Biocontrol of *Penicillium roqueforti* on grain – a comparison of mode of action of several yeast species

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Abstract

The yeast *Pichia anomala* has been found to inhibit the growth of several mould species. In this paper several mechanisms potentially being the mode of action of the biocontrol yeast *P. anomala* was examined. *P. anomala* was compared with a number of yeasts (*Pichia farinosa*, *Clavispora lusitaniae*, *Pichia guillermondii*, *Pichia burtonii*, an unknown yeast, *Hanseniaspora uvarum, Candida fennica, Candida pelliculosa,* and *Candida silvicola*) with respect to four possible mode of actions; killer toxin production, β-1-3-glucanase secretion, production of ethyl acetate and nutrient competition. *P. anomala*, *P. farinosa*, *C. lusitaniae*, *P. guillermondii*, *P. burtonii*, *C. fennica, C. pelliculosa,* and *C. silvicola* inhibited *Penicillium roqueforti* growth in a miniature grain silo with moist wheat (water activity of 0.95). The inhibiting effect was enhanced for several of the yeasts when glucose, maltose, glycerol, or starch was added to the miniature silo. The addition of the different carbon sources did not influence the total yeast cell count in the miniature silos, neither was *P. roqueforti* growth influenced, when cultivated without yeast. *P. anomala, C. silvicultrix, P. farinosa, C. lusitaniae, C. silvicola*, and *C. pelliculosa* were found to produce killer toxin against *P. roqueforti.* Only *P. anomala* produced any detectable ethyl acetate but due to technical or method problems no conclusions can be drawn from that result.
The obtained results did not support killer toxin production, competition for nutrients nor secretion of β-1-3-glucanase as the main mode of action of biocontrol by *P. anomala* in this grain system. *P. farinosa*, *C. silvicultrix*, *P. guillermondii*, *P. burtonii*, *C. fennica, C. pelliculosa, C. lusitaniae,* and *C. silvicola* are potential biocontrol agents in air tight storage of cereal grain. *C. lusitaniae* have not been reported to have biocontrol activity earlier.

Keywords: yeast, biocontrol, biological control, *Pichia*, *Candida*, *Clavispora*, mode of action, *Penicillium roqueforti*

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Introduction

Yeasts have been used by man for different purposes during a long time. Mainly, different *Saccharomyces* strains have been used for wine and beer production, where ethanol is the most important end product and bread were the production of CO_2 is used for leaving of the bread. Yeasts can also be used for other purposes, e.g. feed additives and production of enzymes. During the last decade a lot of research has been done on using yeast as biological control agents. This have shown that yeasts are not only able to inhibit mould growth on post harvest fruit, vegetables, soy beans, grape wine and prevent crop diseases but also prevent stored wood discolouration (Arras et al. 2002, Buzzini & Martini 2001, Chalutz & Wilson 1990, Droby et al. 1989, Droby et al. 1993, Walker et al. 1995, Masih et al. 2001, Paster et al. 1993, Rojas et al. 2003, Rojas et al. 2001, Wilson et al. 1991, Wisniewski et al. 1991, Yonis & Stewart 1999, Punja & Utkhede 2003, Payne et al. 2000, Bruce et al. 2003).

The most common method used for preserving cereal grain is high temperature drying. Storing high-moisture grain in airtight silos without prior drying would be a more cost- and energy effective storage method. However a common problem with airtight silos is that they are not completely airtight and the farmer has to be able to continuously take out grain for feeding. This leads to air leakage, mould can grow and the possibility of toxin production increases. Several studies suggests that addition of the biocontrol yeast *Pichia anomala* J121 might solve this problem (Boysen et al. 2000, Fredlund et al. 2004, Petersson et al. 1999, Petersson & Schnürer 1998, Petersson & Schnürer 1999, Druvefors et al. 2002, Ädel Druvefors 2004). On the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, work has been done on *Pichia (Hansenula) anomala* as biocontrol yeast on high-moisture stored grain. The results have been promising; *P.anomala* inhibits *Penicillium roqueforti* both in miniature silos and larger pilot scale silos with simulated air-leakage (Petersson et al. 1999, Petersson & Schnürer 1998).

Preservation methods

Drying

When harvested in temperate countries as Sweden, cereal grain almost always has too high water content to be stored without some kind of preservation method. The development of mould is determined by water content, temperature and the storage time of the grain. The most common method for conservation in Sweden is high temperature drying (Svedinger 1995) where hot air is used to dry the grain to a water content of 13% before storage. However, this procedure is very energy consuming and consumes up to 60% of the total quantity of fuel used in plant husbandry operations in Sweden (Pick et al. 1989).

Chemical treatment

An alternative to drying for feed grain is acid treatment, by spraying the grain with propionic acid immediately after harvest. This prevents the breathing of the grain and growth of mould. The problem with this method is that the amount of acid has to be very precise and water content and planned storage time have to be considered when the amount is calculated. Also, it is of great importance that the acid is evenly distributed in the grain. Specialised moulds (e.g. *Aspergillus flavus*) can grow at a low pH and increase the pH trough their metabolism and thus clear the way for other microorganisms (Svedinger 1995, Lärn-Nilsson et al. 1997). Inadequately treated grain, colonized by *Aspergillus*, seems to enhance aflatoxin production (Lacey 1989).

Air tight storage

A grain kernel is a living organism and also after harvest it continues breathing, consuming O_2 and produces heat and CO_2. The principle for air tight storing of feed grain relies on these facts, the O_2 is consumed and the CO_2 level increase. Under these conditions most mould cannot grow. To prevent pressure differences inside the silo due to temperature changes and grain outtake, a breather bag is used. The breather bag is a large sack with contact to outside air through a vent. When pressure decrease the lung increases in volume and when the pressure increase, the lung decreases. One of the greatest difficulties with air tight storage of cereal grain is to keep the silo completely airtight. During the winter months there is usually no problem with the quality of the feed due to the low temperature. When spring comes and the temperature varies considerably inside the silo, the quality, i. e. the mould growth, often becomes a problem (Svedinger 1995). When temperature rises and O_2 leaks in *Penicillium* spp. can start to grow. Some strains of *Penicillium* produce ochratoxin (Lärn-Nilsson et al. 1997).

Penicillium roqueforti

P. roqueforti is a well known storage mould. It is the first mould to appear in high moisture stored grain when O_2 concentration increases, preceded only by yeasts. It can grow with CO_2 concentrations up to 80% or O_2 concentrations <1% (Lacey 1989).

Biocontrol

A reason to use yeasts as biocontrol organisms is that the need for fungicides is less or none. Most fungicides are applied directly to fruits and vegetables and man are more subject to direct exposure than to other pesticides. Furthermore, nine oncogenic compounds account for about 90% of all fungicide sales and fungicides constitute 60% of the oncogenic risk among pesticides used on food (Wilson et al. 1991).

Spoilage and toxin-producing moulds can be inhibited by antagonistic fungi during storage. *Saccaromyces cerevisae* and *Debaromyces* spp. have been shown to inhibit *Schlerofoma pithyophila* and *Ophiostoma piceae* growing on wood (Payne

et al. 2000, Bruce et al. 2003). *Debaromyces hansenii* and *P. guillermondii* are antagonists against *P. digitatum* (Droby et al. 1989, Droby et al. 1993).

P. anomala has shown inhibitory effects on strains of *Aspergillus, Fusarium, Penicillium* and *Rhizopus* inoculated on moist wheat kernels (Petersson & Schnürer 1995). It has also been shown to inhibit *P. roqueforti* growth on barley and oat kernels in miniature silos (Petersson & Schnürer 1998). Petersson et al. (1999) have shown that *P. anomala* was as effective in inhibiting mould infection in outdoor pilot scale silos as it was in the miniature silos. Because of the non-homogenous structure of a silo the conditions inside silos can vary. Therefore it is important to know that the yeast used as biocontrol can survive during varying conditions. *P. anomala* can survive in temperatures ranging from -20 ˚C to 37 ˚C and the survival rate after 2 months in -20 ˚C is 48%, sufficient for biocontrol. *P. anomala* can also survive pH values between 2.0 and 12.4, water activity of 0.92 (NaCl) 0.85 (glycerol) and it can assimilate a wide variety of C- and N- sources (Petersson & Schnürer 1998, Fredlund et al. 2002). Several modes of action have been suggested for *P. anomala*; competition for nutrients, killer toxin production, volatile compounds and secretion of β-1-3-glucanases.

Possible mechanisms of fungal inhibition

Killer toxin

P. anomala is a known killer toxin producer (Buzzini & Martini 2001, Guyard et al. 1999, Mathews et al. 1998, Rosini 1983, Fredlund et al. 2002). Makover & Bevan (1963) were the first to describe the killer phenomenon in 1963 within strains of *Saccharomyces cerevisae*. The killer toxins are of proteinaceous nature and their mode of action differs. Cell membrane pore formation can be targeted or the cell cycle can be arrested at the G1 phase (Golubev 1998). There are other genera where the killer property is present, like *Candida, Debaromyces* and *Klyveromyces* (Rosini 1983). The killer toxin activity and production is sensitive to temperature and pH changes (Sawant et al. 1989, Suzuki & Nikkuni 1989).

Fermentation products

During fermentation yeasts produce different fermentation products. *P. anomala* and *Hanseniaspora uvarum* can produce ethyl acetate, isoamyl acetate and 2-phenylethyl acetate, *P. anomala* also produce geranyl acetate (Rojas et al. 2003, Rojas et al. 2001). Under oxygen limitation *P. anomala* produces ethanol (Fredlund et al. 2004). According to Mingorance-Cazorla et al. (2003) *P. anomala* and *Clavispora lusitanae* produce high amounts of acetaldehyde, which is cancerogenic.

Volatiles have been shown to inhibit sapstain fungi, mould and blue-stain fungi (Payne et al. 2000, Bruce et al. 2003). Supplements of different carbohydrates during fermentation can lead to production of volatiles with different composition (Yonis & Stewart 1999).

β-1-3-glucanases

Pichia membranifaciens is an antagonist to *Botrytis cinerea* and its suggested mode of action is secretion of β-1-3-glucanases. The glucanases are supposed to be the most abundant cell wall hydrolases in yeast. Glucose addition seems to have inhibiting effect on the β-1-3-glucanas activity (Masih & Paul 2002, Jijakli & Lepoivre 1998). β-1-3-glucanase activity has been detected in culture filtrates of *P. anomala* and *Pichia guillermondii* produces large amounts (Wisniewski et al. 1991, Jijakli & Lepoivre 1998).

Competition for nutrients

If the antagonist is better adapted to the environment or have a rapid growth rate it can inhibit by competition (Wilson et al. 1991). Droby et al. (1989) showed that *Debaromyces hansenii's* biocontrol activity against *Penicillium digitatum* could be overcome by adding exogenous nutrients.

In order to suitably select, produce, formulate, register and use biocontrol agents under commercial conditions it is important to know their protection mechanisms. In this thesis *P.anomala* is compared with other killer yeasts in order to find out what the mode of action is.

Materials and methods

Fungal isolates

Table 1. *Species used in the experiments*

Species	Number	Origin
Pichia anomala	J121	Grain, CBS 100487
Pichia guillermondii	J167	Not known
Candida silvicultrix	J391	Tunnel of beetle
Pichia burtonii	J464	Grain
Pichia farinosa	J471	Sorbitol solution
Unknown yeast	J536	Orange juice
Clavispora lusitaniae	J537	Orange juice
Hanseniaspora uvarum	J538	Grape must
Penicillium roqueforti	J5	Grain
Candida pelliculosa	U39	Grain
Candida silvicola	U116	Grain
Candida fennica	U128	Sorghum

The strains which were used in the experiments are kept in the culture collection of the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden. *C. fennica*, *C. silvicultrix*, *C. pelliculosa*, *P. farinosa*, *P. burtonii*, *C. silvicola* and *P. guillermondii* has been proved to inhibit mould growth in miniature silos (Ädel Druvefors & Schnürer 2005).

Yeast strains were grown on Malt-Extract Agar (Oxoid, UK) supplemented with 0.01% chloramphenicol (Sigma) (MEAC) plates for 48 hours at 25 °C and then stored at 2 °C. *P. roqueforti* was harvested from 5-day-old colonies grown on MEAC plates. Spores were collected in peptone water (0.2% bacteriological peptone [Oxoid, UK] in distilled water+ two drops of Tween 80 [Kebo AB]) and enumerated by cell counting using a Bürcker cell. Before miniature silo experiments the yeast strains were inoculated with a loopful of cells in an Erlenmeyer-flask with YPD medium (yeast extract, 10 g/litre [Oxoid]; peptone, 20 g/litre [Oxoid]; glucose, 20 g/ litre [Merck]) and then placed on a rotary shaker at 25°C overnight. The next day the yeast concentration was determined by cell counting with a Bürcker cell.

Yeast inhibition experiment

Non-sterile winter wheat (Kosak) was moistened with 9 g tap water/50g wheat and left in 2 °C for 2-3 days to equilibrate the moisture content. In four of the silo trials the water was replaced by 0.1 M carbon source solutions. Carbon sources used were glucose (Merck), maltose (Merck), glycerol (Merck, 87%) and starch (Merck). Water activity (a_w) was measured on the wheat with an AQUA Lab CX-2 (Decagon Devices, Pullman, WA, USA) and were >0.95. The wheat was inoculated with 10^3 *P. roqueforti* spores/g and tested yeast to ~10^5 CFU/g. The mould and yeast suspensions were applied as drops on the wheat and then mixed to obtain an even distribution. Aliquots of inoculated grain were filled into four thick walled test tubes (approx. 17g grain/tube), the tubes were sealed with a rubber plug and to simulate air leakage a syringe needle was set to perforate the plug. The tubes were incubated at 25 °C for seven days. Grain inoculated with only *P. roqueforti* served as control.

On day 7 the silos were opened. The grain was emptied into a stomacher bag, diluted 10-fold with peptone water and homogenized for two minutes at normal speed in a Stomacher 400 (Colworth, UK). Yeast and mould growth was evaluated as CFU/g grain on MEAC and MEACC (supplemented with 5ppm cycloheximide) plates respectively. Cycloheximide was supplemented to suppress yeast growth. The experiment was run in triplicates.

Gas chromatography

Gas chromatography (GC) samples were prepared by shaking 5 ml 99% Decane (Sigma) in a miniature silo for 20 minutes. About one ml was drawn out and filtrated (Acrodisc® LC 13 mm Syringe Filter with 0.45 µm PVDF Membrane) before analyzed by a Hewlett Packard gas chromatograph with a flame ionization detector (250°C, Hewlett Packard Ltd., Cheshire, England) and a capillary column (HP19091S-833 250 µm x 30.0 m). The carrier gas was H_2 at a flow rate of 40.0 ml/min. The column temperature was programmed from 60 to 250°C at a rise rate of 20°C/min and 2 minutes at 250°C. The experiment was run in duplicates.

Killer toxin production

Killer toxin activity was investigated on Methylene Blue Agar (MBA) seeded with sensitive yeast. The MBA plates were prepared in a citrate-phosphate buffer, pH 4.5. To the buffer 2% Agar technical (Oxoid), 2% Saboraud-2%glucose broth (Merck) and 1% tryptone (Oxoid) was added and then heated to 100°C. Methylene blue (0.003 %) and glycerol (5%) was added before the agar was autoclaved (121°C, 15 min). The MBA was cooled to 45°C before sensitive yeast cells were seeded at a density of $5x10^4$ cells/ml. The seeded agar was gently mixed and poured into Petri-dishes. One colony from each yeast strain was streaked on a plate, in three replicates, and plates were incubated at 25 °C for three days. MBA seeded with *P. roqueforti* were also made as described above. The experiment was run in triplicates.

Volatile production

In the centre of a MEA plate 10µl soft agar (0.2%) containing 10^4 *P. roqueforti* spores/ml was placed. Yeast strains were spread on MEAC at a concentration of $1x10^4$ on the plate. The yeast plates were placed "mouth to mouth" with the mould plates. Parafilm was wrapped two revs around the edges of the plates to hold the plates together and prevent air leakage. Days 1 to 5 and 7 the diameters of the *P. roqueforti* colonies were measured. Both the total and the diameter of the "thick" part were estimated, and the "thick" part was defined as the thicker, fluffy, greenish middle part of the mould colony. The experiment was run in triplicates.

Results

Table 3, 4, 5 and 6 shows that addition of carbon sources to the silo increases the yeasts inhibitory effect on *P. roqueforti* J5 compared to a silo moistened with tap water, table 2. Addition of glucose and maltose increases most yeast strains inhibiting effect from +++ to below detection limit. Adding starch and glycerol was less efficient.

Table 2. *Inhibition of* P. roqueforti *growth in grain miniature silo moistened with water and inoculated with different yeast species. Responses compared to non-yeast control are given in five classes as the maximum* P. roqueforti *CFU detected at day 7: - (no inhibition >10^5); + (10^4-10^5); ++ (10^3-10^4); +++ (<10^3 mould CFU/g); below detection limit (bd) <10 CFU/g*

-	+	++	+++	bd
Unknown yeast			*P. anomala*	
H. uvarum			*C. silvicultrix*	
			P. burtonii	
			P. farinosa	
			C. fennica	
			C. pelliculosa	

C. lusitaniae

Table 3. *Inhibition of* P. roqueforti *growth in grain miniature silo moistened with 0.1 M glucose solution and inoculated with different yeast species. Responses compared to non-yeast control are given in five classes as the maximum* P. roqueforti *CFU detected at day 7: - (no inhibition >10^5); + (10^4-10^5); ++ (10^3-10^4); +++ (<10^3 mould CFU/g); below detection limit (bd) <10 CFU/g*

-	+	++	+++	bd
			C. silvicultrix	*P. guillermondii*
			C. pelliculosa	*P. burtonii*
				P. farinosa
				C. silvicola
				C. fennica
				P. anomala

Table 4. *Inhibition of* P. roqueforti *growth in grain miniature silo moistened with 0.1 M maltose solution and inoculated with different yeast species. Responses compared to non-yeast control are given in five classes as the maximum* P. roqueforti *CFU detected at day 7: - (no inhibition >10^5); + (10^4-10^5); ++ (10^3-10^4); +++ (<10^3 mould CFU/g); below detection limit (bd) <10 CFU/g*

-	+	++	+++	bd
			C. silvicultrix	*P. anomala*
			C. pelliculosa	*P. guillermondii*
			C. fennica	*P. burtonii*
				P. farinosa
				C. silvicola

Table 5. *Inhibition of* P. roqueforti *growth in grain miniature silo moistened with 0.1 M starch solution and inoculated with different yeast species. Responses compared to non-yeast control are given in five classes as the maximum* P. roqueforti *CFU detected at day 7: - (no inhibition >10^5); + (10^4-10^5); ++ (10^3-10^4); +++ (<10^3 mould CFU/g); below detection limit (bd) <10 CFU/g*

-	+	++	+++	bd
			C. silvicola	*P. anomala*
			C. silvicultrix	
			C. pelliculosa	
			P. guillermondii	
			P. burtonii	
			P. farinosa	
			C. fennica	

Table 6. *Inhibition of* P. roqueforti *growth in grain miniature silo moistened with 0.1 M glycerol solution and inoculated with different yeast species. Responses compared to non-yeast control are given in five classes as the maximum* P. roqueforti *CFU detected at day 7: - (no inhibition >10^5); + (10^4-10^5); ++ (10^3-10^4); +++ (<10^3 mould CFU/g); below detection limit (bd) <10 CFU/g*

-	+	++	+++	bd
			P. anomala	*P. guillermondii*
			C. silvicola	*C. fennica*
			C. silvicultrix	
			C. pelliculosa	
			P. burtonii	
			P. farinosa	

Table 7 shows the different strains killer toxin production against *P. roqueforti*. *P. anomala*, *P. farinosa* and *C. lusitaniae* show good killer toxin activity while *P. guillermondii*, *P. burtonii*, unknown yeast, *H. uvarum* and *C. fennica* show no activity. Clear zones surrounded by a blue ring of dead cells around the yeast streak were scored as positive result.

Table 7. *Anti-*P. roqueforti *killer toxin production by the different yeast strains.+ small clear zone, ++ large clear zone, - no clear zone and no blue ring of dead cells*

Yeast	Killer toxin production
P. anomala	++
P. guillermondii	-
C. silvicultrix	+
P. burtonii	-
P. farinosa	++
Unknown yeast	-
C. lusitanae	++
H. uvarum	-
C. silvicola	+
C. fennica	-
C. pelliculosa	+

Only *P. anomala* and *C. lusitaniae* showed signs to inhibit *P. roqueforti* J5 on the "mouth-to-mouth" plates where *P. roqueforti* was measured every day. The mould colonies showed signs of stress with a large part thin, outstretched mycelium and achieved a total diameter of 74.5 mm growing with *P. anomala* and 76.5 mm growing with *C. lusitaniae*. The control achieved a total diameter of 59.5 mm and had denser, thick colonies almost without outstretched mycelium. It seems like the inhibiting effect of volatiles is most pronounced in the beginning of the growth period (Figure 1, Figure 2).

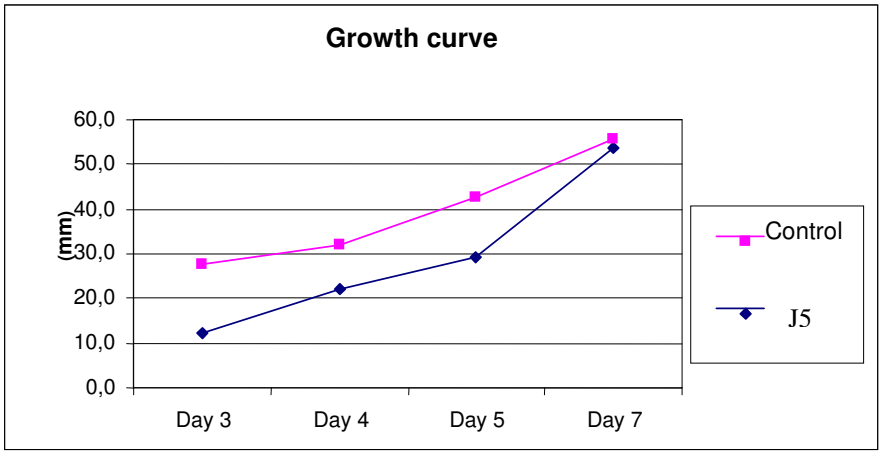

Figure 1. *The diameter of the "thick" part of* P. roqueforti *J5, on the mouth-to-mouth plates with* C. lusitaniae

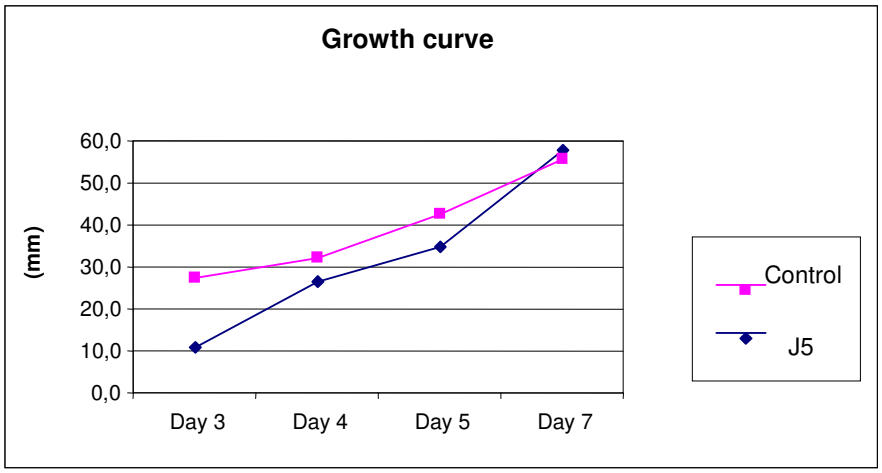

Figure 2. *The diameter of the "thick" part of* P. roqueforti *J5, on the mouth-to-mouth plates with* P. anomala

Of the yeasts tested for ethyl acetate production only *P. anomala* produced amounts over detection level (Table 8).

Table 8. *Ethyl acetate production*

Yeast	Ethyl acetate production
P. anomala	+
P. farinosa	-
Unknown yeast	-
C. lusitaniae	-
H. uvarum	-

All the yeasts which were tested with different carbon sources grew to a similar total CFU count. In the control tubes, *P. roqueforti* had the same CFU count during all the experiments (Appendix, Table 9).

Discussion

In the miniature silo experiment most of the yeasts increased their inhibiting effect on mould growth when simple carbohydrates (glucose or maltose) were added and none of the strains showed a decreased effect. *P. anomala* J121 also showed increased effect when starch was added and *P. guillermondii* and *C. fennica* showed increased effect with added glycerol. This could be related to the different yeasts ability to utilise different carbon sources. These results did not give any support to the competition for nutrients theory. Addition of nutrients to the miniature silos should have decreased the inhibiting effect if nutrient competition is the mode of action of the yeasts. These results are supported by later work done by Ädel Druvefors & Schnürer (2005).

The suggested mode of action for *P. guillermondii* is secretion of β-1-3-glucanase, a cell wall lytic enzyme. Strains of *P. anomala* also secrete this enzyme (Jijakli & Lepoivre 1998). Masih & Paul (2002) have showed that the glucanase production in related yeast was inhibited by addition of glucose. In this study though, *P. guillermondii* and *P. anomala* inhibited *P. roqueforti* below detection level when glucose was added. Furthermore, when the exoglucanase-encoding gene, PaEXG2 coding for β-1-3-glucanase in *P. anomala* strain K was disrupted it did not affect the yeast's biocontrol activity (Grevesse et al. 2003).

The growth of the mould was not altered when the different carbon sources was added without inoculation of *P. anomala.* This suggests there were no inhibitory actions by the carbohydrates themselves or by carbohydrate stimulation of naturally present microorganisms in the grain. The final yeast numbers (CFU) were constant during the experiments, therefore the conclusion can be drawn that crowding by the yeast probably is not occurring. Earlier studies also have showed that inoculated *P. anomala* doubled the time for inoculated *P. roqueforti* to increase from 10^2 to 10^5 CFU/g, without showing observable increase in CFU itself (Petersson et al. 1999).

This would suggest that the mode of action rather is related to the products of sugar metabolism or killer toxin production.

P. anomala, C. silvicultrix, P. farinosa, C. lusitaniae, C. silvicola and *C. pelliculosa* produced killer toxin against *P. roqueforti* and they also inhibited mould growth in the miniature silos. *P. guillermondii, P. burtonii* and *C. fennica* did not produce killer toxin but they too inhibited mould very well in the miniature silos. There did not seem to be any correlation between killer toxin production and inhibiting capacity. The environment inside a real, large silo (e.g. temperature) varies and killer toxins are very sensitive to environmental changes and therefore it

is unlikely that killer toxin production is *P. anomala's* mode of action, at least as the only inhibiting factor.

P. anomala and *C. lusitaniae* inhibited *P. roqueforti* when tested with the mouth-to-mouth method which indicates production of volatiles with antifungal activity. However, of the yeasts tested only *P. anomala* J121 produced any detectable ethyl acetate and in this experiment *P. anomala* produced a low amount. Strain J121 has been proven to produce high amounts of ethyl acetate in other studies (Ädel Druvefors et al. 2005, Fredlund & Ädel Druvefors 2004). This suggested a method problem or a technical problem and there is a possibility that the other yeast species were registered as a false negative. The experiment should be repeated to reject or confirm the results. However, ethyl acetate produced by *P. anomala* J121 has shown antifungal activity in studies made by Ädel Druvefors et al. (2005) which points to that this volatile, perhaps in combination with other volatiles, could be the major mode of action.

There were contamination problems during the experiments. *Rhizopus* spp were found to be growing on some of the MEAC and MEACC plates used to calculate the yeast and mould CFU. The contamination probably was due to spores spread through the air originating from others in the laboratory who where culturing these species. This resulted in that some results from the plates could not be used in calculating CFU, but no whole series of plates were destroyed and the final results are consistent with later findings.

Several of the yeasts used in these experiments are potential biocontrol yeasts, i. e. *P. anomala*, *P. farinosa*, *C. silvicultrix*, *P. guillermondii*, *P. burtonii*, *C. fennica, C. pelliculosa,* and *C. silvicola,* including *C. lusitaniae* which have not been reported to have biocontrol activity earlier. Their potential as biocontrol agents should be investigated further. The possibility for a greater diversity in biocontrol agents would suggest a potential for more robust applications in the future. The positive effect the addition of simple carbohydrates has on the inhibiting effect of *P. anomala* and other species could provide an opportunity to increase the performance of these organisms in a potential commercial formulation.

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Appendix

Table 9. *Total CFU count in miniature silos with different carbon sources. Values are LOG average, n=3*

	Water	Glucose	Maltose	Starch	Glycerol
P. anomala	7,7	7,6	8,1	7,6	7,9
P. guillermondii	*	7,2	7,4	7,3	8,1
C. silvicultrix	7,5	7,6	7,7	7,7	7,5
P. burtonii	7,4	7,1	7,2	7,2	7,4
P. farinosa	7,3	7,2	7,3	7,3	7,4
C. silvicola	*	7,4	7,3	7,2	7,3
C. fennica	7,6	7,3	7,6	7,6	7,8
C. pelliculosa	7,5	7,8	7,8	7,6	7,6
Unknown yeast	6,1	*	*	*	*
C. lusitaniae	7,5	*	*	*	*
H. uvarum	6,3	*	*	*	*
P. roqueforti	6,1	5,7	5,6	5,2	5,7

*not tested