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Yeast Biocontrol of Grain Spoilage Moulds

Mode of Action of *Pichia anomala*

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// Abstract

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The biocontrol yeast *Pichia anomala* J121 prevents mould spoilage by *Penicillium roqueforti* and prolongs the safe storage of moist cereal grain in malfunctioning airtight storage systems. *P. anomala* is naturally occurring on grain and is classified as safe, *i.e.* biosafety level 1 microorganism. Strain J121 does not grow above 37°C and is sensitive to commonly used antifungal drugs.

The ability of *P. anomala* to prevent mould growth during 14 months of wheat grain storage was evaluated in outdoor silos with different air permeability. *P. anomala* rapidly consumed O_2 leaking into the silo during the spring, and *P. roqueforti* did not grow in treatments inoculated with *P. anomala*.

A connection between production of the ester ethyl acetate and biocontrol activity was found. The biocontrol effect was enhanced when complex medium or glucose was added to grain minisilos. The addition did not markedly influence yeast cell numbers, but products of glucose metabolism, ethyl acetate in particular and ethanol, increased. When a diploid and a haploid strain of *Pichia anomala* were tested at two water activities (a_w) for biocontrol ability in grain minisilos, the two yeast strains grew and inhibited mould growth equally well and showed similar patterns of ethyl acetate production at the higher a_w. However, at lower a_w the growth, biocontrol performance and ethyl acetate production of the haploid strain were reduced.

The biocontrol activity of an additional 57 yeast species was evaluated in grain minisilos. Most yeast species grew to levels comparable to that of *P. anomala* J121, but only six other species strongly inhibited *P. roqueforti*. The biocontrol activity of *Candida fennica* and *Candida silvicultrix* is reported for the first time. The ability of 27 yeast species to grow to high CFU values without inhibiting mold growth, and the increase in biocontrol activity after nutrient addition, indicates that nutrient competition is not the main reason for mould inhibition in the system. Instead, a combination of ethyl acetate production and rapid consumption of oxygen leaking into the system could be the main inhibitory mechanism.

Key words: *Pichia anomala*, *Penicillium roqueforti*, ethyl acetate, competition for nutrients, airtight storage, *Candida fennica*, *Candida silvicultrix*, *Pichia farinosa*, antifungal, biocontrol

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Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Fredlund, E., Druvefors, U. Boysen, M.E., Lingsten, K-J. & Schnürer, J. (2002). Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. *FEMS Yeast Research.* 2:395-402.

II. Druvefors, U., Jonsson, N., Boysen, M.E. & Schnürer J. (2002) Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. *FEMS Yeast Research.* 2: 389-394.

III. Druvefors, U.Ä. & Schnürer, J. Mould inhibitory activity of different yeast species during wheat grain storage. (Submitted).

IV. Druvefors, U.Ä., Passoth, V. & Schnürer, J. Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 in cereal grain. (Manuscript).

V. Fredlund, E., Druvefors, U.Ä., Olstorpe, M.N., Passoth, V. & Schnürer, J. Influence of ethyl acetate production and ploidy level on the anti-mould activity of *Pichia anomala*. (Submitted).

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Introduction

Throughout history, storage of cereal grain has provided humans with a buffer against crop failure and starvation.

Genesis 47:56

> [47] During the seven years of abundance the land produced plentifully. [48] Joseph collected all the food produced in those seven years of abundance in Egypt and stored it in the cities. In each city he put the food grown in the fields surrounding it. [49] Joseph stored up huge quantities of grain, like the sand of the sea; it was so much that he stopped keeping records because it was beyond measure….. [56] When the famine had spread over the whole country, Joseph opened the storehouses and sold grain to the Egyptians, for the famine was severe throughout Egypt.

Archaeological findings tell us that the ancient Egyptians established large-scale storage of food and seed reserves approximately five thousand years ago (c. 2920-2770 BC). The first storage systems were large baskets made of reeds or clay jars that were immersed in the soil. During the Middle Kingdom (c. 2040-1785 BC) larger storage facilities, with a capacity for up to 40 000 grain sacks, were employed (Sinha, 1995).

In temperate countries such as Sweden, harvesting cereal grain at high moisture content can protect the grain from prolonged exposure to wet weather conditions, which would otherwise cause weathering and mould infections of grain in the field. Instead, efforts are devoted to preserving the grain in order to maintain its microbiological and nutritional quality. For cereals intended for bread, the baking properties of the flour also have to be considered. High temperature drying is used to preserve the majority of the grain harvested in Sweden today. This is an effective but very energy-consuming method (Pick, Noren & Nielsen, 1989).

For animal feed, airtight storage of the grain is an alternative energy-saving method. However, problems with leakage of air in and out of the silos make the method unreliable and not common on farms. The method relies on a perfect sealing of the silo to retain the carbon dioxide produced by the respiration of the grain and the adhering microorganisms and to keep oxygen out. When this is not achieved, the carbon dioxide leaks out and oxygen enters, especially during the springtime when considerable temperature, and thus pressure, fluctuations occur. This eventually leads to heavy mould growth and the feed grain having to be destroyed (Ekström & Lindgren, 1995).

World-wide, fungal growth destroys large amounts of fruit and vegetables, both pre- and post-harvest (Wilson & Pusey, 1985; Spadaro & Gullino, 2004). Growth of moulds in food and animal feed leads to reduced nutritional values and production of allergenic spores and hazardous mycotoxins (Magan & Lacey, 1987). Traditionally, fungicides have been used to deal with these problems, but factors such as consumer health and environmental concerns, resistance problems and a more strict legislation have made alternatives necessary. During recent

decades, biological control of moulds has evolved as a possibility. Many yeasts and other microorganisms inhibiting plant pathogens have been reported, especially within the fruit and vegetable-producing sector, and several new products have reached the commercial market (Janisiewicz & Korsten, 2002).

The biocontrol ability of the yeast *Pichia anomala* against grain spoilage moulds was first described in 1992 (Ekström, 1992) and since then this yeast has been evaluated at several different scales(Björnberg & Schnürer, 1993; Höglöv & Schnürer, 2002; Petersson, Jonsson & Schnürer, 1999; Petersson & Schnürer, 1995, **II**). The understanding of the mechanism by which a biocontrol organism inhibits the growth of the target pathogen is necessary to be able to register the organism commercially but also for optimizing formulation and application. Production of cell wall degrading enzymes has been suggested as the mode of action of *P. anomala* against *Botrytis cinerea* on apples and grapevine (Jijakli & Lepoivre, 1998; Masih, Alie & Paul, 2000). The mechanisms for *P. anomala* inhibition of *Penicillium roqueforti* in grain storage have not yet been identified.

Aims of the project

The main goal of this thesis work was to understand how *Pichia anomala* prevents growth of *Penicillium roqueforti* in the airtight silo environment.

More specific goals were to:

- Identify characteristic features of *P. anomala* important for mould inhibition
- Investigate the importance of nutrient competition and ethyl acetate production in *P. anomala* biocontrol
- Screen for new potential biocontrol yeasts for use in comparative studies with *P. anomala* or in future yeast combinations

Biological control

Biological control may in simple terms be defined as the use of one living organism to control another. Putting a ladybird on rosebushes infested with greenfly is an obvious example of biological control. Since the first scientific meeting on biocontrol in the 1960s, research and development brought more than 80 biocontrol products to the commercial market by 2001 (Paulitz & Bélanger, 2001). The biological control strategies include natural plant- and animal-derived compounds, as well as antagonistic microorganisms. So far, it seems that the greatest efforts within the research area have concerned fruit, vegetable and flower production. The greenhouses and storage buildings used for these constitute more closed ecosystems, which makes it possible to control and manipulate conditions such as temperature and humidity to optimal conditions for the biocontrol agent. In

addition, greenhouse crops and harvested crops generally have a high economic value and can thus absorb higher cost inputs than many field crops.

Yeast biocontrol

In the end of the 1980s and beginning of the 1990s, a variety of microbial antagonists were reported to control several different pathogens on various fruits. Among these antagonists were many yeasts and yeast-like organisms *e.g. Debaromyces hansenii* (*Pichia guilliermondii,* McLaughlin *et al.*, 1990), for control of post-harvest citrus rot (Wilson & Chalutz, 1989; Droby *et al.*, 1989; Droby, Chalutz & Wilson, 1991; Wisniewski *et al.*, 1991) and several species of *Cryptococcus* for control of post-harvest rot on apples and pears (Roberts, 1990). Both naturally occurring and artificially introduced antagonists were proposed as promising alternatives to fungicide control of post-harvest diseases (Wilson & Wisniewski, 1989; Wisniewski & Wilson, 1992).

Several important properties of yeasts make them useful for biocontrol purposes: For example yeasts do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotic metabolites as possibly produced by bacterial antagonists. Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time. They rapidly utilize available nutrients and can sustain many of the pesticides used in the post-harvest environment. Yeasts can grow rapidly on cheap substrates in fermentors and are therefore easy to produce in large quantities. The suggested modes of action of biocontrol yeasts are not likely to constitute any hazard for the consumer. Furthermore, yeast cells contain high amounts of vitamins, minerals and essential amino acids and several reports on the beneficial effect of yeast addition in both food and feed can be found in the literature (Stringer, 1982; Bui & Galzy, 1990; Hussein *et al.*, 1996).

Commercial yeast biocontrol products

There are currently three commercial yeast biocontrol products available on the market for combating post-harvest decays in fruit. Aspire® (Ecogen, Inc., Langhorne, Pa) is based on the yeast *Candida oleophila* and is used as a spray or dip against post-harvest diseases on pome and citrus fruits (Janisiewicz & Korsten, 2002; Janisiewicz *et al.*, 2003). The product was introduced commercially in the United States in 1996. The commercial product Yield Plus® with *Cryptococcus albidus* as the active antagonist was introduced commercially on the South African market in 1997 by Anchor Yeast. Yield Plus® is used as a biocontrol product against *Botrytis*, *Penicillium* and *Mucor* on apples and pears and is also under evaluation for other crops (Lisa Picard, Anchor Yeast, Cape Town, South Africa, pers. comm.). The recent product Shemer®, registered in Israel, is based on a newly identified yeast *Metschnikowia fructicola* (Kurtzman & Droby, 2001) and is effective against a wide range of pathogens of grape, strawberry and sweet potato (M. Keren Zur, Agrogreen Minrab Group, Ashdod, Israel, pers. comm.)

Mechanisms of yeast antifungal activities

Commonly, the mechanism by which the biocontrol agent inhibits the target pathogen is poorly understood, as it is extremely difficult to construct experiments that can exclude all other possible mechanisms in the complex biocontrol environment. For most biocontrol organisms several modes of action have been suggested, and so far no one single mechanism has been shown to be responsible for the whole biocontrol effect (Janisiewicz & Korsten, 2002).

Competition for nutrients has been suggested as the mode of action of several possible biocontrol organisms, *e.g. P. guilliermondii* against *Penicillium digitatum* (Droby *et al.*, 1989), *Candida guilliermondii*, *Cryptococcus laurentii* and *Metschnikowia pulcherima* against *Botrytis cinerea* and *Penicillium expansum* (Elad, Kohl & Fokkema, 1994; Piano *et al.*, 1997; Roberts, 1990; Saligkarias, Gravanis & Epton, 2002; Vero *et al.*, 2002). This is in most cases a probable and reasonable explanation. However, competition for nutrients is difficult to prove, as it is usually very hard to exclude all other mechanisms. Droby *et al.* (1989) demonstrated that addition of exogenous nutrients reduced the efficacy of *P. guilliermondii* against *P. digitatum.* Filonow (1998) observed that the antagonistic yeasts *C. lauretii* and *Sporobolomyces roseus* had stronger sugar consumption than the pathogen *B. cinerea.* However, no differences in sugar consumption were observed between yeasts with and without biocontrol activity, suggesting that additional factors are part of the inhibiting mechanisms. In a tissue culture plate system with membrane inserts separating the organisms, Janisiewicz (2000) was able to show that *Aureobasidium pullulans* consumes the amino acids and inhibits germination of *P. expansum* in apple juice. Production of inhibitory substances was excluded, using a agar diffusion test of filtrates from the culture plates.

The killer yeast phenomenon was first discovered 40 years ago by Makower and Beavan (1963). They observed that certain strains of *Saccharomyces cerevisiae* produced toxins that killed sensitive strains of the same species. Initially, it was assumed that killer yeasts only killed yeasts belonging to the same or closely related species. However, many killer toxins can affect other yeasts and even bacteria and filamentous fungi (Polonelli *et al.*, 1987; Polonelli & Morace, 1986; Walker, McLeod & Hodgson, 1995; Izgu & Altinbay, 1997). Since its discovery, intensive investigations on the killer system, mainly in *S. cerevisiae*, contributed to the understanding of eukaryotic cell biology, *e.g.* virus-host cell interactions and yeast virology (Schmitt & Breinig, 2002). In addition, several applications within wine and beer fermentation (Passoth & Schnürer, 2003), food preservation (Palpacelli, Ciani & Rosini, 1991) and antifungal therapy in humans and animals (Polonelli *et al.*, 1986; Séguy *et al.*, 1994) have been suggested. However, most killer proteins are only active within a very narrow temperature and pH range, which complicates application in many environments (Suzuki & Nikkuni, 1989).

Production of antibiotic substances is a commonly suggested mode of action for bacterial biocontrol agents (Raaijmakers, Vlami & de Souza, 2002). Avis & Bélanger (2002) have shown that the yeast *Pseudozyma flocculosa* produces extracellular fatty acids that are detrimental to powdery mildew. Biocontrol agents

producing antibiotic metabolites could be effective against a wide range of target organisms, including pathogens that have occurred prior to the application of the biocontrol agent, as well as against latent infections. However, given the current debate about the antibiotic resistance of human pathogens it is doubtful that an antibiotic-producing biocontrol agent would be registered for use on food or feed. Moreover, similar problems with resistant pathogen strains, as experienced today with fungicides, would probably rapidly occur if a one-substance effect was the only mechanism involved.

Parasitism by yeasts has been suggested as a fungus-inhibiting mechanism. The yeast *P. guilliermondii* inhibits *B. cinerea* and adheres strongly to the fungal mycelium (Wisniewski *et al.*, 1991). Jijakli and Lepoivre (1998) have also shown that *P. anomala* strain K has a strong production of ß-1,3-glucanase enzyme that degrades the fungal cell wall.

Droby, Chalutz and Wilson (1991) demonstrated that *P. guilliermondii* can stimulate ethylene production in grapefruit, while Rodov *et al*. (1994) found that it stimulated production of phytoalexins in citrus. *Aureobasidium pullulans* and *Candida saitoana* have been shown to induce accumulation of ß-1,3-glucanases, chitinases and peroxidases in apples (Ippolito *et al.*, 2000; El Ghaouth, Wilson & Wisniewski, 2003). These observations all suggest that the antagonist stimulates some kind of host defence response.

Storage of cereal grains

In Sweden, about 5.5 million tons of cereal grains are produced each year, with feed grain accounting for about 60% (1997-2002, Swedish Board of Agriculture, Jordbruksstatistisk årsbok, 2003). The average moisture content of the harvested grain is about 21% (Jonsson, 1999). This means that all grain harvested needs to be dried before storage. When the grain is to be used as animal feed, preservation by acid treatments or airtight storage is an alternative to drying.

The main proportion (>75%) of the grain harvested in Sweden is preserved by high-temperature drying (Jonsson, 1999). To achieve safe storage, the grain has to be dried to a water content of 13% ($a_w < 0.65$) and this leads to high energy costs. In a report from the FAO European Cooperative Networks on Rural Energy (Pick, Noren & Nielsen, 1989), fuel consumption data from sixteen countries were collected and analysed. In Sweden, up to 60% of the energy used in the total plant husbandry operation was calculated to be spent on grain drying. Several different chemicals have been tested for mould inhibition during storage of cereal grain, including volatile fatty acids, aldehydes and alcohols. Lacey and Magan (1991) conclude that the effects of these chemicals are often promising *in vitro,* but disappointing when finally tested on the cereal grain. Only propionic acid has been used in large amounts on farms. The main difficulty is to obtain sufficient concentrations of the chemical in the whole system. For successful mould control, the optimal amount of acid must be applied in relation to the water content of the grain. The treatment must be uniform, without pockets with non- or under-treated

grains where propionate-tolerant organisms may develop, metabolise the propionic acid and allow growth of more sensitive fungi. *Aspergillus flavus* can tolerate a higher concentrations of propionate than most other fungi, and low concentrations of propionate may even stimulate the production of aflatoxins (Al-Hilli & Smith, 1979).

Airtight grain storage

Harvesting at high moisture content is a common practice to protect the grain from prolonged exposure to wet weather conditions, which otherwise can cause weathering and mould infections of grain in the field. Airtight storage is an alternative storage method that makes it possible to store high moisture grain without prior drying. The method only consumes approximately 2% of the energy consumed in high-temperature drying (Table 1; Pick, Noren & Nielsen, 1989). However, this approach harms the bread-making properties of the grain, *i.e.* the gluten protein is affected in a negative way, and the germination capacity is reduced. Therefore the method is only useful for the storage of animal feed grain. However, in Sweden 60% of all grain harvested is used for animal feed.

Table 1. *Energy consumption (kW/ha) of different cereal grain preservation systems*

	Moisture content at harvest		
Method	20%	22%	25%
Cold air drying	105	150	220
Heated air drying	665	885	1295
Treatment with propionic acid	65	70	75
Airtight storage	15	15	15

Modified from Pick *et al.* (1989)

Airtight storage relies on a perfectly tight silo, in combination with a modified atmosphere. When the freshly harvested grain with adhering microorganisms is stored in the airtight silo, the respiration of the grain itself and of the microorganisms reduces levels of O_2 and increases levels of CO_2. In a well functioning airtight silo, O_2 levels would ideally be reduced to 0.5-1% and CO_2 would simultaneously increase up to at least 50->90% (Busta, Smith & Christensen, 1980; Lacey & Magan, 1991). However, this method has considerable drawbacks. The control of fungal growth depends on the exclusion of atmospheric conditions. Imperfect sealing, daily temperature fluctuations and feed outtake lead to leakage of CO_2 and intake of O_2. In the spring the considerable temperature variations between day and night cause great pressure fluctuations in the silo. The solar radiation during the day heats the silo and the resulting pressure increase causes a movement of air out of the silo through shell perforations, pressure relief valves, or through the emptying auger tube. During the night the pressure decreases and air is sucked in through the same locations (Fig. 1).

Feeding out leads to a continuously diminishing grain bulk, making it difficult for the microbial and grain respiration to maintain the atmospheric composition necessary for safe storage. Microbial development and spontaneous heating may then occur (Lacey & Magan, 1991). Commonly, moist grain in airtight storage can be safely stored during the cold period of the year, but deteriorates if kept until the following summer (Ekström, 1992).

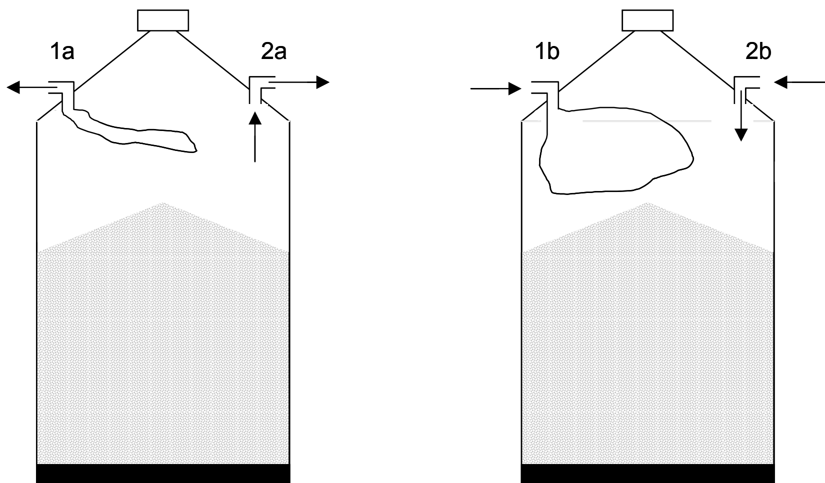

Figure 1. Schematic drawing of the pressure differences between day and night in a grain silo. When heated by the sun, the gas pressure in the silo increases and air is pressed out of the breather bag (1a). When cooling, *e.g.* during the night, pressure decreases and a suction pressure arises. The filling of the breather bag (1b) compensates for this. When the volume changes of the breather bag can no longer compensate for the pressure variations in the silo, the pressure relief valve allows passage of air in or out (2a & 2b). Picture modified from (Simonsson & Göransson, 1982).

Moulds in airtight stored grain

Harvested grain contains a microbial community influenced by climatic and field conditions, as well as by circumstances during the harvest process. The community contains filamentous fungi, as well as yeast and bacteria. Among these, fungi (*Aspergillus* and *Penicillium* spp.) are most tolerant to low water activity (a_w) and are subsequently the most common spoilage organisms. In the beginning of the storage period, while the CO_2 levels are high and the O_2 levels low, yeasts are the predominant fungi (Lacey & Magan, 1991). The growth of the filamentous fungi during storage is influenced by the water availability, temperature and gaseous composition, as well as by interactions with other microorganisms. Mould growth causes undesirable effects such as loss of dry matter, discoloration, reduced nutritional value and digestibility and production of off-odours, and can result in production of mycotoxins (Lacey, 1989; Magan *et al.*, 2003).

Fungi colonizing grain are commonly divided into two major groups, the field and the storage fungi. Field fungi, *e.g.* the genera *Alternaria, Fusarium,* and *Cladosporium*, colonize the grain during field ripening, but seldom develop further

after harvest. Storage fungi mainly belong to the genera *Aspergillus* and *Penicillium*. *Penicillium* spp. are less common before harvest, but start to grow rapidly during storage when appropriate conditions occur. They generally require more water than *Aspergillus* spp., are usually favoured by damper and colder storage conditions and commonly spoil stored cereal grain (Lacey, 1989; Lacey & Magan, 1991).

Penicillium roqueforti

Penicillium roqueforti is a common spoilage fungus in cold stored foods and feed and is often isolated from material stored under limited oxygen supply, *e.g.* airtight storage or silage (Häggblom, 1990). The occurrence of *P. roqueforti* on freshly harvested cereal grain is not as common as that of other *Penicillium* spp., but the ability to grow at low partial pressures of oxygen (< 0.14%, Lacey, 1989), tolerate high levels of carbon dioxide (>80%, Lacey, 1989) and low temperatures (< 5°C, Lacey & Magan, 1991), as well as being a potential mycotoxin producer makes *P. roqueforti* one of the most important spoilage fungi in airtight stored grain.

P. roqueforti produces several mycotoxins: PR-toxin, roquefortine C, mycophenolic acid, patulin and penicillic acid (Pitt & Hocking, 1999). The PR-toxin is regarded as the most important, since it has been reported to cause liver and kidney damage in rats (Scott *et al.*, 1977) and is also potentially carcinogenic (Chang *et al.*, 1991). Chang *et al.* also concluded that PR-toxin production by *P. roqueforti* is stimulated by growth on grain compared to synthetic media. Toxin formation was also increased by the oxygen limitation of a stationary compared to a shaken culture. Roquefortine C and mycophenolic acid are both considered to be mycotoxins with low toxicity and of lesser significance, even though they can occur under natural conditions in feed (Pitt & Hocking, 1999; Pitt & Leistner, 1991). However, Häggblom (1990) isolated an average roquefortine C concentration estimated at 25.3 mg/kg grain from heavily mould-infected grain. Animals feeding on this grain exhibited symptoms such as lack of appetite, ketosis, paralysis and spontaneous abortions. No other *Penicillium* mycotoxins (PR-toxin, patulin or penicillic acid) were detected in the grain. Tawinaki *et al.* (2001) showed that high levels of CO_2 and low levels of O_2 decreased the formation of roquefortine C. Patulin is graded as an important toxin, but is not common in animal feed (Pitt & Leistner, 1991). A wide range of moulds, including *P. roqueforti*, produce penicillic acid, which is commonly found in food. However, the compound is very unstable and is rapidly converted to products that are not biologically active (Smith *et al.*, 1994).

Pichia anomala

According to Gray (1949) *P. anomala* was described for the first time by Hansen (Hansen, E.C. 1894, Compt. Rend. Trav. Lab. Carlsberg 3:44-66) as *Saccharomyces anomalus*. Hansen later transferred *S. anomalus* Hansen together with *Saccharomyces saturnus* Klocker to the new yeast genus *Willia*. However, in

1919, Sydow transferred all species of the genus *Willia* to a new genus *Hansenula* as the name *Willia* had been pre-empted for a genus of mosses (Sydow, H. 1919, Ann. Mycol. 17: 33-47). The genera *Hansenula* and *Pichia* were originally separated on the ability to assimilate nitrate as the only nitrogen source, but in 1984 Kurtzman concluded that this difference was not sufficient to justify the separation and suggested that species of *Hansenula* and *Pichia* with Saturn-shaped ascospores be transferred to the genus *Williopsis* and species of *Hansenula* (*e.g. Hansenula anomala*) with hat-shaped spores be transferred to the genus *Pichia*. Even though the reclassification has now been widely accepted, many reports still refer to *Hansenula anomala* and some argue for a reinstating of the genus *Hansenula* (Naumov, Naumova & Schnürer, 2001).

Pichia anomala is a heterothallic, ascomycetous yeast, forming one to four hat-shaped ascospores. The yeast is commonly isolated as a spoilage organism from food and feed (Kalathenos, Sutherland & Roberts, 1995; Kitamoto *et al.*, 1999; Mingorance-Cazorla *et al.*, 2003) or from fruits and plant material (Kurtzman & Fell, 1998). In a recent genetic study of the yeast, the natural ploidy level was suggested to be diploid (Naumov, Naumova & Schnürer, 2001). Most of the early literature on *P. anomala* concerns ester formation, especially large quantities of ethyl acetate (*e.g.* Gray, 1949; Laurema & Erkama, 1968; Peel, 1951; Tabachnick & Joslyn, 1953).

P. anomala J121 was first isolated from airtight stored grain (Björnberg & Schnürer, 1993) and is stored at the CBS culture collection (Utrecht, The Netherlands) as CBS 100487. The strain is able to grow under completely anaerobic conditions when supplemented with ergosterol and unsaturated fatty acids and can tolerate high concentrations of organic acids **(I)**. It can also grow between 3 ºC and 37 ºC, at pH values between 2.0 and 12.4 and at a water activity (a_w) of 0.92 (NaCl) and 0.85 (glycerol) **(I)**. *P. anomala* J121 produces ethanol, arabitol, glycerol and trehalose as a response to oxygen limitation (Fredlund *et al.*, 2004).

Pichia anomala biocontrol and airtight storage

A study performed on airtight stored grain in Sweden between 1980 and 1987 concluded that addition of the yeast *P. anomala* prolonged the safe storage time of the grain by at least a couple of months and sometimes until the next harvest (Ekström, 1992). In 1993, Björnberg and Schnürer presented *in vivo* results showing that *P. anomala* inhibits *Aspergillus candidus* and *Penicillium roqueforti* in a dose-dependent manner. Both hyphal length and CFU values were reduced. Petersson and Schnürer (1995) further showed that *P. anomala* inhibited *P. roqueforti* growth on wheat grain in semi airtight test-tubes. Since then, the yeast has been found to be active against several target fungi in different environments (Table 2).

Table 2. *Summary of suggested mechanisms for antifungal effects of* P. anomala

Target fungus	System	Suggested mode of action	Ref.*
Aspergillus candidus	plate bioassay	—**	1
Aspergillus flavus	plate bioassay	—, killer toxin	2,13
Aspergillus fumigatus	plate bioassay	—, killer toxin	2,13
Aspergillus nidulans	plate bioassay	killer toxin	13
Aspergillus niger	plate bioassay	killer toxin	1,13
Aspergillus parasiticus	plate bioassay	killer toxin	13
Aurebasidium pullulans	plate bioassay	killer toxin	13
Botrytis cinerea	grape-wine, apple, plate bioassay	cell-wall degrading enzymes., —	2,3,4
Candida albicans	animals, plate bioassay	killer toxin	5,6
Candida glabrata	animals, plate bioassay	killer toxin	5,6
Cladosporium cladosporioides	plate bioassay	—	2
Eurotium amstelodami	plate bioassay	—	2
Fusarium equiseti	plate bioassay	—	7
Fusarium poae	plate bioassay	—	2
Fusarium sporotrichoides	plate bioassay	—	2
Malassezia furfur	animals	killer toxin	12
Malassezia pachydermatis	animals	killer toxin	12
Monascus ruber	plate bioassay	—	2
Mucor hiemalis	plate bioassay	—	2
Paeciliomyces variotii	plate bioassay	—	2
Penicillium camembertii	plate bioassay	killer toxin	13
Penicillium carneum	grain	—	7
Penicillium expansum	plate bioassay	—	2
Penicillium glabrum	plate bioassay	—	2
Penicillium notatum	plate bioassay	killer toxin	12
Penicillium paneum	grain	—	7
Penicillium roqueforti	grain, plate bioassay	—	1,2,7,8,9,10
Penicillium verrucosum	grain	—	11

Pseudallescheria boydii	plate bioassay	killer toxin	12,13
Phialophora verrucosa	plate bioassay	killer toxin	12
Rhizopus microsporus	plate bioassay	killer toxin	12
Rhizopus solani	plate bioassay	Killertoxin	6
Rhizopus stolonifer	plate bioassay	—	2
Scopulariopsis brevicaulis	plate bioassay	killer toxin	12
Sporothrix schenckii	plate bioassay	killer toxin	12,13
Thalaromyces flavus	plate bioassay	—	2

*References: **1**: (Björnberg & Schnürer, 1993), **2**:(Petersson & Schnürer, 1995), **3**: (Masih, Alie & Paul, 2000)**,** **4**: (Jijakli & Lepoivre, 1998), **5**: (Magliani *et al.*, 2002), **6**: (Walker, McLeod & Hodgson, 1995), **7**:(Petersson & Schnürer, 1999), **8**: (Petersson & Schnürer, 1998), **9**: (Petersson, Jonsson & Schnürer, 1999), **10**: **(II)** ; **11**: (Petersson *et al.*, 1998); **12**: (Polonelli & Morace, 1986); **13**: (Polonelli *et al.*, 1987)
** —: No suggestion for possible antifungal mechanism was given in the reference

The ability of *P. anomala* J121 to restrict mould growth and sporulation has been confirmed in small and medium- scale studies (20 g minisilos and 160 kg barrels) (Petersson, Jonsson & Schnürer, 1999; Petersson & Schnürer, 1995; Petersson & Schnürer, 1998, **II**) and tested in large farm silos containing 350 tons of wheat (Höglöv & Schnürer, 2002).

Experimental scale systems

Large-scale commercial silos can generally hold hundreds of tons of cereal grain, making experimental replication difficult. Therefore, to enable the yeast mould interaction in the system to be investigated, one laboratory scale and one pilot scale system were developed.

The minisilo system

The minisilo system was first described by Petersson and Schnürer (1995) and consists of thick-walled glass tubes (27 ml) filled with wheat (18 g, 10ml gas phase) and sealed with a rubber membrane perforated with a needle to simulate the air leakage (Fig. 2a). The wheat used is non-sterile and hydrated with tapwater to appropriate a_w. The system is easy to manipulate with regard to inoculation, temperature, a_w, and atmospheric composition.

The pilot scale silo system

The pilot scale system was developed by the Swedish Institute of Agricultural Engineering and initially used by Petersson, Jonsson & Schnürer (1999). Each silo contains 0.21 m^3 (160 kg) freshly harvested wheat. The silo is equipped with a breather bag and a two-way pressure relief valve to equalize differences between internal and external air pressure (Fig. 2b). The pressure relief valve consists of a U-tube filled with a glycol/water solution. Air passes through the U-tube after the

breather bag has been filled with >14 litres of air or when 7 litres of air have been drained from the bag and the pressure has exceeded ±60 mm static water gauge.

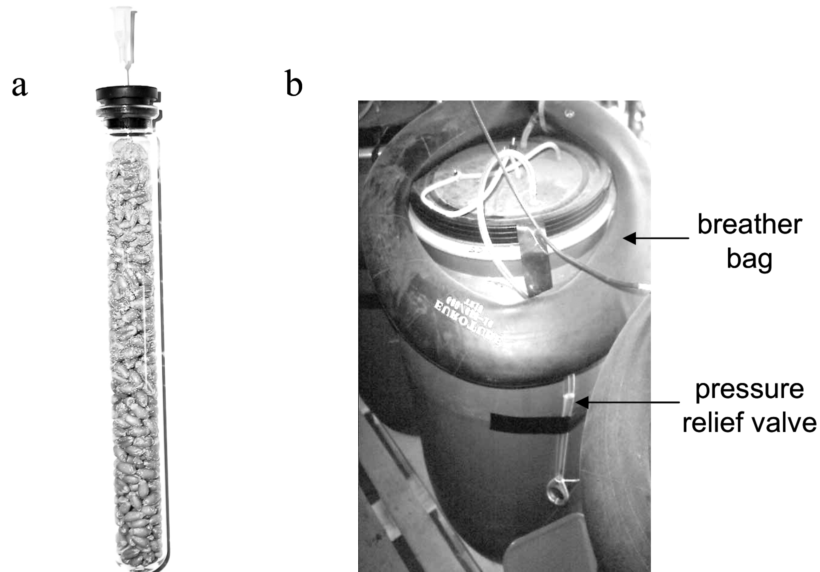

Figure 2. Two experimental scale silos for airtight storage. a) Minisilo containing approximately 18 g hydrated wheat, with rubber membrane perforated with a needle to simulate air leakage. b) Pilot scale silo containing 160 kg moist wheat. Each silo is equipped with a breather bag and a pressure relief valve as well as an emptying auger tube. The permeability to air can also be manipulated in a controlled manner.

Growth of *P. anomala* and *P. roqueforti* during grain storage in air-leaking grain silos

An airtight silo is a harsh environment with low levels of O_2, high levels of CO_2 and large temperature fluctuations. Microorganisms able to grow in this environment have to be very robust and flexible in their demands. The ability to grow and survive well in such an environment is essential for a successful biocontrol organism.

In a study with 160 kg pilot grain silos (Fig. 2; **II**), we constructed a system that behaves like a large-scale airtight silo. The pilot silos were perforated with needles to obtain different degree of leakage. Gas levels detected in our most leaky silos corresponded well with those expected in large-scale silos with a peak CO_2 concentration at 80-90% within one month and a O_2 drop to below detection limit within one day (Hyde & Burell, 1982). The CO_2 level then continuously decreased during the storage period as the silos were emptied to simulate a continuous feed grain outtake.

When inoculated on grain in air-leaking silo systems, *P. anomala* levels rapidly increased. In the minisilo system detectable growth started during the first day (Fig. 3) and in the larger pilot scale within the first month (Fig. 4). In the minisilos the growth continued until it reached a maximum level after two to three days. In the pilot scale silos, yeast growth increased during the autumn, stopped during the cold winter months and reached its maximum during the spring. Also in the barrels not inoculated with strain J121, growth of yeast (*P. anomala*) was observed (Fig. 4). Nine months after the start of the storage, the cell numbers had reached the same levels as in the barrels inoculated with yeast.

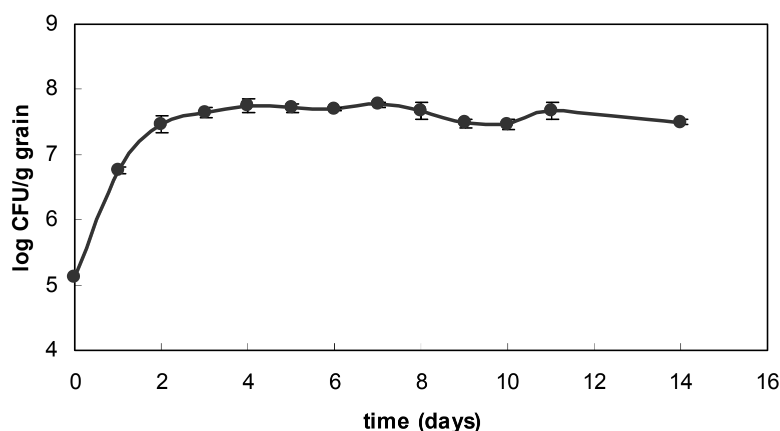

Figure 3. Growth of *P. anomala* J121 on wheat (a_w 0.95, 25°C) in the minisilo system (n=3, ± standard deviation; SD). Modified from **(IV)** with extended storage period.

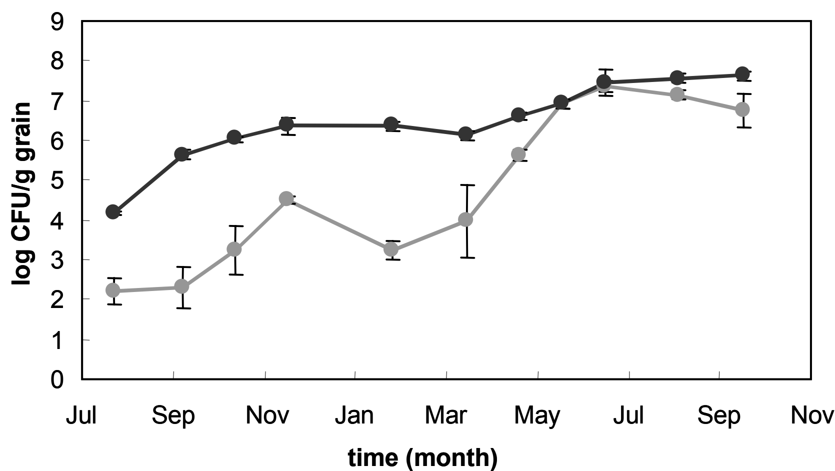

Figure 4. Growth of inoculated (●) and naturally occurring (●) *P. anomala* on wheat grains (a_w 0.96) in pilot scale silos stored outdoors for 14 months (n=3, ± SD). Modified from **(II)**.

Growing on the grain surface the yeast colonies form distinct ‘stacks’ of yeast cells and also seem to fill up scars on the wheat kernel (Fig. 5-6).

Figure 5. Wheat kernel from the minisilo system inoculated with *Pichia anomala* J121.

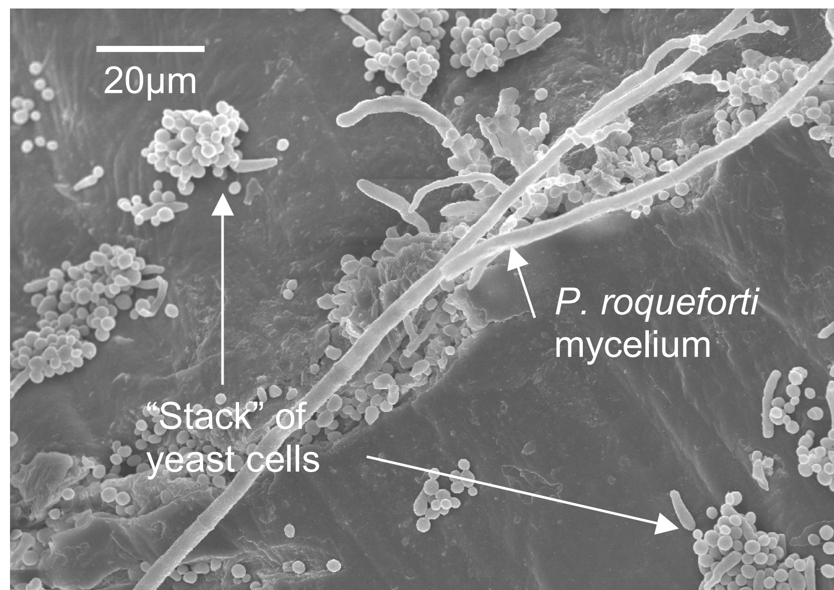

Figure 6. SEM close-up of wheat kernel from the minisilo system, inoculated with *P. anomala* J121 and *P. roqueforti* (Photo: G. Daniels).

In both the minisilo system and the pilot scale silos, a small decrease in *P. roqueforti* CFU was observed in the beginning of the storage period (Figs. 7 and 8). This is probably caused by some kind of shock effect on the conidia after being placed on the grain surface. Generally, mould CFU, if not inhibited by *P. anomala,* increased rapidly after two days in the minisilo system (Fig. 7) and after approximately nine months in the pilot scale silos when the temperature started to increase in spring (Fig. 8).

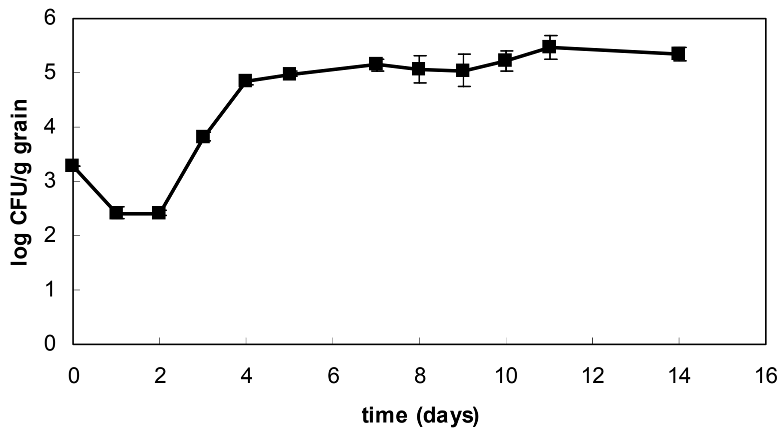

Figure 7. Growth of *P. roqueforti* on wheat (a_w 0.95, 25°C) in the minisilo system (n=3, ± SD). Modified from **(IV)** with extended storage period.

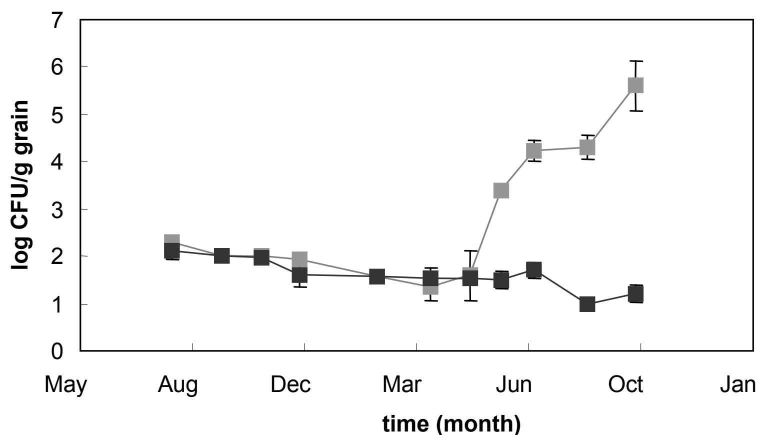

Figure 8. Growth of *P. roqueforti* on wheat grains (a_w 0.96) in the pilot scale silos stored outdoors for 14 months. Yeast inoculated (■) and non-inoculated (■) (n=3, ± SD). Modified from **(II)**.

The results from this pilot scale trial **(II)** confirmed that *P. anomala* J121 is an efficient biocontrol strain. Regardless of degree of air leakage, moulds did not grow in grain inoculated with *P. anomala.* In the non-inoculated silos, however, mould CFU increased drastically in the spring when the temperature increased and the CO_2 levels had decreased to below 30% (Fig. 10). This increase occurred in spite of the O_2 levels being below the detection limit (1%) in all treatments. The treatment not inoculated with yeast showed large fluctuations in oxygen levels during the winter months. On almost all sampling occasions when oxygen was detected in the system, the oxygen levels in the treatment without yeast inoculation were higher than the levels in yeast-inoculated silos (Fig. 10).

One might ask whether the depletion of oxygen or the increase in carbon dioxide is more important for mould inhibition. Moulds are generally considered to be aerobic, but many mould species can grow at very low oxygen levels, suggesting high CO_2 levels as the major factor responsible for mould inhibition (Fig. 9, Paster, 1987; Peterson *et al.*, 1956). Peterson (1956) showed that even nearly anaerobic conditions (0.2% oxygen) failed to prevent the growth of all mould species tested, although it greatly reduced their sporulation. *Penicillium* spp. were more sensitive to oxygen limitation than *e.g. Aspergillus glaucus*. When growth inhibition by CO_2 was tested, there was a sharp decrease in mould growth at CO_2 levels above 12%. Below this level, however, there was even a slight enhancement of mould growth. At CO_2 >50%, mould growth was almost completely inhibited, even at atmospheric oxygen conditions (21% O_2).

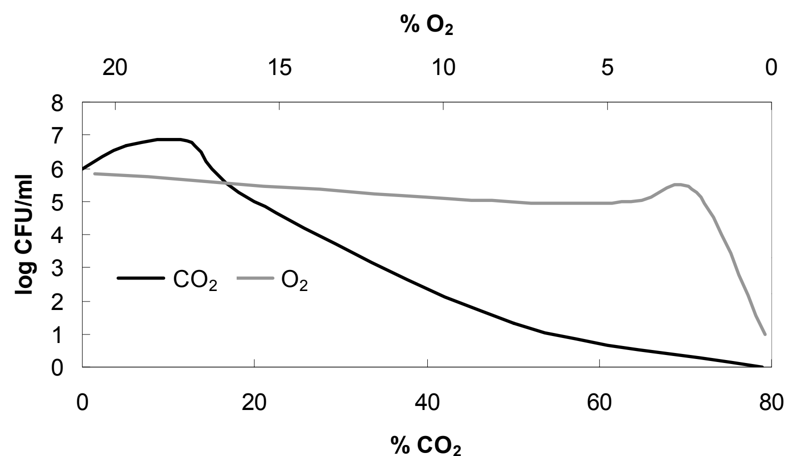

Figure 9. The effect of O_2 and CO_2 levels on fungal growth. Modified from Peterson *et al.*, (1956).

Judging from this, the production of CO_2 would be the factor having the largest influence on mould growth. However, only small differences (~2% units) in the rate of CO_2 decrease were observed between treatments with and without yeast inoculation in the pilot scale silos (Fig. 10). The respiration by the grain bulk is probably the main CO_2 source and the CO_2 production from yeast is nearly negligible in this context. The main difference caused by the yeast addition was probably a faster removal of O_2. During the critical period in the springtime, the temperature increased rapidly and CO_2 concentrations were low both in silos inoculated and not inoculated with yeast. At the same time the oxygen in the non-inoculated silos was close to 3.5%, compared to <1% in the yeast-inoculated silos. The mould CFU then started to increase in an almost exponential way in the non-inoculated silos (**II**, Fig. 10).

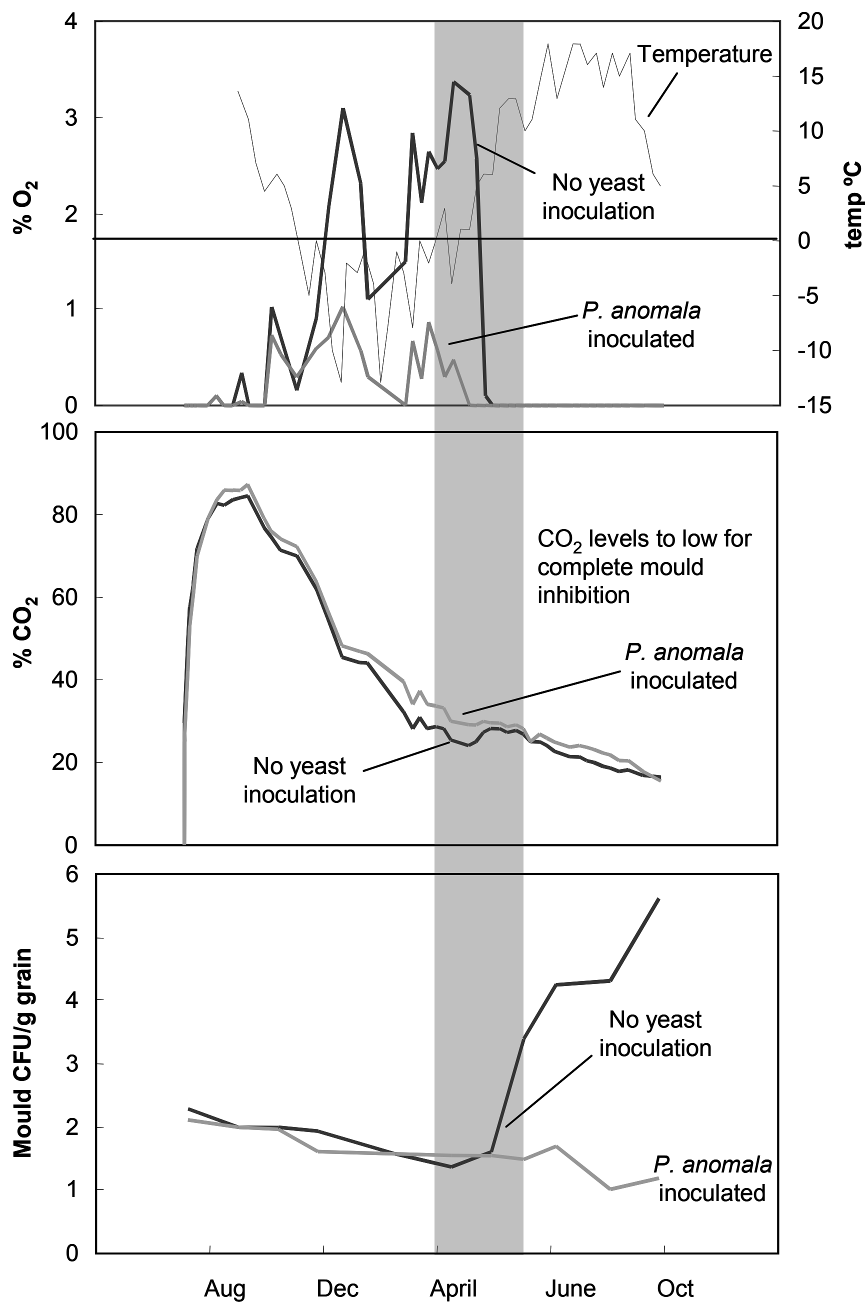

Figure 10. Levels of O_2, CO_2, temperature and mould growth in the pilot silo-system storage during 14month. The grey bar shows the most critical part of the storage period. Modified from **(II)**.

Other yeasts in the airtight biocontrol system

By screening a large number of yeast strains we compared the biocontrol ability of *P. anomala* with that of other yeast strains **(III)**. We wanted to investigate whether the activity of *P. anomala* strain J121 was specific and if other yeast species also exert biocontrol. In total, 29 different *P. anomala* strains were investigated and all had high biocontrol activity. Altogether, 116 strains from 58 species were tested for their biocontrol ability against *P. roqueforti* on wheat in the minisilo system (Fig. 11).

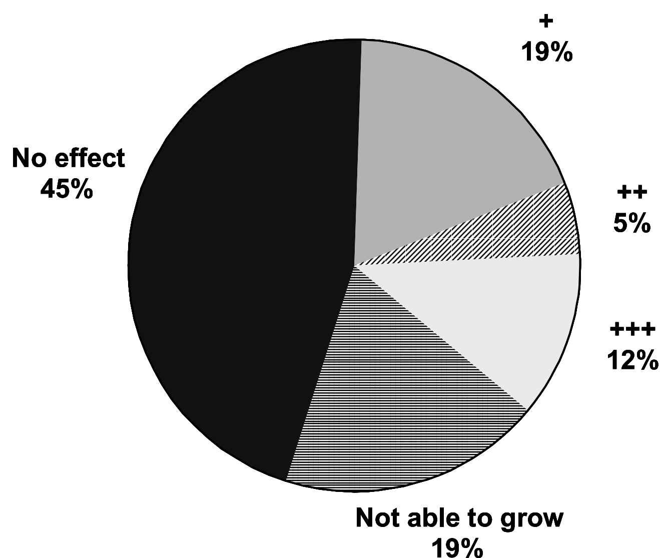

Figure 11. Distribution of ability to inhibit growth of *P. roqueforti* on grain among 59 different yeast species. No inhibition: $>10^5$, +: 10^4-10^5, ++: 10^3-10^4: +++: 10^1-10^3 mould CFU/g grain

Most of the species tested did not show any activity against the mould. However, more than 70% of the species without the ability to inhibit mould in the system were still able to grow, in many cases to levels comparable to those of *P. anomala* **(III)**. This suggests that competition for space and nutrients is not a major component of the mode of action. The fact that *Saccharomyces cerevisiae* is one of the non-active yeasts further implies that neither production of CO_2 nor ethanol by the yeast can be of any prominent importance.

A number of yeasts with documented biocontrol activity in other systems were among the species tested (Table 3), although several of them were unable to grow in the system. Among those that grew in the grain system, the biocontrol activity varied considerably.

Among yeasts with high activity were the three species *Candida fennica*, *Candida silvicultrix* and *Pichia farinosa*, which have not previously been reported as having biocontrol activity. *P. farinosa* has been tested once against *Fusarium* dry rot but was not found to have any significant activity (Schisler *et al.*, 1995).

Table 3*: Inhibitory effect on* P. roqueforti *in grain minisilos of yeast species reported with biocontrol activity in other systems*

Biocontrol Species	Effect	System	Target organism	Ref**
*Cryptococcus albidus**	-	bean, tomato, strawberry, *in vitro*	*Botrytis cinerea* *Penicillium glabrum*	1, 2, 9
*Cryptococcus laurentii**	-	apples	*Penicillium expansum* *B. cinerea*	3, 4, 5, 8
Debaromyces hansenii	-	oranges, grapefruit	*Penicillium digitatum*	6, 7
*Filobasidium floriforme**	-	apples	*B. cinerea*	8
Kluyveromyces marxianus	-	*in vitro*	*P. glabrum*	9
Metschnikowia pulcherrima	+	*in vitro*, apple	*P. glabrum* *P. expansum* *B. cinerea*	9, 10, 11
Pichia burtonii	+++	seed	*Penicillium verrucosum*	12
Pichia guilliermondii	++	tomato, soy bean, grapefruit	*B. cinerea* *Aspergillus flavus* *P. digitatum*	13, 14, 15
Pichia membranaefaciens	+++	grapevine	*B. cinerea*	16
Rhodotorula glutinis	-	bean and tomato plants, apples	*B. cinerea* *P. expansum*	3,1
Saccharomyces cerevisiae	-	*Pinus sylvestris* *in vitro*	Wood decaying fungi, *Alternaria alternata*	17,18
*Sporidiobolus salmonicolor**	-	apples	*B. cinerea*	8
Yarrowia lipolytica	+	apples	*B. cinerea*	8

* Species unable to grow in the minisilo system
** References: **1**: (Elad, Kohl & Fokkema, 1994), **2**:(Helbig, 2002), **3**: (Lima *et al.*, 1998); **4**:(Roberts, 1990), **5**: (Vero *et al.*, 2002), **6**: (Arras, 1996), **7**: (Droby *et al.*, 1989), **8**: (Filonow *et al.*, 1996), **9**: (Sinigaglia, Corbo & Ciccarone, 1998), **10**: (Janisiewicz, Tworkoski & Kurtzman, 2001), **11**: (Piano *et al.*, 1997), **12**: (Ramakrishna, Lacey & Smith, 1996), **13**: (Saligkarias, Gravanis & Epton, 2002), **14**: (Paster *et al.*, 1993), **15**: (Droby *et al.*, 1990), **16**: (Masih & Paul, 2002), **17**: (Payne, Bruce & Staines, 2000), **18**: (Suzzi *et al.*, 1995).

Mechanism of fungal inhibition by *Pichia anomala*

Competition for space and/or nutrients

P. anomala J121 can utilize many different carbon and nitrogen sources and is able to grow under anaerobic conditions, at low water activity (a_w 0.85) and over a wide range of temperatures (3-37 °C) and pH values (2.0-12.4) **(I)**. Many of these

features, *e.g.* the ability to grow at very low oxygen and water tensions, suggest that the organism should be highly competitive in the grain silo ecosystem.

If competition for nutrients plays an important role for the antifungal ability, addition of nutrients should abolish the inhibitory effect (Droby *et al.*, 1990; Droby *et al.*, 1989). The ability of *P. anomala* to inhibit mould growth was tested after addition of different nutrient supplements in the water used to hydrate the grain (Table 4, **IV**). In the first experiment a complex YPD medium was used. The YPD addition surprisingly increased the biocontrol effect, instead of reducing it. In the following experiments the amendment was separated into carbon and nitrogen sources. The addition of a complex nitrogen source or glutamine did not affect the biocontrol effect, while addition of different carbon sources increased the effect.

Table 4. *Effect of nutrient amendments on the biocontrol effect of* P. anomala *against* P. roqueforti

Nutrient addition	Change in inhibition
Yeast Peptone Glucose broth	++*
Glucose	++
Maltose	++
Starch	+
Glycerol	+
Yeast Nitrogen Base	0
Glutamine	0

* ++: increased inhibition; +: weak increase of inhibition, 0: no effect (**IV** and unpublished data)

Enhancement of the biocontrol ability after nutrient addition has been reported in some other cases. Generally, the additives have had inhibiting properties alone or favoured the growth and biomass production of the antagonist (Droby *et al.*, 1990; Janisiewicz, 1994; Janisiewicz, Usall & Bors, 1992). However, in our system no clear effect could be seen on *P. anomala* growth after nutrient amendment (Fig. 12) and simple carbon sources such as glucose and maltose can also be used by the mould.

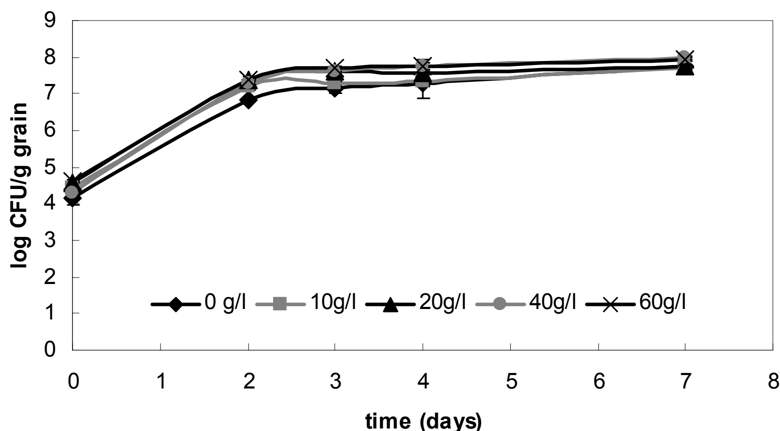

Figure 12. Growth of *P. anomala* J121 on wheat hydrated with glucose solutions of different concentrations (n=3, ± SD, Modified from **IV**).

The conclusion is that competition for nutrients, or at least for sugar or nitrogen, does not significantly contribute to the antifungal activity of *P. anomala* in the grain system. Moreover, the finding that as many as 27 of the 38 species without biocontrol activity (Fig 11) were able to grow to similar levels as *P. anomala* in the minisilo system **(III)** shows that the ability to grow, consume nutrients and compete for space on cereal grains is not enough to prevent the growth of *P. roqueforti.*

Killer toxins

Due to its activity against a wide range of unrelated microorganisms, the killer toxin activity of *P. anomala* has attracted great attention (Magliani *et al.*, 1997; Polonelli *et al.*, 1987; Polonelli *et al.*, 1986; Rosini, 1983). There have been several reports about activity against important opportunistic human pathogens such as *Candida albicans, Malassezia furfur* and *Malassezia pachydermatis* (Cassone *et al.*, 1997; Magliani *et al.*, 2002; Polonelli *et al.*, 1986; Sawant, Abdelal & Ahearn, 1988), but only a few about inhibitory effects against mould species (Polonelli *et al.*, 1987; Polonelli & Morace, 1986; Walker, McLeod & Hodgson, 1995). Other possible applications for the killer phenomenon could be biocontrol of fermentation processes (Palpacelli, Ciani & Rosini, 1991) or characterisation of industrially or clinically interesting strains (Buzzini & Martini, 2000; Polonelli *et al.*, 1983; Polonelli *et al.*, 1989).

P. anomala J121 produce killer toxins active against several yeast and mould species, including *P. roqueforti* (**I**, Table 4). Four of the nine identified species with high biocontrol activity in the grain system **(III)** have documented killer toxin production (*Candida pelliculosa, Pichia burtonii, Pichia farinosa* and *Pichia membranifaciens*). On the other hand, several of the species without activity in the system can also produce killer toxins, *e.g. Saccharomyces cerevisiae*, *Debaromyces hansenii* and *Rhodotorula glutinis*. However, the activity spectra of

killer toxins can vary substantially between different yeast strains (Palpacelli, Ciani & Rosini, 1991; Rosini, 1983).

Table 4. K*iller toxin activity of* P. anomala *J121 against different fungal species compared with that of a known killer toxin producer* P. anomala *CBS 5759* and baker's yeast strain (J191)*

Species	*Pichia anomala* J121	*Pichia anomala* CBS 5759*	*Saccharomyces cervisiae* J191
	Killer effect**		
Pichia guilliermondii CBS 2031	++	+	-
Kluyveromyces marxianus CBS 1089	+++	++++	-
Saccharomyces cervisiae J191	+++	+++	-
Penicillium roqueforti IBT 6754	+++	++++	++
Penicillium camembertii OC 1718	++	+	-
Aspergillus nidulans FGSC A4	++	++	+

* CBS 5759 = K4 (Polonelli *et al.*, 1983; Polonelli & Morace, 1986)
** Killer effects were tested with streak plate assay using Methylene Blue agar according to (Walker, McLeod & Hodgson, 1995). Results are given as -: not determined, +: small clear zone, ++: obvious clear zone, +++:obvious clear zone with thin blue ring, ++++: great clear zone with wide blue ring, -: no effect. Modified from Druvefors (2000) and **(I)**

Killer toxins are mainly produced and active under acidic conditions and activity decreases with increasing pH and temperature conditions (Sawant, Abdelal & Ahearn, 1989; Suzuki & Nikkuni, 1989). Killer toxins isolated from *P. anomala* have been reported to show considerable differences regarding amino acid composition, killer spectrum, optimum pH and temperature (Passoth & Schnürer, 2003). For one toxin reported, killer activity was still observed after incubation at 60 °C for five hours and disappeared only after 25min at 100 °C (Vustin *et al.,* 1989) *cit*. Passoth and Schnürer (2003), while both killer toxins isolated from a halotolerant *P. anomala* strain by Kagiyama *et al*. (1988) were heat labile to different degrees. The main argument against the role of killer toxin in the biocontrol system is the harsh environment in a silo. The pH in the environment would not favour toxin production and the highly variable temperatures would destroy most killer toxins.

Production of cell wall degrading enzymes

The pre- and post-harvest plant pathogen *B. cinerea* is inhibited by *P. anomala* (Jijakli *et al.*, 1993; Masih, Alie & Paul, 2000). Jijakli *et al.* demonstrated that *P. anomala* strain K isolated from apple surfaces had a protective ability against *B. cinerea* and *Penicillium* spp. on wounded 'Golden Delicious' apples. The suggested mode of action was initially thought to be competition for carbohydrates or nitrogen sources, but additional studies implied that exo-ß-1,3-glucanase activity might be involved (Jijakli & Lepoivre, 1998). Even though the mode of

action of *P. anomala* strain K has been extensively studied, it is still not fully clarified. Addition of cell wall products from *B. cinerea* to *P. anomala* strain K suspension stimulated both exo-ß-1,3-glucanase activity and biocontrol ability (Jijakli & Lepoivre, 1998). Morphological changes in *B. cinerea* hyphae have been found after both addition of purified enzyme (Jijakli & Lepoivre, 1998) and co-cultivation on agar plates (Masih, Alie & Paul, 2000). However, even when all detectable exo-ß-1,3-glucanase activity was abolished by disruption of the encoding gene (*PaEXG2*) no decrease in the biocontrol of *B. cinerea* on wounded apples was seen (Grevesse, Lepoivre & Jijakli, 2003).

When *P. anomala* strain J121 was tested for exo-ß-1,3-glucanase and endo-chitinase production, only very low levels (< 0.01 Units) of exo-ß-1,3-glucanase were found in the presence of glucose or lamarin as the sole carbon source and <0.2 Units of endo-chitinase with chitin or glucose as the sole carbon source (H.M. Jijakli 2003, unpublished data). No similar testing for responses to cell wall material from *P. roqueforti* has been carried out, but the observed increase in biocontrol effect after addition of glucose **(IV)**, which would repress glucanase formation (Jijakli & Lepoivre, 1998), argues against cell wall degrading enzymes as the basis of the antifungal activity of *P. anomala* in the grain system.

Production of metabolites

The observation of an enhanced biocontrol effect after addition of different sugars strongly suggests the involvement of products of sugar degradation in the biocontrol activity of *P. anomala*. A good candidate compound may be ethyl acetate, which is produced in large quantities by *P. anomala* and has a considerable antifungal effect **(V)**. In addition, the volatile character of the compound makes it easy to disperse in the non-homogeneous environment of the grain silo. Addition of glucose to the minisilo system increased the biocontrol activity, as well as the production of both ethyl acetate and ethanol (Figs. 13-14).

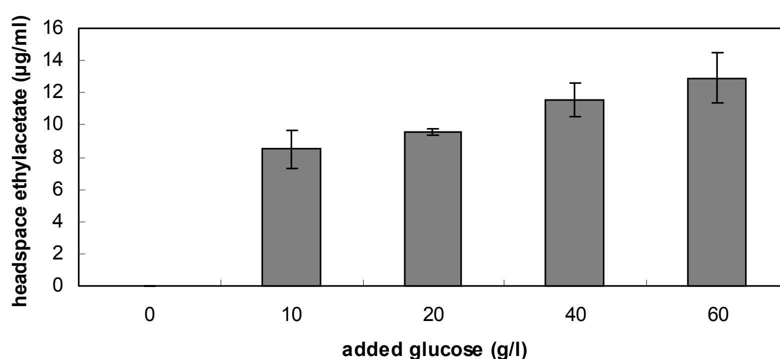

Figure 13. Glucose effects on ethyl acetate concentrations in the minisilo atmosphere. Glucose was added in the solution used to hydrate the dry wheat grain (n=3, ± SD). Modified from **(IV)**.

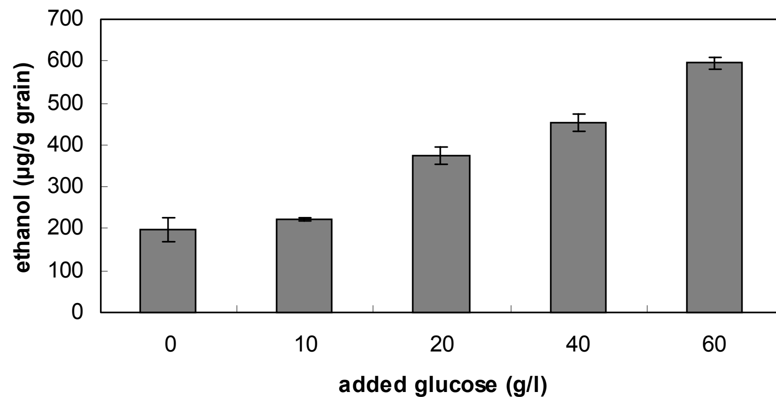

Figure 14. Glucose effect on the amount of ethanol in the minisilo. Glucose was added in the solution used to hydrate the dry wheat grain (n=3, ± SD). Modified from **(IV)**

Ethanol has well-known antimicrobial, and hence antifungal, effects. However, the full effect of glucose addition on biocontrol was observed already after addition of 10g glucose/l (Fig. 15) and at this concentration, the ethanol concentration in the minisilos was only marginally enhanced (Fig. 14). Moreover, *S. cerevisiae* is as an efficient ethanol producer and particularly tolerant to high ethanol concentrations. Therefore if ethanol alone were the critical inhibitory component, *S. cerevisiae* would be the ideal yeast for biocontrol of grain moulds. However, this yeast has not show any biocontrol activity in the grain minisilo system (**III,** Petersson & Schnürer, 1995)

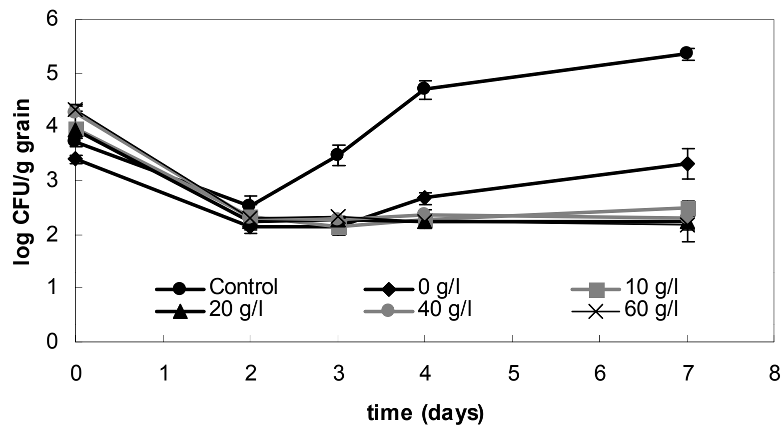

Figure 15. Effect of glucose hydration of wheat on biocontrol of *P. roqueforti* by *P. anomala* (n=3, ± SD). Modified from **IV.**

When a haploid and a diploid strain of *P. anomala* were compared in the minisilo system at a_w 0.95, we found a lower biocontrol ability of the haploid strain. When the a_w of the grain was increased from a_w 0.95 to 0.98, this difference

vanished **(V)**. Further investigations showed a prolonged lag phase of the haploid strain at the lower a_w (Fig. 16). Ethyl acetate was measured in the system and a clear difference could be seen in production when the haploid strain was inoculated on grain with high or low a_w (Fig. 16). When *P. roqueforti* was grown under different ethyl acetate head space concentrations, growth was inhibited at concentrations above 2µg/ml **(V)**. This observations of ester effects of fungal growth agree with those found by Filonow (2003).

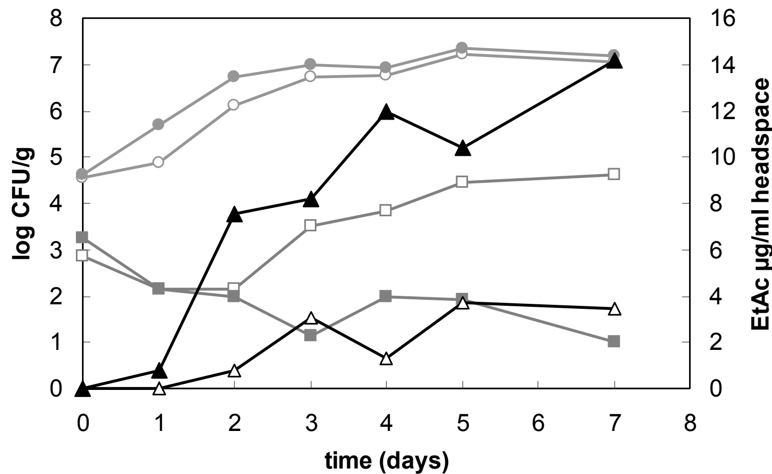

Figure 16. The ability of the haploid *P. anomala* strain CBS 1984 to grow, inhibit mould and produce ethyl acetate in the minisilo system at different a_w. *P. anomala* growth at a_w 0.95: ○ and a_w 0.98: ● *P. roqueforti* growth at a_w 0.95: □ and a_w 0.98: ■. Production of ethyl acetate at a_w 0.95: Δ and a_w 0.98 ▲.

These results suggest that ethyl acetate has a major role in the biocontrol effect of *P. anomala* J121. Based on that, we can also explain earlier findings. The prolonged lag phase of the haploid strain at low a_w can be compared with that of the naturally occurring yeast **(II)**. The slow start probably led to the inability to control the mould growth. However, when a large number of strains were tested for their biocontrol activity several known ethyl acetate producers showed no or low inhibitory capacity **(III)**. An explanation might be that even if the final CFU/g grain was similar to that of *P. anomala,* the initial growth rate was too slow or the ethyl acetate production was reduced due to harsh growth conditions in the grain silos.

Safety of biological control agents

An increasing number of biological control products are on the market or awaiting regulatory approval. This has initiated a debate on safety aspects. On one hand, the biocontrol advocate suggests biological control as a superior and more environmental friendly alternative to chemical control. The suggested advantages compared to synthetic fungicides are fewer non-target and environmental effects and less probability of resistance problems. On the other hand, more doubtful minds point to potential harmful effects of introducing non-indigenous species in sensitive ecosystems.

Indeed, nothing can be regarded to be absolutely safe and the question therefore must be: What can be defined as safe and which criteria should be used for measuring safety? It is almost impossible to conquer a pest or a pathogen population without influencing the surrounding ecosystem, no matter what method or strategy is used. Cook (1996) identified four safety issues with potential unintended and adverse effects of microbial biocontrol agents: Competitive displacement; allergenicity; toxigenicity of antibiotics and other biologically active metabolites; and pathogenicity. Competitive displacement, specific toxigenicity and pathogenicity are also all modes of action of many biocontrol organisms.

Safety of *P. anomala* J121

The occurrence of non-target effects is naturally also a question of choice of biocontrol organism, mode of action, application method, *etc.* As stated earlier, yeasts do not produce spores or mycotoxins as mould species do, nor do they produce antibiotic metabolites as bacterial antagonists probably do. The risk of allergenicity is also considered to be low. Furthermore, none of the suggested modes of action of the yeast are likely to constitute any hazard for consumers.

Pichia anomala is classified as a biosafety level 1 organism, without any restrictions on handling, and is considered to be safe for healthy individuals (de Hoog, 1996). However, infection outbreaks in immunocompromised hosts do occur and there have been reports of increasing numbers of outbreaks in recent years. Generally, these *P. anomala* infections have occurred in environments with patients immunocompromised as a result of disease or therapeutic procedures (Aragao *et al.*, 2001; Chakrabarti *et al.*, 2001; Kalenic *et al.*, 2001; Thuler *et al.*, 1997; Yamada *et al.*, 1995). Therefore this increase in outbreaks observed in the literature is more likely to reflect an increased number of immunocompromised patients than an enhanced level of virulence of the yeast. *P. anomala* J121 was found to be sensitive to commonly used antimycotic compounds **(I)**.

Inoculation of the yeast to the man-made grain ecosystem of a storage silo represents a reinforcement of a species already present rather than addition of a non-indigenous organism. *P. anomala* is naturally occurring on grain and is already favoured by the conditions in the silo. In our studies we have even seen that naturally occurring *P. anomala* in non-inoculated silos grows to the same levels as in inoculated systems. At this point, competitive displacement is certainly

a mode of action of the biocontrol effect and the risk for non- target effects within the closed silo environment should be low. However, it is important to take precautions during production, formulation and inoculation to avoid overexposure of personnel (Murphy & Kavanagh, 1999).

Summary of the current knowledge of *P. anomala* biocontrol

Generally, it is difficult to fully identify the mode of action of a biocontrol organism, and more than one mechanism is likely to be responsible. This is also true for the mode of action of *P. anomala*. I believe that a combination of different characteristics of the yeast inhibits mould growth in a sort of 'hurdle' effect. The main mechanisms may even differ in different biocontrol environments or be dependent on the target organism.

In all cases when yeast or mould was inoculated into the minisilo system, the yeast growth started within one day after inoculation and mould growth was detectable after two days (Figs. 2 and 4). Therefore the period of the first two days, *i.e.* the beginning of the storage period, might be the main determinant of the biocontrol ability in the system.

As stated earlier, maintaining high CO_2 levels might be more important than securing low O_2 levels. However, if oxygen leaks into the system, higher concentrations of CO_2 will subsequently be needed to inhibit the mould growth. The role of *P. anomala* is postulated thus:

I) In the beginning of the storage period, large amounts of CO_2 are produced at the same time as O_2 is consumed. This prevents mould growth. Yeast takes part in this process, but not as the major producer/consumer. This occurs during all airtight storage due to the respiration of the grain and epiphytic microorganisms.

II) When the atmosphere changes to more oxygen-limited conditions, *P. anomala* starts to produce large amounts of ethyl acetate. This further inhibits mould growth and sporulation of the mould before the winter.

III) In the spring, when the maximum gas exchange takes place in the silo, *P. anomala* rapidly consumes the oxygen. This prevents the sporulation of the mould, compared to silos where yeasts have not been inoculated. If the oxygen level becomes too high, moulds grow and sporulate when the temperature increases.

IV) When the constantly decreasing grain bulk is not able to maintain CO_2 levels >50%, oxygen consumption and ethyl acetate production by *P. anomala* prevent the growth and sporulation of the mould.

V) Competition for available nutrients may still contribute to the mould inhibition by *P. anomala*, even though no evidence for this was found in this study.

Concluding remarks

Storing moist grain in airtight silos is an energy-saving method compared to the alternative, high temperature drying. However, problems with air leakage and mould growth in the grain before the next harvest have made many farmers sceptical and the method is not commonly used in Sweden today.

By addition of *P. anomala* to the grain when put into the silo, the system can become less sensitive and the time of safe storage prolonged. *P. anomala* is naturally occurring on grain and the main effect of the yeast is to strengthen the antifungal effect of the airtight storage, *i.e.* to increase CO_2 and decrease O_2. In addition *P. anomala* produces ethyl acetate which inhibits mould growth. Ethyl acetate is a highly volatile compound that easily disseminates through the system and that evaporates quickly from the grain when it is taken out for feed.

Research is ongoing on formulation and storage stability of the yeast in order to get an effective product on the market. I believe that if this succeeds, airtight storage of feed grain can become a promising alternative to the more expensive and energy-consuming high temperature drying. Furthermore, Vora and Satyanarayana have in several reports pointed out that *P. anomala* produces a thermo- and acid-stable phytase suitable for supplementing animal feed, either purified as enzyme or as living yeast cells (Vohra & Satyanarayana, 2001, 2002a, b). Cereal grain contains phosphate-rich phytic acid, which is poorly digested by animals. Addition of yeast phytases to animal feed can be a more economical and environmentally friendly alternative to inorganic phosphate supplementation in *e.g.* swine and poultry diets, as these hydrolytic enzymes are able to help the release of phosphate from phytate (Lei & Stahl, 2001; Stahl *et al.*, 2000).

Within this project several new potential biocontrol yeasts for grain storage are presented. The biocontrol ability of two of these, *Candida fennica* and *Candida silvicultrix*, had not been demonstrated previously. The resistance problems that arise after treatment with chemical preservatives and fungicides are believed to be less likely for biocontrol agents due to their complex inhibitory mechanisms. In addition, access to several biocontrol yeasts offers a possibility to use combinations and to make use of their different biological properties, thus making the system more robust.

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I. Did experimental work concerning a_w, temperature, pH and killer toxin production. Participated in data evaluation and writing of the manuscript

II. Prepared inocula and participated in the mould/yeast determinations. Had the main responsibility for evaluation of the results and writing of the manuscript.

III. Did all the experimental work and had the main responsibility for planning, data evaluation and writing of the manuscript.

IV. Did all the experimental work and had the main responsibility for planning, data evaluation and writing of the manuscript.

V. Performed growth study in minisilos and analysis of ethyl acetate production. Participated in evaluation and writing of the manuscript.

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