتر الواحد.

الكلمات المفتاح : التفاح، أمراض ما بعد الجني، العفونة الزرقاء، المكافحة الحيوية، *Penicillium expansum*، الخميرة، التجفيف.

Abstract

A previously isolated yeast strain 1113-5 and proven to be highly inhibitory to Penicillium expansum and Botrytis cinerea; major wound pathogens on postharvest apple fruit in Morocco, was used in this study. The objective of the present study was to evaluate and optimise the potentialities for mass production of the yeast strain in a laboratory-scale fermentor. The effect of storage conditions on the stability of fluid-bed-dried cells of the yeast strain 1113-5 and the antagonistic activity against P. expansum on apple fruits a pilot scale were also evaluated. The high cell density fermentation could be achieved with inhibitory strain 1113-5 using glucose fed-batch fermentation and has yielded a final biomass dry weight of 107g- l^{-1}. The biomass produced in the fed batch fermentation was dried by using the fluid bed technology. An optimum viability of 60% has been obtained. After rehydratation, the starter culture has maintained its antimicrobial activity for up to 7 months of storage at 4 °C. The highest antagonistic activity against P. expansum was achieved with the application of a 1_10^{8} c.f.u. ml^{-1} suspension of the yeast strain 1113-5 on wounded apple fruit.

Key-words: Biocontrol, antagonistic activity, yeast, apple, biofongicide, biomass, blue mold disease, *Penicillium expansum*.

Introduction

Morocco is considered as the second biggest apple producer in Africa after South Africa and its contribution to apple production in the continent is estimated at 30%. Apple production reachs 315 422 tons/year (average value on the five last years) counts for 10% of the total fruit production and is sharing the third place with the grapes (10%). The first place is devoted to citrus fruits (31%) and the second one to olives (18%). (D.E.R.D., 2006).

Postharvest diseases cause substantial losses (60%) in apple production (Bondoux, 1992). These losses vary from 5% to more than 20% in the United States and can be higher than 50% in developing countries (Janisiewicz & Korsten 2002). Postharvest fungal pathogens cause severe losses on apple during postharvest storage and commercialization (Jijakli & Lepoivre 2004). The most deleterious fungal pathogens were reported to be *Penicillium expansum*, *Botrytis cinerea*, *Monilinia* spp. and *Phlyctaena vagabunda* (=*Gleosporium album*) (Lima *et al.* 2003). In particular, *P. expansum*, is the causal agent of apple blue mold disease that results in an aesthetic depreciation of the fruits thereby reducing its marketing opportunities leading to a great economic losses. Furthermore, the blue mold disease is difficult to control efficiently with the fungicides commonly used in the chemical treatment of apple due to the resistance of *P. expansum* (Caffarelli *et al.* 1999). Therefore, search for alternative means to control the disease in order to increase the production and to obtain a premium quality fruits. Biocontrol using microbial preparations are one of the most promising means for fruit protection and the most accepted by consumers as they provide healthier product and free from pesticide residues.

Effective antagonists have been used successfully to control major postharvest diseases of many fruits including apple, stone, citrus, and subtropical and tropical fruits worldwide (Janisiewicz & Korsten 2002).

The epiphytic yeasts naturally occurring on the surface of fruits or vegetables have been the focus of research as potential antagonists of postharvest diseases (Janisiewcz *et al.* 1994, El Ghaouth *et al.* 1998, Jijakli *et al.* 1999, Lima *et al.* 2003) essentially due to their high ability to colonize and survive on skin of fruits, their non fastidious nature and their resistance to the most of commercial fungicides.

The evaluation of such yeasts in suitable formulations is essential to predict their potential effectiveness when used as biocontrol agents (Abadias *et al.* 2003). Several biofungicide formulations based on yeast have been developed and commercialized in the USA, Israel (Aspire", based on *Candida oleophila*, strain I-182) (Droby *et al.* 1998) and South Africa (Yield Plus", *Cryptococcus albidus*) (Droby *et al.* 2002).

Recently, a yeast strain 1113-5, isolated from the surface of apple fruit in Morocco, has proven to be highly inhibitory against *P. expansum* and *B. cinerea*, two major wound pathogens causing serious alterations of *Golden Delicious* apples during storage (Achbani

et al. 2005). In this context, the objectives of the present study were to develop an active biomass formulation through the optimization of the fermentation process with subsequent drying. Stability and the antagonistic activity against *P. expansum* of the dried cell-mass were evaluated during storage at 4 °C.

Material and methods

Strains

The yeast 1113-5 (INRA, Meknès, Morocco) was maintained on PDA medium (Potato Dextrose Agar; LAB MTM, Germany) at 4 °C and subcultured biweekly.

P. expansum strain 880 (INRA, Meknès, Morocco) has been isolated from decayed apples as the most aggressive strain. It was maintained also on Potato Dextrose Agar medium at 4 °C. To maintain its agressivity, it was inoculated periodically (3 months) on apples and re-isolated.

Stock Cultures

For long-term storage, microbial cultures were stored at -80 °C (freezer Revco Ultima II, USA) in YEPD broth (10 g l^{-1} yeast extract (Merk), 10 g l^{-1} peptone (Merk) and 20 gl^{-1} D-Glucose monohydrate (Merk)) containing 40% (v/v) glycerol.

Inoculum preparation

For fermentation experiments, inocula were prepared by transferring one millilitre of each stock culture in a 500 ml conical flask containing 250 ml of YEPD broth and incubated at 28 °C for 48 h under continuous shaking at 200 rpm in a rotary shaking incubator (Innova 4230, Refrigerated incubator shaker, USA).

Biomass production

The biomass production of strain 1113-5 was carried out at 28 °C by fed-batch fermentation using a 10-litres Biostat ® ED bioreactor (B. Braun Biotech International GmbH, Melsungen, Germany). Fed batch solution consisted in sterile solution of glucose (50% (w/w)) which was sterilized by autoclaving at 121 °C for 20 min. The fermentation medium contained per litre : 5 ml of mineral salts concentrated solution (0.32 g l^{-1} $MnCl_2$ 4 H_2O, 0.49 g l^{-1} $CuSO_4$ $5H_2O$, 5.75 g l^{-1} $ZnSO_4$ $7H_2O$, 0.48 g l^{-1} $CoCl_2$ $6H_2O$, 0.49 g l^{-1} Na_2MoO_4 2H2O, 15 g l^{-1} EDTA, 2.94 g l^{-1} $CaCl_2$ $2H_2O$ and 2.78 g l^{-1} $FeSO_4$ $7H_2O$), 30 g yeast extract, 30 g soy peptone, 0.37 g Na_2SO_4, 4.5 g K_2SO_4, 6 g KH_2PO_4, 3 g $MgSO_4$ $7H_2O$ and 700 ml distilled water. A 250-ml glucose solution (110 g of glucose in 250 ml of distilled water) and 5 ml of filter-sterilized concentrated solution of vitamins (1 g l^{-1} thiamine HCl, 1 g l^{-1} pyridoxine HCl, 1 g l^{-1} nicotinic acid, 1 g l^{-1} D-biotin, 1 g l^{-1} Ca-D-pantothenat, 0.2 g l^{-1} p-aminobenzoïc acid, 5 g l^{-1} inositol) were added to the fermen-

tation medium before inoculation. During fermentation, the medium was aerated with 1.5 vvm (air volume/liquid volume/min) and agitated by stirring at 600 rpm. The pH of the culture was maintained constant at 5.0 by automatic addition of 2 M NaOH or 10% (v/v) H_3PO_4. The bioreactor was inoculated with 250 ml of active culture prepared as described above.

During cultivation, pH, temperature, agitation and dissolved oxygen, pO2 were constantly registered with the Micro Fermentation Control System (MFCS) unit (B. Braun Biotech International GmbH, Melsungen, Germany).

Analytical Methods

Samples were withdrawn aseptically from the fermentor at regular intervals to determine the optical density (OD) and by dry cell weight determination (DCW). To determine the OD, the samples were diluted in order to achieve the linear response range of absorbance and the OD was measured at 660 nm in a Jenway spectrophometer model 6100 (Dunmow, England). As for the dry cell weight (DCW) a 100-ml sample was centrifuged at 12 000¥g for 15 in a Sorvall-RC-5B centrifuge (Wilmington, USA). The pellet was washed successively with saline solution (0.9% (w/v)) (twice) and with distilled water. The cell-pellet was, then, oven-dried at 105 °C until constant weight to determine the CDW as described by Kim *et al.* (2003).

In addition, residual glucose and ethanol content were also determined during the course of fermentation at the same intervals. A 1-ml sample was centrifuged for 10 min at 2000 g and the pellet was decanted. The supernatant was used for glucose and ethanol measurements.

Glucose was determined by High Performance Liquid Chromatography (HPLC), using a Prevail carbohydrate ES column (Alltech, USA), mobile phase 75% v/v acetonitrile; flow rate 1.0 ml min^{-1}; at 30 °C; with an injected volume of 25l; refractometer detector 410 (Waters, USA). Ethanol also was determined by HPLC, using a SH-1011 Shodex column (USA), mobile phase 0,01N H_2SO_4; flow rate 0.8 ml min^{-1}; at 50 °C; with an injected volume of 25l; refractometer detector 410 (Waters, USA). Millenium 32 Chromatography Manager Software (Waters, USA) was used for data treatment.

Solid formulation

At the end of fermentation (48 h), the biomass of the yeast strain 1113-5 was harvested by centrifugation in a Sorvall" RC 12 BP centrifuge at 4500 x g, for 30 min and dried in a fluid bed dryer (Niro-Aeromatic, Compenhagen, Denmark).

The maize starch (30 % (w/w)) was used as the loading agent. Extrusion of the paste was accomplished immediately in the dryer through holes of a 1-mm diameter. During the drying process, the air flow and temperature were controlled at 150 m^3 h^{-1} and 30 °C, res-

pectively, inside the bed. The yeast was dried until a final moisture of 10.5% (w/w) was achieved.

Viability of the dried yeast strain

Dry yeast was stored at 4 °C and its viability was monitored throughout the storage period. At regular intervals, a 1-g sample was taken and resuspended in 10 ml of an isotonic solution (0.9% (w/w) NaCl and 0.05% (w/w) of soy peptone in demineralised water). This suspension was stirred for 2 h to obtain an optimal rehydration of the dry yeast and then used to determine the viable cell count. The viable counts were monitored by the standard plate count technique after serial dilutions in saline solution (0.9% NaCl) using YEPD agar and incubation at 25 °C for 72 h. The viability of 1113-5 cells was expressed as a percentage of surviving cells compared with the initial number of cells.

Antagonistic activity of dried 1113-5 cells against *P. expansum* on apples

The effectiveness of the dried yeast in controlling *P. expansum* after 7 months of storage at 4 °C was evaluated on *Golden delicious* apple fruit. A suspension of *P. expansum* spores was prepared by adding 10 ml of sterile distilled water on the surface of a 10-days-old culture grown on PDA plates and scrapping the surface with a sterile hockey stick glass rod. The spores were counted in the Bürker cell and diluted to obtain a concentration of $1x10^5$ c.f.u. ml^{-1} to be used in the challenge experiments.

Apples were wounded at four equidistant points. The wounds were 2 mm wide and 4 mm deep. The inter-wounds distance was 20 mm. The wounded fruits were then dipped in the antimicrobial yeast suspensions at concentrations of $1x10^6$, $1x10^7$ or $1x10^8$ c.f.u. ml^{-1} for two minutes. Twenty four hours after yeasts application, wounded fruits were artificially contaminated by pulverization of 2 ml suspension of *P. expansum* spores per apple at a concentration of $1x10^5$ c.f.u. ml^{-1}. Treated fruits were stored at 5 or at 25 °C under relative humidity of 94-98% in closed plastic trays. Each treatment was applied to three replicates of 20 fruits (240 wounds for each treatment). The lesion severity (lesion diameter in mm) caused by *P. expansum* was measured for apples stored at 25 °C after 5 and 7 days and after 20 and 28 days for apples stored at 5 °C.

Parameters and statistical analysis

Statistical analysis of the severity of decay due to the pathogen infection as assessed by the diameters of the alteration spots corresponding to the fungal growth was done by the analysis of variance (ANOVA) using the general linear model procedure of Statistical Analysis System (SAS Institute, Cary, NC). Statistical significance was judged at the $P<0.05$ level. When the analysis revealed statistically significant differences, Duncan's

Multiple-Range Test was used to test the mean separations among mean values of each treatment.

The protective levels (Y %) were calculated with respect to the following formula: $D_T - D_X / D_T \times 100 = Y\ \%$, where D_T = diameter lesion in control samples and D_X = diameter lesion in samples treated with the antagonistic yeast strain.

Results and discussion

Biomass production by fed-batch fermentation

After 48 h of fermentation in a fed-batch fermentor at a constant pH (5.0) and temperature (28 °C), a biomass dry weight of 107 g l^{-1} was achieved. The use of fed-batch culture takes the advantage of the fact that the concentration of the limiting substrate may be maintained at a very low level, thus avoiding the repressive effects of high substrate concentration. It is also used in controlled glucose-feeding regimes to maximize respiratory growth (Abadias *et al.* 2003; Hyung-Pil *et al.* 2004).

The yield factor ($Y_{X/S}$) was equivalent to 0.67 confirming that the fed-batch mode was more favourable to the biomass production by avoiding of products synthesis as ethanol and by consequence most of the carbon source was allocated to cell mass production.

Viability of the dried yeast

The drying process was accomplished using fluidised bed drying technology. The evolution of the viability during the drying process is presented in Fig. 1. The final product showed a high rate of viability (60%) corresponding to a residual moisture of 10.5% (w/w), suggesting that the fluidised bed drying is a suitable drying technology to stabilise the yeast strain 1113-5.

Such a viability ratio is acceptable and may justify a possible industrial transposition of the production of this yeast strain as a biocontrol agent for fruit preservation. After drying, the yeast granules were stored at 4 °C to maintain metabolic activity at a low level thereby extending the shelf life (Selmer-Olsen *et al.* 1999). After 7 months of cold storage, a viable count of $1.5.10^{10}$ c.f.u. g^{-1} of the dried yeast strain was recorded corresponding to 16% of the initial viable count. The decrease in viability occurred mainly in the first 30 days of storage and the viability remained constant throughout the remaining period of the study.

Antagonistic activity of 1113-5 formulation against *P. expansum* on apples

Fermentation and formulation processes are important steps in the development of a biocontrol product. In fact, the objective is not only to produce a high biomass, but also that such a biomass should conserve it capability to control effectively the disease during the processing and as much as possible during storage (Alabouvette & Lemanceau 1997).

The dried formulation of biocontrol yeast strain 1113-5 was evaluated for its antagonistic activity against the *P. expansum* responsible of the blue mold disease on apples at a pilot scale for two storage temperatures, 5 °C and 25 °C. In both cases, the inoculation of the apple was carried out with the same concentration of pathogen.

Statistically, the lesion diameters caused by *P. expansum* in all treatments were significantly reduced ($p>0.05$) for all biological treatments with 1113-5 at different concentrations (ie., 1.10^6, 1.10^7 and 1.10^8 c.f.u. ml^{-1}) compared to the untreated control for both temperature of storage, 5 °C and 25 °C.

The concentrations of antagonist had significant effects on biocontrol effectiveness ; the best control of blue mold caused by P. expansum inoculated at a concentration of 1.10^5 c.f.u. ml-1 was achieved by application of 1113-5 at a concentration of 1.10^8 c.f.u. ml^{-1} (Fig. 2).

For apples stored at 25 °C, the different concentrations of dried formulation of strain 1113-5 used allowed to reduce the severity of the disease (Fig. 3a). The use of dried formulation of strain 1113-5 at 1.10^8 C-F-U ml^{-1} showed the highest protective level of 89% after 7 days of storage (Fig. 3a) whereas both lower concentrations (1.10^6 and 1.10^7 c.f.u. ml^{-1}) offered a protective level ranged between 48% and 76%. Our results are in accordance with those reported by El-Ghaout *et al.* (2000) who observed a more effective control of postharvest decay with antagonistic yeasts applied at 1.10^8 c.f.u. ml^{-1} and often no control of decay when biocontrol agents were applied at 1.10^5 c.f.u. ml^{-1}.

Similar results were also observed for apples stored at 5 °C. The use of dried formulation of strain 1113-5 at 1.10^8 c.f.u. ml^{-1} showed also the highest protective level of 89% after 28 days of storage (Fig. 3b) whereas both lower concentrations (1.10^6 and 1.10^7 c.f.u. ml^{-1}) offered a protective level ranged between 47% and 81%.

For apples stored at 5 °C, the first symptoms of disease appeared only after 13 days. This lack of lesion development after 12 days in untreated control and in biological treatments with 1113-5 may have resulted from either fruit still being resistant to the pathogen in this condition of storage or the low temperature 5 °C regime, which prevented decay development. The protective level of dried formulation of strain 1113-5 at 1.10^8 c.f.u. ml^{-1} obtained at day 20 was of 98% and decreased to 89% after 28 days (Fig. 3b).

The efficiency of the biological control agent to inhibit the development of the pathogen for long period of storage was greater at 5 °C than at 25 °C. It is, therefore, important to

keep apple fruit at a low temperature to reach high level of protection. Similar performances were observed by He et al. (2003) who studied the effect of different storage temperatures (10, 15 and 20 °C) and who showed that low storage temperature significantly retarded the decay of apples treated with *Cryptococcus laurentii* and contaminated by *P. expansum*.

Conclusions

In this study, we can conclude that the biomass formulation of the yeast 1113-5, isolated from the surface of apple fruit, did not affect the efficacy of the biological control agent of apple postharvest diseases (*Penicillium expansum*) after 7 months of storage at 4 °C.

In the challenge experiments, the yeast strain 1113-5 was able to reduce postharvest blue mold development on apple. The antagonist efficacy at 25 °C and 5 °C indicated an excellent adaptation of this strain to cold storage temperatures, what is an important feature for postharvest biocontrol agents.

The level of efficacy of 1113-5 at 1.10^8 c.f.u. ml^{-1} on postharvest apple diseases obtained in our laboratory was high and opens a good opportunity to use this strain as biocontrol agent for apple preservation. Further studies at large scale are needed to confirm these results.

Biological control in the postharvest environment has significant advantages over that under field conditions because the two most important factors affecting biocontrol, temperature and relative humidity are constant and under strict control.

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Figure captions

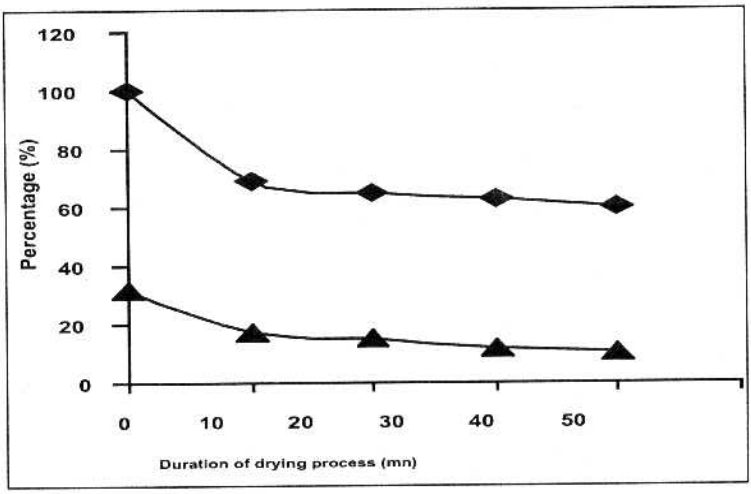

Fig. 1. Viability (%) (◆) and residual moisture (% w/w) (▲) of antagonistic yeast strain 1113-5 during drying by the fluid bed technique.

Dried cells 1113 5 Treatment at concentration of 1_10^8 c.f.u. ml^{-1}

The biomass formulation of 1113-5 was effective in controlling blue mold under laboratory conditions and its efficacy could be improved by a concentration 1_10^8 c.f.u. ml^{-1}

Untreated Control

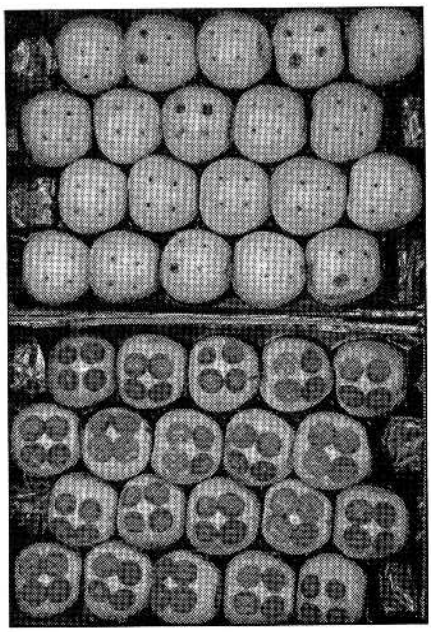

Fig. 2. Photo illustrate the difference in protective level between dried biocontrol agent cells 1113-5-treated *Golden Delicious* apples and an untreated control. The photo was taken after 5 days of storage at 25 °C.

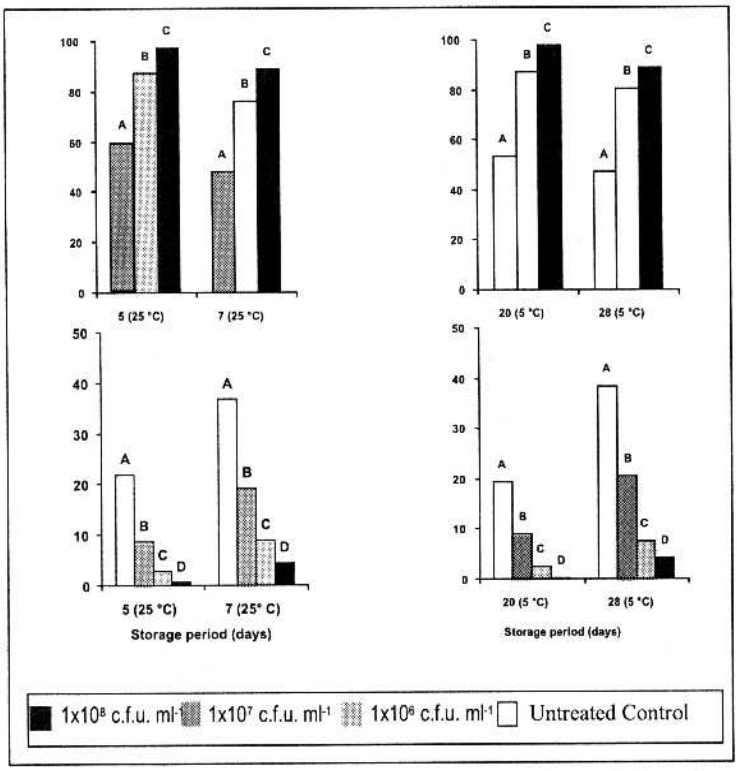

Fig. 3. Biocontrol activity of the fluid bed-dried yeast strain 1113-5 against *Penicillium expansum* (880) at a pilot scale on wounded apples (*Golden delicious*). The yeast suspension was applied at various concentrations (i.e., 1.10^6, 1.10^7 or 1.10^8 c.f.u. ml^{-1}) and the apples were artificially contaminated by *P. expansum* (1.10^5 c.f.u. ml^{-1}). The protective level and severity of decay were based on three replicates of 20 fruit each and were determined after 5 and 7 days of storage at 25 °C (a) and after 20 and 28 days of storage at 5 °C (b). Columns with the same letter within the same time interval are not significantly different according to Duncan's Multiple-Range Test, $P<0.05$ level.