**Biocontrol Science and Technology, Vol. 18, No. 10, 2008, pp1063-1073.**
DOI: 10.1080/09583150802585769

# Physiological manipulation and formulation of the biocontrol yeast *Pichia anomala* for control of *Penicillium verrucosum* and ochratoxin A contamination of moist grain

Stella Mokiou* and Naresh Magan

*Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 0AL, U.K.*

Address for correspondence: Prof. N. Magan, Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 0AL, U.K. Tel: +44 1234 758303; Fax: +44 1234 758370; E-mail: n.magan@cranfield.ac.uk

Key words: yeast cell quality, formulations, sugar alcohols, fluidised bed drying, spoilage fungi, ochratoxin, stored grain

* Present address: NAGREF, Thessaloniki, Greece

**Abstract**

The major hurdle in the production of commercial biocontrol agents (BCAs) has been the lack of production of appropriate formulations. Of particular importance is the conservation of viability and ecological competence after application. With this in mind studies were conducted to develop formulations of *P. anomala* which would have these attributes. Cells were grown in molasses-based medium modified with proline to different water availability levels (0.98 and 0.96) which significantly increased (up to 50%) the content of trehalose and arabitol in the yeast cells during liquid broth fermentation. The use of isotonic solutions for harvesting the yeast cells further increased the endogenous content of these compatible solutes as well as glycerol. Fluidised bed drying of cells at 30-80$^{o}$C was carried out for 10 and 20 mins and showed that viability was significantly decreased at 70-80$^{o}$C. A temperature of 50$^{o}$C for 20 mins was found to be best for viability (70%) and moisture content of <10%. Several additives for conservation of viability showed that cotton seed flour+skimmed milk was the best treatment when dried at 50$^{o}$C. The biocontrol efficacy of formulated *P. anomala* cells was tested in laboratory scale studies and this showed that they inhibited growth of *Penicillium verrucosum* and reduce ochratoxin A production in moist wheat grain under some combinations of water availability. Physiologically modified formulated yeast cells with increased levels of trehalose and arabitol gave similar efficacy as fresh cells. This suggests that ecophysiological manipulation of such BCAs can result in improved ecological competence of such formulations and effective biocontrol.

## Introduction

For animal feed, airtight storage of the cereal grain in silos is an alternative energy-saving method compared to high temperature drying of temperate cereals. However, temporal fluctuation of carbon dioxide and oxygen levels do occur and this eventually leads to heavy mould growth and poor quality animal feed grain. *Pichia anomala* has been frequently isolated from airtight-stored grains and Björnberg & Schnürer (1993) first showed that strain J121 effectively reduced growth of *Penicillium roqueforti* in vitro in a dose-dependent manner. *P. anomala* was also shown to reduce ochratoxin A (OTA) accumulation in co-culture with *Penicillium verrucosum*.

The major obstacle in the commercialisation of biocontrol agents (BCA) products is the development of a shelf-life-stable formulated product that retains efficacy similar to those of fresh BCA cells (Janisiewicz & Jeffers, 1997; Magan, 2006). BCAs are living organisms and their economic production process, formulation, distribution and application are of great importance and require special considerations. Drying microorganisms enables preservation of the inoculum over a long period of time, maintaining high viability, and does not require cool temperatures during storage and distribution.

Magan (2001; 2006) has demonstrated that it is possible to utilise physiological stress conditions to enable a significant increase in the endogenous synthesis of compatible solutes such as trehalose and low molecular weight sugar alcohols (erythritol or glycerol). This was shown to result in improved viability especially under water stress conditions for yeast BCAs such as *Candida sake* (Teixido et al., 1998) and filamentous fungi (Magan, 2001; 2006). This can produce characterised BCA inocula which have a better ecological competence, especially in the phyllosphere.

Recent studies have furthermore shown that the use of isotonic solutions for harvesting of such characterised BCAs can result in a significant increase in retention of trehalose and sugar alcohols in yeast cells and spores (Abadias et al., 2003; Ypsilos and Magan, 2004). The question is whether these aspects can be combined to develop appropriate formulations which can conserve these improved ecophysiological quality.

Fluidised bed-drying has been extensively used to manufacture active dry yeast on a large scale (Bayrock & Ingledew, 1997). Hallsworth & Magan (1995) showed that elevated concentrations of the dissacharide trehalose, in response to osmotic stress, in conidia of entomopathogenic fungi prolonged shelf-life. Mokiou & Magan (2002) showed that *P. anomala* cells intracelularly accumulated trehalose when exposed to water stress by addition of proline and NaCl to molasses-based media. Formulations of other biocontrol agents (e.g. *Candida sake*, *Penicillium oxalicum*, *Penicillium frequentans*, *Epicoccum nigrum*, *Pantoea agglomerans*) have also been examined using fluidised bed drying (Larena et al., 2003, Guijarro et al., 2006; Magan 2006). Recently, Melin et al. (2006) compared different formulations and showed that some liquid formulations containing exogenously supplied commercial trehalose were effective for long term storage of *Pichia anomala* and control of *P. roqueforti* in moist sealed feed grain.

Ochratoxin A (OTA) is a class 2b carcinogen for which EU legislation exists in a range of food raw materials and products including cereals (Magan & Olsen, 2004). The origin of OTA in cool and temperate climates is generally attributed to *P. verrucosum*, whereas in warm temperate and tropical zones it is now commonly associated with *A. ochraceus* and the black aspergilli. Recent studies have identified the water and temperature requirements for

growth and OTA production by *P. verrucosum* (Cairns-Fuller et al., 2005). There is interest in minimising the contamination of such toxins in the food and feed chains. However, few if any studies have been carried out under different steady state intermediate moisture conditions to examine whether ochratoxin A can be effectively controlled using formulations of BCAs during post-harvest storage.

The objectives of this study were to (a) produce characterised *P. anomala* cells in liquid broth culture with elevated compatible solutes for stress tolerance, (b) evaluate the addition of different additives to these treatments prior to fluidised-bed drying on protection of cell viability and (c) to test the best formulations in controlling *P. verrucosum* and OTA in moist grain at intermediate water availability conditions.

## Materials and methods

*Microorganism, fermentation media and extraction of polyols and sugars*

The microorganism used in this study was *Pichia anomala* (strain J121). This was kindly supplied by Prof. J. Schnürer, Agricultural University, Uppsala, Sweden. Cane molasses-based medium made of cane molasses 40 $gl^{-1}$ and urea 1.2 $gl^{-1}$ was used as the basic medium. Molasses was kindly supplied by UdL-IRTA, Lleida, Spain; pH 6.1/$a_w$ 0.993-0.996. Modification of media water activity ($a_w$, 0.98 and 0.96) was made by the addition of proline. Extraction of sugar alcohols (mannitol, arabitol, glycerol and arabitol) and sugars (trehalose, glucose) was done as detailed in Ypsilos and Magan (2004). The yeast pellets were washed in HPLC grade water or isotonic solutions and centrifuged to remove any residual liquid medium. Between 50-100 mg of fresh weight of the yeast cells were extracted. One millilitre of HPLC grade water was added and samples were sonicated with a 4 mm sonicator probe for 2 min at an amplitude of 26 lm (Soniprep 150; Sanyo, Loughborough, UK). After immersion

in a boiling water bath for 5 min, the samples were left to cool, and an adequate volume of acetonitrile (ACN) was added to each sample to obtain the same ratio of ACN : water as the mobile phase (40 : 60). The Eppendorf tubes were centrifuged for 10 min at 1150 g and the supernatant was filtered through a 0.22 μm filter into HPLC vials sealed with plastic septa. Sugars and sugar alcohols were analysed and quantified by HPLC using a Hamilton HC-75 $Ca^{2+}$ column (Anachem Ltd, Luton, UK) and a Gilson refractive index detector (Anachem), specifically for sugar:polyol separation. The mobile phase used was a mixture of 40:60 degassed ACN:water. The peak areas were integrated and compared with calibration curves constructed with standards of 100–800 ppm of each solute analysed. The sugars analysed were trehalose and glucose and the sugar alcohols were glycerol, erythritol, arabitol and mannitol. The results were expressed as milligrams of intracellular component accumulated per gram of fresh weight *P. anomala* cells.

*Fluidised bed-drying of the treatments*

Initial studies were carried out with fresh cells. Thus, 100 ml of the chosen molasses based treatment containing proline to modify $a_w$ to 0.98 and 0.96 were used in 250 ml conical flasks. These were inoculated with 1ml aliquot of $10^6$ *P. anomala* cells $ml^{-1}$ and were cultured with agitation on a rotatory shaker (150rpm) at 25$^o$C. After 48 h of incubation subsamples of each conical flask were distributed in 50ml sterile Centrifuge tubes and centrifuged immediately for 15 min at 3300 rpm in a MSE Centaur 2 (Norwich, U.K.) centrifuge. The supernatant was discarded and yeast cells were washed twice in 50 mls of sterile water. The wet pastes were placed on filter paper to remove excess water before placing in the four tubes of the fluidised bed dryer 350S (Burkard Manufacturing Co. Ltd, Hertfordshire, UK) and dried for 10 and 20 mins at 30-80$^o$C. The final moisture content was determined by oven drying at 80$^o$C overnight.

*P. anomala* cells were grown in the proline modified molasses-based medium at 0.98 and 0.96 $a_w$ in shake cultures, harvested and washed twice in HPLC grade water or in NaCl (0.98 or 0.96 $a_w$ isotonic solutions) and centrifuged. Carriers [corn meal (CM), cottonseed flour (CSF), and wheat starch (WS)] were added in a proportion 1:1 (w/w, *P. anomala* cells/carrier) and adjuvants (skimmed milk for CSF and WS and glycerol for CM) at 10% (w/w). The pastes were extruded through a 1 ml syringe and then the mixtures (2 g) placed in each of four tubes of the fluidised bed-dryer 350s and dried at 50$^{o}$C for 20 mins. Viability was assessed using a viablue stain (Hutchenson *et al.*, 1998) which was shown to give a good correlation between cell viability and staining ($r^2$=0.99). All experiments were carried out twice with four replicates per treatment.

*Control of* P. verrucosum *and ochratoxin contamination*

Irradiated wheat grain with retained germinative capacity was used in this study (Cairns et al., 2005). This was modified by the addition of Sterile Distilled Water (SDW) to achieve 0.96 and 0.93 $a_w$ levels. Inoculation of the grain was done so as to achieve a final concentration of $10^3$ *P. verrucosum* (strain OTA11) and $1x10^5$ CFUs $g^{-1}$ grain of *P. anomala*. The biocontrol yeast and the mycotoxigenic species were inoculated individually or in co-cultures. Grain was stored in Petri dishes (15 g; 12 replicates per treatment) and three replicates destructively sampled after 1, 10, 20, 30 days at 25$^{o}$C. Treatments were placed in plastic boxes with isotonic glycerol solutions to maintain the required atmospheric relative humidity at 25$^{o}$C. At each sampling time, three replicate 1g samples of grain were removed and placed in empty, sterile Universal bottles. 9 ml of diluent (SDW+tween 20+ 0.01% agar) was added and shaken for 2 mins. A serial dilution series was performed and 0.1 ml plated at appropriate dilutions and spread plated on Nutrient Yeast Dextrose Agar (NYDA) modified to 0.93 $a_w$ with PEG

200. Growth of the biocontrol agent and *P. verrucosum* was recorded as CFU $g^{-1}$.The rest of the sample was used for extraction and OTA analyses. The experiments were all carried out twice.

*Ochratoxin A (OTA) extraction and quantification*

Extraction was by shaking treatments containing the solvent methanol for 24 hrs. Samples were subsequently filtered through Whatman No1 paper placed inside a filter funnel containing 1 g of celite agent. 1 ml aliquots of the methanol extract were placed in a 1.5ml plastic Eppendorf tubes and centrifuged in a bench top microcentrifuge (Beckman Lite, Ser 98E 4000, Beckman Inc. Columbia, U.S.A.) at 13000 rpm for 10 minutes. The pellet produced was discarded and the supernatant put into 1ml amber HPLC vials (Fisher) and stored at -20ºC until processed. OTA contents were measured using High Performance Liquid Chromatography (HPLC). An HPLC (Waters 600E System controller) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used (Cairns-Fuller et al., 2005). Under these experimental conditions, a fluorescence detection limit of 1-1.2 μg $ml^{-1}$ OTA was possible.

*Statistical analysis*

Data were analysed using Genstat software (Genstat 5th edition). Analysis of Variance (ANOVA Table) was used to compare different treatments. Percentage data was LOGIT transformed prior to statistical analysis. Statistical significance was judged at the $P<0.05$ level. When the analysis was statistically significant the LSDs of the means were used to compare the latter.

**Results**

*Intracellular sugar and polyol accumulation in* P. anomala *cells grown in molasses media harvested in water and in isotonic solutions*

Figure 1a shows the comparison of unmodified and modification of $a_w$ of molasses medium by addition of proline imposed water stress on *P. anomala* cells at 0.98 and 0.96 $a_w$. This shows the change in accumulation/synthesis of sugars and sugar alcohols. In the unmodified molasses media, trehalose was found to be the predominant intracellular compatible solute followed by arabitol. In the proline/0.98 $a_w$ treatment, a significantly increased (P=0.05) intracellular accumulation of both trehalose and arabitol was found when compared with the unmodified treatment (0.996 $a_w$). Cells from the proline 0.96 $a_w$ treatment accumulated high amounts of glycerol, trehalose and arabitol. Figure 1b shows that when harvesting the yeast cells in isotonic solutions of NaCl then the amounts of the desiccant protectant trehalose, and key compatible solutes such as glycerol and arabitol were significantly (P=0.05) increased, often to twice the amounts accumulated when water was used to harvest the yeast cells. One of the best treatments (0.98 $a_w$) with the highest accumulated endogenous reserves was used for fluidised bed drying experiments to identify the best temperature and time required for optimising conservation of viability.

*Fluidised bed drying of* P. anomala *cells with use of several additives and isotonic solutions*

Figure 2a shows the effect of temperature and time of fluidised bed drying on the fresh *P. anomala* yeast cells from the optimised proline (0.98 $a_w$) treatment. This shows that viability of the cells decreased significantly at 70-80$^{o}$C in both 10 and 20 mins fluidised bed drying regimes. The effect of these fluidised bed drying treatments on the actual moisture content of the yeast cells at the different temperatures showed that 40-80$^{o}$C and 20 mins gave the lowest

moisture content levels. Based on these studies it was decided to use 50$^{o}$C and 20 mins as the standard fluidised bed treatment as it gave good viability and a moisture content of <10%.

Figure 3 shows the effect of the addition of additives to the optimised proline (0.98 $a_w$) treatment used as carriers (100% or 1:1 w/w) including corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability after drying for 20 mins. Viability of *P. anomala* formulation treatments significantly differed from the control treatment (unformulated cells). Of all the treatments evaluated, CSF:1+10%SM (w/w, additives/fresh cells) resulted in significantly (P=0.05) increased final cell viability. Addition of several adjuvants to carriers resulted in increased or similar cell viability. The final cell moisture content of the CSF:1+10%SM treatment was 4.3% (data not shown). Washing yeast cells with a NaCl isotonic solution (osmoprotection) prior to drying resulted in retention of the highest intracellular accumulation of endogenous solutes.

*Control of* P. verrucosum *and Ochratoxin A production in moist grain*

The effect of *P. anomala* on *P. verrucosum* population growth on wheat grain (0.93 $a_w$ water activity) at 25$^{o}$C over 30 days is shown on Figure 4a. The changes in populations of *P. verrucosum* over the 30 day period showed that there were significant (P<0.05) interactions between different treatments used; wheat grain, $a_w$ and time. The main treatments and interaction between two factors were also statistically significant. *P. verrucosum* alone reached 4.5 x $10^6$ CFUs $g^{-1}$ at 0.93 $a_w$ after 30 days starting from 1 x $10^3$ CFUs $g^{-1}$ one day after inoculation. When co-cultured with *P. anomala*, it reached a level of 3.2-3.75 x $10^5$ CFUs $g^{-1}$ with no statistical differences between the different treatments. However, there were statistical differences between treatments after 10 days. Figure 4b shows the increase in

populations of *P. anomala* cells over the same 30 day storage period. There was an increase in populations over time with all treatments reaching $\log_{10}8$ CFUs $g^{-1}$ grain.

Figure 5 shows the impact of these treatments on OTA accumulation. There was a three-way significant ($P<0.05$) interaction between the different treatments used; wheat grain, $a_w$ and time. One and two-way interactions were also significant. OTA contamination significantly increased after 30 days in all treatments. When *P. verrucosum* was present alone, OTA accumulated after 30 days reaching 28700 µg $kg^{-1}$. Co-culture with *P. anomala* resulted in a significant decrease in OTA contamination of the grain with treatments A, B and D.

**Discussion**

This study was carried out to examine the production of physiologically characterised *P. anomala* cells, the use of isotonic solutions for optimised harvesting, the best temperature and time for fluidised bed drying of the best cell treatments and the formulated yeasts with additives and carriers for controlling spoilage and OTA production in moist grain.

This has demonstrated that although *P. anomala* is an osmotolerant yeast it is possible to physiologically stress the cells to enhance the endogenous accumulation of useful compatible solute accumulation when grown in a molasses medium, which is cheap and readily available. This was particularly demonstrated when proline was used to modify the $a_w$ level of the medium to 0.98 $a_w$. The significant increase in trehalose under the water stress treatments was an important result as this is a key compound which can be beneficial during subsequent drying and formulation. Since trehalose is a very expensive compound to add exogenously, the natural biosynthesis shown here is very beneficial for subsequent processing.

The increase in synthesis of the disaccharide trehalose is important as it acts as a membrane-protecting agent for yeast cells during environmental stress conditions such as heat treatment, dehydration and freezing (Zayed & Roos, 2004). Synthesis and accumulation of trehalose by yeast cells correlates with increased survival following dehydration or freezing (Beker & Rapoport, 1987). The proposed mechanism of desiccation protection by trehalose is known as the "water replacement hypothesis", whereby trehalose replace water molecules in the membranes and form hydrogen bonds with the phospholipids, thus preventing collapse of the membrane upon water removal (Crowe et al., 1987).

The use of isotonic solutions is a very interesting finding. In most cases biocontrol agents are harvested in water regardless of the liquid or solid medium used for production. The present study has shown how important the harvesting process can be where the aim is optimisation of BCA inoculum quality and viability. This confirms previous studies with the entomopathogen *Metarhizium anisopliae* where isotonic solutions significantly enhanced retention of sugar alcohols such as erythritol and mannitol. This resulted in increased germination of blastospores, even under water stress conditions (0.96 $a_w$; Ypsilos and Magan, 2004). Isotonic harvesting was also found to be an effective treatment for the biocontrol yeast *Candida sake* for control of post-harvest fungal pathogens of pome fruits (Abadias et al., 2003).

Fluidised bed drying has been successfully used for the production of formulations of *C. sake* and *Epicoccum nigrum* (Abadias et al., 2003; Larena et al., 2003). Indeed with *E. nigrum*, it was found that fluidised bed drying gave 100% viability of spores for up to 90 days. In contrast, freeze drying required the use of additives. In contrast, spray drying resulted in only 10% viability. Recent work with *P. anomala* compared vacuum, fluidised bed, freeze and

liquid formulations and effects on storage viability over periods of 1 year (Melin et al., 2007). This study suggested that freeze dried cells could be stored at up to 30$^{o}$C for 1 year with minimal loss of viability while other techniques required <10$^{o}$C to conserve viability. They used exogenous trehalose to enhance viability during formulation. The present study concentrated on the use of stress physiology as a method of enhancing endogenous synthesis of trehalose and other beneficial sugar alcohols. Our study also suggests that the provision of additives during fluidised bed drying is important to maximise final formulations. Studies of our best treatments suggest that viability under ambient conditions can be conserved for up to 6 months (unpublished data). We believe that maintaining a final moisture content of <10% of the formulated product is critical to conserving viability. The key criterion is the ability of the formulated product to give efficacy similar to or better than that provided by freshly harvested cells.

In the present study *P. anomala* cells reduced populations of *P. verrucosum*, based on CFU counts, although after 30 days there were no significant differences between treatments during co-culture in wheat. However, some differences were observed after 10 days probably due to better yeast colonisation and growth on wheat during the early stages in the storage experiments. A decrease in mould populations coincided with a decrease in OTA production. Osmoprotected fluidised bed dried cells reduced OTA production. The isotonic solution itself had an influence on OTA production but had no differential influence on fungal populations. This study has also shown that formulation additives had no adverse effect on mould growth and OTA production. Our studies did not involve storage under airtight or microaerophilic environmental conditions where this yeast has been previously shown to be very effective against the spoilage fungus *P. roqueforti* (Björnberg and Schnürer, 1993; Druverfors et al.,

2002). However, the laboratory storage experiments suggests that even under normal oxygen conditions the yeast formulations are able to provide control of OTA production.

For effective use in controlling the growth and mycotoxin production by spoilage fungi it is essential that potential BCAs can tolerate a wide range of environmental factors, particularly intermediate moisture conditions. The approach presented here can be used to develop ecologically competent BCA candidates such that the potential for success fan be improved. This could provide opportunities for wider implemention of such approaches for minimising mycotoxin contamination of food and feed by utlilizing this ecophysiological strategy.

**ACKNOWLEDGEMENTS**

We are very grateful to Prof. J. Schnürer for the *P. anomala* J121 strain. Stella Mokiou is also grateful to the Konstantinos Lazaridis and Alexander Onassis foundations for support.

**REFERENCES**

Abadias M, Usall J, Teixido N, Vinas I. 2003. Liquid formulation of postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. Phytopathology 93: 436–442.

Andersen M, Magan N, Mead A, Chandler D. 2006. Development of a population based threshold model of conidial germination for analysing the effects of physiological manipulation on the stress tolerance and infectivity of insect pathogenic fungi. Environmental Microbiology 8:1625-1634.

Bayrock D, Ingledew WM. 1997. Mechanism of viability loss during fluidised bed drying of baker's yeast. Food Research International 30**:**417-425.

Beker MJ, Rapoport AI. 1987. Conservation of yeasts by dehydration. Advances in Biochemichal Engineering and Biotechnology 35:127-171.

Björnberg A, Schnürer J. 1993. Inhibition of the growth of grain-storage moulds in vitro by the yeast *Pichia anomala* (Hansen) Kurtzman. Canadian Journal of Microbiology 39**:** 623-628.

Cairns-Fuller V, Aldred D, Magan N. 2005. Water, temperature and gas composition interactions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain. Journal of Applied Microbiology 99:1215-1221.

Crowe JH, Crowe LM, Carpenter JF, Winstrom CA. 1987. Stabilization of dry phospholipids bilayers and proteins by sugar. Biochemical Journal 242**:** 1-10.

Druverfors U, Jonsson N, Boysen ME, Schnürer J. 2002. Efficacy of the biocontrol yeast *Pichia anolmala* during long term storage of moist feed grain under different oxygen and carbon dioxide regimens. FEMS Yeast Research 2: 389-394.

Guijarro B, Larena I, Melgarejo P, De Cal A. 2006. Effect of drying on conidial viability of *Penicillium frequentans,* a biological control agent against peach brown rot disease caused by *Monilinia* spp.1. Biocontrol Science and Technology 16: 257-269.

Hallsworth JE, Magan N. 1995. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. Microbiology 141: 1109-1115.

Hutcheson TC, McKay T, Farr L, Seddon B. 1988. Evaluation of the stain viablue for the rapid estimation of viable yeast cells. Letters in Applied Microbiology 6: 85-88.

Janisiewicz WJ, Jeffers, SN. 1997. Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. Crop Protection 16**:** 629-633.

Larena I, De Cal A, Linan M, Melgarejo P. 2003. Drying of *Epicoccum nigrum* conidia for obtaining a shelf-life stable biological product against brown rot disease. Journal of Applied Microbiology 94: 508-514.

Magan, N. (2001). Physiological approaches to improving ecological fitness of biocontrol agents. Chapter 9 in *Fungal Biocontrol Agents: Progress, Problems and Potential,* eds. T.Butt, C.Jackson & N.Magan. Cabi BioSciences. Pp.239-252.

Magan N. 2006. Ecophysiology of biocontrol agents for improved competence in the phyllosphere. In Microbial Ecology of Aerial Plant Surfaces, eds. M.J. Bailey, A.K. Lilley, T.M. Timms-Wilson and P.T.N. Spencer-Phillips. CABI International, Wallingford, U.K., pp. 150-164.

Magan N, Olsen M. 2004. Mycotoxins in food: detection and control. Woodhead, Oxford. U.K.

Mokiou S, Magan N. 2002. Ecophysiological manipulation of the fermentation process improves viability of the biocontrol yeast *Pichia anomala*. Biocontrol of Fungal and Bacterial Plant Pathogens, IOBC wprs Bulletin 25: 395-398.

Melin P, Hakansson S, Eberhard TH, Schnurer J. 2006. Survival of the biocontrol yeast *Pichia anolmala* after long term storage in liquid formulations and different temperatures, assessed by flow cytometry. Journal of Applied Microbiology 100: 264-271.

Melin P, Hakansson S, Schnürer J. 2007. Optimisation and comparison of liquid and dry formulations of the biocontrol yeast *Pichia anomala* J121. Applied Microbiology and Biotechnology 73: 1008-1016.

Teixido, N, Vinas I, Usall J, Magan N. 1998. Control of blue mould of apples by preharvest application of *Candida sake* grown on media with modified water activity. Phytopathology 88: 960-964.

Ypsilos IK, Magan N. 2004. The effect of water activity, modifying solute and culture age on *Metarhizium anisopliae* blastospore production, quality and germinability. Mycological Research 108: 1337-1345**.**

Zayed G, Roos YH. 2004. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Proceedings of Biochemistry 39: 1081–1086.

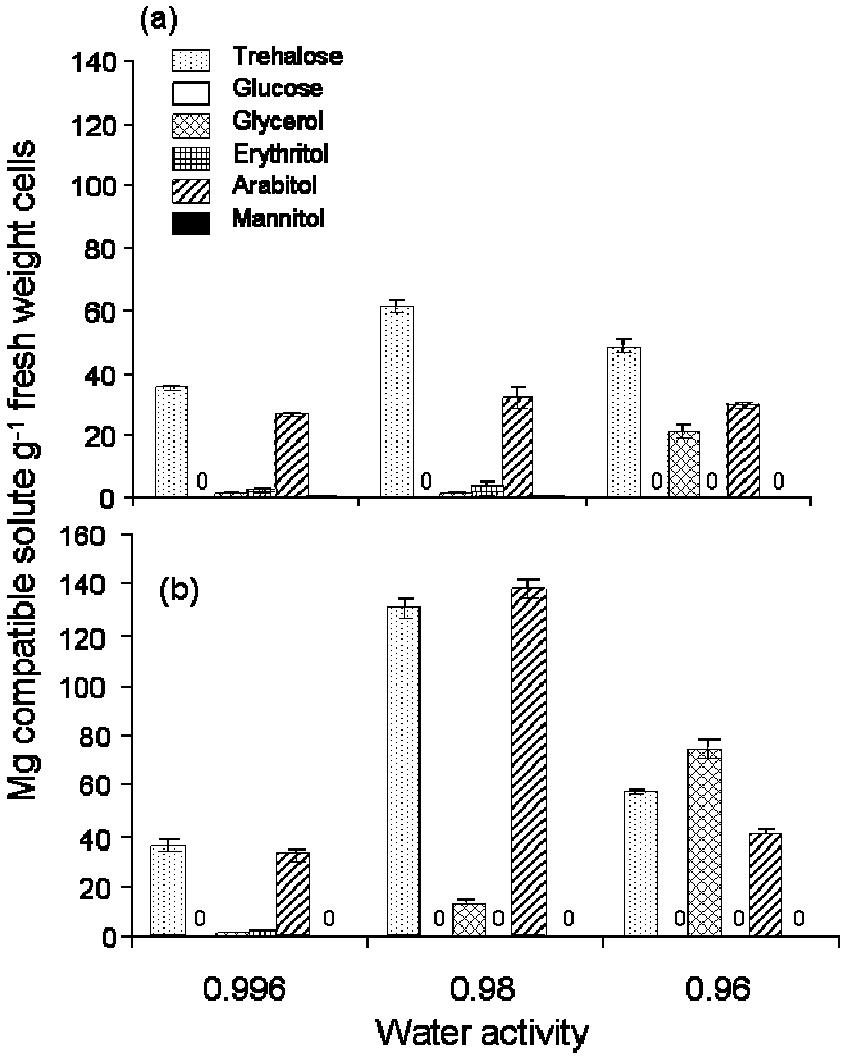
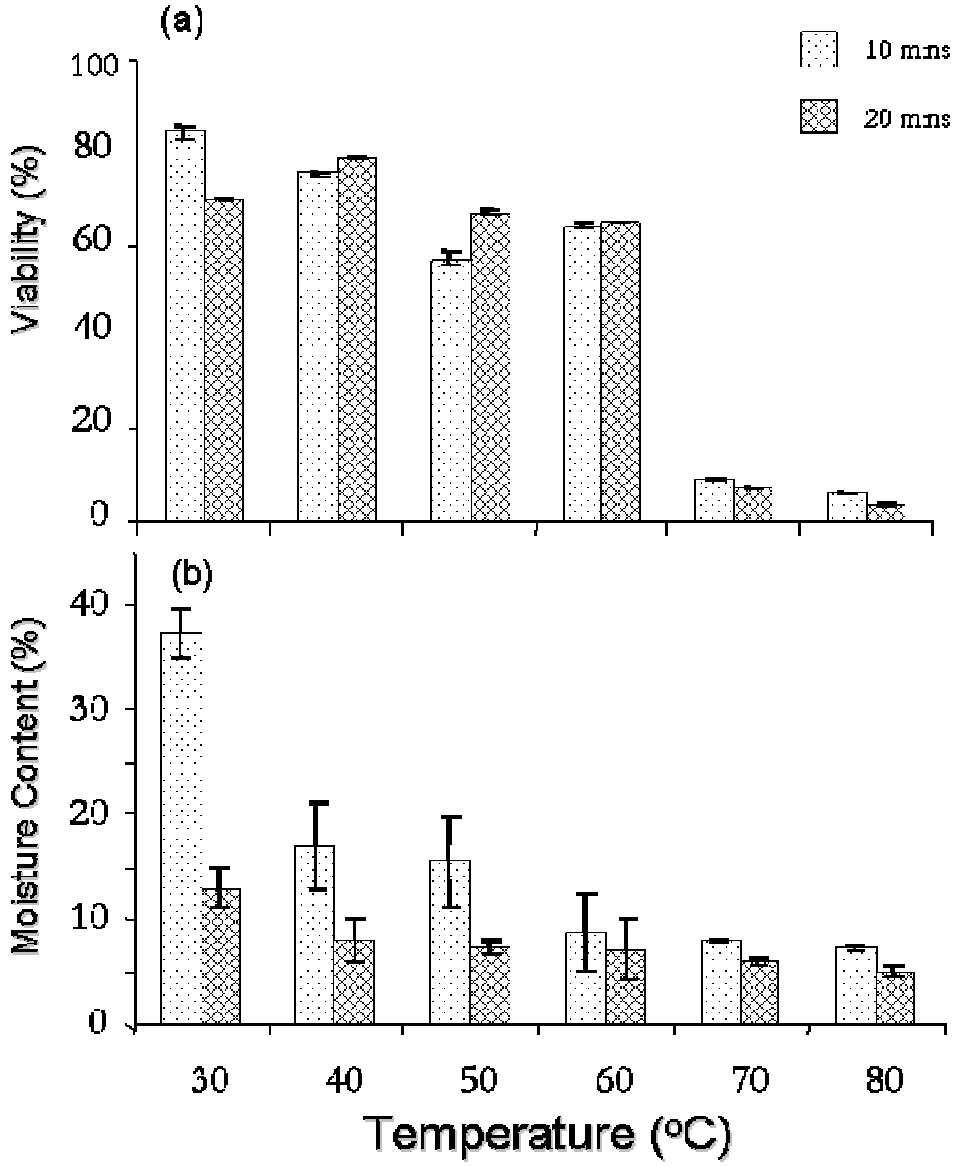



Figure 1. Mokiou & Magan

Figure 1. Comparison of endogenous sugar alcohols and sugar reserves in *Pichia anomola* cells after growth on an unmodified molasses-based medium and that modified with proline to 0.98 and 0.96 $a_w$ at 25$^{o}$C for 48 hrs. (a) cells harvested in water and (b) cells harvested with isotonic NaCl solutions. Bars indicate standard error of the means.




Figuro 2. Mokiou & Maçan

Figure 2. Effect of temperature and time on (a) cell viability and (b) moisture content of *Pichia anomola* cells dried in the fluidised bed dryer. Cells were grown on a molasses-based medium modified to 0.98 $a_w$ with proline at 25°C for 48 hrs. Bars indicate standard error of the means.

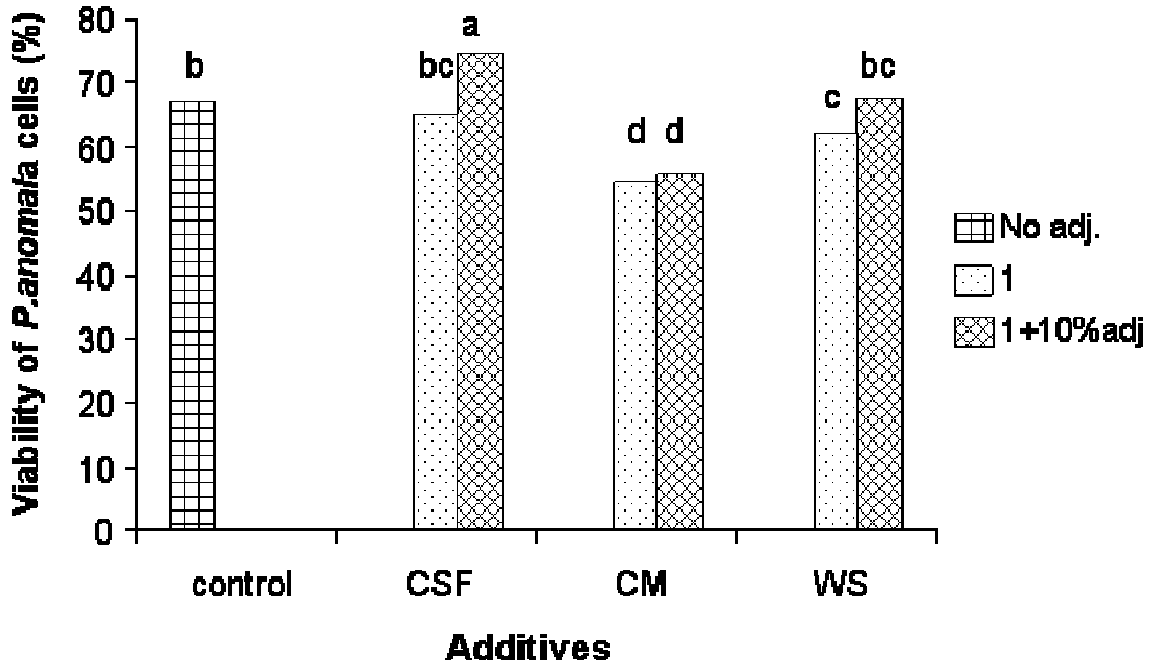



Figure 3. Mokiou & Magan

Figure 3. Effect of different additives used as carriers (100% or 1:1 w/w) such as corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability after drying at 50$^{o}$C for 20 mins using a fluidized bed-dryer. Control is untreated *P.anomala* cells. Different letters indicate statistical differences ($P<0.05$) between means.

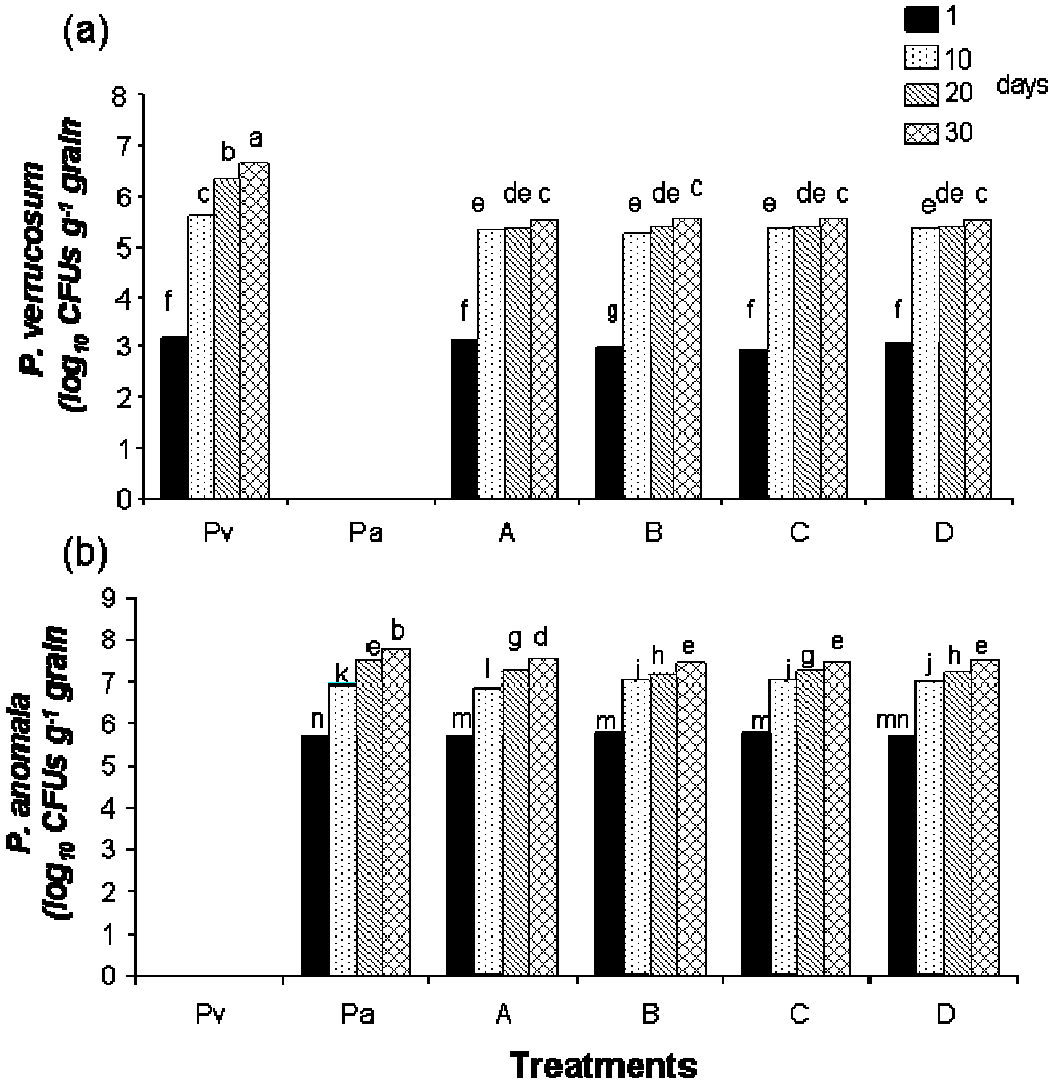
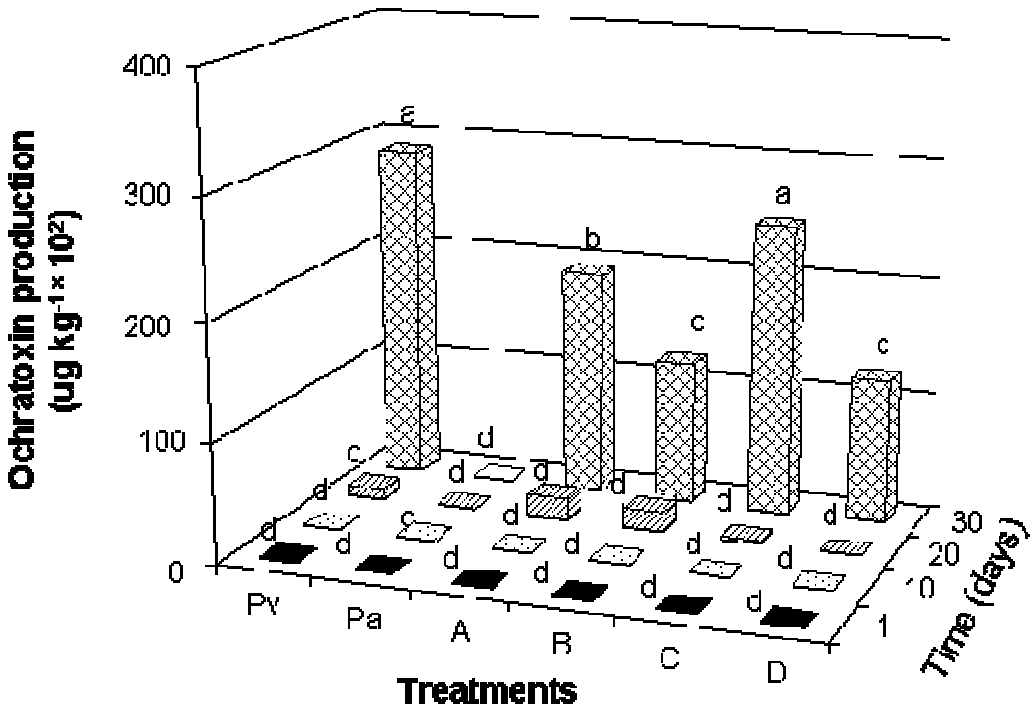



Figure 4. Mokiou & Magan

Figure 4. Temporal changes in populations of (a) *P. verrucosum* and (b) *P. anomala* in stored wheat grain treatments over a 30 day period. Different letters indicate significant differences (P=0.05). Treatments were: *P. verrucosum* alone (Pv, control) or *P. anomala* (Pa, control) unmodified fresh cells alone; (A), *P. anomala* fresh cells grown on modified proline medium washed with NaCl isotonic solution; (B), *P. anomala* unmodified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation; (C), *P. anomala* proline modified cells dried as cottonseed flour:1+10% skimmed milk formulation washed with water; (D), *P. anomala* proline modified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with NaCl isotonic solution. A, B, C, D treatments were co-cultures with *P. verrucosum*. Different letters indicate statistical (P<0.05) differences between means.




F gure 5. Mokiou & Magan

Figure 5. Effect of different treatments on ochratoxin A (OTA; μg $kg^{-1}$) in co-cultures on wheat grain [0.93 $a_w$] at 25°C for up to 30 days. Different treatments were *P. verrucosum* alone (control), *P. anomala* unmodified fresh cells alone; (A), *P. anomala* proline modified fresh cells washed with NaCl isotonic solution; (B), *P. anomala* unmodified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation; (C), *P. anomala* proline modified cells dried as cottonseed flour:1+10% skimmed milk formulation washed with water; (D), *P. anomala* proline modified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with NaCl isotonic solution. A, B, C, D treatments were co-cultures with *P. verrucosum*. Different letters indicate statistical (P=0.05) differences between means.

https://dspace.org/

# Physiological manipulation and formulation of the biocontrol yeast Pichia anomala for control of Penicillium verrucosum and ochratoxin A contamination of moist grain.

**Mokiou, Stella; Magan, Naresh**

**2008**
Taylor and Francis
http://dx.doi.org/10.1080/09583150802585769
Stella Mokiou, Naresh Magan, Physiological manipulation and formulation of the biocontrol yeast Pichia anomala for control of Penicillium verrucosum and ochratoxin A contamination of moist grain. Biocontrol Science and Technology, Volume 18, Issue 10, 2008, pp 1063-1073

Downloaded from a DSpace repository. https://dspace.org/