Inhibition of *Enterobacteriaceae* by *Pichia anomala* during moist grain storage

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Abstract

Enterobacteriaceae one of the bacteria families found in the grain, cause problems later in the food chain. Different methods are applied to give safe storage of harvested grain. Technique like airtight storage in combination with biopreservation provides a good opportunity to assure long term storage of harvested grain. *Pichia anomala* (J121) a biocontrol yeast with proved inhibitory effects on different mold species was cultivated together with 14 strains belonging to 13 *Enterobacteriaceae* species (*Pantoea agglomerans, Enterobacter sakazakii, Enterobacter cloacae, Serratia marcescens, Klebsiella oxytoca, Klebsiella pneumonia, Proteus mirabilis, Enterobacter aerogenes, Morganella morganii, Citrobacter freundii, Hafnia alvei, Erwinia carotovora, Escherichia coli*) in mini silos to stimulate airtight storage. *Enterobacteriaceae* CFU values were calculated at different time points (at the beginning, after two weeks and after two month´s). Results obtained from the study showed that in most cases an inhibition of bacterial growth was obtained. *P. agglomerans, E. sakazakii, E. cloacae, E. coli (no 13), E. coli, E carotovora* and *S. marcescens* growth was reduced under the detection level (CFU 10^1) after two month´s storage. Reduction in CFU levels (to 10^1 or higher) was observed for *K. oxytoca, K. pneumonia, P mirabilis, E. aerogenes* and *M. morganii*. Not clear results could be obtained for *C. freundii* and *H. alvei* because the death of the bacteria after two months storage period occurred in both minisilos cultivated with, and without *P. anomala*. Yeast CFU levels in mini silos with co-cultivated *Enterobacteriaceae* species as well as mini silos with no co-cultivation increased after two weeks. After two months the growth stayed at 10^6 CFU g^{-1}. Results show that the *P. anomala* have capacity of reducing growth of *Enterobacteriaceae*. This ability could improve the quality of the stored grain.

Keywords: *Pichia anomala* J121, *Enterobacteriaceae*, biocontrol, air tight storage, minisilo system, inhibition.

Contents

Aim of the study

The aim of the project is to evaluate if the yeast *Pichia anomala* inhibits growth of different *Enterobacteriaceae* species in moist cereal grain during air tight storage.

Introduction

Microbial spoilage occurs due to incomplete conservation (Fleet, 1992). Different methods are applied to enable long term storage of harvested grain. The most frequently used method in Sweden is preservation by drying (Jonsson and Pettersson, 2000). The high energy requirement of drying leads to development of new methods which are more effective and need less energy.

Microbial content in grain storage system

The grain can be colonized by different microorganisms such as bacteria or fungi. The colonization of microbial species begins already in the pre-harvested grain and change during the storage time (Flanningen, 1987; Lacey and Magan, 1991; Magan et al., 2003). Changes in microbial flora depend on the nutrient composition and on physical and chemical conditions (Lacey, 1989).

Enterobacteriaceae species

Escherichia coli can be found in many different environmental habitats, like in the intestine of animals. Several strains can be harmful and cause food poise (Denny et al 2007). *Erwinia carotovora* is a plant pathogen which can infect a wide range of plants (tomato, carrot, potato etc.) and lead to economical loses (Bell et al 2004). *Pantoea agglomerans* another plant pathogen (Cruz et al 2007) is successfully used as biocontrol agent on apples (Nunes et al 2002). Another *Enterobacteriaceae, Klebsiella oxytoca* is a bacterium which is used in industrial research for ethanol production (Dien et al 2003) but it is also a pathogen causing colitis, meaning inflammation of the colon (Högenauer et al 2006).In humans *P. agglomerans* can cause diseases, like septic monoarthritis leading to inflammation of one joints (De Champs et al 2000, Flatauer et al 1978). Another species in the *Enterobacteriaceae* family, *Enterobacter sakazakii* causes meningitis, bacteraemia and necrotizing enterocolitis leading to high death rate in human infants (Lai 2001, Bowen and Braden 2006). *Enterobacter cloacae* have been reported as pathogen causing infections in respiratory and urinary tract (Sanders et al 1997). Urinary tract infection can be cause also by *Proteus mirabilis* (Li et al 2004) or *Enterobacter aerogenes,* a soil or waste living bacterium. *Enterobacter aerogenes* occurs also in human gastrointestinal tract (Atlas et al 1998). Urinary infection occurs mostly in individuals with impaired immune system due to different hospital treatments (Bornet et al 2000). Fermentation by the bacterium leads to production of hydrogen gas which can be used as energy resource (Yokoi et al 1995). *Morganella morganii* apart from urinary tract infection, can be found in mammals intestines and cause infections in respiratory tract, wound (Kilcoyneet al 2002). *Serratia marcescens*, which is found on starch rich food and mostly associated with hospital environments (Hejazi and Falkiner 1997) also belongs to this group and apart from infections from respiratory tract to urinary tract, it causes also meningitis and septicemia (Gouin et al 1993, Cox 1985, Komer et al 1994). *Citrobacter freundii* bacterium commonly living in soil, water etc. but it can be even found in human intestine. It cans cause infection of membranes which function as protection of brain and spinal cord in human offspring (infant meningitis) and blood infection (sepsis) (Drelichman

and Band 1985, Badger et al 1999). *Klebsiella pneumoniae* is a bacterium which normally belongs to skin, mouth and intestine flora (Ryan and Ray 2004). It cans function as a pathogen and cause klebsiella pneumonia in individuals with impaired immune system due alcohol problems etc. (Podschun and Ullman 1998). *Hafnia alvei* is water and food living bacterium, and a part of human gastrointestinal flora. It can be pathogenic in humans with immunodeficiency (Podschun et al 2001, Günthard and Pennekamp 1996, Conte et al 1996) and is associated with diseases as pneumonia and peritonitis (Günthard and Pennekamp 1996, Conte et al 1996). *Enterobacteriaceae* species can affect feed quality and by reducing the bacterial flora in stored post-harvested material the risk of spreading to food production is minimized (Brooks et al., 2001). Hence the biocontrol organism is needed to inhibit the growth of potential pathogen bacterium found in post-harvested material.

Enterobacteriaceae species and grain storage

During grain storage different kinds of *Enterobacteriaceae* species can be found. The tolerable levels of *Enterobacteriaceae* in cereals have not been stated due to the assumption that the bacteria cannot multiply at low moisture content (Adams and Moss 2000). Olstorpe et al. (2010) showed that the *Enterobacteriaceae* can grow above the Swedish guideline values even at low moisture content (mc 16%). The same study also showed inhibition of the two species *P. agglomerans* (95%) and *E.coli* (5%) when they were co-cultivated with *P. anomala*. Reduction of *Enterobacteriaceae* in feed may reduce the levels later in the food production chain (Brooks et al 2001).

Preservation methods for the grain

Occurrence of microorganisms such as molds and bacteria, lead to spoilage of the grain. New techniques which consume less energy are required, to enable the long term storage of the grain.

Airtight storage and Biocontrol

Airtight storage is used to maintain long-term storage of the grain. This method only consumes approximately 2% of energy when compared to hot air drying (Pick et al 1989). The concept of the airtight storage includes anaerobic conditions under which the grain kernels and microorganisms breath and consume remaining O_2, leading to high levels of CO_2 (Lacey and Magan 1991, Magan et al 2003). Higher levels of CO_2 prevent mold growth. The problem with this technique is to maintain airtight sealing. Due to leakage of air into the silo, growth of aerobic microorganisms like molds can occur. Also the variation of the temperature during the day/night under the spring and summer give pressure changes that can increase O_2 content and thereafter lead to microbial changes (Lacey and Magan 1991, Petersson et al 1999, Druvefors et al 2002).

Preservation by using biocontrol is simply adding microorganisms, like yeasts, to the grain storage system to inhibit growth of spoilage organism and preserve the good quality of the grain (Druvefors et al 2002). Such properties have been observed is several studies. *P. anomala* antibacterial characteristics were observed in study by Olstorpe et al (2010). The inhibition of the *Enterobacteriaceae* during storage of moist cereal grain inoculated with *P. anomala* was obtained. The observation included two species, *P. agglomerans* and *E.coli*. CFU levels of both species were reduced in grain inoculated with *P. anomala,* but not in stored grain without yeast. Combination of airtight storage and biocontrol gives opportunity to obtain improved preservation and chance to obtain the good quality of the stored grain.

P. anomala J121 as a biocontrol yeast

The *P. anomala* yeast is a very useful biocontrol agent. It cans survive under different conditions such as low water activity (a_w=0,85), pH between 2.0-12.4, low levels of O_2 and temperatures which vary between 3°C to 37°C. Additional advantages with *P. anomala* are its ability to grow under anaerobic conditions (Fredlund et al. 2002). *P. anomala* can inhibit mold growth by several ways: production of killer toxins, secretion of β-1-3-glucanases, production of ethyl acetate or the competition for nutrients (Fredlund et al. 2004, Druvefors et al. 2005 Masih & Paul 2002; Wilson et al. 1991).

Materials and methods

Bacterial and yeast isolates

The bacterial and yeast strains used in this project were provided from the culture collection of the Department of Microbiology at the Swedish University of Agricultural Sciences (SLU), Uppsala Sweden. The bacterial strains were stored at -70°C. The *P. anomala* strain J121 was stored in refrigerator on MEA agar plates.

Table 2. *Enterobacteriaceae* strains used in the experiment.

***Enterobacteriaceae* strains**	**Number /strains origin**
Citrobacter freundii	ATCC 8090
Enterobacter sakazakii	ATCC 29544
Enterobacter cloacae	ATCC 13047
Klebsiella oxytoca	ATCC 13182
Klebsiella pneumoniae	no 14*
Proteus mirabilis	ATCC 29906
Enterobacter aerogenes	ATCC 13048
Escherichia coli	no 13*
Morganella morganii	ATCC 25830
Hafnia alvei	ATCC 13337
Serratia marcescens	ATCC 13880
Erwinia carotovora	SCC 3193*
Pantoea agglomerans	ATCC 27155
Escherichia coli	ATCC 11775

* ATCC – American Type Culture Collection/ SCC – Swedish Culture Collection / *K. pneumoniae* (no 14) *E.coli* (no 13) SLU cultures.

Preparation of the grain

The grain was prepared and mixed with distilled water to establish the appropriate water content, which can be observed at harvest. 750 grams of cereal grain was placed in Erlenmeyer flask and 180 ml of distilled water was added. The Erlenmeyer flask was sealed with parafilm (Pechiney Plastic Packaging Company, Chicago IL) and stored in 2°C. After three days the water activity and temperature was measured by using CX-2 AquaLab instrument (Decangon Devices, Washington, USA). The water activity (a_w) should be 0,95. The grains were divided into three portions of approximately 240 grams and placed in three beakers respectively.

Preparation of the bacterial and yeast cultures

A loop of *P. anomala* J121 strain was re-suspend with YPD Medium [yeast extract 10 g/l (Oxoid, UK), bacteriological peptone 20 g/l (Oxoid, UK), glucose 20 g/l (Merck), distilled water] in 15 ml Falcon tube and incubated in 25°C on a rotary shaker at 150 rpm over-night. The bacterial strains were inoculated with LB medium in a 15 ml Falcon tube and store in 37°C incubation rum over-night. The *E. carotovora* was incubated at 25 °C. The concentration of both the yeast and bacterial cultures were established using the Bűrcker cell counting chamber.

Inoculation of mini silos

Determination of the yeast and bacteria concentrations in the over-night cultures was performed by using Bűrcker cell counting chamber. Prepared grain was inoculated with 10^3 CFU/g of *Enterobacteriaceae* and/or 10^5 CFU/g of *P. anomala*. For each *Enterobacteriaceae* spp. four different treatments were made; treatment 1: only *P. anomala* (Pa), treatment 2: only *Enterobacteriaceae* (Eb), treatment 3: *Enterobacteriaceae* in co-cultivation with *P. anomala* (PaEb) and treatment 4: non- inoculated grain. Triplicates (A, B, C) were made (Only yeast: Pa_{ABC}, yeast in combination with bacteria: $PaEb_{ABC}$, only bacteria: Eb_{ABC} and not inoculated grain Sp_{ABC}). The yeast and bacteria were mixed with grain and approximately 18 gram of grain was respectively put into a stomacher bag for each treatment. This was the first measuring point (0t) and was examined directly after inoculation. The remaining grain was respectively divided into 18 grams portions and placed into thick walled test tubes. Then the tubes were sealed with a rubber plug and a syringe was inserted for air leakage stimulation. Each treatment was made in triplicates. All the tubes were incubated at 25°C samples were examined after two weeks (2w) and the remaining tubes was examined after two months (2m).

Table 3. *Enterobacteriaceae* /Yeast used in experiment.

***Enterobacteriaceae* /Yeast**	**0 test**	**2 week test**	**2 month test**
C. freundii/P.anomala	$Eb21_{ABC\ [0\ t]}$	$Eb21_{ABC\ [2\ w]}$	$Eb21_{ABC\ [2\ m]}$
	$Pa21_{ABC\ [0\ t]}$	$Pa21_{ABC\ [2\ w]}$	$Pa21_{BCA\ [2\ m]}$
	$Pa/Eb21_{ABC\ [0\ t]}$	$Pa/Eb21_{ABC\ [2\ w]}$	$Pa/Eb21_{ABC\ [2\ m]}$
E. sakazakii /P.anomala	$Eb22_{ABC\ [0\ t]}$	$Eb22_{ABC\ [2\ w]}$	$Eb22_{ABC\ [2\ m]}$
	$Pa22_{ABC\ [0\ t]}$	$Pa22_{ABC\ [2\ w]}$	$Pa22_{ABC\ [2\ m]}$
	$Pa/Eb22_{ABC\ [0\ t]}$	$Pa/Eb22_{ABC\ [2\ w]}$	$Pa/Eb22_{ABC\ [2\ m]}$
E. cloacae /P.anomala	$Eb23_{ABC\ [0\ t]}$	$Eb23_{ABC\ [2\ w]}$	$Eb23_{ABC\ [2\ m]}$
	$Pa23_{ABC\ [0\ t]}$	$Pa23_{ABC\ [2\ w]}$	$Pa23_{ABC\ [2\ m]}$
	$Pa/Eb23_{ABC\ [0\ t]}$	$Pa/Eb23_{ABC\ [2\ w]}$	$Pa/Eb23_{ABC\ [2\ m]}$
K. oxytoca/P.anomala	$Eb24_{ABC\ [0\ t]}$	$Eb24_{ABC\ [2\ w]}$	$Eb24_{ABC\ [2\ m]}$
	$Pa24_{ABC\ [0\ t]}$	$Pa24_{ABC\ [2\ w]}$	$Pa24_{ABC\ [2\ m]}$
	$Pa/Eb24_{ABC\ [0\ t]}$	$Pa/Eb24_{ABC\ [2\ w]}$	$Pa/Eb24_{ABC\ [2\ m]}$
K. pneumoniae/P.anomala	$Eb25_{ABC\ [0\ t]}$	$Eb25_{ABC\ [2\ w]}$	$Eb25_{ABC\ [2\ m]}$
	$Pa25_{ABC\ [0\ t]}$	$Pa25_{ABC\ [2\ w]}$	$Pa25_{ABC\ [2\ m]}$
	$Pa/Eb25_{ABC\ [0\ t]}$	$Pa/Eb25_{ABC\ [2\ w]}$	$Pa/Eb25_{ABC\ [2\ m]}$
P. mirabilis /P.anomala	$Eb26_{ABC\ [0\ t]}$	$Eb26_{ABC\ [2\ w]}$	$Eb26_{ABC\ [2\ m]}$
	$Pa26_{ABC\ [0\ t]}$	$Pa26_{ABC\ [2\ w]}$	$Pa26_{ABC\ [2\ m]}$
	$Pa/Eb26_{ABC\ [0\ t]}$	$Pa/Eb26_{ABC\ [2\ w]}$	$Pa/Eb26_{ABC\ [2\ m]}$
E. aerogenes/P.anomala	$Eb27_{ABC\ [0\ t]}$	$Eb27_{ABC\ [2\ w]}$	$Eb27_{ABC\ [2\ m]}$
	$Pa27_{ABC\ [0\ t]}$	$Pa27_{ABC\ [2\ w]}$	$Pa27_{ABC\ [2\ m]}$
	$Pa/Eb27_{ABC\ [0\ t]}$	$Pa/Eb27_{ABC\ [2\ w]}$	$Pa/Eb27_{ABC\ [2\ m]}$
E. coli(no 13) /P.anomala	$Eb28_{ABC\ [0\ t]}$	$Eb28_{ABC\ [2\ w]}$	$Eb28_{ABC\ [2\ m]}$
	$Pa28_{ABC\ [0\ t]}$	$Pa28_{ABC\ [2\ w]}$	$Pa28_{ABC\ [2\ m]}$
	$Pa/Eb28_{ABC\ [0\ t]}$	$Pa/Eb28_{ABC\ [2\ w]}$	$Pa/Eb28_{ABC\ [2\ m]}$
M. morganii /P.anomala	$Eb29_{ABC\ [0\ t]}$	$Eb29_{ABC\ [2\ w]}$	$Eb29_{ABC\ [2\ m]}$
	$Pa29_{ABC\ [0\ t]}$	$Pa29_{ABC\ [2\ w]}$	$Pa29_{ABC\ [2\ m]}$
	$Pa/Eb29_{ABC\ [0\ t]}$	$Pa/Eb29_{ABC\ [2\ w]}$	$Pa/Eb29_{ABC\ [2\ m]}$
H. alvei /P.anomala	$Eb30_{ABC\ [0\ t]}$	$Eb30_{ABC\ [2\ w]}$	$Eb30_{ABC\ [2\ m]}$
	$Pa30_{ABC\ [0\ t]}$	$Pa30_{ABC\ [2\ w]}$	$Pa30_{ABC\ [2\ m]}$
	$Pa/Eb30_{ABC\ [0\ t]}$	$Pa/Eb30_{ABC\ [2\ w]}$	$Pa/Eb30_{ABC\ [2\ m]}$
S. marcescens/P.anomala	$Eb31_{ABC\ [0\ t]}$	$Eb31_{ABC\ [2\ w]}$	$Eb31_{ABC\ [2\ m]}$
	$Pa31_{ABC\ [0\ t]}$	$Pa31_{ABC\ [2\ w]}$	$Pa31_{ABC\ [2\ m]}$
	$Pa/Eb31_{ABC\ [0\ t]}$	$Pa/Eb31_{ABC\ [2\ w]}$	$Pa/Eb31_{ABC\ [2\ m]}$
E. carotovora /P.anomala	$Eb33_{ABC\ [0\ t]}$	$Eb33_{ABC\ [2\ w]}$	$Eb33_{ABC\ [2\ m]}$
	$Pa33_{ABC\ [0\ t]}$	$Pa33_{ABC\ [2\ w]}$	$Pa33_{ABC\ [2\ m]}$
	$Pa/Eb33_{ABC\ [0\ t]}$	$Pa/Eb33_{ABC\ [2\ w]}$	$Pa/Eb33_{ABC\ [2\ m]}$
P. agglomerans/P.anomala	$Eb34_{ABC\ [0\ t]}$	$Eb34_{ABC\ [2\ w]}$	$Eb34_{ABC\ [2\ m]}$
	$Pa34_{ABC\ [0\ t]}$	$Pa34_{ABC\ [2\ w]}$	$Pa34_{ABC\ [2\ m]}$
	$Pa/Eb34_{ABC\ [0\ t]}$	$Pa/Eb34_{ABC\ [2\ w]}$	$Pa/Eb34_{ABC\ [2\ m]}$
E. coli /P.anomala	$Eb35_{ABC\ [0\ t]}$	$Eb35_{ABC\ [2\ w]}$	$Eb35_{ABC\ [2\ m]}$
	$Pa35_{ABC\ [0\ t]}$	$Pa35_{ABC\ [2\ w]}$	$Pa35_{ABC\ [2\ m]}$
	$Pa/Eb35_{ABC\ [0\ t]}$	$Pa/Eb35_{ABC\ [2\ w]}$	$Pa/Eb35_{ABC\ [2\ m]}$

[0 t] – 0 test [2 w] – 2 week test [2 m] – 2 month test Eb21 – identification number for each *Enterobacteriaceae

Evaluation of microbial growth

Each sample was put into stomacher bags and diluted 10 times with peptone water [2 g/l bacteriological peptone (Oxoid, UK), 900 ml dH_2O]. The bags were runned in a Stomacher 400 (Colworth, UK) for 120 seconds to homogenize the content. A dilution series was made on the homogenize content and both yeast and bacteria was cultured on selective plates. The bacteria concentration was evaluated by adding 1 ml of each tube content in dilution series to a petri dish and mixing with approximately 20 grams of VRBG medium [Violet Red Bile Agar(Oxoid, UK): 30,8 g VRBG; 800 ml dH_2O] which is a selective medium for the isolation and enumeration of *Enterobacteriaceae*. When the medium stiffened, approximately additional 10 grams of VRBG medium was overlaid. The plates were incubated at 30°C and CFU was calculated after 24 hours. The *P. anomala* growth was evaluated on MEAC plates [Malt Extract Agar (MEA) 45 g (Oxoid, UK); 0,009 g chloramphenicol (Boehringer Mannheim, Germany); 900 ml dH_2O] by surface plating 100 µl of content from the dilution series. The MEAC plates were incubated for 5 days at 25°C.

Overlay method

Grain (2 kg) was divided into stomacher bags (50 g/stomacher bag) and 150 ml of dH_20 was added to the grain. Stomacher bags with content was then stored at 37°C under 1 hour. The bags were then placed into a Stomacher 400 (Colworth, UK) and run for 120 seconds to homogenize the content. A sieve was used to separate the fluid from the solid particles. The fluid was centrifuged with JA14 under 10 min at 5k. The fluid was filtered through double coffee filters. Afterward the filtered fluid was sterilized with NUNC filters [NUNC, Denmark] to 400 ml sterile flasks. The sterile cereal grain water (CGW) were stored at +2°C. The CGW was used for plate preparation (grain medium: 100 ml dH_2O, 900 ml CGW, 10 g technical agar [Oxoid, UK].The grain medium was autoclaved and stored in water bath before usage. Thereafter the plates were inoculated with over-night culture of *P. anomala* (10^5cells/ml) (fig.1). The plates were incubated 2 days at 25°C. Soft agar was prepared (100 ml dH_2O, 900 ml sterile grain fluid, 10 g technical agar [Oxoid, UK]) by boiling and then putting into water bath (40°C). Before adding of the *Enterobacteriacea* the concentration was counted by using mc Farland 2 (10^5cells/ml).1 ml of each *Enterobacteriaceae* strains was added to 9 ml of soft agar in sterile tubes, carefully mixed and then added to the plates. The plates were incubated at 37°C for 1 week.

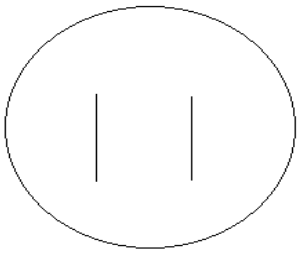

Fig.1 Inoculation of the plate with over-night culture of *P. anomala* (10^5cells/ml).

Results

Growth of *Enterobacteriaceae*

14 strains belonging to 13 bacterial species from the *Enterobacteriaceae* family were cultivated in a mini silo system together or without biocontrol yeast *Pichia anomala* (J121). The growth was monitored by calculating CFU/g grain of the bacteria and yeasts during the storage time.

Table 4. *Enterobacteriaceae* growth log CFU/g at the beginning, after 2 weeks and 2 months storage.

Enterobacteriaceae	**0 test (log)**	**2 week test (log)**	**2 month test (log)**
C. freundii 21	Eb21 [0 t]: 6.6 Pa/Eb21 [0 t]: 5.7	Eb21 [2 w]: 4.9 Pa/Eb21 [2 w]: 4.8	Eb21 [2 m]: 0 Pa/Eb21 [2 m]: 0
E. sakazakii 22	Eb22 [0 t]: 6.5 Pa/Eb22 [0 t]: 6.6	Eb22 [2 w]: 3.9 Pa/Eb22 [2 w]: 4.6	Eb22 [2 m]: 5.9 Pa/Eb22 [2 m]: 0
E. cloacae 23	Eb23 [0 t]: 5.0 Pa/Eb23 [0 t]: 5.8	Eb23 [2 w]: 4.1 Pa/Eb23 [2 w]: 5.6	Eb23 [2 m]: 4.8 Pa/Eb23 [2 m]: 0
K. oxytoca 24	Eb24 [0 t]: 6.7 Pa/Eb24 [0 t]: 6.8	Eb24 [2 w]: 7.1 Pa/Eb24 [2 w]: 7.8	Eb24 [2 m]: 7.0 Pa/Eb24 [2 m]: 3.0
K. pneumoniae 25	Eb25 [0 t]: 4.4 Pa/Eb25 [0 t]: 5.7	Eb25 [2 w]: 6.8 Pa/Eb25 [2 w]: 7.0	Eb25 [2 m]: 7.0 Pa/Eb25 [2 m]: 3.0
P. mirabilis 26	Eb26 [0 t]: 6.4 Pa/Eb26 [0 t]: 6.6	Eb26 [2 w]: 7.1 Pa/Eb26 [2 w]: 7.1	Eb26 [2 m]: 7.2 Pa/Eb26 [2 m]: 3.8
E. aerogenes 27	Eb27 [0 t]: 6.4 Pa/Eb27 [0 t]: 6.6	Eb27 [2 w]: 6.9 Pa/Eb27 [2 w]: 6.5	Eb27 [2 m]: 7.0 Pa/Eb27 [2 m]: 4.0
E. coli (no 13) 28	Eb28 [0 t]. 5.9 Pa/Eb28 [0 t]: 6.2	Eb28 [2 w]: 3.1 Pa/Eb28[2 w]: 2.9	Eb28 [2 m]: 3.8 Pa/Eb28 [2 m]: 0
M. morganii 29	Eb29 [0 t]: 3.8 Pa/Eb29 [0 t]: 5.3	Eb29 [2 w]: 5.1 Pa/Eb29 [2 w]: 5.7	Eb29 [2 m]: 6.6 Pa/Eb29 [2 m]: 3.4
H. alvei 30	Eb30 [0 t]: 3.3 Pa/Eb30 [0 t]: 4.5	Eb30 [2 w]: 2.0 Pa/Eb30 [2 w]: 2.3	Eb30[2 m]: 0 Pa/Eb30 [2 m]: 0
S. marcescens 31	Eb31 [0 t]: 6.0 Pa/Eb31[0 t]: 6.0	Eb31 [2 w]: 6.2 Pa/Eb31 [2 w]: 5.8	Eb31 [2 m]: 6.3 Pa/Eb31 [2 m]: 0
E. carotovora 33	Eb33 [0 t]: 6.4 Pa/Eb33 [0 t]: 6.5	Eb33 [2 w]: 3.3 Pa/Eb33 [2 w]: 4.6	Eb33 [2 m]: 3.4 Pa/Eb33 [2 m]: 0
P. agglomerans 34	Eb34 [0 t]: 6.4 Pa/Eb34 [0 t]: 6.4	Eb34 [2 w]: 7.9 Pa/Eb34 [2 w]: 6.9	Eb34 [2 m]: 5.5 Pa/Eb34 [2 m]: 0
E. coli 35	Eb35 [0 t]: 6.8 Pa/Eb35 [0 t]: 7.0	Eb35 [2 w]: 6.9 Pa/Eb35 [2 w]: 6.0	Eb35 [2 m]: 5.8 Pa/Eb35 [2 m]: 0

[0 t] – 0 test [2 w] – 2 week test [2 m] – 2 month test / Eb – Enterobacteriaceae, PaEb – Enterobacteriaceae/ *P. anomala

The obtained results showed different CFU levels of growth after two weeks and two months. The decrease in CFU levels (2w test, no co-cultivation with *P. anomala*) was observed in six strains (*C. freundii, E. sakazakii, E. cloacae, E. coli (no 13), H. alvei, E. carotovora*). Remaining eight *Enterobacteriaceae* strains (*K. oxytoca, K. pneumoniae, P. mirabilis, E. aerogenes, M. morganii, S. marcescens, P. agglomerans*, *E. coli*) showed increase in CFU levels (table 4).

After two months (no co-cultivation with *P. anomala*), further increase in CFU levels was observed in 9 strains (*E. sakazakii, E. cloacae, K. pneumoniae, P. mirabilis, E. aerogenes, E. coli (no 13), M. morganii, S. marcescens*, *E. carotovora*).The remaining three strains showed decrease (*K. oxytoca, P. agglomerans*, *E. coli* , table 4).

The CFU levels of two *Enterobacteriaceae* strains (*C. freundii, H. alvei*) could not be detected after two months (table 4). The reason was the death of these two strains after two months.

Nine *Enterobacteriaceae* strains (*C. freundii, E. sakazakii, E. cloacae, E. aerogenes, E. coli (no 13), H. alvei, S. marcescens, E. carotovora* and *E. coli*) co-cultivated with *P. anomala,* showed decrease in growth after two weeks test. The remaining five strains (*K. oxytoca, K. pneumoniae, P. mirabilis, M. morganii*, *P. agglomerans*) increased after two weeks (table 4).

A reduction in growth below detection level (10^1) after two months was observed in seven *Enterobacteriaceae* strains (*E. sakazakii, E. cloacae*, *S. marcescens, P. agglomerans, E. coli, E. coli (no 13), E. carotovora*). Remaining five *Enterobacteriaceae* (*K. oxytoca, K. pneumoniae, P. mirabilis, E. aerogenes, M. morganii*) was reduced in growth but not below 10^1.

C. freundii and *H. alvei* results (2 month test) cannot be taken in consideration due to results obtained in mini silos where the was no co-cultivation (table 4).

Growth of *Pichia anomala*

In all mini silos (co-cultivated/ not co-cultivated with *Enterobacteriaceae*) *P. anomala* growth increased after two weeks storage period. The two months storage showed some variation in growth of the yeast. Both higher and lower CFU levels were observed in mini silos. For example the *Pichia anomala* which was not co-cultivated with *Enterobacter sakazakii* increased from log 5.8 to log 8.6. No decrease could be observed in CFU g^{-1} after two months. In minisilos with co-cultivated *E. sakazakii,* the increase in yeast CFU levels could be observed after two weeks as well as after two months storage time (test $_{[0\ t]}$ – log 4.8, test $_{[2\ w]}$ - log 7.6 test$_{[2\ m]}$ – log 8.4).

As mentioned before the *P. anomala* CFU levels in mini silos with co-cultivated *Enterobacteriaceae* strains as well as mini silos with no co-cultivation showed at the beginning (0 test) levels at $10^2 – 10^3$ CFU. After two weeks the values of *P. anomala* increased to 10^5- 10^6 CFU g^{-1}. After two months the CFU levels stayed at 10^6.

Results for overlay method could not be obtained due to not working procedure.

Discussion

Olstorpe et al (2010) observed inhibition of the *Enterobacteriaceae* during storage of moist cereal grain inoculated with *P. anomala*. The observation included two species, *P. agglomerans* (95%) and *E.coli* (5%). CFU levels of both species were reduced in grain inoculated with *P. anomala,* but not in stored grain without yeast.

In this study the similar results were obtained. In some cases reduction below detection level (10^1) was obtained. In the remaining cases, the reduction in *Enterobacteriaceae* growth was also obtained, but did not decreased below 10^1 CFU.

Not clear conclusions could be made for *C. freundii* and *H. alvei*. The bacterial death occurrence in two month´s treatments (no co-cultivation with yeast), probably occurred also in mini silos where the co-cultivation with yeast was performed. The reason for death of both species is not clear, but one of the possible explanations could be theirs sensitivity to water activity.

The death could not depend on mold contamination, because the other species probably would also be affected and no bacterial growth would be observed. The contamination in two month treatments comprised approximately 10-20% of the content and was seen almost in all mini silos. One can assume that the mould could affect the growth of all tested bacterial species but not in such way, that the growth was totally inhibited.

P. anomala can reduce the growth of molds by several ways: production of killer toxins, secretion of β-1-3-glucanases, production of the ethyl acetate or by competition for nutrients (Fredlund et al. 2004, Druvefors et al. 2005; Masih & Paul 2002; Wilson et al. 1991). One of these inhibition methods can be applied on tested *Enterobacteriaceae* strains. The production of cell wall degrading enzyme (β-1-3-glucanases) by *P. anomala* should be assumed as one of the possible ways to reduce the growth of the *Enterobacteriaceae*. By secreting the β-1-3-glucanases, would be an effective method to kill bacterial species by degrading theirs cell walls.

P. anomala antibacterial characteristics were observed in study by Polonelli and Morace (1986) on *Erwinia* species and *Enterobacteriaceae*. The study included *C. freundii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae* and *S. marcescens* among others. All the named strains growth was reduced by production of the killer toxins by *P. anomala*. It is important to point out as report mention that the reduction in bacteria growth could be affected by metabolic products and not by toxins. Another study performed by Conti et al. (2002) on *Streptococci* showed also that the bacteria growth affects by toxin production. It took longer time for *P. anomala* to reduce growth of the *Enterobacteriaceae* (2 month period) than to reduce growth of molds (1 week). Competition for nutrients is another way for *P. anomala* to reduce mold growth, but in this situation the competition for oxygen or nitrate cannot play a role. For the first the *Enterobacteriaceae* species are facultative anaerobic organisms which can use nitrate instead of oxygen. For the second the *Enterobacteriaceae* species can develop fumarate respiration so the competition for the nitrate is not significant.

The production of killer toxins by *P. anomala* is likely one of the mostly possible ways to reduce the growth of *Enterobacteriaceae*. Obtained results from this study, show only that the *Enterobacteriaceae* growth can be reduced by co-cultivation with *P. anomala.* Additional studies should be made where the toxins from *P. anomala* are purified and used against *Enterobacteriaceae*.

Members of the *Enterobacteriaceae* family can be found in water and soils. Many of them are found in the animal and human intestines. Some of them are human or plant pathogens (Bell et al 2004, Cruz et al 2007 and Bornet et al 2000). In this study the growth of the few tested *Enterobacteriaceae* species was reduced below detection level. The reduction in growth after two months varied between *E. aerogenes* and the three other species (*P. agglomerans, E. sakazakii, E. cloacae*) belonging to the same genus (*Enterobacter*).

The growth of *Enterobacter aerogenes,* a bacterium found in soil or waste (Atlas et al 1998) and *Klebsiella* species (*K. oxytoca, K. pneumonia*) found also in soil, surface waters (Bagley 1985) was reduced after two month´s storage period (not below 10^1). Results for *C. freundii* and *H. alvei* found in the same environmental places as the *Klebsiella* species (Drelichman and Band 1985, Badger et al 1999, Podschun et al 2001) could be not taken in account in this study. Results for these two species (*C. freundii* and *H. alvei*) could give better explanation if species living under the same or similar environmental conditions, are affected by *P. anomala* in the same way. Hence is too hard to explain if the are connection between *P. anomalas* inhibitory effects and reduction of bacterial species belonging to the same genus or different species living under the same environmental conditions. Hence the several species for each genus or several different species living under the same environmental conditions, would give better explanation.

In the mini silos where the only *Enterobacteriaceae* species were cultivated the contamination occurred. The contamination was observed in 95% of all mini silos. In these silos the growth of two molds *Aspergillus* and *Fusarium* was observed. This contamination did not affected calculation of *Enterobacteriaceae* CFU. Another contamination occurred in one of the plate with *P. anomala.* This resulted in difficulties with counting of yeast colonies on six other plates (2 month´s treatment).

The overlay method did not work properly, due to high growth of the yeast. The high yeast growth was probably due to difficulties matching the turbidity against mc Farland 2.

Conclusions

The obtained results show that *P. anomala* has inhibitory properties against most of the tested *Enterobacteriaceae* species. Further studies about which mechanism of yeast inhibit/reduce growth of *Enterobacteriaceae* would be appropriate.

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