Corvinus University of Budapest
Faculty of Food Science

INHIBITION OF FRUIT AND VEGETABLE SPOILAGE MOULD *PENICILLIUM EXPANSUM* WITH YEASTS

ANDREA TACZMAN-BRÜCKNER

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Name: PhD School of Food Science

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Head: Prof. András Fekete
Corvinus University of Budapest

Supervisor: Csilla Mohácsi-Farkas
Corvinus University of Budapest

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Head of PhD School Supervisor

BACKGROUND

Postharvest losses of fruits and vegetables range widely from 10% to 40% (WORLD RESOURCES, 1998). Microbiological spoilage – mainly decay caused by fungi – might represent also food safety risks besides high economic losses.

Fungicid treatments during cultivation and storage are the primary methods for controlling infections and spoilage. Public and scientific concern about synthetic chemicals in our food and environment has been increasing in the past decades. Moreover, several fungicides have been banned in the USA, and this trend is continuing to decrease the use of chemicals during the postharvest period (WISNIEWSKI and WILSON, 1992). For this reason it was and it has been important to develop new methods for controlling postharvest diseases.

Concept and subsequently the practice of biological control – using antagonistic microorgansims to inhibit plant pathogenic moulds – during fruit-, vegetable- and grain storage has become more and more widespread in the last decades. Biocontrol yeasts and bacteria derive mainly from crops that are attacked by pathogenic fungi. Therefore the use of the antagonistic organisms is generally limited to the variety of the fruit or vegetable, or to the region.

Biological control is only part of plant cultivation and integrated plant protection in Hungary. Promising results of application of antagonistic organisms to reduce losses all over the world urge to make known and realize this method in the Hungarian fruit and vegetable storage, too.

AIMS OF THE WORK

1. To outline the international literature referring to spoilage during fruit and vegetable storage, methods applied for prevention of spoilage with emphasis on biological control and the methodology of investigations connected with biocontrol agents.
2. Screening yeasts antagonistic against *Penicillium expansum*: (a) yeasts isolated from fruits; (b) selecting yeast species not deriving from fruits.
3. Investigation of antagonistic efficiency, mode of action, and applicability in practice (on fruit, under circumstances modelling storage) of the selected yeast strain or isolate.

Culture media

Malt-glucose medium (MG); Potato-dextrose medium (PDA); yeast extract-peptone-dextrose medium (YEPD); methylene blue medium; apple medium; Rose bengal-chloramphenicol medium (RBC).

Microorganisms

a) Moulds
   Penicillium expansum NCAIM F00811
   Penicillium expansum NCAIM F00601
b) Yeasts
   Kluyveromyces lactis NCAIM Y01080, NCAIM Y0258, NCAIM Y00260
   Metschnikowia pulcherrima NCAIM Y00681
   Sporobolomyces roseus NCAIM Y00693
   Pichia anomala (Hansen) Kurtzman (J121)
   Saccharomyces cerevisiae S6 killer sensitive strain

Methods

Yeast isolation from apple

Yeast strains were isolated from 4 apple varieties cultivated in Fejér county and Pest county. API ID 32 C test was applied for yeast identification.

***Screening yeasts antagonistic against* Penicillium expansum**: from strains deriving from culture collections, Tokaj wine, aszú berries or isolated from apple.

Investigation of inhibitory effect of antagonistic yeasts

List of yeasts applied against mould strains in the inhibition experiments:

Mould strain	Antagonistic yeast strain
Penicillium expansum F00811	*Metschnikowia pulcherrima* Y00681 *Sporobolomyces roseus* Y00693 *Pichia anomala* (J121) *Kluyveromyces lactis* Y00260, Y0258, Y01080 *Metschnikowia pulcherrima* isolated from apple
Penicillium expansum F00601	*Metschnikowia pulcherrima* Y00681 *Sporobolomyces roseus* Y00693 *Pichia anomala* (J121) *Kluyveromyces lactis* Y00260 *Metschnikowia pulcherrima* isolated from apple

According to the method of BJÖRNBERG and SCHNÜRER (1993) at 25°C, 15°C and 5°C, on MG and PDA media.. Mould colony diameters were measured, diameters of whole colonies (aerial mycelium + substrate mycelium + conidia forming part) and that of the conidia forming part were distinguished.

***Investigations of mode of action of* Kluyveromyces lactis**

- Effect of cell free culture filtrate
- Killertoxin production of *Kl. lactis*
- Effect of gaseous compounds produced by *Kl. lactis*
  Inhibitory effect of volatile compounds was investigated according to the method of DRUVEFORS (personal communication) in „mouth-to-mouth" plates: antagonistic yeast was inoculated on the lower plate, and mould was inoculated on the upper plate. Mould colony diameters were measured. Main components of the volatile compounds in the mouth-to-mouth plates were identified by GC/MS method. Effect of single components on *P. expansum* F00811 was investigated with the application of the commercially available compounds in 1 and 10% concentration. In order to determine the CO_2 quantity produced by *Kl. lactis* CO_2/O_2 concentration was measured with gas analyser on day 2 and 5 of incubation at 25°C.
- Microscopic investigations on direct effect of *Kl. lactis* on mould conidia germination and growth
- Investigations on growth of *Kl. lactis* strains on MG and PDA medium

***Effect of yeasts on patulin production by* P. expansum**

Patulin production of mould strains was investigated in two steps: (1) mycotoxin production of the *P. expansum* strains was observed as a function of incubation time; (2) effect of antagonistic yeasts (*Kl. lactis*, *M. pulcherrima*) was investigated on patulin production of *P. expansum* F00811. Mycotoxin content of samples was determined by HPLC.

Effect of combined treatments

Effect of antagonistic yeasts combined with modified atmosphere storage (6% and 22% CO_2) was investigated as described in chapter *Investigation of inhibitory effect of antagonistic yeasts*.

In vivo experiments

- Inhibitory effect of two antagonistic yeasts against *P. expansum* strains on apple medium was carried out as in chapter *Investigation of inhibitory effect of antagonistic yeasts*.
- Effect of antagonistic yeasts on artificially wounded apple. Lesion diameters were measured.

Statistical methods

Statistical functions of Microsoft Excel, STATGRAPHICS 5.1 were used: Duncan's t-test (P=0,05), Multifactor Variance Analysis.

RESULTS

***Screening yeasts antagonistic against* Penicillium expansum**

Partial (inhibition of conidia formation) or total (inhibition of conidia and aerial hyphae formation) inhibition was shown in the presence of *Kl. lactis* Y00260, *Kl. lactis* Y0258, *Kl. lactis* Y00251, *Pich. anomala* J121, *Geotrichum candidum*, and of 4 isolates deriving from apple and identified as *Metschnikowia pulcherrima*.

Antagonistic efficiency of yeast strains

- Antagonistic efficiency of three biocontrol yeasts

The inhibitory effect of *M. pulcherrima*, *Sp. roseus* and *Pich. anomala* – known as biocontrol agents – was analysed in the function of *P. expansum* strain, concentration of antagonistic yeast suspension, applied culture media and temperature.

- The antagonistic efficiency of *Kluyveromyces lactis*

No practically significant difference was observed among antagonsitic activities of three *Kl. lactis* strains at any temperature investigated. Therefore for further comparison the strain Y00260 was selected.

Inhibitory effect of *Kl. lactis* was highly influenced by the factors of the experiment, similarly as in case of the three above mentioned biocontrol yeasts: *Kl. lactis* showed minimal inhibitory effect against the whole colonies of *P. expansum* strain F00811 on MG medium. On the contrary, whole colonies of the mould strain F00601 were significantly inhibited by *Kl. lactis* on the same culture medium. *Kl. lactis* was more effective on PDA medium against both mould strains.

Considering the inhibition on the conidia forming part *Kl. lactis* was very effective against *P. expansum* strains under any set of conditions.
Inhibitory effect of *Kl. lactis* increased when higher concentrations of yeast suspension were applied. Furthermore, application of antagonistic yeast in combination with reduced storage temperature (15°C, 5°C) enhanced the inhibition of *P. expansum* growth compared to the effect of the temperature or the yeast only.
Biocontrol activity of *Kl. lactis* NCAIM Y00260 was similar to strains of *M. pulcherrima*, *Sp. roseus* and *Pich. anomala,* yeasts used as antagonistic agents against mould growth *in vitro* and *in vivo*. Therefore *Kl. lactis* is a possible biocontrol agent.

***Mode of action of* Kluyveromyces lactis *against* Penicillium expansum**

No killertoxin production of *Kl. lactis* Y00260 was detected. Microscopic investigations did not refer to direct interaction between yeast cells and mould conidia or hyphae. Investigating the effect of cell free culture filtrate of *Kl. lactis* against *P. expansum*, slight inhibition was detected in case of *P. expansum* strain F00601. Applying the concentrated supernatant no inhibition was observed. Therefore, the mode of action of *Kl. lactis* can not be explained with the production of antibiotic substances.
Investigating the effect of volatile and gaseous compounds produced by *Kl. lactis* strains, significant inhibition was detected in case of both *P. expansum* strains at 22°C, on MG medium. The same inhibitory effect on PDA medium was developed only by strain Y00260. At low temperature storage volatile and gaseous compounds did not show significant inhibition.
Analysing the volatile compounds produced by *Kl. lactis* Y00260, five components – iso-valeric acid, iso-butyric acid, fenil-iso-butyrate, iso-amyl alcohol and ethyl acetate – were identified. Investigating the effect of these components separately, smaller inhibition was observed as applying *Kl. lactis* in the mouth-to-mouth plates.
Atmosphere in the mouth-to-mouth plates was strongly influenced by metabolism of *Kl. lactis* Y00260. Carbon-dioxid concentration increased from 0.1% to 8-10%, and oxygen concentration decreased from 20.5% to 13-14%.
Investigating the effect of culture media (MG and PDA) on growth of *Kl. lactis* strains no significant difference was shown between the two media at 25°C. Differences detected at low temperature storage (5°C) did not influence the inhibitory efficiency of the yeasts.

***Effect of yeasts on patulin production of* Penicillium expansum**

P. expansum strain F00811 produced, while strain F00601 did not produce high amounts of patulin under the same conditions. Intensive patulin production of strain F00811 was observed for the first six days of incubation, but the quantity of patulin was less on day 9. The quantity of patulin was ten times less in mixed cultures of *P. expansum* and antagonistic yeasts (*Kl. lactis* or *M. pulcherrima*) than in the pure mould culture.

Application of combined treatments

Applying modified atmosphere storage and *Kl. lactis* Y00260 together, smaller mould colony diameters were measured at higher CO_2 level in the atmosphere. Using *M. pulcherrima* as antagonistic organism mostly no growing effect of increased CO_2 concentration was detected.

Additive inhibitory effect was observed when lower temperature storage (15°C, 5°C) and antagonistic yeasts were applied in combination. Storage at 5°C and applying *Kl. lactis* did not develop additive effect.

Results of in vivo experiments

In case of *Kl. lactis* greater inhibition was observed on apple medium than on MG or PDA medium almost at any set of condition. Inhibitory effect of *M. pulcherrima* isolated from apple was greater than that of *Kl. lactis* on MG and PDA media, but similar on apple medium. Inhibitory effect of *M. pulcherrima* strain isolated from apple was more significantly higher than that of the strain derived from the culture collection.

Inhibition of *P. expansum* on apples from summer cultivar was observed applying *M. pulcherrima* at low temperature storage (7°C).

M. pulcherrima isolated from apple and *Kl. lactis* Y00260 developed significant inhibition against *P. expansum* at both storage temperatures (25°C, 7°C) on apples from cold storage. The efficiency of the two yeast strains was similar but smaller than on apple medium.

CONCLUSION AND PROPOSAL

Investigating the antagonistic effect of several yeasts the influence of the different factors – mould strain, concentration of antagonistic yeast suspension, culture media, storage temperature – was observed. The two *P. expansum* strains showed great differences in sensitivity against the yeasts, therefore investigations clearing the genetic background of these mould

strains would give more knowledge about interaction between mould and antagonistic yeasts. Direct relationship was observed between the inhibitory efficiency of yeasts and the applied cell concentration, or storage temperature. Similar to the results of MCLAUGHLIN and co-workers (1990), and FAN and TIAN (2001): the mould inhibition increased when yeast suspension of higher cell density was applied. Combined treatment with lower temperature (15°C, 5°C) and antagonistic yeasts resulted additive inhibitory effect that was reported also by BJÖRNBERG and SCHNÜRER (1993).

Applied strains of *Kl. lactis* can be determined as potential biocontrol agents on the basis of comparison of the inhibitory effect with biocontrol yeasts. The investigated *Kl. lactis* strains having good inhibitory effect were not isolated from fruits, vegetables or other crops, therefore its application may not get limited to a given crop or region.

Investigating the mode of action of *Kl. lactis* Y00260 there are only indirect proofs for competition for nutrients similarly as reported by MCLAUGHLIN and co-workers (1990) and PIANO and co-workers (1997): on one hand because inhibitory efficiency of *Kl. lactis* was growing when the concentration of the applied yeast suspension was higher, on the other hand because using culture media of various composition differences in inhibition were detected. Different nutrients present in culture media did not influence yeast growth, but some of the components may be the bottle-neck for mould growth. Investigating the antagonistic effect of *M. pulcherrima* isolates on six different culture media SPADARO and co-workers (2002) found that nutrients do not influence only the competition between yeast and mould, but the metabolism of the organism as well.
Investigations on the effect of volatile compounds prove that volatiles produced by *Kl. lactis* (iso-valeric acid, iso-butyric acid, iso-amyl alcohol, ethyl acetate and fenil-iso-butyrate) contribute to inhibition. Comparing the inhibitory efficiency of the volatiles applied separately and produced by *Kl. lactis* we can conclude that the mixture of volatiles produced constantly by the yeast in relatively low concentration and develop a greater inhibition than single volatiles applied once in higher concentration. Changes in composition of the gas atmosphere (decrease in O_2 / increase in CO_2 concentration) as a result of microbial metabolism might contribute to the inhibitory effect of volatile compounds but are not the only reason for inhibition. Inhibition developed by volatile compounds is confirmed also

by DRUVEFORS (2004) who observed the mould inhibitory effect of ethyl acetate produced by *Pichia anomala*.
Combined treatments (antagonistic yeast + modified atmosphere storage, or antagonistic yeast + cold storage at 15°C) enhanced mould inhibition developed by *Kl. lactis* same like in case of biocontrol yeasts. Enhanced inhibitory effect of *Candida sake* against *P. expansum* on CA stored apple was reported by USALL and co-workers (2000). Disadventage of *Kl. lactis* regarding practical application is that no additive effect in inhibition was observed when it was applied at 5°C. Therefore, main factors of fruit storage should be considered when new antagonistic yeasts are going to be screened (e.g. screening at low temperature, isolating yeasts from cold stored apples).
Results of investigations with volatiles and of combined treatments suggest that the most effective application of *Kl. lactis* as biocontrol agent could be realized in modified atmosphere packaged products considering the inhibitory effect of antagonistic yeasts enhanced by higher CO_2 and lower O_2 level and the appropriate concentration of volatiles present in the smaller air space.

Practical application of *Kl. lactis* was confirmed by the inhibition observed on stored apples. In the future it is essential to modell practical application more realistically: to investigate the antagonistic effect of the yeast also on the whole surface of the fruit.
Although smaller inhibition was achieved on apple than on apple medium, it is important to use culture media based on fruit or vegetable composition to get a closer prognosis about the yeast – mould interaction on the crop.
Promising results were achived when the effect of antagonistic yeasts on patulin production of *P. expansum* was investigated. It would be worth-while to continue these investigations regarding food safety and basic research aspects.
Genetic background of the antagonistic organism, the mould or the mode of action of the inhibition was rarely investigated in connection with biological control during fruit and vegetable storage. More emphasis should be set on this kind of research in order to get more knowledge on biocontrol organisms, to extend the practical applicability, or to enhance the inhibitory effect due to genetic intervention.

NEW SCIENTIFIC RESULTS

1. In this work the antagonistic effect of a yeast not derived from fruits or vegetables was investigated for the first time. *Kluyveromyces lactis* is a potential biocontrol agent regarding the results of *in vitro* experiments and investigations modelling practical application (combined treatment, using apple).

2. Besides competition for nutrients, volatile compounds and CO_2 produced by *Kluyveromyces lactis* play important part in the mode of action of inhibition.

3. Considering the effect of *Kluyveromyces lactis* and *Metschnikowia pulcherrima* on patulin production of *Penicillium expansum* application of antagonistic yeast is advantageous not only of economical point of view but in terms of food safety as well.

4. Yeast strains effective against *Penicillium expansum* isolated from apple were identified as *Metschnikowia pulcherrima* strains. *Metschnikowia pulcherrima* isolated from apple was more effective in controlling mould than the *Metschnikowia pulcherrima* strain derived from culture collection.

REFERENCES

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

DRUVEFORS, U.A. (2004): Yeast biocontrol of grain spoilage moulds. Mode of action of *Pichia anomala*. Doctoral thesis. Swedish University of Agricultural Sciences. Uppsala. p. 29.

FAN, Q., TIAN, S. (2001): Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus* (Saito) Skinner. *Postharvest Biology and Technology*. 21, 341-350.

MCLAUGHLIN, R.J.; WISNIEWSKI, M.E.; WILSON, C.L.; CHALUTZ, E. (1990): Effects of inoculum concentration and salt solutions on biological control of postharvest diseases of apples with *Candida* sp. *Phytopathology.* 80, 456-461.

PIANO, S., NEYROTTI, V., MIGHELI, Q., GULLINO, M. L. (1997): Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. *Postharvest Biology and Technology*. 11, 131-140.

SPADARO, D., VOLA, R., PIANO, S., GULLINO, M.L. (2002): Mechanism of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. *Postharvest Biology and Technology*. 24, 123-134.

USALL, J.; TEIXIDÓ, N.; FONS, E.; VIÑAS, I. (2000): Biological control of blue mould on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *International Journal of Food Microbiology*. 58, 83-92.

WISNIEWSKI, M.E.; WILSON, C.L. (1992): Biological control of postharvest diseases of fruits and vegetables: recent advances. *Hortscience*. 27, 94-98.

WORLD RESOURCES (1998): Environmental change and human health. – Disappearing food. How big are postharvest losses? http://population.wri.org/pubs_content_print.cfm?ContentID=1385

PUBLICATIONS

Scientific journals

- Taczman-Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs.; Kiskó, G.: Comparison of biocontrol activity of *Kluyveromyces lactis* with other yeast strains against *Penicillium expansum*. Acta Alimentaria, 34. (1), 71-80.
- Taczman-Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs.; Kiskó, G.: Mode of action of *Kluyveromyces lactis* in biocontrol of *Penicillium expansum*. Acta Alimentaria, 34. (2), 153-160.

Abstracts

- Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: *Penicillium expansum* gátlása élesztőkkel in vitro és in vivo körülmények között. Magyar Mikrobiológiai Társaság Évi Nagygyűlés, Balatonfüred, 2002. október 8-10. Előadások és poszterek összefoglalói 17.
- Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: Investigation of the biocontrol effect of yeasts on *Penicillium expansum*. Croatian, Hungarian and Slovakian Symposium on Industrial Microbiology and Microbal Ecology. Power of Microbes in Industry and Environment. Opatija, Croatia. 7-9. June, 2002. Book of Abstracts p.73.
- Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: Biocontrol effect of *Kluyveromyces lactis* strains and *Pichia anomala* against *Penicillium expansum* 1st FEMS Congress of European Microbiologists. Ljubljana, Slovenia. 29. June – 3. July, 2003. Book of Abstracts p. 433.
- Brückner, A.; Mohácsi-Farkas, Cs.; Kiskó, G.; Balla, Cs: Antagonistic effect of some yeast strains against *Penicillium expansum*. 23rd

International Specialised Symposium on Yeasts. Budapest, Hungary. 26-29. August, 2003. Book of Abstracts p. 110.

- Taczman-Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: Inhibition of *Penicillium expansum* with combined treatments. 14th International Congress of the Hungarian Society for Microbiology. Balatonfüred, Hungary. 9-11. October, 2003. Book of Abstracts p. 204.
- Taczman-Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: Mode of action of inhibition of *Penicillium expansum* with *Kluyveromyces lactis* strains. Lippay János – Ormos Imre – Vas Károly Scientific Symposium. Budapest, Hungary. 6-7 November, 2003. Book of Abstracts p.164-165.
- Taczman-Brückner, A.; Mohácsi-Farkas, Cs.; Balla, Cs: Inhibition of mold growth with CO_2 and volatile compounds produced by *Kluyveromyces lactis*. 2nd Central European Congress of Food. Budapest, Hungary.26-28. April, 2004. Book of Abstracts p.275.
- Taczman-Brückner, A., Kiskó, G., Mohácsi-Farkas, Cs., Balla, Cs., Horváth, O.: Effect of antagonistic yeasts on patulin production of *Penicillium expansum*. The Safe Consortium Seminar series 5., Contaminants and influence of agricultural practices. March 18-19, 2004, Brussels, Book of Abstracts 53.