Feed Grain Improvement through Biopreservation and Bioprocessing

Microbial Diversity, Energy Conservation and Animal Nutrition Aspects

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Feed Grain Improvement through Biopreservation and Bioprocessing. Microbial Diversity, Energy Conservation and Animal Nutrition Aspects

Abstract

Fermentation is an environmentally friendly method to improve feed quality. Fermented liquid feed and airtight stored moist crimped cereal grain systems that are of increasing importance in agricultural practice were studied. Both rely on spontaneous microbial developments with poorly understood population dynamics, resulting in unpredictable final quality. Temperature, fermentation time and ingredients affected final properties of the feed. Molecular-based species identification showed that microbial populations changed, even when total viable counts were stable. Moisture contents in farm-stored crimped cereal grain were mostly below recommended values, resulting in insufficient lactic acid formation and growth of undesirable microbes.

Adding starter cultures to fermentation systems influenced microbial populations. A grass silage lactic acid bacteria starter culture was added to liquid feed fermentations. *Lactobacillus plantarum* of the starter consortium grew well, but *Lactobacillus panis* from the feed ingredients dominated both inoculated and non-inoculated fermentations. The starter culture did not influence yeast populations. Starter strains should be adapted to the system and may include appropriate yeasts. The lactic acid bacteria *Lactobacillus fermentum*, *L. panis* and *Pedicoccus pentosaceus,* and the yeasts *Pichia fermentum* and *Pichia anomala* may be included in such starter cultures.

Adding the biocontrol yeast *P. anomala* to moist crimped cereal grain decreased numbers of undesirable moulds and reduced *Enterobacteriaceae* by 5 log units during storage. Feeding animals with *P. anomala* inoculated grain did not adversely affect performance, but only marginally improved weight gain. The yeast contribution to protein content was insufficient to describe the grain as protein feed. This is possibly due to cell density dependent growth inhibition of the yeast, as maximum *P. anomala* levels on grain, regardless of treatments, never exceeded 8.5 log units. Inoculation of *P. anomala* with high phytase activity into crimped cereal grain reduced grain phytate levels. Bound phosphorus, in the form of inositol phosphates, was not detected in any of the liquid diets after fermentation.

Keywords: Cereal grain, bioprocessing, biopreservation, storage, microbial diversity, yeast, lactic acid bacteria, moulds, feed hygiene, phytate, phytase, protein

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Dedication

To all of you who struggle with the concept of reading and writing. As one dyslexian to another, I hope that you have friends and family, like mine, who support you, giving you the strength and inspiration that you need to overcome the difficulties of words.

Jag tittar på de svarta krumelurerna som framträder på skärmen. Svarta tecken som känns kalla och hårda. Formationer som ska sammanlänkas i något så fint som ett ord och därefter kopplas ihop med flera ord. Resultatet skall bli en mening med innebörd som förmedlar något till läsaren. Innebörden av meningen kan sedan uppfattas som något lärorikt eller känslofyllt. Jag hoppas du som läser känner närhet till det du läser att du förstår vad det står och att det ger dig något.

Matilda Olstorpe

"Ur ingenting är allting möjligt"

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lyberg, K., Olstorpe, M., Passoth, V., Schnürer, J. and Lindberg, J.E. (2008). Biochemical and microbiological properties of a cereal mix fermented with whey, wet wheat distillers' grain or water at different temperatures. *Animal Feed Science Technology*. 144(1-2), 137-148.

II Olstorpe, M., Lyberg, K., Lindberg, J.E., Schnürer, J. and Passoth, V. (2008). Population diversity of yeast and lactic acid bacteria in pig feed fermented with whey, wet wheat distillers' grain or water at different temperatures. *Applied and Environmental Microbiology*. 74(6), 1696-1703.

III Olstorpe, M., Schnürer, J. and Passoth, V. Screening of yeast strains for phytase activity (submitted).

IV Olstorpe, M., Schnürer, J. and Passoth, V. Microbial changes during storage of moist crimped cereal grains under Swedish farm conditions (submitted).

V Olstorpe, M., Borling, J., Schnürer, J. and Passoth, V. The biocontrol yeast *Pichia anomala* improves feed hygiene during storage of moist crimped cereal grain under Swedish farm conditions (submitted).

VI Olstorpe, M., Axelsson, L., Schnürer, J. and Passoth, V. Effect of starter culture on feed hygiene and microbial population development in cereal grain mix fermented with wet wheat distillers' grain (submitted).

Abbreviations

a_w	Water activity
DDG	Dried distillers' grain
DM	Dry matter
IP	Inositol phosphates
LAB	Lactic acid bacteria / bacterium
MC	Moisture content
SCP	Single cell protein
SSF	Solid substrate fermentation
WWDG	Wet wheat distillers' grain

1 Introduction

Cereal grain is a major component of animal feed in large parts of the world. Swedish farmers produce approximately 3 million tons of cereal grains each year for animal feed (SJV, 2007). On cereal grains and their by-products, microbes may interfere with feed hygiene, storage stability, may reduce palatability of the feed and bioavailability of minerals and protein depending on the composition of the microbial population. It is, therefore, of primary interest to manage/control the microbial species present in animal feed.

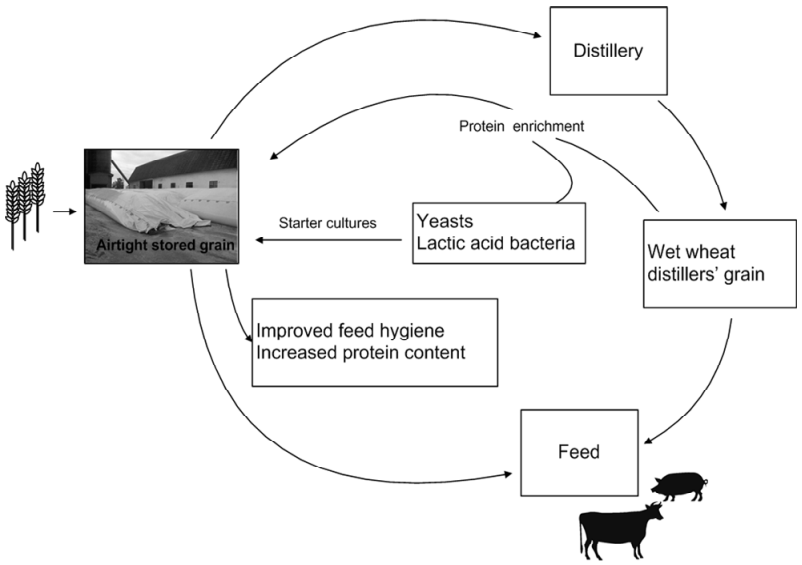

Figure 1. Integration of feed grain, biopreservation, bioprocessing and feed improvement within a sustainable system.

In Sweden, the average grain moisture content at harvest is approximately 21%. High-temperature drying is the primary preservation technique to achieve long-term safe storage (Jonsson & Pettersson, 2000). This requires much energy. With increasing energy prices and climate concerns, interest in finding alternative preservation methods has intensified. Cereal grain can be stored moist, but feed hygiene is then easily impaired due to growth of deteriorative and/or hazardous microorganisms. However, addition of biopreservative organisms may ensure feed hygiene.

The mineral bioavailability from cereal products is poor due to the presence of phytate, which is the major phosphorus storage compound in the grain (Reddy, 2001; Reddy *et al.*, 1989). Phytate also binds minerals and proteins, resulting in insoluble complexes that are unavailable for absorption in the intestine of monogastric animals. The phytate content may be reduced by phytase enzymes, via supplementing the feed with pure enzymes or with phytase-producing microorganisms, and/or via activation of endogenous phytase present in the cereal grain (Carlson & Poulsen, 2003; Han *et al.*, 1997; Nelson, 1967).

Cereals are high in starch, dietary fiber, vitamins and minerals, but typical amounts and quality of protein present do not fulfil the nutritional requirements of animals (McDonald *et al.*, 2002). Thus, cereal based feeds are amended with additional protein sources to attain the required protein levels. The addition and proliferation of microorganisms in feed may improve the nutritional quality for the animals (Ravindra, 2000). From a Swedish, and indeed a European perspective, such protein feed could be produced nationally, reducing intercontinental transportation and usage of soya protein, the production of which is commonly associated with negative environmental and socioeconomic consequences (Bertrand, 2006).

Increased competition among food, feed and biofuels in the usage of agricultural products is caused by the increasing human population and a higher consumption of protein food of animal origin, as well as the search for carbon dioxide neutral biomass sources for energy production. The production of biofuels, such as ethanol, will likely impact the feed industry, in terms of both supply and type of feedstuffs available. Co-products derived from the bioethanol industry are rich in protein and dietary fibers (Patience *et al.*, 2007). Utilising these co-products as feed components can further reduce the dependence on imported protein supplements.

1.1 Aims

The fundamental hypothesis of this thesis work has been that it is possible to manage microbial communities to obtain a safe and nutritionally optimal feed. The particular aims have been to:

- Describe the microbial diversity with molecular and cultivation methods in two cereal based feeds: fermented liquid feed and crimped moist cereal grains.
- Elucidate how microbial fermentations influence the hygienic and nutritional feed quality of fermented liquid feed and crimped moist cereal grains.
- Investigate whether it is possible to influence microbial community structure and feed quality by adding yeast and lactic acid bacteria starter cultures to cereal based fermentation systems.

2 Cereal grain

2.1 Swedish cereal grain production in numbers

The total harvest of cereal grain has increased from 2 356 thousand metric tons in the late 1920s to 5 444 thousand metric tons at the beginning of the 21st century (SJV, 2005). This is due to an increase in hectare yields of approximately 180%. The total area used for cereal grain production has varied markedly over the decades, but is today approximately 1.15 million hectares. Allocation of land to different cereal grains crops has also varied. During the late 19th century, 90% of the area used for cereal grain was cultivated with oats, rye and barley crops, whereas today, the main cultivars are wheat, barley and oats (SJV, 2005). In Sweden, approximately 5.5 million tons of cereal grain are produced annually (1995-2007), of which approximately 60% is used for animal feed (SJV, 2007).

2.2 Cereal grain as feed component

Cereal grain is a major constituent of animal feed. This plant material essentially consists of carbohydrate concentrates, with starch as the main component of the dry matter. It is, therefore, considered as an energy feed and has a long tradition of production, associated with highly developed technology of production and storage (McDonald *et al.*, 2002). Cereal grains such as corn, barley, oats, wheat, triticale, sorghum and millet are used in different parts of the world (Hammes *et al.*, 2005). In Sweden, the most common cereals in feed are barley, oats, wheat, triticale and rye, based on tradition and suitability for growth in the Swedish climate (SJV, 2005). Different cereals can be included in animal diets in varying proportions, depending on cereal cultivar, and species and production capacity of the animal. Calves, pigs and poultry depend upon cereal grains for their main

source of energy, and as much as 90% of their diet may consist of cereals and cereal by-products (McDonald *et al.*, 2002).

Wheat (*Triticum aestivum*) is an excellent fodder cereal, with high energy and low bran content, but the protein quality is generally fairly poor (Odal, 2000; Shewry, 2007). The high starch content also limits the use of wheat in ruminant feeds, as it might perturb the rumen fermentation (McDonald *et al.*, 2002). Barley (*Hordeum sativum*) is well liked by animals and can be used as the sole cereal in the feed. However, both energy and protein contents fluctuate substantially between batches of barley. This needs to be considered when planning feeding regimes (Odal, 2000). More specifically, a validation of the protein quality is necessary, as the proportion of essential amino acids often decreases when crude protein content increases (Shewry, 2007; Simonsson, 1995). Oat grains (*Avena sativa*) have a balanced amino acid composition, with higher lysine and methionine contents than wheat and barley (McDonald *et al.*, 2002). The crude fat level is high, which limits the use of oats as feed to pigs. Feeding unsaturated fatty acids to pigs results in weak fat that easily turns rancid in the carcass (Simonsson, 1995). The use of rye (*Secale cereale*) is relatively limited in animal diets. Rye grain is very similar to wheat in composition, although rye protein is higher in lysine but lower in tryptophan than wheat protein. Rye is regarded as the least palatable of the cereal feed grains and could also cause digestive upsets. Therefore, its inclusion in feed is carefully considered, and it is typically fed in restricted amounts (McDonald *et al.*, 2002; Simonsson, 1995). Triticale is a hybrid cereal derived from crossing wheat with rye. The objective was to create a grain with enhanced disease resistance, quality, vigour and hardiness (Odal, 2000). However, triticale also contains different anti-nutritional factors that have been associated with poor palatability and performance in pigs. It is generally recommended that triticale should be limited to 50% of the grain in diets for farm animals (McDonald *et al.*, 2002). Common to all cereal grains is the low quality of the protein, being particularly deficient in essential amino acids, e.g. lysine and methionine. Thus, additional feed protein sources are required to meet the animals' needs.

3 Protein feed

3.1 Protein amendments to animal diets

In Sweden, animal diets are, to some extent, amended with different legumes to increase the protein content. However, there are practical limitations due to acceptability, anti-nutritional substances and influences on carcass quality. Peas and rapeseeds are the domestically available protein fodders. In 2007, the total Swedish production of peas and field beans was 49 000 tons, and of oil seed crops, 229 000 tons (SJV, 2008). Peas have fairly high crude protein and lysine contents, but their amino acid composition is not considered to be adequate (Odal, 2000). Furthermore, due to the acrid taste, animals often reject peas in the fodder. Peas also contain tannins that impede the protein turnover (Simonsson, 1995) Rapeseeds have a high protein content and an advantageous amino acid composition. However, their use is limited due to the high content of highly unsaturated fatty acids (Odal, 2000) and glycosides (McDonald *et al.*, 2002). The glycosides are cleaved during metabolism and the intermediates may inhibit growth of the production animals (Simonsson, 1995). Currently, Europe is less than 30% self-sufficient in the production of protein feed (Chudaske, 2007). The world market for protein feed has grown continuously over the last few years, with over 200 million tons traded annually and with soya as the primary cultivar (Chudaske, 2007). Soya meal is generally regarded as one of the best sources of protein available to animals. Soya protein contains all the essential amino acids, although the concentrations of cysteine and methionine are suboptimal (McDonald *et al.*, 2002). The production of soya is associated with negative environmental and socioeconomic issues (Bertrand, 2006). Export may further increase the negative impact on the environment, as no recirculation of plant nutrients occurs when crops are moved to importing countries. Fossil fuels used in transporting soybeans

further increase the environmental load. Addition of microorganisms to the feed can increase the protein content and quality of the feed, thus, providing a more local (national) protein feed base.

3.1.1 Protein enrichment with single cell protein

Single cell protein (SCP) is the protein extracted from cultivated microbial biomass. The first purposeful SCP production originated in Germany during World War I, when bakers' yeast, *Saccharomyces cerevisiae*, was grown on molasses and ammonium salts for consumption as a protein supplement. Later during World War II, *Candida utilis* was cultivated on different waste products from the paper industry and used as a protein source for both humans and animals (Litchfield, 1983). Currently, SCP is produced from many species of microorganisms, including algae, fungi and bacteria. These are cultured on abundantly available agricultural and industrial wastes (Jin *et al.*, 1999; Leathers, 2003; Villas-Bôas *et al.*, 2003; Villas-Bôas *et al.*, 2002; Yang *et al.*, 1993). Although these organisms are grown primarily for their protein contents, microbial cells also contain carbohydrates, lipids, vitamins, minerals, and non-protein nitrogen material, such as nucleic acids. The feed value and usefulness of SCP is based on its composition. The protein and lipid contents in the SCP are determined by the composition of the medium. Fungi have higher lipid and lower protein contents when grown on media rich in carbon sources and poor in nitrogen. Fundamental problems when using SCP include the deficiency of sulphur-containing amino acids, and the belief among nutritionists that microbial growth conditions have a limited influence on amino acid content (Giec & Skupin, 1988). However, when the yeast *Pichia anomala*[1] was grown on different liquid substrates, freeze dried and then analysed for its essential amino acid content, fairly large differences in the amount of crude protein and the composition of amino acids were observed, indicating that culture conditions substantially influenced quality of the yeast protein (Table 1).

[1] According to Kurtzman *et al.*, (2008) *P. anomala* is proposed to be included in a new genus, *Wickerhamomyces*, and in the future will probably be called *Wickerhamomyces anomala*. However, throughout this thesis, this yeast will be referred to as *P. anomala*. Kurtzman, C.P., Robnett, C.J. & Basehoar-Powers, E. (2008). Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Willopsis* determined from multigene sequence analysis, and the proposal of *Barnettozyma* gen nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen nov. *FEMS Yeast Resarch* 8, 939-954.

Table 1. *Amino acid composition of lyophilised* P. anomala *cells grown in the liquid substrates Yeast Nitrogen Base or Malt Extract. Data are given as g amino acid kg^{-1} dry matter.* ⋆*Essential amino acid.*

Amino acid	Yeast Nitrogen Base	Malt extract
Cysteine	3.9	1.4
Methionine⋆	5.3	1.1
Aspartic acid	29.2	10.7
Threonine⋆	15	6.4
Serine	18.9	7.2
Glutamic acid	56.2	16.2
Proline	10.4	4.3
Glycine	14.6	5.5
Alanine	18.9	6
Valine⋆	16.6	6.1
Isoleucine⋆	16.5	5.9
Leucine⋆	22.2	7.7
Tyrosine (calculated)	11.1	3.7
Phenylalanine⋆	12.6	4.6
Histidine⋆	6.6	2.4
Ornitine	1	0.1
Lysine⋆	22.7	7.8
Arginine	26.4	4.7
Hydroxiproline	0.1	0.1
Crude protein	353	123

Fermentation of apple pomace (pulp, seeds, peel) with *C. utilis* increased crude protein from 4.1% to 8.3% in 6 days. Co-inoculation with *Pleurotus ostreatus* further increased crude protein contents to 10.5% over 30 days' fermentation (Villas-Bôas *et al.*, 2003). Co-inoculation of starch rich sweet potato residues with *Aspergillus niger* and a *Rhizopus* sp. yielded crude protein contents that increased from 3.5% to 32% over 4 days (Yang *et al.*, 1993). Addition of inorganic nitrogen sources considerably improved the final protein content during protein enrichment of sweet potato residues (Yang *et al.*, 1993). Addition of inorganic nitrogen (N) and phosphorus (P) to sugar beet stillage fermented with *Pichia* spp. increased SCP biomass yield by approximately 33% and also increased the crude protein content (Shojaosadati *et al.*, 1999). SCP from mixed cultures of *Trichoderma reesei* and *Kluyveromyces marxianus* grown on beet pulp had an amino acid profile meeting the Food and Agriculture Organization guidelines and comparable to that of soya bean meal (Ghanem, 1992). Feeding trials with pigs and

chickens suggest that 10-20% of the protein in feeds can be replaced by SCP (Giec & Skupin, 1988).

The presence of fungal cell wall material has been identified as a potential problem. Cell walls consist of glucans, mannans and chitin, that efficiently decrease the availability and digestibility of SCP (Giec & Skupin, 1988). High nucleic acid contents are also of concern. Yeast contain levels of nucleic acids from 50 to 120 g^{-1} kg DM, bacteria, from 80 to 160 g^{-1} kg DM (McDonald *et al.*, 2002). Nucleic acids in the diet increase the uric acid concentration in the blood and urine of humans and monogastric animals (Kamel & Kramer, 1979; McDonald *et al.*, 2002). However, high amounts of nucleic acid do not have such a negative influence in ruminants, and are also better tolerated by non-ruminants than by humans (Giec & Skupin, 1988).

3.1.2 Protein enrichment of cereal grain

Traditionally, microbial biomass is produced either by submerged or solid state fermentation (SSF). After fermentation, biomass is harvested and subjected to downstream processing (Villas-Bôas *et al.*, 2002). Growth of microorganisms *in situ* on the cereal grain would be a more cost effective method to produce SCP, as no further processing would be needed. Protein enrichment of feed grain through SSF with selected microfungi and mineral nitrogen amendments could provide a domestic alternative to imported feed protein. The microfungi used should preferably have a history of previous use in food or feed, and have the status Generally Regarded as Safe (GRAS). This would minimise the risk of selecting toxin-producing or otherwise hazardous fungi. They should also grow well on cereal grain and preferably outcompete spoilage microorganisms, but be able to co-exist with lactic acid bacteria (LAB). In the course of this study different microfungi were tested for growth on cereal grain to produce SCP *in situ*. *Rhizopus oligosporus* is a fast growing filamentous fungus that is used in food fermentations, such as barley tempeh, and that grows in co-culture with LAB (Feng *et al.*, 2005). Bakers' yeast, *S. cerevisiae*, has been domesticated for thousands of years, co-exists with LAB in sourdough (Meroth *et al.*, 2003; Paramithiotis *et al.*, 2006), and is the most intensively studied fungus, with the complete genome sequences available (Goffeau *et al.*, 1996). *Saccharomyces boulardii* is a probiotic yeast preventing bacterial intestinal disorders, approved for human use (Precosa®, Astra Läkemedel AB, Södertälje, Sweden), but also known to exert beneficial effects in the gastrointestinal tract of pigs (Schroeder *et al.*, 2004). However, there are strong indications that *S. boulardii* is a variant of *S. cerevisiae* and not a separate species (van der Kühle & Jespersen, 2003).

P. anomala is a biocontrol yeast able to prevent growth of spoilage moulds (Björnberg & Schnürer, 1993) and *Enterobacteriaceae* in stored feed grain (**V**). *P. anomala* has considerable phytase activity (Vohra & Satyanarayana, 2001; **III**) and may co-exist with LAB (Daniel *et al.*, 2008; **II**).

The biomass production of different microfungi on wheat (*Triticum aestivum*) at different water activities (a_w) 0.92, 0.95 and 0.98 was determined. The moisture content (MC) of cereal grains was equilibrated by adding tap water. Cells of *P. anomala* (J121), *S. boulardii* (J552), *S. cerevisiae* (J543) and spores from *R. oligosporus* (J401) were inoculated at 10^5 cfu g^{-1} grain. After 5 days, cfu g^{-1} grain was quantified on malt extract agar as previously described (Petersson & Schnürer, 1995). Incubation temperature was set to the optimum for each organism, and biomass production on whole or rolled cereal grain was compared.

S. boulardii and *S. cerevisiae* numbers did not increase on either whole or rolled cereal grains. At the higher cereal grain a_w, mould growth was detected. As no biocontrol effect was observed for *S. boulardii* and *S. cerevisiae*, and due to their poor growth on cereal grain, they were not further evaluated for production of SCP *in situ* on cereal grain. The cfu of the filamentous fungus *R. oligosporus* increased by approximately 1.5 log units on cereal grain, independent of a_w, and with a significant increase (4 log units) on rolled grain at a_w 0.98, compared to that on whole cereal grain. However, the increase was related to heavy sporulation of *R. oligosporus*. This may be hazardous both to animal and humans, due to allergenic reactions and irritation of the lungs. This species was, therefore, not considered an acceptable candidate for SCP enrichment *in situ* on cereal grain.

P. anomala was the only yeast that grew well on cereal grain, increasing in cfu numbers during 5 days incubation on both rolled and whole grain, at all a_w evaluated, and independent of inoculum level (10^3, 10^5 and 10^7 cfu g^{-1} grain). The maximum cfu number reached in all tested conditions was about 8 log units. To increase the protein content, different nitrogen sources – urea, ammonium-sulphate and ammonium-phosphate – were added at 0.5% w/w nitrogen to the cereal grains before inoculation. However, nitrogen supplementation did not increase yeast growth above that achieved on unamended cereal grains (Table 2). Higher levels of nitrogen amendments had a negative effect on *P. anomala* growth, presumably due to osmotic stress.

Table 2. *Final cfu (log_{10}) of P. anomala grown on cereal grain under different a_w, inoculum and amendment conditions. The inoculum level was 10^5 cfu g^{-1} grain, the a_w 0.95, and the grain incubated at 25 °C for 5 days, if not stated otherwise. Data are given as mean values ± SD (n=3) (Olstorpe et al., unpublished).*

	Treatment	Cfu g^{-1} grain
Whole cereal grain	0.92 a_w	8.1 ± 0.3
	0.95	8.3 ± 0.2
	0.98	8.4 ± 0.3
Rolled cereal grain	0.92 a_w	7.9 ± 0.3
	0.95	8.2 ± 0.2
	0.98	8.5 ± 0.4
Yeast inoculation level	10^3	7.6 ± 0.3
	10^5	8.4 ± 0.2
	10^7	6.5 ± 0.5
Nitrogen amendment	Urea	8.1 ± 0.3
(0.5% N w/w)	Ammonium sulphate	8.2 ± 0.1
	Ammonium phosphate	8.2 ± 0.2
Storage time	5 days	8.0 ± 0.3
	3 months	8.1 ± 0.1
	1 year	7.9 ± 0.5
Air admission before closure of	0 h	7.9 ± 0.3
storage vessel	24 h	8.0 ± 0.2
	48 h	8.1 ± 0.3

Adding glucose to the grain moistening solution also did not significantly affect the final yeast cfu levels (Druvefors *et al.*, 2005). It appears that cell density regulates and restricts growth in *P. anomala*. This phenomenon complicates the SCP production *in situ* on cereal grains, as the amount of protein generated is insufficient for the feed to be acknowledged as a protein feed (250 g protein kg^{-1} feed).

3.1.3 Yeast colony morphology on cereal grain

I tried to elucidate whether the yeast distribution pattern on cereal grain explained why yeast cell growth was inhibited at a certain cfu level. The cereal grain was hydrated to 0.98 a_w with either water or liquid YPD medium. The grain was inoculated with *P. anomala* at different levels, resulting in four treatments, where sample A and B were moistened with water and inoculated with 10^3 and 10^6 cfu g^{-1} grain, respectively. Sample C and D were moistened with YPD and inoculated with 10^3 and 10^6 cfu g^{-1} grain, respectively. The cereal grain was incubated at 25 °C for 5 days, then the number of yeast cfu was assayed according to Petersson & Schnürer,

(1995). Final yeast cell numbers varied only slightly between treatments (Table 3).

Table 3. *Final amounts of* P. anomala *inoculated at two different levels on wheat grain moistened with either water or complex YPD medium and incubated at 25 °C for 5 days. Data are given as mean values of* log_{10} *cfu* g^{-1} *grain ± SD (n=3) (Olstorpe et al., unpublished).*

Treatments	10^3 cfu g^{-1} grain	10^6 cfu g^{-1} grain
Water	(A) 8.0 ± 0.1	(B) 8.1 ± 0.0
YPD	(C) 8.2 ± 0.2	(D) 8.4 ± 0.1

P. anomala inoculated cereal grain was photographed both through a stereo microscope and during cryoscanning electron microscopy by Dr Jan Dijksterhuis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). The distributions of colonies over the kernels clearly differed among treatments (Figure 2).

Figure 2. Cereal grains moistened with water (A and B) or YPD (C and D) and inoculated with *P. anomala* at different levels, 10^3 (A and C) and 10^6 (B and D) cfu g^{-1} grain. Photographed by Dr Jan Dijksterhuis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

Treatments B and D, with higher inoculum levels, showed more extensive "powdering" than A and C, that had more distinct colonies, indicating that the inoculum level affected colony morphology. Samples C and D moistened with YPD showed two different colony types: whitish, which are

typical *P. anomala* colonies; and yellowish, probably another species present on the non-sterile cereal grain, that grew on the extra nutrients present. Their presence was not as pronounced in sample D as in sample C.

Cryoscan electron microscopy pictures of the inoculated cereal grain showed that in treatment A, yeast cells with varying morphology occurred, indicating vegetative cells and possible ascospores. Hyphae and *Penicillium*-type conidiophores were also visible on the grain (not shown in Figure 3). Treatment B, with a higher inoculation level of *P. anomala*, showed tree-like yeast growth structures ascending 50-100 μm into the air from the kernel surface. There were hyphae present on the grain, but to a lesser extent (not shown in Figure 3).

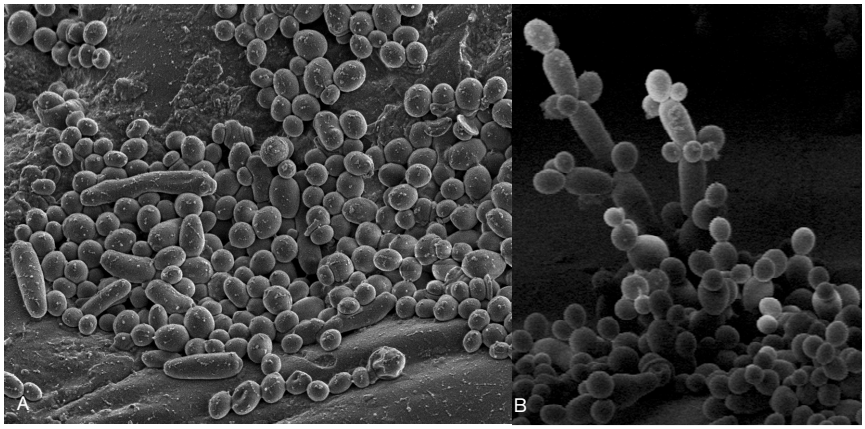

Figure 3. Morphology of yeast cells from treatment A and B, where *P. anomala* was grown on cereal grain moistened with water. In treatment A (inoculation level 10^3 cfu g^{-1} grain) there were "typical" rounded yeast cells forming buds, but also elongated cells and possible ascospores. Treatment B (inoculation level 10^6 cfu g^{-1} grain) showed branched and tree-like yeast structures that grew into the air phase. Cryoscan microphotographs by Dr Jan Dijksterhuis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

In sample C, the amount of hyphae present on the grain was estimated to be similar to sample B. Yeast morphology was also similar between treatments C and B, as yeast grew with tree-like structures. In sample D, yeast cells covered the grain kernel more extensively. The cells did not elongate into the air and the classical rounded yeast cells with buds and scars on the surface were dominant.

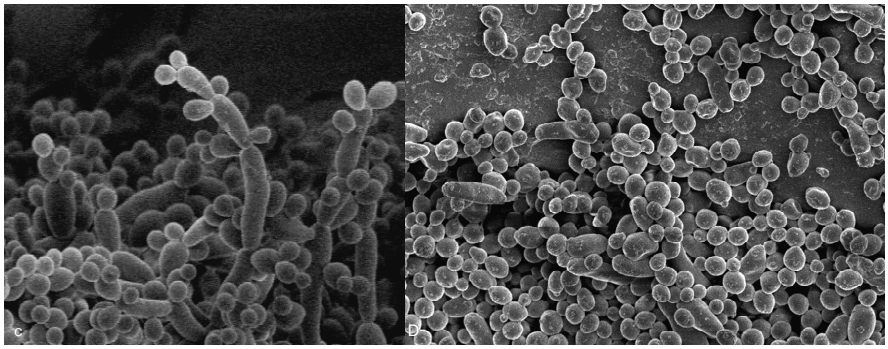

Figure 4. Morphology of yeast cells from treatment C and D where *P. anomala* was grown on cereal grain moistened with YPD. Treatment C (inoculation level 10^3 cfu g^{-1}) showed similar morphology to treatment B. Treatment D (inoculation level 10^6 cfu g^{-1}) showed more extensive covering of the grain kernel with classical rounded yeast cells. Cryoscan microphotographs by Dr Jan Dijksterhuis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

Variations in cell morphology may have influenced determination of cfu numbers. We could not distinguish whether the growth arrest at a certain yeast cfu concentration was due to morphology or competition for space, as there were always uncolonised areas on the kernel surface.

3.1.4 Quorum sensing

Available space (inoculum level), carbon or nitrogen supplementation did not markedly affect the final cell number; thus, the hypothesis was proposed that the cells in some way communicate with each other. Signalling mechanisms that govern physiological and morphological responses to changes in cell density are common in bacteria. These signal transduction processes are called quorum sensing, and involve the production, release and response to hormone-like molecules that accumulate in the external environment when the cell population grows (Fuqua *et al.*, 1994; Pappas *et al.*, 2004).

If such cell density dependent growth inhibition exists, cells at high density would multiply more slowly than cells at low density when equivalent amounts of fresh nutrients were added. To investigate this, I grew *P. anomala* J121 to stationary phase (48 h) in a highly aerated culture in complete medium (450 ml YM in a 3000 ml Erlenmeyer flask at 25 °C on a rotary shaker at 150 rpm). From this culture, 50 ml aliquots were transferred to six new Erlenmeyer flasks. Fresh nutrients were supplied by adding 5 ml of twofold (A), fivefold (B) and tenfold (C) concentrated YM, each to two bottles. To one bottle of each pair, 145 ml of sterile, ultra pure water was

added (Aw, Bw and Cw), creating a fourfold increased culture volume with the same total amount of nutrients for each pair. If there is a cell density dependent inhibition of growth, cultures with higher volumes should yield more biomass. Indeed, within each pair, those bottles with a higher volume yielded more biomass after 48 h incubation (Figure 5). Biomass was calculated from measured OD_{600} and the OD-biomass correlation reported by Fredlund *et al.*, (2004b). Moreover, low amounts of nutrients were converted to biomass more effectively than high amounts of nutrients. This also points to cell density related growth inhibition.

To determine whether there is an inhibitory factor secreted into the medium, I harvested 100 ml of stationary phase cells and washed them with sterile NaCl solution. The cells were then resuspended in 100 ml of fresh YM medium, and divided in two portions of 50 ml (D). To one of these cultures, water was added (Dw) to increase the volume fourfold, as described above. In these cultures, no difference was observed in the total biomass formed (Figure 5). This strongly indicates that there is a factor dissolved in the medium that is responsible for the growth inhibition.

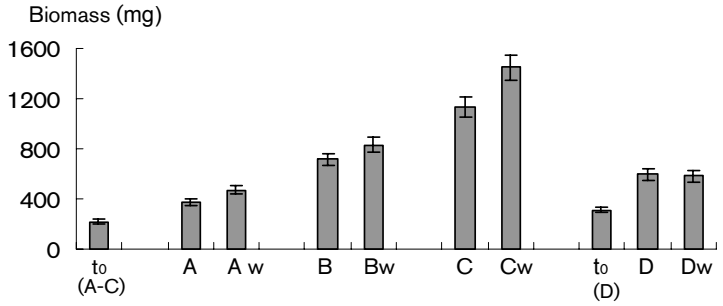

Figure 5. *Biomass formed by* P. anomala *grown to stationary phase and supplemented with fresh nutrients (see text). t_0: initial biomass of stationary phase cells. After incubation for an additional 48 h, A: addition of 5 ml twofold concentrated YM, B: addition of 5 ml fivefold concentrated YM, C: addition of tenfold concentrated YM, D: washed cells resuspended in the original volume of fresh YM. w: indicates a fourfold increase in culture volume by addition of sterile water. Data are given as mean values ± SD (n=3) (Olstorpe et al., unpublished).*

The cell density growth regulation in *P. anomala* would probably be more pronounced in its "natural habitats" compared to growth in liquid medium. The cooperation of microorganisms that would form a multicellular structure on a surface appears to be controlled by mechanisms that are, in some cases, not functioning in individual microbial cells grown in shaken liquid cultures (Palková & Váchová, 2006). Quorum sensing allows

microorganisms to monitor their density and consequently leads to a specific response by the whole population. Farnesol has been identified as a quorum sensing molecule responsible for hyphal formation in stationary phase cells of *Candida albicans* (Hornby *et al.*, 2001). Yeast filament formation may represent a foraging strategy that aids in nutrient acquisition by directing growth away from a dense microbial colony (Hogan, 2006). A similar process might be the basis for the response observed in *P. anomala* grown on cereal grain inoculated at higher levels and with nutrient amendments.

Ammonia signals of aging colonies trigger metabolic changes that localise cell death to the colony centre. The remaining population can thereby exploit the released nutrients and survive during long term development of the colony (Váchová & Palková, 2005). The system of quorum sensing allows the amount of a signalling compound to increase more efficiently than could be achieved by a simple increase in the number of non-induced cells. Therefore, the population can change its behaviour before reaching a critical density (Chen *et al.*, 2004; Palková, 2004).

Tyrosol has been identified as a molecule involved in abolishing the lag phase preceding the resumption of growth of stationary phase cells (Chen *et al.*, 2004). Chen & Fink (2006) showed that the quorum sensing molecules regulate transcription of a small set of genes (~150). About 70% of these genes have previously been shown to be up-regulated upon entry into stationary phase. The induction of these genes suggests that quorum sensing molecules may also play a role in signalling the transition to stationary phase. To be able to increase protein content by adding yeast to cereal grain, there is a need to further elucidate why growth stops at a certain limit of yeast cells present on the grain surface.

4 Phytate degradation

4.1 Phytate and phytase

Phytate (*myo*-inositolphosphate 1,2,3,4,5,6-hexakisphosphate, phytic acid, IP_6) is the primary storage form of phosphate and inositol in cereal grains. It is considered to be the most important anti-nutritional factor for the bioavailability of minerals in food and feed (Bouis, 2000). During seed development, phytate rapidly accumulates and can account for several percent of the seed dry weight. The amount of phytate varies from 0.14 to 2.22% in different cereals (Reddy, 2001; Reddy *et al.*, 1989). About 60% to 70% of the P present in cereal grain is stored as IP_6, which is mainly deposited in the outer layers of the seeds: the pericarp and aleurone layers. During germination, it is digested by *de novo* synthesised phytases of the grain (Brinch-Pedersen *et al.*, 2007; Reddy *et al.*, 1989). Phytate, and to some extent, the lower phosphorylated inositol phosphates, IP_5, IP_4 and IP_3, are strong chelators and bind positively charged proteins, amino acids and minerals, forming insoluble salt complexes (Brinch-Pedersen & Hatzack, 2006; Kies *et al.*, 2006; Sandberg & Andlid, 2002). Degradation of phytate is thus of nutritional importance, because when phosphate groups are removed from the inositol ring, minerals are less tightly bound and the solubility increases. This results in increased bioavailability of other essential dietary minerals, such as zinc, calcium, iron and magnesium (Cheryan, 1980; Türk, 1999).

The basic mechanisms of gastrointestinal P metabolism differ substantially between ruminants and monogastric animals. In contrast to monogastric animals, ruminants have a ruminal microflora that can break down IP_6. Some microbial degradation of IP_6 occurs in the colon of monogastrics, but degradation is considered to have only a limited effect on digestibility of the feed, as the major site of absorption is the small intestine (Sandberg &

Andlid, 2002; Sandberg *et al.*, 1993). This results in the release of phytate into the environment, where it is degraded to phosphate by microorganisms, and eventually, P is transported to water environments. Excess plant nutrients in bodies of water stimulate blooms of algae and nuisance plants (Rausch & Beleya, 2006).

The low bioavailability of the P in the phytate also necessitates addition of inorganic P to animal feed, in particular to pig and poultry feed. Inorganic P must be imported, which in itself is an environmental burden. Furthermore, in animal feeds, P is the third most expensive nutrient, after amino acids and components providing energy. Therefore, the low digestibility of phytate-bound P contributes to higher feed costs. Phytate content in feed may be reduced by different processing regimes, such as milling and polishing, which, unfortunately also reduce the mineral content (Reddy *et al.*, 1989). Phytate hydrolysis may also occur during feed preparation and production, and in the intestine, through the action of phytase from plants, yeasts or other microorganisms. Feed processing techniques that increase the activity of phytases include soaking, malting, hydrothermal treatment and fermentation (Bergman *et al.*, 1999; Fageer *et al.*, 2004; Lyberg, 2006; Sandberg & Andlid, 2002; **I**, **V** and **VI**).

Phytases are phosphatases that hydrolyse the phosphate esters of phytic acid and phytates (Gibson & Ullah, 1990). Depending on the phytase used, different *myo*-inositol phosphates can be formed during degradation of phytate. The degree of phosphorylation of the inositol ring may affect the nutritional quality of the feed (Brearley & Hanke, 1996; Greiner & Egli, 2003; Skoglund, 1998). Regarding the position for initial hydrolysis, two types of phytases, 3- and 6- phytases, are known to start the dephosphorylation of IP_6 at position *D*-3 or *L*-6 of the inositol ring, respectively. The 3-phytases (EC 3.1.3.8) dominate among microbial phytases, whereas 6-phytases (EC 3.1.3.26) are considered to be characteristic for the seeds of higher plants (Cosgrove, 1980). However, these are merely generalisations.

4.1.1 Plant phytase

Phytases have been isolated and characterised from a number of plant sources (Eeckhout & Depaepe, 1994). The first preparation was made from rice bran (Suzuki *et al.*, 1907). Plant phytases exhibit activity between pH 4.5 to 6.5 and have a temperature optimum of 45 to 60 °C, depending on origin of the enzyme (Reddy *et al.*, 1989). The phytase content varies substantially among different cereals, and is highest in rye. Typical phytase activities of rye, triticale, wheat, barley and oats have been reported to be

5130, 1688, 1193, 584 and 41 U kg^{-1} cereal (on 88% DM basis), respectively (1 U is the amount of enzyme that releases 1 μmol orthophosphate min^{-1} from phytate; Eeckhout & Depaepe, 1994).

4.1.2 Activation of plant phytase in feed

Processing of animal feed formulations during pelleting generally results in complete inactivation of endogenous phytases, as processing temperature reaches approximately 85 °C. In animal feed not subjected to any heat treatments, the plant phytases are activated when the feed is ingested (Moore & Tyler, 1955; Sandberg *et al.*, 1993). This could also be achieved by feed treatments prior to ingestion. Activating plant enzymes by steeping raw materials is a method to increase the bioavailability of P in feed, which could be incorporated into liquid feeding system. The naturally occurring phytases in some cereal grains and seeds are activated when the raw materials are soaked (Carlson & Poulsen, 2003; Skoglund *et al.*, 1997). One hour of soaking a cereal-based diet was shown to reduce IP_6 content; this may sufficiently increase availability of P in pig diets containing significant amounts of intrinsic phytase, so that the need for inorganic P supplementation is reduced (Lyberg, 2006). However, microbial fermentation commences rapidly when the feed is mixed with water, thus phytate dephosphorylation in liquid feed may be due to both endogenous and exogenous i.e. microbial phytase activities (Canibe & Jensen, 2003; **I** and **VI**).

4.1.3 Microbial phytase

Microbial phytase may be utilised in animal nutrition to increase the bioavailability of minerals and proteins present in animal feed. The first concentrated efforts to produce microbial phytase commercially started in 1962, at International Minerals and Chemicals, Illinois, USA. The intent of the project was to replace the use of inorganic phosphate in animal diets by adding phytase to the feed of monogastric animals, in a manner that is economically and environmentally competitive. Phytases have been isolated from many organisms, with PhyA produced by *Aspergillus niger* being the first characterised enzyme (van Hartingsveldt *et al.*, 1993). Cloning studies, gene sequencing and overexpression were subsequently performed by Gist-Brocades, The Netherlands. Based on these studies and their own bioengineered strain, the company then developed an enzyme product (Natuphos) that is being used as a feed additive. Currently, five different Natuphos® products are available in the EU as zootechnical additives for seven categories of animals (piglets, pigs for fattening, sows, chicken for

fattening, laying hens, turkey and ducks) (EUR-Lex, 2008). A number of microorganisms have been investigated for phytase production. The incidences of phytase production are highest in *Aspergillus* spp., but the enzymes have also been found in both bacteria and yeasts. Phytases from different microbial sources display a wide range of pH (2.5–7.5) and temperature optima (35–63 °C), and are produced both intra- or extracellularly (Wodzinski & Ullah, 1996).

4.1.4 Addition of microbial phytase to feed

Addition of microbial phytase increases the availability of phosphorus and proteins for animal digestion by degrading the phytate present in feed, and improves the growth rate and feed conversion ratio in monogastric animals (Beers & Jongbloed, 1991; Han *et al.*, 1997; Kies *et al.*, 2006; Kornegay & Qian, 1996; Lei *et al.*, 1993; Pandey *et al.*, 2001; Simons *et al.*, 1990). Pre-treatment of animal feed with phytase may also decrease the phosphorus pollution from animal manure by up to 50% (Lonsane & Ghildyal, 1993; Pandey *et al.*, 1999; Tengerdy, 1996). In expanding the use of microbial phytase, another important factor has been achieved. Conservation of the world's deposits of phosphate is recognised as important for future generations. Unlike nitrogen, P does not have a cycle to continuously replenish its supply. Therefore, utilisation of phytase may help to conserve the world's phosphate reserves, as addition of phytase to feed at 250 to 1000 U kg^{-1} feed can fully replace P supplementation in cereals for monogastric animals (Chudaske, 2007).

Most phytase research now concentrates on engineering a heat-stable enzyme that survives the elevated temperatures during feed pelleting, with improved temperature and pH optima, substrate specificity and enzyme stability. The optimum temperature for PhyA is 58 °C (Pasamontes *et al.*, 1997), which is approximately 20 °C higher than the body temperature of poultry and pigs at which maximum activity is desirable. Having a pH optimum similar to the pH level found in the digestive tract of the animals is also essential for maximum efficacy of the enzyme. A broad pH optimum, pH 2.5 to 7.5, has been reported for *Aspergillus fumigatus* phytases, expanding their potential usefulness as a feed additive. However, increasing the thermostability and improving enzyme activity over a broad range of temperatures and pH values would only be advantageous for compound feed preparations.

Today, large-scale fermentation operations employ overexpression and other biotechnological methods to produce nearly all the recombinant phytase used in the animal feed industry. Currently, the high price of

commercial phytase prevents its widespread use as a feed supplement. A more economical alternative for phytase addition would be SSF of the feed, in which phytase is produced *in situ* during SSF by growth of fungi on selected feed components (Pandey *et al.*, 1999; Ullah & Gibson, 1987). Yeasts, in contrast to filamentous fungi, do not produce mycotoxins (Fleet, 1992), and so, would be preferable for SSF. Several yeast species produce extracellular and/or intracellular phytases, for example *Arxula adeninivorans*, *Candida bombi*, *Candida lambica*, *Candida vartiovaarae*, *Cryptococcus laurentii*, *P. anomala*, *Pichia spartinae*, *Pichia rhodanensis*, *Schwanniomyces castellii* and *S. cerevisiae* (Andlid *et al.*, 2004; Nakamura *et al.*, 2000; Sano *et al.*, 1999; Segueilha *et al.*, 1992; Vohra & Satyanarayana, 2001; **III**).

4.2 Screening fungi for phytase activity

Different screening methods have been developed to find yeast species with phytase activities. In an agar-based assay, a translucent zone around colonies indicated the ability to produce extracellular phytase (Bae *et al.*, 1999; Lambrechts *et al.*, 1992). However, it was then shown that the size of the halo could not be quantitatively correlated with the amount of secreted phytase (Fredriksson *et al.*, 2002; Nakamura *et al.*, 2000). Lambrechts *et al.* (1992) stated that *P. anomala* did not hydrolyse phytate, as no clearing zone was detected. However, in the agar assay used in paper **III,** *P. anomala* grew on phytate as sole P source; thus, it must possess phytase activity.

Using the agar-based method presented in paper **III**, it was difficult or impossible to visually differentiate growth capacities of different yeast strains, as some growth also occurred in the negative controls. There is need for confirmatory assays using both solid and liquid media, as microbes producing phytase on solid phytate media may not produce the enzyme in liquid broth, and vice versa (Mukesh *et al.*, 2004; Tseng *et al.*, 1994). A liquid culture assay in microtiter plates was developed, and although slight growth was still observed in the negative controls, this method yielded more useful data. Here, growth could be followed over time and pronounced differences between the phytate test and the negative control could be detected. However, formation of yeast pseudohyphae that scattered more light than yeast cells might lead to overestimates of phytate degradation (**III**).

Synthesis of various phosphatases in yeasts and filamentous fungi is repressed by inorganic phosphorus (Wodzinski & Ullah, 1996). This was confirmed in paper **III**, where intracellular phytase activity was strongly suppressed and extracellular phytase activity completely inhibited for all ten tested yeast strains when grown in phosphate containing medium.

A. adeninivorans has previously been reported to have extracellular phytase activity (Sano *et al.*, 1999), but it also showed a very high intracellular activity (**III**). *P. anomala* is reported to have high activity of intracellular phytase, and an insignificant extracellular phytase activity (Vohra & Satyanarayana, 2001). The results of paper **III** indicate considerable differences between strains of *P. anomala*, regarding both intracellular and extracellular specific phytase activities.

Another interesting observation was that the crude extract preparations used to measure phytase activity showed a reduction in phosphate concentrations during the first few minutes of the assay. This reduction indicates the presence of a phosphate consuming reaction in the crude extract. Most phytase activity measurements have been conducted by estimating the difference between phosphate concentrations at only two time points in the assay (Mukesh *et al.*, 2004; Nakamura *et al.*, 2000; Sano *et al.*, 1999; Shieh & Ware, 1968). Such an approach will not detect antagonistic reactions, as long as the phytase activity releases more phosphate than is removed by the phosphate-consuming reaction. This must be taken into account when determining phytase activities (**III**).

5 Microbial dynamics and feed hygiene in cereal grain

5.1 Pre and post harvest ecology of fungi and bacteria in grain

The colonisation of plants by microorganisms starts almost as soon as leaves are exposed to the air. Bacteria usually colonise first, rapidly followed by yeasts, and then by pathogenic and saprophytic fungi. Filamentous fungi usually continue to develop at all stages of plant growth, including seed ripening (Flannigan, 1987; Lacey & Magan, 1991; Magan *et al.*, 2003). Fungi present on plants before harvest are traditionally termed 'field fungi'. Typically, these include species of *Cladosporium, Alternaria, Epicoccum* and *Fusarium* (Flannigan, 1987; Lacey & Magan, 1991; Magan & Lacey, 1984). *Cladosporium* species are among the most abundant components of daytime summer air-borne spores. Species of this genus are widespread on the ears of cereals at harvest. *Alternaria alternata* is, after *Cladosporium* spp., probably the most common airborne fungal spore. *Alternaria* may colonise cereal crops soon after emergence and penetrate the kernel sub-epidermally. This makes it tolerant of fungicides, and *Alternaria* species can be isolated from most grains at harvest (Lacey, 1989). *Fusarium* spp. are important pathogens of cereal grain, causing various infections such as scab, ear rot or head blight. They may also produce mycotoxins, such as deoxynivalenol and various trichothecenes, in the grain, both pre- and post harvest (Aldred & Magan, 2004; Lacey *et al.*, 1999). Pigs are more sensitive to trichothecenes than other farm animals. The main symptoms are reduced feed intake and weight gain, but impairment of the immune system has also been observed in pigs (Eriksen & Pettersson, 2004; Smith, 1992). Depending on the storage conditions of cereal grain, growth of typical storage fungi may occur. These fungi are present at low levels before harvest, and are principally species of *Aspergillus* and *Penicillium*. *Penicillium roqueforti,* a species also used in cheese

manufacture, is an important spoilage fungus in airtight storage systems (Lacey & Magan, 1991). In Sweden, this mould has been found in acid-preserved cereals, as well as in airtight stored grain with insufficient oxygen exclusion (Kaspersson *et al.*, 1988). Ruminants eating *P. roqueforti* infested feed displayed symptoms such as lack of appetite, ketosis, paralysis and spontaneous abortions (Häggblom, 1990). *Aspergillus* spp. are characteristic colonisers of stored products, different species vary considerably in their growth requirements; thus, the dominance of certain species may be indicative of previous storage conditions (Lacey, 1989).

On the other hand, it is clear that the concept of field and storage flora should not be carried too far. The terminology was first used in northern temperate regions, whereas in warmer, more humid climates, the species distribution between field and storage fungi differs (Lacey & Magan, 1991). The species composition of field and storage flora may also vary with grain storage method. For example, observations of partial persistence of the field flora in some moist storage systems were noted. *Cladosporium*, typically regarded as field flora, was detected after grain storage at three investigated farms and *Fusarium* spp. at one farm (**IV**), in accordance with previous findings (Kaspersson *et al.*, 1988). Lacey and Magan (1991) stated that *Fusarium* spp. could occur as storage flora, when a_w is high and temperatures are low. However, this correlation could not be substantiated in paper **IV,** as a_w was generally low.

Fungi seldom occur on grains in isolation, but usually as a mixed consortium of bacteria, yeasts and filamentous fungi (Magan *et al.*, 2003). Yeasts are best known for their contribution to society through their fermentation of bread, alcoholic beverages, and other products. Many studies have also been published on the spoilage of food and feed by yeasts (Fleet, 1992; Loureiro & Malfeito-Ferreira, 2003; Middelhoven & van Balen, 1988). Yeasts of different genera such as *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula* and *Sporobolomyces* have been isolated from grains at harvest (Flannigan, 1987; **IV** and **V**). However, the significance of their presence has not been examined in cereal grains, as filamentous fungi are usually considered to be the main agents of pre- and postharvest spoilage of grain (Lacey, 1989; Lacey & Magan, 1991). Spoilage of grain postharvest is initiated by insufficient drying or by subsequent moisture increases due to poor storage equipment. However, the importance of yeasts associated with pre- and postharvest deterioration needs further investigation. It is evident that yeasts play a significant role in the production and spoilage of fermented grain (Fleet, 1990). Although LAB are primarily responsible for silage fermentation, yeasts may compete with LAB for fermentable growth

substrates. Air ingress activates yeast growth that may lead to rapid deterioration, as some yeasts can metabolise lactic and acetic acid, causing the pH to increase and encouraging the growth of spoilage bacteria and moulds (Fleet, 1992). Yeasts may, on the other hand, inhibit mould growth (Passoth & Schnürer, 2003), be utilised as a SCP (Ravindra, 2000), and may have positive effects in the gastrointestinal tract (Schroeder *et al.*, 2004).

At harvest, cereals normally are at a_w 0.86 to 0.97, corresponding to a water content of 15–25%. In the field prior to harvest, cereals normally contain bacteria at approximately 6.5 log units g^{-1} fresh material. However, these numbers may vary substantially between different microbial groups and places of production (**IV** and **V**). Generally, control measures during grain storage do not focus on bacteria, as they are not regarded to be problem organisms. The minimum a_w that support active growth of most Gram-negative and Gram-positive bacteria are 0.97 and 0.90, respectively (Adams & Moss, 2000). Bacterial status is usually evaluated by determining the total counts of aerobic bacteria on general substrates, as an indication of the hygiene status of the feed. The dominant bacterial flora on plant surfaces are Gram-negatives – *Erwinia*, *Pseudomonas* and *Xanthomonas* (Flannigan, 1987) – with a smaller number of Gram-positive bacteria, such as *Lactobacillus* and *Leuconostoc* (Adams & Moss, 2000; Kaspersson *et al.*, 1988) that may become important in the production of fermented feed. The presence of *Lactobacillus* and *Leuconostoc* on cereal grain was confirmed in papers **IV** and **V**. However, species of *Bacillus*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Weisella* were also present on cereal grain at harvest (**IV** and **V**).

Presence of *Enterobacteriaceae* is indicative of the general hygiene status of the feed. High cfu values imply that further studies of potentially harmful organisms are required. However, these bacteria are usually not evaluated, as the a_w of cereal grain does not support their growth. We found that their presence on cereal grain at harvest was substantial (**IV**), far exceeding Swedish guideline values for silage (Table 4). This indicates the importance of including this microbial group during evaluation of feed hygiene of cereal grain stored using different technical systems.

5.2 Microbial feed hygiene

The hygienic quality of feeds is estimated by evaluating their general microbial status. The microbial quality of a feed or feed component may negatively influence both production and performance of animals. Different feeds may pose various risks, depending on management, composition,

production site, weather conditions etc. In Sweden, the acceptable maximum levels of microorganisms in different feeds are only stated as recommendations, i.e. not as legislated maximum levels (AnalyCenAB, 2008; Statute-book, 2007). However, these levels do provide an indication of the desired hygienic status. Exceeding the value does not necessarily indicate hazardous feed, but suggests that the risk for harmful feed is increased, and that a case-by-case assessment is required.

Table 4. *Swedish guidelines for acceptable levels of microorganisms in different feeds (log cfu g^{-1} feed)* [a] *AnalyCen AB,* [b] *Swedish code of statutes SJVFS 2006:81 (Statute-book, 2007).*

Microbial group	Silage [a] (MC<60%)	Hay/Straw [a]	Dried cereals [b]
Total mould	<4.5	<5.5	<5.0
Yeast	<6.0		
Enterobacteriaceae	<6.0		
Total aerobic bacteria		<8.0	<7.7

No data are available on permissible levels of various microorganisms for all feedstuffs used. Hence, evaluations of hygiene in certain feeds are based on estimations. Feed hygiene is mainly influenced by water content, as high a_w may permit bacterial and fungal growth during storage, thereby reducing the quality of the feed (Artursson, 2007).

5.2.1 Methodology and cultivation

Determining the diversity of microbes has always been a challenge. Classical approaches, which rely on culture-based techniques, may only recover a fraction of the microorganisms in the environment. Current estimates for soil and water place the number of uncultivable microbes at approximately 99% (Amann *et al.*, 1995), although some investigations have reported a recovery of up to 4% of total microbes (Olsen & Bakken, 1987). Moreover, the process of cultivating microbes, although improving due to development of new methodologies, is time consuming, labour intensive, and gives only limited insight into the spatial distribution of microorganisms. Yet this is the only way to isolate strains of different organisms that may be further used as starter cultures (**II**, **IV**, **V** and **VI**).

The culture-based techniques used during feed hygiene evaluation were applied, following the recommendations of the Nordic Committee on Food Analysis (NMKL). Feed samples were prepared by generating dilution series according to general microbiological principles. The number of viable microorganisms per gram of feed was calculated based on the number of

colonies counted on selected plates (NMKLNo.91, 2002). Enumeration of LAB was performed on de Man, Rogosa and Sharp agar (MRS). The methodology represents a general approach applicable to all types of foods and feeds. However, optimum growth requirements of LAB species may differ considerably; hence, this method may not always equally support the growth of all LAB (NMKLNo.140, 2007). The amount of LAB in the feed provides an indication of the fermentation process.

Tryptone Glucose Extract Agar (TGEA) is a general substrate for enumeration of aerobic bacteria, providing a generalised picture of the bacterial load in the feed. The aerobic plate count is determined by pour-plating into molten agar in Petri dishes (NMKLNo.86, 2006). This method was deemed inappropriate for determining hygiene in fermented feed, as 16S rRNA gene-based identification revealed that the majority of colonies on TGEA were LAB, which are desirable in feed (**VI**).

Enterobacteriaceae are a family of Gram-negative rods that are facultatively anaerobic. Enumeration is performed in Violet Red Bile Glucose Agar (VRBG) according to (NMKLNo.144, 2005). *Enterobacteriaceae* counts are used as a general indicator of hygienic quality. Thermotolerant coliform bacteria, such as *Escherichia coli*, are also enumerated on VRBG by incubating at an increased temperature (NMKLNo.125, 2005). The presence of *E. coli* in feedstuffs strongly suggests some history of faecal contamination. *Clostridium* is enumerated on Reinforced Clostridial Medium (RCM). This estimates the total number of anaerobic spore forming bacteria, as samples are held at 80 °C for 13 minutes before plating.

Yeasts and moulds are quantitatively determined according to (NMKLNo.98, 2005) on dichloran glycerol agar (DG18) for feedstuffs at reduced a_w (< 0.95). The cfu are determined by dilution plating. Moulds and yeast are counted separately after incubation. Malt Extract Agar (MEA) may also be used to isolate moulds and yeasts, if reduced water activity is not required. Enumeration methods provide numbers, rather than information about community phylogeny or diversity. However, as cells are counted as separate colonies on a solid medium, single colonies can readily be isolated and subjected to further analysis (**I**, **IV**, **V** and **VI**).

Real time PCR – another method to quantify species

Real time PCR may be used to quantify different species in food and feed samples (Bleve *et al.*, 2003; Geisen *et al.*, 2004; Mackay, 2002). It has been proven to rapidly and selectively quantify fungal species in mixed species fermentation of inhomogeneous substrate (Feng *et al.*, 2007). The distinguishing feature of real time PCR is that the process of amplification is

monitored in real time using fluorescence. By comparison with known standards, the amplification curves obtained can be used to quantify the initial amounts of template molecules with high precision. As real time PCR provides the whole amplification profile, individual reactions deviating in their amplification efficiency may easily be detected (Wilhelm & Pingoud, 2003). However, the method may not be used directly on feed samples, as primer design is required to detect the species of interest. As microbial composition is more or less unknown in feed samples, this method can not be directly applied. One the other hand, the method would be most useful in quantifying known species of interest, e.g. during starter culture evaluation.

5.2.2 Species identification by molecular methods

Isolated LAB and yeasts strains were identified to species level by PCR-amplification of rRNA genes: sequences that are often used for taxonomic differentiation. Prior to species identification through sequence analysis, the isolated strains in papers **II**, **IV**, **V** and **VI** were differentiated based on genotype. Banding patterns generated by repetitive-DNA-element PCR fingerprinting with the microsatellite primer $(GTG)_5$ (Lieckfeldt *et al.*, 1993) were analysed using the GelCompar II V4.5 software (Applied Maths, Kortrijk, Belgium). A dendrogram was constructed and a representative strain from each cluster was selected for identification.

The rRNA genes of representative strains were sequenced to identify the species present in the cereal grains. For sequence analysis of selected yeasts, the D1/D2 region (approximately 600 bp) in the 25S rRNA gene was amplified using primers NL1 and NL4 (Valente *et al.*, 1999). For LAB, nearly the entire 16S rRNA gene was amplified using primers specific for the domain Bacteria: 16SS and 16SR (Pedersen *et al.*, 2004). Strains were identified by comparing the sequences with those of known species in the genetic database of EMBL, Nucleotide Sequence Database (http://www.ebi.ac.uk/).

Determining community structures – RFLP, T-RFLP and DGGE

Analysis of restriction fragment length polymorphisms (RFLP) is a simple procedure in which a standard restriction digest is performed on PCR-amplified rDNA (Spiegelman *et al.*, 2005). First, the rRNA genes of the community sample are amplified by PCR. Then, various restriction enzymes are used to digest the amplified community DNA. Divergence in the rRNA gene sequence of different species will create different restriction sites for various enzymes. The use of appropriate restriction enzymes will

result in a unique fingerprint for each species or strain (Vaneechoutte *et al.*, 1992). When run on a gel, the digested DNA produces a pattern of fragment sizes characteristic for that community. The method does not allow detection or identification of specific phylogenetic groups within the community profile. However, the bands from the RFLP gel may be transferred to a membrane and probed for presence of specific sequences (Lovell & Hui, 1991).

Terminal-restriction fragment length polymorphism (T-RFLP) is a method modified from RFLP. T-RFLP uses fluorescent PCR primers and automated scanners to quantitatively visualise a community restriction pattern. One primer is labelled with a fluorescent dye. This enables measurements of both size and intensity of the terminally-labelled restriction fragments with digital detection equipment. The method is useful both for creating a community fingerprint that can be compared to other communities, and for estimating species richness and diversity within a community (Spiegelman *et al.*, 2005).

Denaturing gradient gel electrophoresis (DGGE) analysis of microbial communities produces a complex profile, which can be rather sensitive to spatial and temporal sampling variations (Murray *et al.*, 1998). The method separates PCR-amplified rDNA according to differences in the G-C content. Amplicons representative of the community structure are run on a polyacrylamide gel with a gradient of DNA-denaturing compounds. As DNA is passing through the gradient, strands are successively separated. In the primers used during PCR, a GC clamp is incorporated into the amplified DNA. The GC clamp prevents complete denaturation of the DNA. However, increasing denaturation of the DNA decreases its mobility, and it finally it comes to rest when it is almost fully denatured. Thus, differences in the G-C content cause amplicons to migrate to different positions in the gel. Accurately calibrated DGGE is sensitive enough to show single base-pair differences in the amplicons (Miller *et al.*, 1999). For taxonomic identification, bands can be sequenced. DGGE is at a disadvantage here, as DNA fragments are typically below 500 bp in size, which restricts the amount of sequence available for identification. As with all PCR-based rDNA fingerprinting methods, lack of precision exists in associating a single band in the community with a single microbial species, as multiple amplicons may co-migrate to the same location in the gel (Nübel *et al.*, 1997).

6 Biopreservation of cereal grain

6.1 Preservative systems for cereal grain

When storing cereal grain, the objective is to minimise losses and maintain nutritional value. Improper handling of harvested cereal grain causes significant quantitative and qualitative losses, ranging from 9% to 50% (Sinha, 1995). At harvest, cereal grains contain almost always too much moisture for safe storage, thus preservative measures are needed. The main preservation methods are drying, acid treatment and airtight storage (Jonsson, 1996).

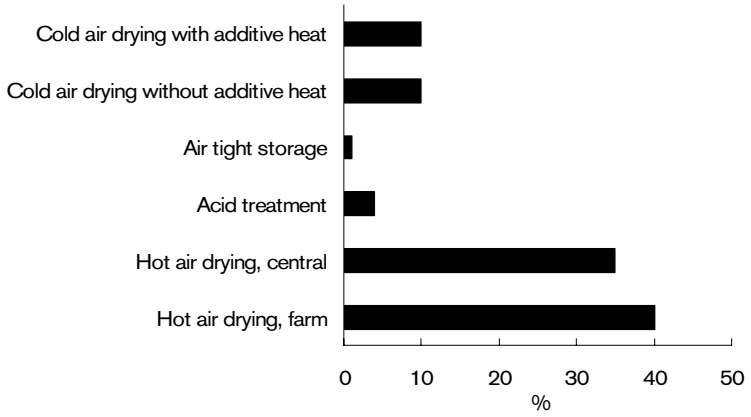

Figure 6. Relative use of different cereal grain storage methods in Sweden (Data from Jonsson, 1996).

The water content of the cereal grain at harvest determines how quickly the cereal grain needs to be preserved (Flannigan, 1987; Magan *et al.*, 2003).

This water content can vary substantially in different years, depending on the local weather conditions before and during harvest (Ekström & Lindgren, 1995; Jonsson, 1996).

6.1.1 Acid treatment

Acid treatment of cereal grain was introduced in Sweden during the late 1960s. This preservation method inactivates the sprout and interferes with the baking process. Thus, acid treatment may only be used for storage of cereal grain intended for feed (Jonsson, 1997). Acid application is a delicate process and needs to be monitored accurately. Addition of the correct concentrations of acid depends on the water content of the cereal grain (Lacey & Magan, 1991). Uneven distribution of acid over the kernel surface may permit mould growth during storage. Initial mould growth may then affect surrounding grains, as acid is less effective on actively growing microorganisms (Jonsson, 1997). In Sweden, propionate is the only acid permitted for feed applications, as formic acid has been forbidden due to the risk of aflatoxin production in the event on inaccurate dosage (Clevström *et al.*, 1989). Low concentrations of propionate may also stimulate the production of aflatoxins (Al-Hilli & Smith, 1979).

6.1.2 Drying

Most of the cereal grain harvested in Sweden is preserved by drying: with cold air; cold air with additional heating; or with hot air. Insufficient drying is the main cause of deterioration in grain quality during storage. Cold air drying often results in uneven water content in the cereal grain, indicating that the drying zone had not passed through the entire batch in the drier. Drying without heat could also be ineffective due to high moisture levels in the air, as commonly occurs in September and October in Sweden. Cereal grains dried with heat addition or with hot air usually show much lower MC (Jonsson, 1996). To achieve safe storage, the grain has to be dried to a water content of 13% ($a_w < 0.65$), which consumes much energy. In Sweden, approximately 60% of the energy used during total plant husbandry operations was calculated to be spent on grain drying (Pick *et al.*, 1989).

The agricultural sector produces food, feed, and biomass for energy. A large proportion of the required energy input is met by fossil fuels such as oil, diesel and gasoline. The use of fossil fuel needs to be reduced, due to both diminishing resources and because combustion gives rise to pollution that contributes to eutrophication, acidification and climate change (SJV, 2008). One way to reduce energy consumption in agriculture is to use safe and energy-efficient long term storage methods for moist cereal grain.

Preserving cereal grain while moist dramatically reduces the consumption of fossil fuels used for drying of the grain. If all feed grain were to be stored moist under airtight conditions, instead of being hot-air dried, this would save 34 000 m^3 diesel oil per year in Sweden alone (calculated from Edström *et al.*, 2005). Farmers have also indicated an interest in preserving cereal grain with higher MC, to generate feed with improved structure and less dust.

6.1.3 Airtight storage

Airtight storage of high moisture feed grain requires only ~ 2% of the energy consumed in high-temperature drying (Pick *et al.*, 1989). Safe storage of grains relies on a perfectly airtight silo with a modified atmosphere, enabling storage of the cereal grain at higher MC. Respiration of the grain and the endogenous microflora reduces levels of O_2 and increases levels of CO_2 (Lacey and Magan, 1991; Magan *et al.*, 2003). However, the control of spoilage microorganisms depends on maintaining the modified atmosphere. Temperature fluctuations may, in turn, generate pressure fluctuations in the silo (Druvefors *et al.*, 2002). Also, imperfect sealing and feed outtake lead to gas leakage. Feed outtakes also result in a continuously diminishing grain bulk, making it difficult for the microbial and grain respiration to sustain the modified atmosphere needed for safe storage. Deteriorative microbial development and spontaneous heating may then occur (Lacey & Magan, 1991).

6.1.4 Moist crimped cereal grain

Moist grain crimping, a preservation method to store feed grain and convert it into livestock fodder by fermentation, is now becoming widely used (Finch *et al.*, 2002). Harvest of cereals intended for crimping should preferentially occur during grain yellow ripeness and while the kernels have a MC of 30-45%. Prior to storage, the cereals are preferably rolled to facilitate packing and thereby reduce air-space between the kernels. At sufficiently high MC, a spontaneous fermentation starts and an ensiled cereal grain is obtained. The stability of moist crimped cereal grain is a result of acid fermentation by the natural microbial flora on the cereals. The success of natural fermentation is dependent on a number of factors, for example: the strains of indigenous LAB and yeasts present and their population density; cultivation; crop management; and conditions of harvest and storage. Moist crimping of cereal grain allows the harvest of grain at higher MC. This may protect the crops from prolonged exposure to inclement

weather, which might otherwise lead to weathering and mould infections of the grain in the field (Lacey & Magan, 1991).

Moist crimped cereal grain cannot be stored in silos, as a MC above 25% impedes the feed outtake system (Jonsson, 1996). However, other structures could be used, such as permanent clamps or bunkers, or plastic tubes. The use of plastic tubes has increased in the last few years. This storage system is interesting from an economical point of view, as oil prices are estimated to reach 200 USD per barrel within 3 years, increasing the cost of hot air drying. In addition, capital and maintenance costs for permanent storage space have been replaced by mobile costs (Sundberg, 2007). In northern Sweden, most cereal grain intended for feed was previously bought and transported from the south. Increasing grain prices and higher transportation costs have now motivated farmers to produce their own cereal grain for fodder. Life cycle analyses have also shown that local production of cereal grains i.e. on individual farms, reduces the environmental impacts of grain production (Cederberg *et al.*, 2007). Due to the weather conditions and shorter growth period, the moist crimped cereal grain storage method would be ideal for this region.

6.2 Microbial changes during storage of moist crimped cereal grain

Not much is known about the microbial population in moist crimped cereal grain. Eight farms at different locations in Sweden were investigated, providing the first data on the microbial composition of crimped cereal grain stored moist under farm conditions (**IV**). Currently, permissible levels of various microorganisms in moist crimped cereal grain have not been stipulated or recommended, and there are no data available about typical values in this storage system. Another aspect of the study was to identify key organisms in this process (**IV**), as constant cfu values do not necessarily indicate a stable population, because the species composition may change (**I** and **II**). The diversity of the LAB and yeasts identified may offer new opportunities to upgrade technology and develop products to benefit crimped grain storage systems. Such species may potentially be used as starter cultures in a controlled fermentation of moist cereal grains.

During the investigation, most of the stored grain systems contained less moisture than the recommended 30–45%. However, MC in this study were within the bounds of typical farm practice (15–25%). The grain pH of the tested storage systems was only slightly reduced during storage. It is likely that low concentrations of accessible and easily degradable sugars in the

grain did not support a substantial decrease in pH during the storage period. Thus, moist grain storage is very different from established grass silage systems, where storage stability is primarily achieved by the decrease in pH. The storage stability of the crimped feed grain might instead be due to the low MC at harvest, or the exclusion of oxygen in combination with a high CO_2 atmosphere (Magan *et al.*, 2003).

It is of note that *Lactobacillus fermentum* was always the dominant LAB at the end of storage in eight tested farms, independent of MC, temperature, pH, type of grain or number of microorganisms in the systems (**IV**). Strains of *L. fermentum* have probiotic properties and may be antagonistic against other bacteria, such as *E. coli*, *Salmonella* spp., *Shigella* spp. and enterotoxigenic S*taphylococcus aureus* (Lin *et al.*, 2007). The competitiveness of *L. fermentum* on cereal grain, irrespective of moisture, makes such strains interesting candidates for evaluation as starter organisms, to improve the microbial stability of the grain, and possibly even the nutritional characteristics of the material.

Similar to LAB, the dominant yeast species changed during the storage period, but in contrast to the LAB, to variable species. No general trends were observed, e.g. a yeast dominant in one farm before storage was dominant post-storage elsewhere. Even among farms with similar storage conditions, the dominant species in grain from one farm was outcompeted at another farm. This variation could not be explained by current data, and further studies need to be implemented to understand the yeast diversity in these grain storage systems (**IV**). However, Magan *et al.* (2003) stated that the interactions and dominance of species are dynamic processes, and emphasised the importance of taking into account all different environmental conditions, in order to understand the dominance of certain species.

The flora of filamentous fungi also changed during storage. Species of field flora – *Cladosporium* and *Fusarium* – were observed after storage (**IV**). Lacey and Magan (1991) stated that *Fusarium* spp. could occur as storage flora, at high a_w and low temperatures. Also, *Fusarium* spp. were shown to survive better than most field fungi in moist barley stored under a controlled atmosphere (Kaspersson *et al.*, 1988). However, no relationships between a_w and the presence of field flora after storage were observed, as these species were detected in cereal grain with low a_w (**IV**). However, field flora do not necessarily persist during storage of moist crimped cereal grain (**V**).

6.3 Inoculation of starter culture to increase feed hygiene in moist cereal grain

Varying microbial composition in cereal grains from different farms suggests a need for a starter culture to influence the predictable development of all relevant microbial groups. Under practical farm conditions, obtaining the optimum MC in cereal grain intended for crimping appeared to be problematic (**IV** and **V**). Storage of moist grain increased the risk of mould growth, unless moisture content was high enough to initiate LAB fermentation (**IV**).

It was therefore of interest to secure feed hygiene in cereal grain with MC lower than 30%. Inoculation of the biocontrol yeast *P. anomala* was conducted to evaluate feed hygiene of moist grain intended for storage in large plastic tubes as moist crimped cereal grain (**V**). This yeast has previously been shown to prevent growth of spoilage moulds, both *in vitro* and in model systems of airtight storage of moist feed grain (Björnberg & Schnürer, 1993; Petersson & Schnürer, 1998). In the inoculated grain, the mould flora was reduced during the storage period (**V**), confirming previous results (Druvefors *et al.*, 2005; Druvefors *et al.*, 2002; Petersson *et al.*, 1999; Petersson & Schnürer, 1995; Petersson & Schnürer, 1998).

Several different mechanisms of fungal inhibition have been suggested for *P. anomala*. Generally, competition for limited nutrients and space (Janisiewicz & Korsten, 2002), production of killer toxins (Walker *et al.*, 1995) and production of cell wall degrading enzymes (Jijakli & Lepoivre, 1998) are seen as important strategies deployed by yeasts. However, these may not be the main mode of action of biocontrol by *P. anomala* (Druvefors *et al.*, 2005; Druvefors & Schnürer, 2005). Instead, formation of ethyl acetate, a product of glucose metabolism in yeast, is concluded to be a major component of the mould-inhibiting activity of *P. anomala*, (Druvefors *et al.*, 2005; Fredlund *et al.*, 2004a). The numbers of moulds were not reduced in the control cereal grain, in which only low levels of naturally occurring *P. anomala* were present (**V**).

A surprising result was that in *P. anomala* inoculated grain, the number of *Enterobacteriaceae* decreased below detection level (10 cfu g^{-1} grains). The identified *Enterobacteriaceae* population in the cereal grain at harvest comprised 95% *Pantoea agglomerans* and 5% *E. coli*. *P. agglomerans* is used as a biocontrol agent in apples (Nunes *et al.*, 2002), but can also be a plant pathogen and cause human disease (Cruz *et al.*, 2007). *E. coli* is commonly found in the intestine of warm-blooded animals. Most strains are harmless, but certain serotypes cause serious food poisoning (Denny *et al.*, 2007). Guidelines for acceptable levels of *Enterobacteriaceae* in hay and dried cereals

do not exist, because it is assumed that these bacteria cannot grow at low MC (Adams & Moss, 2000). However, these bacteria could grow in cereal grain (**IV** and **V**), exceeding the guideline values for silage, even though MC values were relatively low. *Enterobacteriaceae* were initially present in both inoculated (MC 18%) and control (MC 16%) cereal grain, but their numbers were only reduced in inoculated grain (**V**). This is the first report of an inhibitory effect of *P. anomala* on *Enterobacteriaceae* in cereal grain. This finding is of great relevance to feed hygiene, as it has previously been shown that reducing the number of *Enterobacteriaceae* in feed leads to a reduction in *Enterobacteriaceae* present later in the food chain (Brooks *et al.*, 2001).

7 Bioprocessing of cereal grains

7.1 Co-products from bioethanol production

Distillers' grain is a by-product derived from the ethanol industry. Bioethanol is defined as ethanol derived from fermentation of sugars, and is used for human consumption, by industry, and as a fuel (Chudaske, 2007). Different agricultural products can be used to produce bioethanol. In Sweden, wheat is the main raw material (www.agroetanol.se; www.reppe.se; www.vsgroup.com). Bioethanol production utilises the starch in cereal grain, leaving a protein and fiber rich residue. The production of 1 ton of ethanol requires about 3 tons of cereal grains, resulting in large quantities of distillers' grain (Chudaske, 2007). Production of bioethanol is increasing, due to the accelerating interest in producing energy from sustainable sources. Hence, feedstuffs derived from by-products from those processes will appear in even larger quantities in the future (EurObserv'ER, 2007). As by-products may be utilised as a protein feedstuff by compound feed manufacturers, as well as for direct feeding on the farm, they should rather be viewed as a value-added co-products. Such use of co-products avoids wasteful disposal, and can decrease feed costs and the environmental burden (Brooks *et al.*, 2001; Brooks *et al.*, 2003; Scholten & Verdoes, 1997). Composting organic products causes biological emissions of ammonia, nitrous oxide and methane. These emissions can be significant if gas cleaning equipment is not used. Thus, utilisation of co-products indirectly benefits the environment, by reducing emission of greenhouse gases and of pollutants that contribute to eutrophication (Farrell, 2006).

Production of fuel ethanol derived from farm crops is widely disputed, as food products are used as raw materials for ethanol production. Historically, livestock agriculture was also disputed, as it competed with the human food market for raw materials. But the livestock sector is able to address

competition from the human food conglomerate, as it produces competitive products (meat, milk and eggs) that are desired by consumers. The entry of yet another large and powerful competitor – the bioethanol industry – into the market place is indeed cause for concern. Bioethanol production is also disputed based on energy efficiency, as the energy input required during processing is quite high. Use of co-products as protein feed could improve the energy efficiency of bioethanol production. Replacement of imported soy protein, and the resulting reductions in transport, could also be included in the energy analysis, further increasing energy efficiency (Börjesson & Mattiasson, 2007).

Adding phytases in the production of bioethanol derived from cereals would yield major benefits, both in the ethanol process and in co-product value. The removal of phosphate from phytate releases inositols, which play a major role in yeast physiology, especially in the synthesis of structural components in the cellular membranes. This, in turn, results in faster ethanol production and increased tolerance to higher ethanol concentrations (Chi *et al.*, 1999). Increased availability of P in co-products would reduce phosphate pollution and lower the need of P addition in feed formulations, yielding both economic and environmental benefits (Shetty *et al.*, 2008). Further environmental benefits could be gained by using cereals that have been stored moist instead of hot air dried. Apart from the considerably lower energy demands during grain storage, moist stored grain also supported fermentation with a substantially improved ethanol yield of more then 10% (Passoth *et al.*, unpublished).

7.2 Utilisation of co-products

Distillers' grain, and other co-products from human food production, can be highly variable in composition, limiting its utility in animal feed diets. The feedstock used in the production of ethanol will influence the nutrient profile of the resulting co-products. Therefore, the specific material used by a supplier must be certified (Patience *et al.*, 2007). An accurate nutrient profile for the distillers' grain is a desirable for its effective use in practical diet formulation. For example, crude protein content has been reported to vary from 23.9% to 40.1% in wheat distillers' grain with approximately 90% DM (Beyer *et al.*, 2003; Nyachoti *et al.*, 2005; Widyaratne & Zijlstra, 2007). Despite the variability of the co-products, they can be used efficiently and without detrimental effects on pig performance, if the diets are accurately reformulated to account for batch to batch variation (Scholten & Verdoes, 1997).

7.2.1 Dry or wet formulations of distillers' grain

Distillers' grain could either be utilised as liquid feed, or be dehydrated and pelleted. Drying distillers' grain is cost and energy intensive, but yields a commodity with 90% DM content that is easy to handle and transport, and requires less storage space. The dried distillers' grain (DDG) is primarily used as feed for ruminants, which prefer fibrous material. Pelleted DDG is also used as pig feed. However, there is a need to further evaluate the influence of high fiber content on the feed value of DDG for pigs (Nyachoti *et al.*, 2005). There is also an indication of increased incidence of *Salmonella* in pig herds given pelleted dry feed instead of non-pelleted feed. The incidence was even lower when non-pelleted wet feed was provided (Brooks *et al.*, 2001). If wet wheat distillers' grain (WWDG) is fed in its liquid form, there is no necessity for drying.

Figure 7. Pelleted distillers' grain from Agroetanol (Norrköping, Sweden). Wet wheat distillers' grain from Absolut Spirits (Åhus, Sweden) used in paper **I**, **II** and **VI**. (Photo: Niclas Olstorpe)

This reduces the energy requirements during production of pig meat, decreasing cost and reducing dependence on non-renewable energy sources. On the other hand, using liquid co-products increases transport costs, as the commodity primarily consists of water (8-30% DM) (www.agroetanol.se; www.reppe.se; www.vsgroup.com). Transport-associated demands for energy increase with distance. Therefore, feeding WWDG may only be efficient where pig production is situated close to the source of supply.

The WWDG is mostly utilised in pig production, where liquid feeding is adapted to the production system. Pigs accept liquid feed faster than dry feed. This may partly be due to better water supply, as weaned piglets may not take in sufficient water through water nipple drinkers (Pedersen & Lindberg, 2003). The utilisation of co-products may increase the animals' water consumption further, as they have high mineral contents (Brooks & Carpenter, 1990). A wide variety of feeds and fermentation techniques are used in different countries. Both individual feedstuffs and complete compound diets are fermented (Scholten *et al.*, 1999). Use of fermented liquid feed has been shown enhance performance in pigs: daily weight gain, feed conversion ratios and gastrointestinal health are improved and susceptibility to diarrhoea is reduced (Brooks *et al.*, 2003; Jensen & Mikkelsen, 1998; Lyberg *et al.*, 2005). Piglets fed on liquid diets have better developed *villi* in their small intestine (Mikkelsen & Jensen, 2001), which is of major importance for efficient nutrient uptake (Pluske, 2001).

7.3 Fermented co-products as animal feed

Fermented feed is sometimes categorised as liquid or wet feed, although soaking *vs* fermentation processes are not clearly distinguished in literature. However, when a feed is mixed with liquid, fermentation is rapidly initiated (Canibe & Jensen, 2003). In this way, most liquid diets are, more or less, affected by microbial activities. The chemical and microbial characteristics of fermented liquid feed have been shown to differ among fermentations (Beal *et al.*, 2005; Moran *et al.*, 2006; Scholten *et al.*, 2001a; Scholten *et al.*, 2001b; **I**, **II**, and **VI**). There is considerable concern about the incidence of zoonoses in animal feeds, in particular, regarding possible transmission of *Enterobacteriaceae* such as *Salmonella* into the food chain. LAB and yeast present during fermentation of the feed may suppress unwanted contaminants (Magnusson *et al.*, 2003; Passoth & Schnürer, 2003; Pedersen *et al.*, 2005). The microorganisms involved in fermentation produce organic acids, such as lactic and acetic acid, which may reduce the pH to approximately 3.5–4.5. A low pH and a high concentration of lactic and acetic acid in liquid fermented feed can prevent proliferation of *Enterobacteriaceae* in the feed and along the animal gastrointestinal tract (Geary *et al.*, 1999; Geary *et al.*, 1996; Mikkelsen & Jensen, 1998; Russell *et al.*, 1996; Scholten *et al.*, 1999; **I**).

Differing microbial flora in the fermented feed may influence the organic acid profile, which, in turn, affects palatability and feed hygiene. Pigs tolerate dietary lactic acid concentrations up to 200 mmol l^{-1} fermented

liquid feed (Beal *et al.*, 2000). To inhibit *Salmonella* in fermented liquid feed, lactic acid at 75 mmol l^{-1} and a pH below 4.5 has been shown to be sufficient (Beal *et al.*, 2002). But there are also indications that pH below 4.0 and a high lactic acid concentration do not necessarily ensure product safety, as the exposure time in these conditions is obviously an important factor to exclude *Enterobacteriaceae* (Moran *et al.*, 2006). Consequently, properly fermented liquid feed could make a significant contribution to food safety. High levels of acetic acid might negatively affect palatability for pigs (Beal *et al.*, 2005). Thus, fermentative microorganisms in the feed should produce an organic acid profile that increases feed hygiene, but also ensures palatability.

Yeasts are sometimes considered undesirable in liquid diets. For example, some yeasts may produce off-flavours and taints that reduce palatability of the feed. Yeast metabolism can also convert starch into alcohol and carbon dioxide, resulting in high ethanol contents and energy losses as carbon dioxide (Brooks *et al.*, 2001; Brooks *et al.*, 2003; Jensen & Mikkelsen, 1998). However, it has also been shown that high yeast numbers do not necessarily decrease feed quality. During cereal grain fermentations with liquid added in the form of WWDG, whey or water, only low levels of ethanol and slight weight losses in the feed were observed (**I** and **VI**). Yeasts may contribute to the intestinal health effect seen in weaned pigs, as administration of live yeast improved *villus* height in the small intestine (Lallès *et al.*, 2007). Fermentation of feed has also been shown to increase nutrient availability through phytate degradation. Extensive degradation of IP_6 in fermented feed (**I** and **VI**), which possibly improves the digestibility of P (Lyberg, 2006), may reduce the need for supplementation with feed phosphates or commercial microbial phytase. Fermentation of liquid feed would, thus, reduce the cost and environmental impact of pig production.

7.4 Microbial population instability in WWDG

The characteristics of a fermented diet depend on the activity and nature of the microbial populations, which are affected by temperature, substrate and time (**I**, **II**). WWDG inherently contains high numbers of microorganisms that can stimulate the development of a beneficial microbial population in the feed (Scholten *et al.*, 1999). In reality, microbial populations from different batches of WWDG vary in both numbers and species composition, even when coming from the same distillery (Pedersen *et al.*, 2004; **II** and **VI**), resulting in unpredictable feed fermentations. Both during cold storage of WWDG and fermentation of formulated diets, the population diversity of

LAB and yeast species shifted. Even though cfu values of yeasts and LAB appeared to be stable during fermentation, species identification indicated changes in microbial populations. This shows that cfu numbers alone cannot be regarded as a reliable indicator of population stability and nutritional and hygienic properties of the fermented feed (**I**, **II** and **VI**). The changing microbiological and biochemical properties of fermented liquid diets highlight a need for starter cultures to ensure both feed hygiene and an organic acid profile appropriate for pig feed.

7.4.1 Stabilisation of the microbial population

A silage starter culture containing species of *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactococcus lactis* and *Pediococcus pentosaceus* was applied to initiate fermentation of a feed prepared from WWDG and a cereal grain mix. *L. plantarum* was the only starter culture organism to became dominant; the other dominant LAB, *L. panis,* likely originated from the WWDG. Both organisms are heterofermentative, producing lactic acid together with other organic compounds such as ethanol and acetic acid (Axelsson, 1998; Wiese *et al.*, 1996). Strains of *L. panis* also possess probiotic properties (Pedersen *et al.*, 2004) that may be advantageous for the intestinal health of pigs.

The cfu numbers of different LAB populations, as well as the presence of other organisms, influence the amounts of organic acids in feed, which may not always reduce the number of *Enterobacteriaceae* (**I**, **II** and **VI**). To increase the concentration of lactic acid in the feed, inclusion of a homofermentative LAB could be appropriate, for example, *P. pentosaceus*. Both LAB, *L. plantarum* and *P. pentosaceus*, have been used as starter culture in laboratory trials of fermented pig feed, indicating their potential to ensure feed hygiene (Beal *et al.*, 2002; Moran *et al.*, 2006). *P. pentosaceus* was present in the starter culture, but did not proliferate during fermentation (**VI**). It was however, previously identified as the dominant LAB in a batch of WWDG, and continued to dominate in the feed fermentation generated with this WWDG (**II**). This may indicate the importance of using strains isolated from the fermentation process, which are adapted to the specific conditions.

The yeast flora was not influenced by adding the LAB-starter culture. The number of yeast was similar in all treatments, and *P. fermentans* became dominant in all experiments, independent of whether starter culture was added or not (**VI**). Uncontrolled yeast growth in feed may lead to feed losses and reduced palatability; but desirable yeast can be added to the starter culture, to contribute to the fermentation without causing negative changes.

8 Concluding remarks

The aim of this thesis was to investigate if microbial fermentations can improve animal feed quality. Feed fermentation can be done with the direct intent to improve feed quality (as during generation of fermented pig feed) or to save energy during storage of cereal grain. Feed quality is determined by several parameters, e.g. protein content, energy content, hygiene status (i.e. the amount of potentially dangerous microorganisms), and bioavailability of nutrients and minerals. During the work described in this thesis, I have investigated two different systems of feed treatment: airtight storage of moist crimped cereal grain; and fermented liquid pig feed. Both systems can greatly benefit the environment compared to conventional feed handling. Storage of moist grain saves the energy required for drying, (up to 60% of the total process energy); feed fermentation can utilise by-products from food or ethanol production, thereby reducing waste streams. Drying of wet distillers' grain also requires energy, which can be saved by using it directly in the fermentation.

I found that fermentation methods improved the nutritional and hygienic parameters of the feed. The amount of phytate decreased in both investigated systems, and the protein content increased in the airtight stored grain. The numbers of *Enterobacteriaceae* were reduced both in fermented feed and in airtight stored wet grain inoculated with the biocontrol yeast, *P. anomala*. The latter was especially remarkable, as antibacterial activity, in addition to its well-known antifungal potential, has not previously been shown for this yeast in farming systems.

However, the results in my thesis also show the limitations of the two feed fermentation systems. Both rely on spontaneous fermentations, caused by the microorganisms present on the feed ingredients and/or the equipment. It became obvious during the project that the microbial populations differed considerably from batch to batch. Microbial species that

were dominant in one batch were outcompeted in another batch. Thus, microbial successions in these systems seem to be determined by characteristics of particular strains, rather than by species. Airtight stored grain under practical farm conditions seldom reached the moisture content required to obtain sufficient lactic acid fermentation, resulting in the growth of undesirable moulds and bacteria. The spontaneous character of the studied fermentations is a considerable economic risk for the farmer.

One conclusion from my thesis is that feed fermentations should be handled as directed fermentations, instead of relying on spontaneous processes. This can be achieved by adding starter cultures containing appropriate LAB and yeasts to the systems. My investigations showed that it is important to use appropriate strains for the starter cultures. Microorganisms that I isolated from the storage systems, belonging to the LAB species *L. fermentum*, *L. panis*, and *P. pentosaceus,* and the yeasts *P. fermentum* and *P. anomala*, may qualify to be included in such starter cultures.

The yeast, *P. anomala*, appears to be well suited for inclusion in a starter consortium for the conservation of airtight stored moist grain. In addition to its antimicrobial characteristics, it also showed considerable phytase activity and a nutritionally advantageous amino acid composition. Interestingly, the phytase activities were highly variable among different strains of this species, once more indicating the importance of selecting the right strain for developing a good starter consortium. Adding *P. anomala* to the cereal grain did not significantly enhance the protein content of the fermented grain. This might be due to a cell density depending growth inhibition of *P. anomala* that was observed on grain and in other cultivation systems. More research is required to understand and eventually overcome this effect.

My research also showed that it is important to develop new methods to evaluate feed quality. Molecular species identification of isolates showed that there are substantial changes in microbial populations that traditional plate counting methods would have shown to be "stable". Selection for particular microbial groups failed in "selective" cultivation methods, demonstrating that results from these methods appear to be of limited value in evaluating the quality of fermented feed.

8.1 Future perspectives

In conclusion, future developments should concentrate on isolating additional beneficial strains from relevant fermentation systems. Functional consortia of appropriate microorganisms should then be developed, and

interactions among the different strains investigated. Culture-independent methods need to be established to monitor the bacterial and fungal population dynamics in the storage systems, and to quantify specific strains. Protein content in feed grain may be boosted through isolating yeast strains or mutants unaffected by the cell density dependent growth inhibition observed in *P. anomala*. Alternatively, yeasts can be grown on other waste materials, e.g. stillage water from ethanol distilleries and their biomass added as an extra protein source to the feed. In this way, one can envision the development of sustainable biorefinery concepts, where both protein-rich animal feed and biofuels can be produced in ways that are energy-efficient and preserve plant nutrients.

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