Pre-treatment of grain for ethanol production during storage

Ingrid Almgren

Uppsala BioCenter
Department of Microbiology
Faculty of Natural Resources and Agriculture Sciences
Swedish University of Agricultural Sciences

Uppsala

Independent project 2010:10

ISSN 1101-8151
ISRN SLU-MIKRO-EX–10/10–SE

Pre-treatment of grain for ethanol production during storage

Ingrid Almgren

Supervisor: Karin Jacobsson
Examiner: Volkmar Passoth

Keywords: ***ethanol, starch, amylase, Lactobacillus plantarum, Pichia anomala J121, airtight storage, microbial pre-treatment***

EX0564 Independent project in Biology
30 HEC in Engineering Biology at Linköping Institute of Technology, Linköping University
Performed at the Department of Microbiology, SLU

Uppsala BioCenter
Department of Microbiology
Faculty of Natural Resources and Agriculture Sciences
Swedish University of Agricultural Sciences

Uppsala

Independent project 2010:10

ISSN 1101-8151
ISRN SLU-MIKRO-EX–10/10–SE

Abstract

Ethanol for automotive purposes is an issue much debated and researchers disagree about the benefits of bioethanol. Converting energy-rich crops into ethanol suitable as motor fuel is a complex process and continuous development and improvements of processes and material used are important steps in the work of developing environmental friendly fuels. Grain is usually preserved by drying which consumes a lot of energy. Alternative storage of high-moisture grain in airtight storage systems reduce the energy required to produce bioethanol and studies have also shown that ethanol yields are increased more than 10% in ethanol fermentations of moist grain compared to dry. Airtight storage is often not perfectly airtight so biocontrol agents are necessary in order to preserve the grain. The yeast *Pichia anomala* has anti-microbial activity and inhibits mould growth in airtight stored moist grain with some air leakage and is therefore an attractive alternative for biocontrol.

Starch has to be degraded by enzymes into fermentable sugars before it can be fermented into ethanol. Storing high-moisture grain improves enzymatic degradation which results in higher ethanol yields and the aim of this study was to investigate if ethanol yields could be increased further by pre-treating the grain with amylase producing *Lactobacillus plantarum* strains in combination with *P. anomala* J121. Amylase is an enzyme that degrades starch into sugar units and pre-treating grain with bacteria that has amylase activity might have a positive effect on glucose concentrations in grain before and after starch is enzymatically degraded and ultimately result in increased ethanol yields. Also, lactic acid bacteria have been observed to have antifungal activity.

Amylase producing *L. plantarum* strains Amy 1 to 7 were screened for their ability to degrade starch and for possible antifungal activity. No inhibiting activity against *P. anomala* J121 or *Penicillium roqueforti* J9 was detected. *L. plantarum* Amy 1 to 7 did inhibit growth of *Fusarium culmorium* J617. *L. plantarum* Amy 1, was used in a two months storage study to investigate how ethanol yields are affected by microbial pretreatment during storage. Grain was inoculated with *L. plantarum* Amy 1 or *P. anomala* J121 alone or a combination of the two and fermentations were performed on the grain after one month and two months of storage. Inoculation with *L. plantarum* Amy 1 did not increase ethanol yields, but slightly higher yields were observed in grain pretreated with *P. anomala* J121. Two different moisture contents of the grain were used in the study; 25% and 40%. Increased ethanol production rates were observed in the wetter grain but final ethanol yields were similar in both moisture contents. Earlier findings that ethanol yields are increased in fermentations of grain stored moist was confirmed. To study if pretreatment with *L. plantarum* Amy 1 can increase storage stability of moist grain a storage study with *P. roqueforti* J9 was performed and also to ensure that any glucose released during pretreatment does not increase mould growth. Increased storage stability by inoculating grain with *L. plantarum* Amy 1 could not be concluded from this study. However, no increased mould growth was observed in grain treated with *L. plantarum* Amy 1.

Keywords: ethanol, starch, amylase, *Lactobacillus plantarum, Pichia anomala* J121, airtight storage, microbial pretreatment

Sammanfattning

Etanol som fordonsbränsle är mycket omdebatterat och det råder delade meningar om dess nytta. Omvandlingen av energirika grödor till etanolbränsle är en komplex process och ständig utveckling av ingående processer och av de råvaror som används är viktigt för att öka bioetanols nytta och miljövinst. Vanligen konserveras spannmål genom att skörden torkas innan lagring vilket kräver mycket energi. Ett energisnålare alternativ är att lagra fuktig spannmål i lufttäta lagringssystem. Energiåtgången minskas genom att torkning då inte är nödvändig och dessutom har studier visat att etanolutbyten ökas med över 10% då spannmål som lagrats fuktigt fermenteras till etanol. Lufttät lagring är ofta inte helt perfekt så för att säkerställa att spannmålet konserveras kan ytterligare åtgärder behövas. *Pichia anomala* är en jäst lämplig för biokontroll eftersom den har antimikrobiell aktivitet som inhiberar mögelväxt i spannmål som lagras i dåligt fungerande lufttäta system.

Stärkelse måste brytas ned till fermenterbara sockerenheter genom enzymatisk behandling innan stärkelsen kan fermenteras till etanol. Fuktig lagring av spannmål ger ökad enzymatisk nedbrytning av stärkelse vilket resulterar i högre etanolutbyten och syftet med denna studie var att undersöka om etanolutbytet kan förbättras ytterligare genom att förbehandla spannmålet med *Lactobacillus plantarum* stammar som producerar enzymet amylas i kombination med *P. anomala* J121. Enzymet amylas bryter ner stärkelse och förbehandling under lagring med bakterier som producerar amylas kan ha positiv inverkan på glukoskoncentrationer i spannmålet före och efter enzymatisk förbehandling och i bästa fall leda till ökade etanolutbyten. Antifugal aktivitet har också observerats hos mjölksyrabakterier.

De amylasproducerande stammarna *L. plantarum* Amy 1 till 7 undersöktes med avseende på förmåga att växa på stärkelse och eventuell antifugal aktivitet. Ingen inhiberande verkan mot *P. anomala* J121 eller *Penicillium roqueforti* J9 kunde detekteras men *L. plantarum* Amy 1 till 7 hämmade växt av *Fusarium culmorium* J617. *L. plantarum* Amy 1 användes i en två månader lång lagringsstudie för att undersöka hur den mikrobiella förbehandlingen påverkar etanolutbytet. Spannmål ympades med endast *L. plantarum* Amy 1 eller *P. anomala* J121 eller med en kombination av dem och efter en månad och två månaders lagring fermenterades spannmålet till etanol. Förbehandling med *L. plantarum* Amy 1 ökade inte etanolutbytet, dock observerades något högre utbyten för spannmål som förbehandlats med *P. anomala* J121. I studien undersöktes två fukthalter; 25% och 40%, och för den högre fukthalten producerades etanol snabbare men den slutliga etanolkoncentrationer var likvärdiga för de både fukthalterna. Tidigare observationer att spannmål som lagrats fuktigt ger ökade etanolutbyten bekräftades i studien. En lagringsstudie med *P. roqueforti* J9 gjordes för att undersöka om förbehandling med *L. plantarum* Amy 1 kan öka stabiliteten i lagringen och för att säkerställa att inte mögelväxt ökar på grund av frigjord glukos. Några slutsatser om ökad lagringsstabilitet på grund av förbehandling med *L. plantarum* Amy 1 kunde inte dras i denna studie men ökad mögelväxten var inte observerad hos spannmål som ympats med *L. plantarum* Amy 1.

Nyckelord: etanol, stärkelse, amylas, *Lactobacillus plantarum, Pichia anomala* J121, lufttät lagring, mikrobiell förbehandling

Table of contents

1. Aim of the study

The aim of this study was to investigate if ethanol yields can be increased by pre-treating grain with a combination of the yeast *Pichia anomala* J121 and amylase producing strains of *Lactobacillus plantarum* during airtight storage. A storage study with *Penicillium roqueforti* J9 was also performed to study storage stability and ensure that any release of glucose during starch-degradation does not increase mould growth.

2. Introduction

The use of ethanol as automotive fuel has now been in practice for some years, either as a blend with gasoline or as pure ethanol. In the 1970s the Brazilian government introduced the Pro-Alcohol Program that aimed at replacing imported gasoline as motor fuel with ethanol derived from sugarcanes (Weiss, 1990). The technology needed to succeed worked but the program had some financial issues. Oil derived fuels are often cheaper to produce than ethanol fuels. The process of converting agriculture crops into ethanol is complex and includes steps of pre-treating the raw material, fermentation by microorganisms and also concentrating and dehydrating the aqueous ethanol solution into a final form suitable for automotive purposes (Cardona and Sánchez, 2007).

2.1 Bioethanol - a climate neutral alternative

Fossil fuels are often cheaper to produce than ethanol fuels but there are other motives for the use of ethanol and the main reason is of concern for the environment. Fuels based on energy-rich crops should have no net effect on the carbon concentration in the atmosphere since the carbon released during combustion was recently fixed in photosynthesis by the crops in contrast to fossil fuels where the released carbon has not been a part of the carbon cycle for a very long time (Reijnders and Huijbregts, 2007). Hence, ethanol is called climate neutral but this is a much debated question and researchers have different opinions regarding the total energy balance. When energy balances are estimated the energy content of the end product is considered in proportion to the energy consumed producing the fuel. Energy inputs from activities like transportation, farming and manufacturing of fertilizers are considered as well as handling of byproducts which can be used as a resource to increase the energy output (Börjesson, 2006).

2.1.1 Energy balance of bioethanol

There are researchers claiming that the energy balance of bioethanol is negative and that more energy is consumed during the production process than the amount of energy found in the end product (Pimentel and Patzek, 2005; Pimentel, 2003). Other studies show the opposite and states that the energy balance of ethanol fuel is positive (Hill *et al.,* 2006; Börjesson, 2004). A report by Börjesson (2006) concludes that the mean value is positive when energy balances reported in different studies are compared but using results from different studies are not unproblematic. Energy balances varies a lot depending on the data used, considered inputs and outputs, differences in system boundaries, local differences in the ethanol production process, agriculture methods, climate etc (Börjesson, 2006). The energy output is also dependent on the raw material. Sugar cane and beet which are rich in sucrose are often used for ethanol production. Starchy material like grains are also used, especially wheat and corn, but the material must then be pretreated to hydrolyze polysaccharides into sugars. Lignocellulose, a complex of several polysaccharides, is a promising material for bioethanol that is under development (Cardona and Sánchez, 2007). Lignocellulose is the material that seems to have the highest energy output followed by sugar cane that has a slightly higher output than grain and corn (Börjesson, 2006).

2.1.2 Hopes for the future

Switching to bioethanol as automotive fuel alone is not a realistic goal, especially not since estimating the agriculture area needed to substitute the gasoline consumed in the U.S. with corn based ethanol shows that the total U.S. cropland today would not be enough (Kheshgi *et al.,* 2000). Improving agriculture methods to increase harvest yields and decrease the energy consumption used per area unit cropland would enhance the efficiency in which solar energy is converted into bioethanol (Börjesson, 2006). New technology could also make it possible to use a larger fraction of the crops than possible today. Lignocellulosic materials including fast-growing trees, energy crops, agriculture residues and industrial byproducts are attractive resources for ethanol production but there are technical problems and more research is necessary (Liu *et al.,* 2008). Using what today is considered waste in the agriculture and forest industry would be a valuable achievement. Another way of improving the energy balance of bioethanol is to use the distillers grains from the ethanol fermentation to produce biogas instead of drying and use it as animal feed. Energy used when drying the residues can then be saved and about 75% instead of 55% of the grains energy content will be transformed into biofuels (Börjesson, 2006).

It is quite obvious that bioethanol as automotive fuel is not the solution of the world´s problem regarding oil crises and global warming but it might be a part of the solution. Improving existing production processes to increase ethanol yields and develop technologies for the use of innovative raw materials are important steps in the work of developing environmental friendly fuels.

2.2 Storage of high-moisture grain under airtight conditions

Preserving grain after harvest is in Sweden usually done by hot air drying which consumes a lot of energy (Olstorpe *et al.,* 2010). An alternative is to store moist grain under airtight conditions in plastic tubes or sealed silos. The grain and microorganisms in the grain will consume oxygen by respiration and anaerobic conditions are obtained in the airtight system. Microorganism requiring oxygen cannot grow and facultative organisms are inhibited. However, the sealing is often not perfect and it is common that the grain has to be accessed at several different occasions during the storage which means that oxygen will reenter the system and that additions of biocontrol agents are necessary in order to preserve the grain (Petersson *et al.,* 1999).

2.2.1 *Pichia anomala* as a biocontrol organism

Studies have showed that the yeast *Pichia anomala* can inhibit mould growth and sporulation on agar plates and mould growth in moist grain stored under airtight conditions with some air leakage (Boysen *et al.,* 2000; Petersson *et al.,* 1999; Petersson and Schnürer, 1995; Druvefors *et al.,* 2005). *P. anomala* is therefore an attractive alternative for biocontrol to increase storage stability of high-moisture grain.

P. anomala can grow at pH values between 2.0 and 12.4, under oxygen-limited conditions and even under anaerobic conditions in the presence of ergosterol and fatty acids (Fredlund *et al*., 2002). Other characteristics that make *P. anomala* a robust microorganism suitable for biocontrol is its ability to grow at both high and low temperatures (3°C - 37°C on solid substrates) and on a wide range of carbon and nitrogen sources (Fredlund *et al*., 2002).

P. anomala inhibits growth of *Penicillium roqueforti* in high-moisture grain stored in airtight storage systems with some air leakage in laboratory test systems and in large scale studies (Petersson *et al.,* 1999; Petersson and Schnürer, 1995). *Penicillium* species are important spoilage organisms found in stored grain and especially *P. roqueforti* since this species can tolerate high carbon dioxide concentrations and low oxygen levels (Lacey, 1989). In malfunctioning airtight storage systems, oxygen leakage will enable mould growth and the

first *Penicillium* species to appear with increased oxygen concentration is *P. roqueforti* (Lacey, 1989). Druvefors and coworkers concluded that production of the sugar metabolites ethyl acetate and ethanol is probably one of the mechanisms responsible for inhibition of *P. roqueforti* in airtight stored grain by *P. anomala*. Other mechanisms that might contribute to antifungal activity are for example competition for oxygen and high levels of carbon dioxide (Druvefors *et al.,* 2005).

2.2.2 Increased ethanol yields with high-moisture grain

Storing grain in airtight systems saves a lot of energy since there is no need of hot-drying the grain after harvest, but it also increases ethanol yields when the high-moisture grain is used as raw-material in ethanol fermentations (Passoth *et al.,* 2009). Passoth *et al.* (2009) showed that glucose concentrations after enzymatic degradation of starch was higher and starch content lower in high-moisture grain than in dry grain which resulted in 14% higher ethanol yields. Using high-moisture grain for ethanol production improves energy output of bioethanol by saving energy needed in the preservation process after harvest and increasing the amount ethanol obtained from the raw material.

2.3 Ethanol produced from starch

Starch is the main storage and reserve substance of carbohydrate in plants (Lineback, n.d.). There are two types of starch, storage and transient starch. Transient starch is a kind of short storage for daily use and storage starch is important to manage growth, seasonal variations and stress (Geigor and Servaites, 2001).

Starch consists of glucose units linked together to form two types of polymers, amylose and amylopectin. In amylose the glucose units are linked by α-1,4 bonds to form long polymers that in many ways behave like a linear molecule (Lineback n.d.). Amylopectin has a branched structure in which glucose is linked by α-1,4 linkage as in amylose but in addition there are α-1,6 bonds at branch points (Nigam and Singh, 1995). The average length of the chains in amylose is between 900 and 1000 glucose units and the corresponding figure for amylopectin is 20 to 26 units (Lineback n.d.).

2.3.1 Pretreatment

Before starch can be fermented into ethanol it has to be pretreated into fermentable sugar units. The first step is to mill the material and then gelatinize it (Nigam and Singh, 1995). Gelatinization is obtained by heating or cooking hydrated starch. The granules containing amylose and amylopectin will lose their structure when the temperature is increased (Lineback, n.d.). The starchy material is then treated with the enzymes α-amylase and glucoamylase to break the polymers into sugar units. α-amylase is an endo-acting amylase that cleaves internal α-1,4 bonds in both amylase and amylopectin resulting in shorter sugar chains called dextrin. Glucoamylase is an exo-acting amylase that hydrolyze terminal α-1,4 bonds in dextrin and also α-1,6 bonds to form the fermentable sugar units glucose and maltose (Nichols *et al.,* 2008).

2.3.2 Ethanol fermentation

Saccharomyces cerevisiae is the yeast generally used in ethanol production. The yeast converts glucose, maltose and fructose into pyruvate. Maltose has to be hydrolyzed into glucose before it can enter the metabolic pathway glycolysis where glucose and fructose are broken down into puruvate molecules. Pyruvate is decarboxylated into acetaldehyde which is reduced to ethanol. In the fermentation process two ethanol molecules and two carbon dioxide molecules are formed from one glucose unit (Nichols *et al.,* 2008). The yeast could switch

into respiration and metabolize ethanol under aerobic conditions if the glucose concentrations are low (Postma *et al.,* 1989). Oxygen free conditions are therefore to prefer during ethanol production since alcohol fermentation is the only metabolic pathway providing energy when oxygen is not present.

2.4 Lactic acid bacteria

Lactic acid bacteria are bacteria that produce lactic acid as their main fermentation product and this group includes the genera *Streptococcus, Leuconostoc, Pediococcus, Lactobacillus, Enterococcus* and *Lactococcus*. Lactic acid bacteria are generally aerotolerant anaerobes which mean that they are not sensitive to the presence of oxygen and can even grow under aerobic conditions. There are two groups of lactic acid bacteria, homofermentatives and heterofermentatives. The group homofermentative produce lactic acid as their only fermentation product in contrast to the heterofermentative which produces other products than lactic acid, for example acetate, carbon dioxide and ethanol (Madigan *et al.,* 2003).

2.4.1 Antifungal activity by lactic acid bacteria

Lactic acid fermentation is a naturally occurring process traditionally used to preserve food. Yoghurt, sour cream and cheese are typical products produced from milk processed by lactic acid bacteria (Müller, 2008). This group of bacteria is also involved in the preservation of feed for example silage (McEniry *et al.,* 2010).

Lactic acid bacteria produce organic acids like lactic and acetic acid that have a general preserving effect on food and feed. Growth of many microorganisms is inhibited by low pH, especially bacterial growth since most bacteria cannot grow at pH below 4.5 (Stratford and Eklund, 2003). Moulds are often more tolerant to low pH so organic acids must have other inhibiting mechanisms. Furthermore, weak acids like lactic acid have stronger antimicrobial effects than strong acids (Stratford and Eklund, 2003). Lactic acid molecules can diffuse through cell membranes because they are lipid soluble and the acid will then dissociate inside the cell. Charged ions on the other hand cannot pass through the membrane and will accumulate inside the cell and lower pH of the cytoplasm leading to acidification of the cell (Stratford and Eklund, 2003).

Lactic acid bacteria also produce other compounds with antifungal properties, for example hydrogen peroxide, reuterin and bacteriocins, but studying specific antifungal mechanisms are difficult because of the general inhibitory effect of lowered pH and probably interactions between different mechanisms (rewieved by Schnürer and Magnusson, 2005).

3. Materials and methods

3.1 Microorganisms

All the bacterial, yeast and fungal isolates used in this study were from the culture collection at the Department of Microbiology, Swedish University of Agriculture Science in Uppsala. Microorganisms used in the study and their growth conditions are summarized in Table 1.

Lactobacillus strains were grown on de Man Rogosa Sharp (MRS) medium (Oxoid, Basingstoke, UK) which was developed to support growth of lactobacilli (de Man *et al.* 1960). MRS-agar with 0.1 g/l delvocid (Gist-brocades, Delft, the Netherlands) (MRS-D) was used as selective medium since delvocid inhibits fungal growth. Malt extract agar (Oxoid) with 0.1 g/l chloramphenicol (Boehringer Mannheim GmbH, Germany) (MEA-C) was used as medium for *Pichia anomala.* Chloramphenicol inhibits growth of bacteria. MEA with 0.1 g/l chloramphenicol and 10 mg/l cycloheximide (MEA-CC) was the medium used for selective growth of *Penicillium roqueforti* since 10 mg/l cycloheximide inhibits growth of *P. anomala* but not *P. roqueforti* (Björnberg and Schnürer, 1993).

Yeast peptone dextrose medium (appendix 1) with 0.1 g/l chloramphenicol (YPD-C) was used to monitor growth of *Saccharomyces cerevisiae* in ethanol fermentations. Medium used for general bacterial growth was trypton soya agar (Oxoid) with 0.1 g/l delvocid (TSA-D).

Table 1 Growth conditions and medium used for microorganisms in the study

Organism	**Medium**	**Growth conditions**
Fusarium culmorum J617	Oatmeal agar (BD, Le Point de Claix, France)	25°C in ultraviolet light
Lactobacillus fermentum T14	MRS-agar	25°C for 48h, anaerobic
Lactobacillus plantarum MiLab393 and amylase producing strains	MRS-agar	25°C for 48h, anaerobic
	MRS-broth	25°C for 48h, anaerobic
Penicillium roqueforti J9	MEA-agar	25°C for 48h and longer, aerobic
Pichia anomala J121	YPD-broth (appendix 1)	30°C for 24h, aerobic
	MEA-agar	30°C for 48h, aerobic
	YPD-agar	30°C for 48h, aerobic
Saccharomyces cerevisiae J672	YPD-agar	30°C for 48h, aerobic
	YPD-broth	30°C over night (ON), aerobic

3.2 Degradation of starch by Lactobacillus plantarum *Amy 1-7*

Seven strains of *L. plantarum*, denoted Amy 1 to 7, were screened for their ability to degrade starch by production of the enzyme amylase. *L. plantarum* Amy 1 to 7 were grown on MRS agar where glucose was replaced by starch, appendix 1 (Table 15). How efficiently starch is degraded might vary between starches of different origin and therefore six different starch sources were used; potato and pea starch from Emsland Stärke GmbH (Emlichheim, Germany) and starch from Merck (Darmstadt, Germany) with four different article numbers.

L. plantarum was suspended in 0.9% NaCl (BDH ProLabo, Leuven, Berlgium) to a density of MacFarland 2 and applied in two parallel streaks on plates containing starch and no glucose. The plates were incubated for 48h at 25°C under anaerobic conditions.

A potassium iodide-iodine (KI-I_2) solution of 2.0 g/l iodine (Merck) and 20.0 g/l potassium iodine (Merck) (Schmieder and Keeney, 1980) was added to the agar plates to facilitate reading of the result by staining starch dark purple. The size of the clear zone surrounding the bacteria was taken as an indication of how effective the particular *L. plantarum* strain was at degrading starch.

3.3 Antifungal activity by Lactobacillus plantarum *Amy 1-7*

L. plantarum Amy 1 to 7 were also screened for their ability to inhibit growth of *P. roqueforti* J9, *F. culmorum* J617 and *P. anomala* J121 according to the overlay-method (Magnusson and Schnürer, 2001).

L. plantarum Amy 1 to 7 were suspended in 0.9% NaCl to a density of MacFarland 2 and applied in two parallel streaks on MRS plates. The plates were incubated for 48h at 30°C under anaerobic conditions. *P. roqueforti* J9 was grown on MEA slants at 25°C until sporulation and *F. culmorum* J617 on oatmeal agar under ultraviolet light at 25°C. *P. anomala* J121 was grown in YPD-broth over night (ON) at 30°C, 150 rpm. Cell/spore concentrations were determined in a Bürker counting chamber (Marienfeld, Lauda-Königshofen, Germany) and checked on MEA-plates by viable counts.

P. roqueforti J9, *F. culmorum* J617 and *P. anomala* J121 were diluted in sterile peptone water (0.2% bacterial peptone (Oxoid), 0.01% Tween 80 (Merck) to a concentration of 10^5 cells/spores per ml and 1 ml was mixed with 9 ml MEA soft agar (0.2% malt extract (Oxoid) and 1% agar (Oxoid)) in sterile cultivation tubes. The soft agar mixture was spread on the previously prepared MRS plates with *L. plantarum* Amy 1 to 7 and incubated for 72h at 25°C.

3.4 Pre-storage study

L. plantarum Amy 1, 3 and 5 seemed to have the best ability to produce amylase and were used in a two week pre-storage study to find the strain best suited for further studies. The non-amylase producing *L. plantarum* strain MiLab393 was used as control.

Dried wheat kernels were rehydrated into approximate moisture contents of 25% and 40% (section 3.7.1). The rehydrated grain was inoculated with about 10^6 cells/g grain after growing *L. plantarum* Amy 1, 3 and 5 in MRS-broth for 48h in 25˚C. Water activity and actual moisture content was measured as described in 3.7.2 before the grain was packed in mini-silos, see 3.7.3. The mini-silos were stored at 25°C and samples were taken at 0h, 5 days, 8 days and 14 days to measure bacterial growth, pH and concentrations of organic acids.

3.4.1 Sample-processing

Five g grain from each mini-silo was mixed with 45 ml sterile peptone water and processed by a Stomacher 400, Laboratory Blender (Seward) for two minutes at medium speed. The

suspension was used to determine concentrations of *L. plantarum* Amy 1, 3. 5 and MiLab393 on MRS-D and measure pH with a PHM92 pH meter (Radiometer, Copenhagen, Denmark).

Another 10 g of grain was milled in a food processor "Mini-Hacke" and mixed with 90 ml deionized water. The mixture was processed by a Stomacher at medium speed for four minutes and about 2 ml was used for HPLC analysis of organic acids (section 3.7.4).

3.5 Storage study

L. plantarum Amy 1 seemed to be best suited for further storage studies based on results from the pre-storage study and antifungal screening and was used in a two months storage study to investigate if pre-treatment with microorganisms during storage can increase ethanol yields.

Grain was rehydrated to approximate 25% and 40% moisture content (section 3.7.1). The grain was inoculated with *L. plantarum* Amy 1, *P. anomalia* J121 or a combination of the two before packing the grain in mini-silos (section 3.7.3). Inoculation concentration for *L. plantarum* Amy 1 was 10^6 cells/g grain and the corresponding concentration for *P. anomala* J121 was 10^5 cells/g. Grain not treated with any microorganism was used as control. The different treatments are summarized in Table 2.

Table 2 Microbial pre-treatments used in the storage study

Treatment
L. plantarum Amy 1 (10^6 cfu/g)
L. plantarum Amy 1 (10^6 cfu/g) and *P. anomala* J121 *(*10^5 cfu/g)
P. anomala Amy 1 *(*10^5 cfu/g)
No microbial pre-treatment

Concentration of bacteria and yeasts was determined in a Bürker counting chamber after growing *L. plantarum* Amy 1 in MRS-broth at 25°C for 48h and *P. anomala* J121 in YPD-broth ON at 30°C at 150 rpm. The estimated titer of *L. plantarum* Amy 1 was confirmed on MRS-D and *P. anomala* J121 on YPD-C.

Water activity and moisture content of the grain was measured as described in section 3.7.2. The mini-silos were stored at 25°C and samples were taken at 0 h, 4 weeks and 7 weeks to measure concentration of organic acids and pH. The stored grain was used in ethanol fermentations to study if there were any differences in ethanol yield.

3.5.1 Sample-processing

Stored grain was milled in a food processor and 5 g was mixed with deionized water and processed for four minutes by a Stomacher at medium speed. pH was measured and samples for HPLC analysis of organic acids was collected (section 3.7.4). The samples were stored in a freezer until HPLC analysis. The remaining milled grain was used to produce ethanol.

3.5.2 Enzymatic pre-treatment

Starch has to be pre-treated into fermentable sugars before it can be used in ethanol fermentations.

Ten g of milled grain was mixed with 40 ml deionized water adjusted to pH 5 with HCl in sterile serum bottles. The hydrated grain was gelatinised by heating the samples in water bath, (100°C for 25 minutes). Eighty ml of water with pH 5 was added to the bottles and also 3.5 µl/g grain of the enzyme mixture Stargen 001 (a kind gift from Mats Sandgren). The samples were incubated at 37°C, 100 rpm for 24 h, and after incubation water with pH 5 was added to the mixture to a final volume of 100 ml.

Samples for HPLC analysis of glucose content was collected after the pre-treatment.

3.5.3 Inoculation with *Saccharomyces cerevisiae* J672

S. cerevisiae J672 was grown ON in 250 ml YPD-broth at 30˚C and 140 rpm. To wash the yeast cells and remove nutrients from the medium the culture was centrifuged at 8000 G for 20 minutes. The pellet was re-suspended in 1/10 volumes 0.9 % NaCl and each bottle was inoculated with 1 ml yeast suspension.

Figure 1 Ethanol fermentations were performed in serum bottles sealed with rubber corks perforates with needles to allow leakage of CO_2 (Photo: Ingrid Almgren, 2010).

3.5.4 Ethanol fermentation

The serum bottles were sealed with rubber corks perforated with needles, diameter 0.6 mm (Becton Dickinson S.A., Fraga, Spain), to allow leakage of carbon dioxide (Figure 1). Ethanol fermentations were performed at 30°C on a horizontal rotary shaker, 150 rpm and samples were taken at 24h and 48h.

Yeast concentration in samples taken at 48h was determined on YPD-C medium to verify that *S. cerevisiae* J672 was present and viable. The bacterial concentration was also studied on TSA-D since contaminating bacteria can interfere with ethanol production by consuming glucose and compete for trace nutrients (Nichols *et al.*, 2008).

3.5.5 PCR-fingerprinting

PCR-fingerprinting was used to investigate if microorganisms detected in the fermentations could be identified as *P. anomala* J121, *S. cerevisiae* J672 or *L. plantarum* Amy 1. The primer used corresponds to a natural occurring sequence that exists at different locations in the genome depending on the organism and therefore can different microorganisms be distinguished by their polymerase chain reaction (PCR) profile (Versalovic *et al.,* 1991).

PuRe Taq Ready-To-Go PCR beads (GE Healtcare, Buckinghamshire, UK) were used to prepare a master mix containing all reagents required for the reaction (water (25 µl per bead) and primer (10 pmol per 100 µl)). Randomly picked colonies were used as templates. The primer used had the following sequence: 5'-GTGGTGGTGGTGGTG-3. The DNA was amplified by PCR in a MiniCycler (MJ research, USA) according to protocol in Table 3. The PCR products were separated on a 1% agarose (Abgene, New York, USA) gel in TBE buffer for 30 minutes at 50 V followed by 3h at 70 V.

Table 3 PCR program used to amplify DNA material for PCR fingerprinting

Step	Temperature	Time
1.	95˚C	7 min
2.	90˚C	30 sec
3.	95˚C	1 min
4.	40˚C	1 min
5.	65˚C	4 min
6. Step 2 to 5 are repeated 29 more times		
7.	65˚C	16 min

3.5.6 Analysis of ethanol yield by HPLC

Concentrations of ethanol, glucose and maltose were measured by HPLC (Agilent 1100/1200 system, Agilent Technologies, Stockholm, Sweden). The samples were filtered through a sterile filter (Sarstedt, Nümbrecht, Germany) with pore size 0.45 µm and analyzed on a Rezex-ROA-Organic Acid H+ column (Skandinaviska Genetec, Västra Frölunda, Sweden) at 60˚C with 5 mM H_2SO_4 as mobile phase and flow rate 0.6 ml/min. Mixtures of ethanol, glucose and maltose at concentrations 1.0 g/l, 5.0 g/l, 10 g/l, 20 g/l and 50 g/l were included as standard solutions and resulting data was analyzed in ChemStation for LC systems (Agilent Technologies).

3.6 Inhibition of Penicillium roqueforti

A storage study with *P. roqueforti* J9 was performed to investigate if storage stability can be improved by pre-treating the grain with *L. plantarum* Amy 1 and *P. anomala* J121 in different combinations, and to ensure that any release of glucose during starch degradation does not increase mould growth.

Grain was rehydrated into approximate moisture content 40% as described in section 3.7.1 and water activity and moisture content was measured (section 3.7.1). The grain was inoculated with *L. plantarum* Amy 1, *L. fermentum* T14, *P. anomala* J121 and *P. roqueforti* J9 according to Table 4.

Table 4 Microbial treatments and inoculation levels in cells/spores per g grain used in the storage study

Treatment / Microorganism	***P. roqueforti,***	***P. roqueforti* and *P. anomala***	***P. roqueforti, P. anomala* and *L. plantarum***	***P. roqueforti, P. anomala, L. plantarum* and *L. fermentum***
***P. roqueforti* (10^4)**	+	+	+	+
***P. anomala* (10^5)**		+	+	+
***L. plantarum* (10^6)**			+	+
***L. fermentum* (10^6)**				+

Grain packed in mini-silos was stored at 25˚C and samples were taken after 15 days and 26 days. Samples were studied to determine microbial growth, pH and concentrations of organic acids. To study the aerobic stability, grain stored for 26 days were packed in new sterile mini-silos after 15 days to let oxygen reenter the system.

3.6.1 Sample-processing

Ten g grain was mixed with 90 ml sterile peptone water and processed in a Stomacher for 2 minutes at medium speed. Concentrations of *L. plantarum* Amy 1 and *L. fermentum* T14 were determined on MRS-D and *P. anomala* J121 on MEA-C. *P. roqueforti* J9 was quantified on MEA-CC. The suspension was also used to measure pH.

Five g grain was milled and mixed with 45 ml deionized water and processed in a Stomacher for four minutes. Samples were taken for HPLC analysis of organic acids (section 3.7.4).

3.7 Methods used in storage studies

3.7.1 Rehydrating grain

Dried grain and tap water was mixed in sterile glass jars. To achieve approximate moisture content 25%, 500 g wheat was mixed with 85 ml H_2O. In the same way was approximate moisture content 40% obtained by mixing 250 ml H_2O and 500 g wheat. The rehydrated grain was incubated for 48 h at 2 °C and frequently mixed.

3.7.2 Water activity and moisture content

Actual moisture content of the grain was estimated by the difference in weight before and after drying 10 g grain at 105°C for 16h.

Water activity was measured at room air temperature by a CX-2 AquaLab instrument (Decagon Devices, Washington, USA).

3.7.3 Mini-silos

So called mini-silos were used to model airtight storage systems (Petterson and Schürer, 1995). Sterile glass tubes were packed with approximate 17 g grain and sealed with rubber corks (Figure 2). A syringe needle with diameter 0.4 mm (B. Braun, Melsungen, Germany) inserted through the rubber cork simulated air leakage since airtight storage usually is not perfectly airtight.

Figure 2 Model system designed to mimic airtight storage of grain and used in this study. (Photo: Ingrid Almgren, 2010)

3.7.4 Analysis of sugars and organic acids by HPLC

Concentrations of glucose, maltose, succinic acid, propionic acid, butyric acid, lactic acid and acetic acid were measured by HPLC. The samples were filtered through a sterile filter with pore size 0.45 µm and analyzed on a Rezex-ROA-Organic Acid H+ column at 60˚C with 5 mM H_2SO_4 as mobile phase and flow rate 0.6 ml/min. Mixtures of analyzed substances at concentrations 0.1 g/l, 1.0 g/l, 10 g/l and 20 g/l were included as standard solutions.

4. Results and discussion

4.1 Degradation of starch by Lactobacillus plantarum *Amy1-7*

Seven different strains of *L. plantarum* (denoted Amy 1 to 7) were ranked by their ability to produce amylase and grow on starch. Figure 3 illustrates how the zone surrounding the bacteria is less stained by the KI-I_2 solution since the starch content is lower in this area. Bacteria that produce a lot of amylase will be surrounded by a large clear zone.

The sizes of the clear zones surrounding *L. plantarum* Amy 1 to 7 were compared and the strains ranked by their amylase producing ability. The result is presented in appendix 2 (Tables 16-18). To summarize, *L. plantarum* Amy 1, 3 and 5 were found at the top of the ranking for all six different starches which means that these strains seemed to be the most capable strains of producing amylase and also able to degrade a wide range of starches. Therefore, *L. plantarum* strains Amy 1, 3 and 5 were the choice for further studies of their ability to degrade starch in grain.

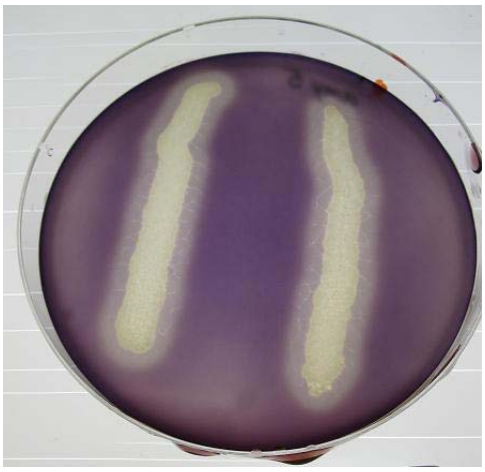


Figure 3 Amy 5 growing on starch from Merck after staining starch blue by treating the plate with KI-I_2 solution. (Photo: Ingrid Almgren, 2010)

4.2 Antifungal activity by Lactobacillus plantarum *Amy 1-7*

None of the *L. plantarum* strains Amy 1 to 7 were able to inhibit growth of *P. anomala* J121 or *P. roqueforti* J9 (data not shown). Antifungal activity against *P. roqueforti* J9 is desired since *Penicillium* species are important spoilage organisms in stored grain (Lacey, 1989), unfortunately only very weak antifungal activity was detected for all seven strains.

Growth of *F. culmorum* J617 was inhibited by all seven *L. plantarum* strains. Antifungal activity against *F. culmorum* J617 is positive since *Fusarium* species are important plant pathogens that can produce mycotoxins in stored grain (Nicholson, 2009).

4.3 Pre-storage study

Rehydrated grain inoculated with *L. plantarum* Amy 1, Amy 3, Amy 5 or MiLab393 was stored in mini-silos for 0h, 5 days, 8 days or 14 days.

4.3.1 Water activity and moisture content

Moisture content (MC) was confirmed to be approximate 40% and almost 25% (Table 5). Observed moisture content was used when converting yields into g/g dry matter (DM).

Table 5 Water activity and moisture content of grain rehydrated into two moisture content levels and inoculated or not with *L. plantarum* Amy 1, 3, 5 or MiLab393. Data presented are mean values (n=2)

Desired moisture content	Sample	a_w	Moisture content
40 %	After inoculation of *L. plantarum*	0.995	40.0 %
40 %	Before inoculation of *L. plantarum*	0.998	38.9 %
25 %	After inoculation of *L. plantarum*	0.944	23.7 %
25 %	Before inoculation of *L. plantarum*	0.933	23.4 %

Water activity (a_w) is the ratio of vapor pressure measured for a sample at a given temperature and vapor pressure of pure water at the same temperature (Reid, 2008). When the water activity is high, more water is available for microorganisms than at low water activities. Moulds, yeasts and bacteria require that water activity falls in a certain interval to be able to grow, maximum and minimum levels depend on the species. Moulds can usually grow at lower water activities than yeasts and bacteria, and bacteria are generally more sensitive to low water activity (Labuza and Altunakar, 2008). About the same amount of water activity was recorded in grain inoculated with *L. plantarum* Amy 1, 3, 5 or MiLab393 and not inoculated.

4.3.2 Bacterial growth

L. plantarum Amy 1, Amy 3, Amy 5 and MiLab393 all reached a concentration of approximately 10^7 cfu/g after two weeks of storage when growing on grain with moisture content 25% and 10^9 cfu/g in grain with moisture content 40%. Hence, no clear difference in bacterial growth could be detected between the three amylase producing strains of *L. plantarum* and the control *L. plantarum* MiLab393.

4.3.3 Organic acids and sugar content in grain

Organic acids were produced and sugar consumed to the same extent in all samples with the same moisture content irrespective of which *L. plantarum* strain the grain was pretreated with. Concentrations of glucose and maltose decreased during two weeks of storage and there were no real difference in final sugar concentration between the control *L. plantarum* MiLab393 and amylase producing strains (Table 6). Glucose concentration in grain with moisture content 40% was slightly lower after 14 days in grain treated with *L. plantarum* MiLab393 and slightly higher in grain treated with *L. plantarum* Amy 1. In grain treated with *L. plantarum* Amy 1 was 9.3 mg/g DM glucose detected after 14 days compared to 8.3 and 8.6 mg/g DM for *L. plantarum* Amy 3 and 5 (Table 6). Only one sample of each treatment was analyzed and therefore can the results only show indications of which strain of *L. plantarum* that seems to have the best ability to grow on and degrade starch in grain.

Table 6 Concentration of glucose and maltose in samples taken after 0h and 14 days of storage, n=1

	Glucose (mg/g DM)				**Maltose (mg/g DM)**			
	Moisture content 40%		**Moisture content 25%**		**Moisture content 40%**		**Moisture content 25%**	
***L. plantarum* strain**	**0h**	**14 days**	**0h**	**14 days**	**0h**	**14 days**	**0h**	**14 days**
Amy 1	10.4	9.3	10.4	9.5	7.2	7.2	7.2	6.8
Amy 3		8.3		9.1		7.0		6.7
Amy 5		8.6		9.6		6.9		7.2
MiLab393		7.8		9.6		6.8		6.9

More lactic acid was produced in grain with moisture content 40% (Table 7) and this can be related to the larger bacterial growth in grain of the higher moisture content where bacterial concentrations were about a hundred times higher. Acetic acid is only present in samples of the wetter grain except in grain inoculated with *L. plantarum* Amy 1 where 4.3 mg/g DM was detected in the 25% sample (Table 7). The presence of acetic acid in grain with the lower moisture was the only observed result where the amount of measured substances differed between the studied *L. plantarum* strains.

Table 7 Concentration of lactic and acetic acid in samples taken after 0h and 14 days of storage, n=1

	Lactic acid (mg/g DM)				Acetic acid (mg/g DM)			
	Moisture content 40%		Moisture content 25%		Moisture content 40%		Moisture content 25%	
***L. plantarum* strain**	**0h**	**14 days**	**0h**	**14 days**	**0h**	**14 days**	**0h**	**14 days**
Amy 1	n.d.*	24.5	n.d.	4.3	n.d.	6.4	n.d.	4.3
Amy 3		24.4		4.4		6.6		n.d.
Amy 5		24.0		4.3		6.5		n.d.
MiLab393		25.3		4.5		4.0		n.d.

* Not detectable

Propionic, succinic or butyric acid were not detected in any of the samples

Grain with moisture content 25 % had pH 5.7 at 0h which increased slightly to just over pH 6 during two weeks of storage (Table 8). The larger production of organic acids in grain with moisture content 40% resulted in decrease in pH from 5.8 to about 4.

Table 8 pH of samples with moisture content 40% and 25% at 0h and after 14 days of storage, n=1

***L. plantarum* strain**	**Moisture content 40%**		**Moisture content 25%**	
	0h	**14 days**	**0h**	**14 days**
Amy 1	5.8	4.0	5.7	6.2
Amy 3		4.0		6.2
Amy 5		4.1		6.1
MiLab393		4.0		6.1

To conclude, no real differences could be detected between *L. plantarum* Amy 1, Amy 3 and Amy 5 regarding bacterial growth, concentrations of organic acids and sugars. Neither when comparing the amylase producing strains with *L. plantarum* MiLab393 was any differences observed. The only result that differed between the compared strains was the presence of acetic acid in grain with moisture content of 25% when the grain was pretreated with *L. plantarum* Amy 1. Therefore, *L. plantarum* Amy 1 was the strain used in a longer and larger storage study because weak acids like acetic acid have anti-microbial effect (Stratford and Eklund, 2003) which can increase storage stability.

A possible explanation why no differences could be detected between *L. plantarum* MiLab393 and *L. plantarum* Amy 1, 3 or 5 is that glucose in the grain might repress amylase production and activity. Glucose inhibition of amylase activity has been observed for other microorganisms that produce the enzyme (Adinarayana Reddy *et al.,* 1986; Markeberg *et al.,* 1995). For example, amylase activity is totally repressed when growing amylase producing *Aspergillus niger* in cultures with 10-12 mg/ml glucose (Adinarayana Reddy *et al.,* 1986). It is possible that the presence of free glucose in the grain inhibits amylase activity, although detected glucose concentrations were only about 8 mg/g DM in grain of moisture content 40% and about 7 mg/g DM for moisture content 25%. It is also possible that the starch degrading ability of *L. plantarum* Amy 1, 3 and 5 is so poor that the amylase producing strains do not have any advantage against non-amylase producing *L. plantarum* MiLab393.

4.4 Storage study

Rehydrated grain inoculated with *P. anomala* J121, *L. plantarum* Amy 1 or a combination of *P. anomala* J121 and *L. plantarum* Amy 1 was stored in mini-silos for about two months.

4.4.1 Water activity and moisture content

Actual moisture contents were slightly lower than the desired 40% and 25% (Table 9). Observed moisture content was used to convert yields and concentrations into g/g DM.

Table 9 Water activity and moisture content in grain rehydrated to moisture content 25% or 40% and inoculated or not with *P. anomala* J121 and *L. plantarum* Amy 1. Data presented are mean values where n=2

Desired moisture content	Sample	a_w	Moisture content
40%	No microbial treatment	0.994	38.6%
40%	After inoculation of *P. anomala* or *L. plantarum*	0.999	38.8%
40%	After inoculation of *P. anomala* and *L. plantarum*	0.996	36.9%
25%	No microbial treatment	0.931	23.7%
25%	After inoculation of *P. anomala* or *L. plantarum*	0.935	23.9%
25%	After inoculation of *P. anomala* and *L. plantarum*	0.943	24.5%

4.4.2 Organic acids and sugar content in grain

Concentrations of propionic, succinic, butyric, lactic and acetic acid were analyzed by HPLC. Maltose and glucose concentrations were also measured in the grain.

Propionic, succinic and butyric acid were not detected in any of the samples.

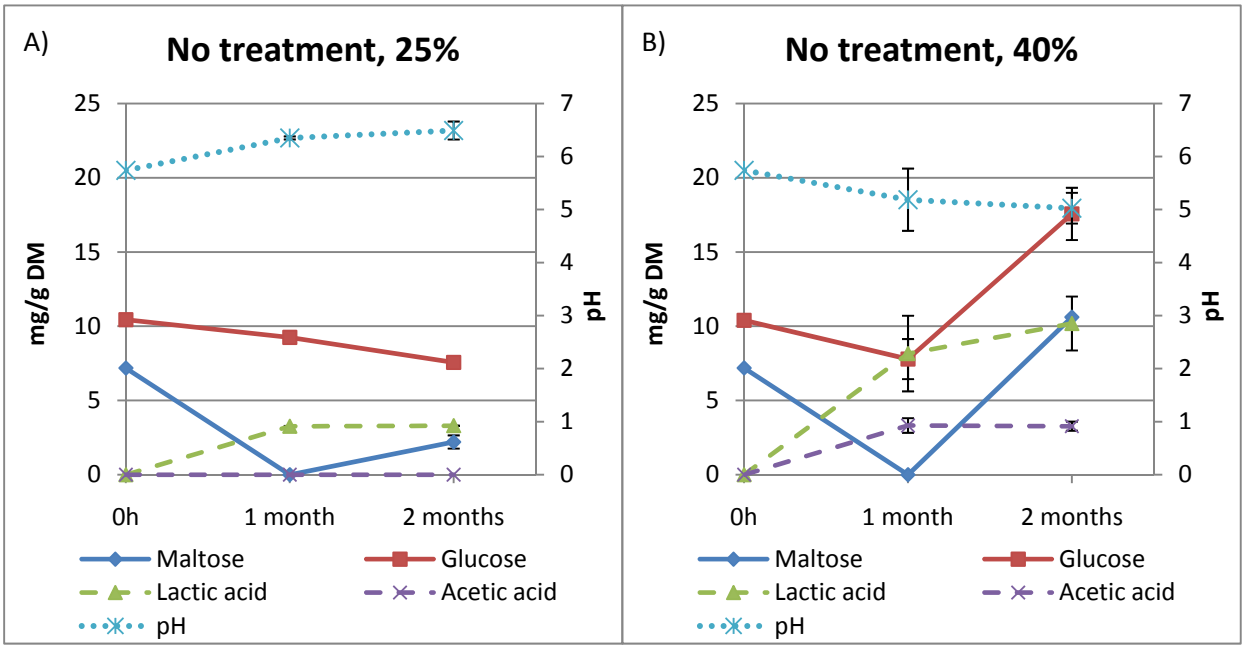


Figure 4 Concentrations of maltose, glucose, lactic acid and acetic acid in grain with no microbial treatment and moisture content A) 25% and B) 40%. (Data presented are mean values where n=2.)

No acetic acid and only small amounts of lactic acid was produced in grain with moisture content of 25%, no microbial treatment (Figure 4). High moisture content will promote growth of naturally occurring lactic acid bacteria and other microorganism in the grain. Thus, more lactic acid and acetic acid was produced in grain with a moisture content of 40%, and pH decreased slightly during storage (5.74 to 5.03), (Figure 4).

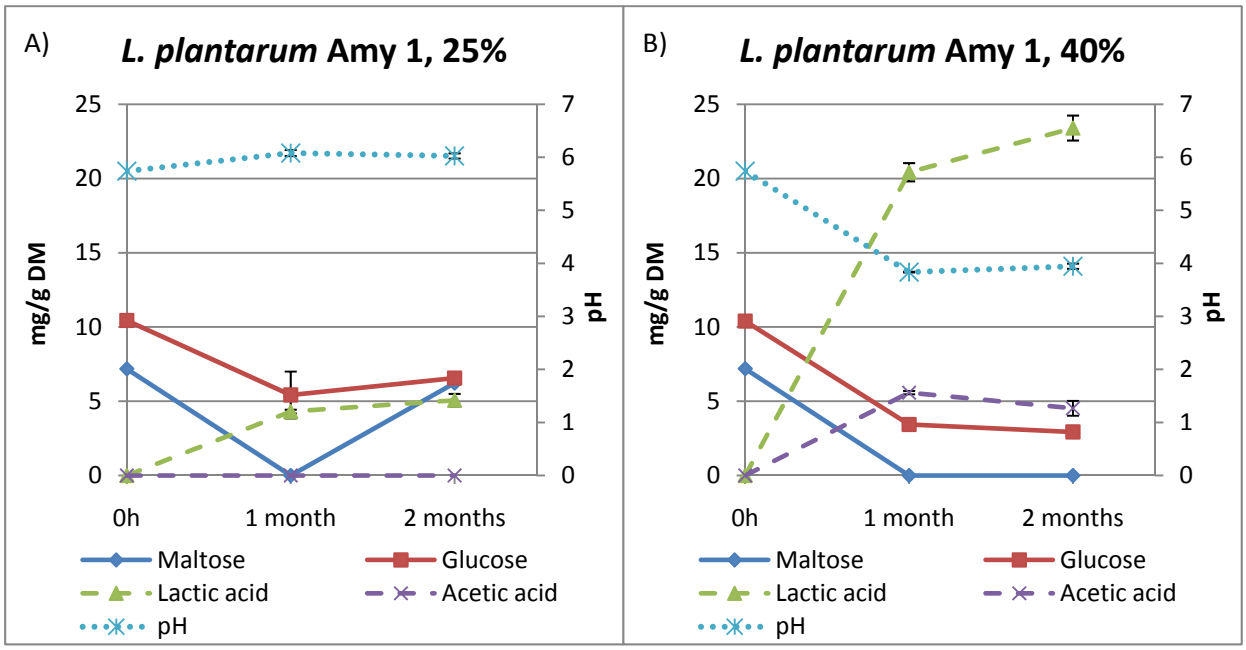


Figure 5 Concentrations of maltose, glucose, lactic acid and acetic acid in grain pretreated with *L. plantarum* Amy 1 and moisture content A) 25% and B) 40%. (Data presented are mean values where n=2.)

Pre-treating grain with *L. plantarum* Amy 1 resulted in higher concentration of lactic acid compared to no microbial treatment (Figure 5). This was observed for both moisture contents but the effect of *L. plantarum* Amy 1 was much larger in the wetter grain where 23.4 mg/g DM was recorded at two months compared to 10.1 mg/g DM in the drier grain.

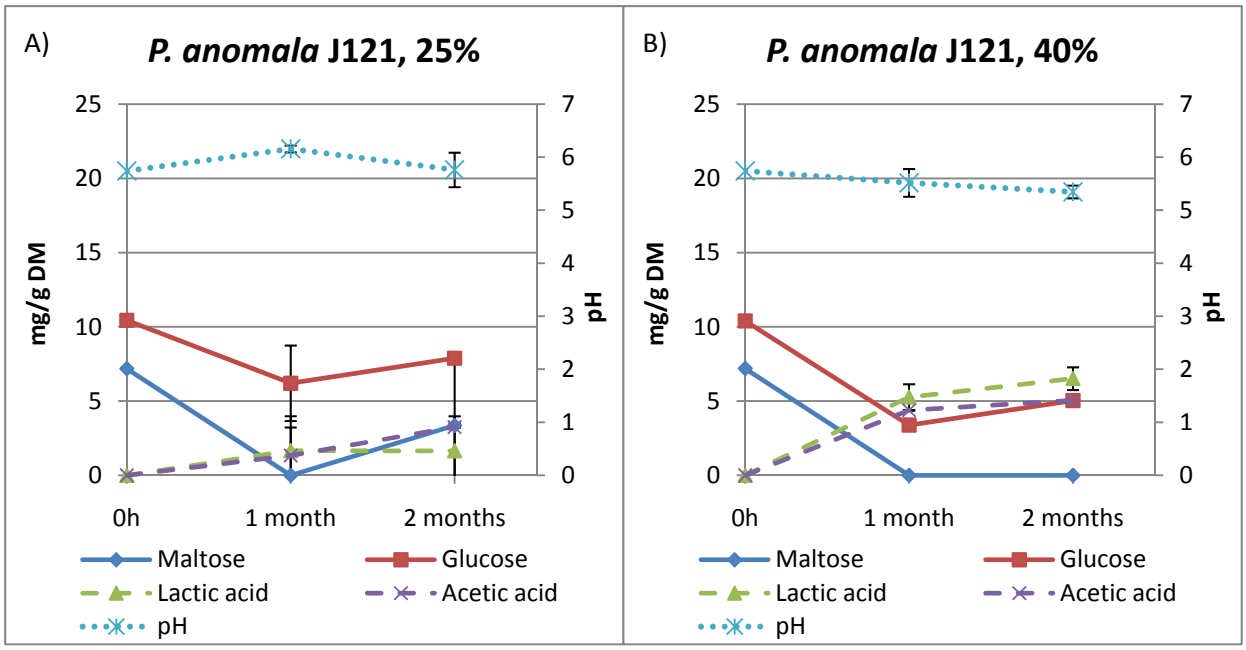


Figure 6 Concentrations of maltose, glucose, lactic acid and acetic acid in grain pretreated with *P. anomala* J121 and moisture content A) 25% and B) 40%. (Data presented are mean values where n=2 for moisture content 25% and n=1 for moisture content 40%.)

Production of organic acids in grain pretreated with *P. anomala* J121 follows the same pattern as in grain with no microbial treatment except that acetic acid is produced in larger amounts (Figure 6).

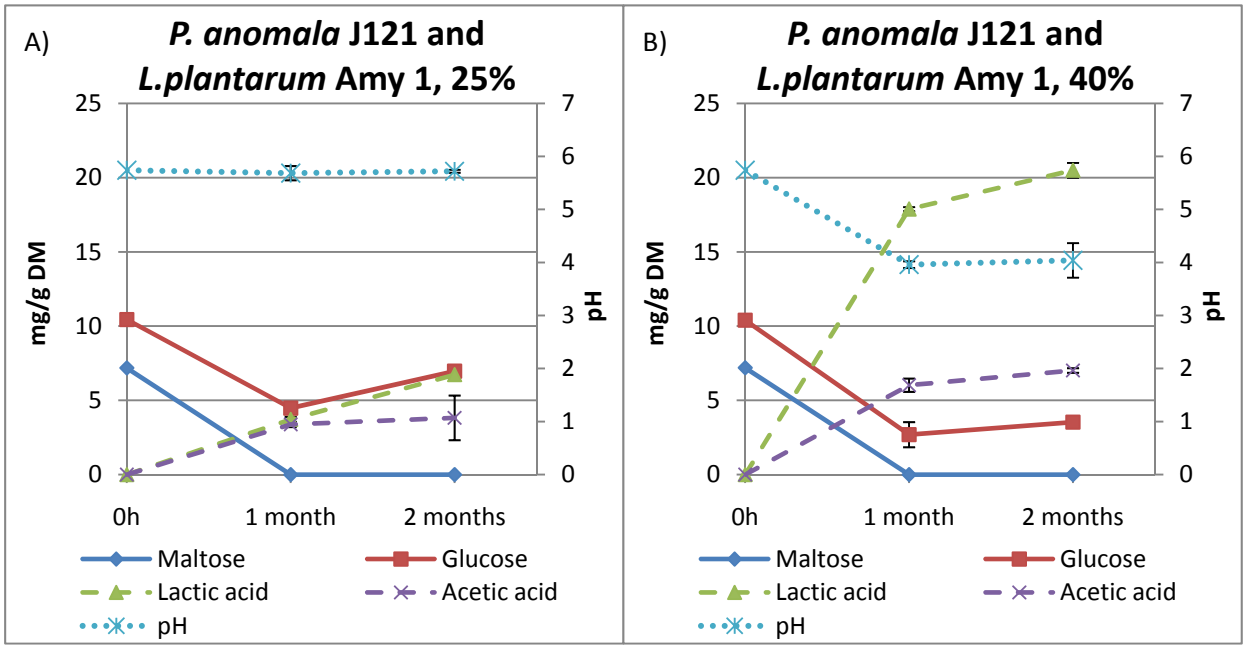


Figure 7 Concentrations of maltose, glucose, lactic acid and acetic acid in grain pretreated with *P. anomala* J121 and *L.* plantarum Amy 1, moisture content A) 25% and B) 40%. (Data presented are mean values where n=2**)**

Lactic acid production in grain inoculated with both *P. anomala* J121 and *L. plantarum* Amy 1 followed the same pattern as treatment with *L. plantarum* Amy 1 alone and acetic acid production as treatment with *P anomala* J121 alone (Figure 7).

General observations were that more lactic acid was produced in the wetter grain and pre-treating grain with *L. plantarum* Amy 1 resulted in the highest concentrations as

expected. When the pretreatment included *P. anomala* J121 acetic acid was detected in samples of both moisture contents, otherwise it was only detected in the wetter grain.

4.4.2.1 Comparison of glucose concentration

Figure 8 shows a comparison of glucose concentrations after one month and two months of storage for the different microbial pretreatments to illustrate the effect of storage time on sugar level.

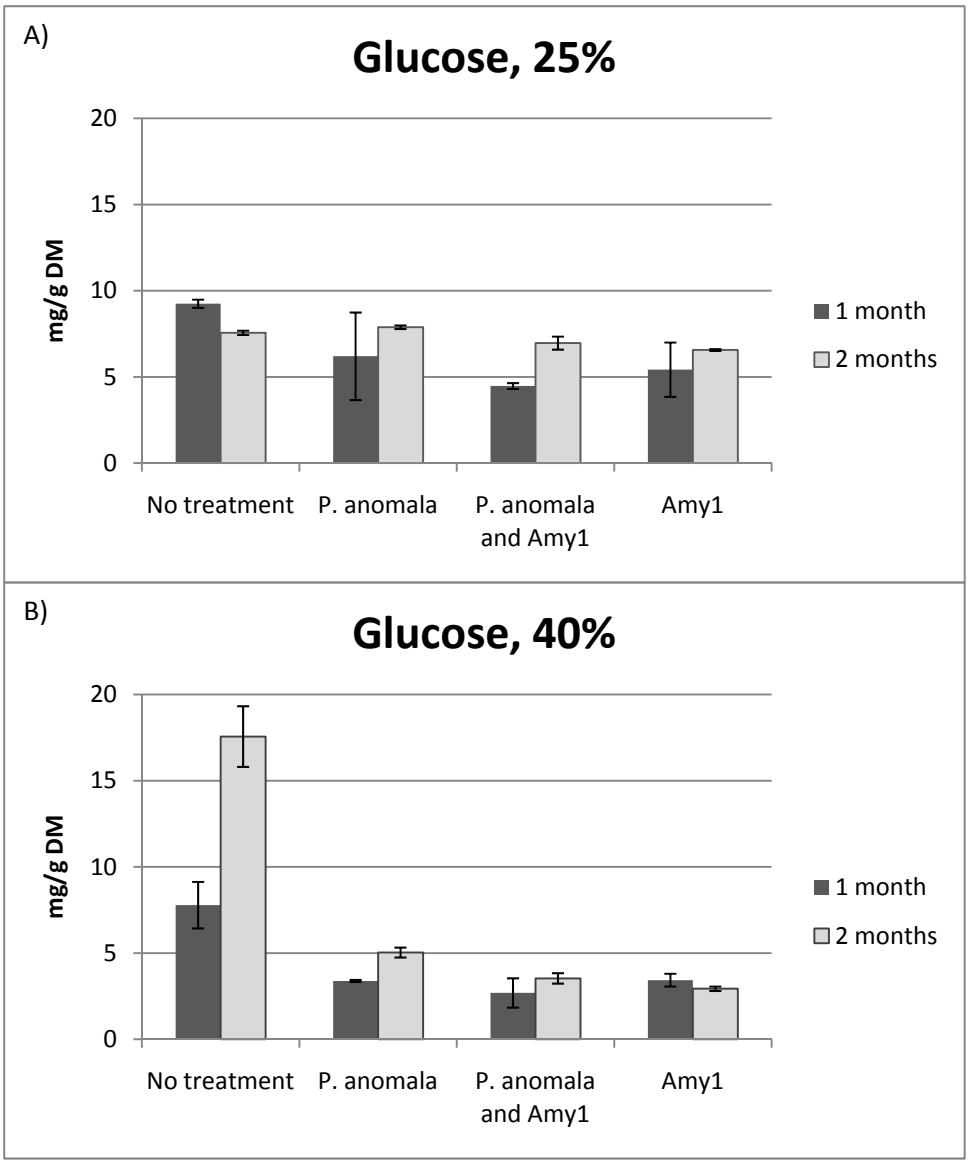


Figure 8 Glucose concentrations after one month and two months of storage in grain with moisture content A) 25% and B) 40%. (Data presented are mean values where n=2.)

Glucose concentrations tend to be higher in samples taken after two months of storage than samples from one month (Figure 8). The exceptions are grains treated with *L. plantarum* Amy 1, 40% and no microbial treatment, 25% but it has to be noted that variations in some of the data is quite high. Some of the treatments showed the same pattern for maltose (Figure 4-6).

Microbial growth consumes glucose and it is quite natural that glucose concentrations fall between 0h and one month in all treatments (Figure 5-7). Increased levels of glucose after two months might be a result of the rehydration of the grain, see also

Figure 4-6 where maltose concentrations also tend to be slightly higher after two months than after one month. Passoth *et al.* (2009) observed that glucose concentrations after enzymatic pretreatment prior ethanol fermentation were higher in grain stored moist because the starch was more accessible and easily degraded by the enzymes. Enzymatic activity by added microorganisms might also benefit from the more accessible starch in moist grain and therefore increase glucose concentrations during storage. The high glucose concentration in no microbial treatment, 40% is difficult to explain and has to be verified.

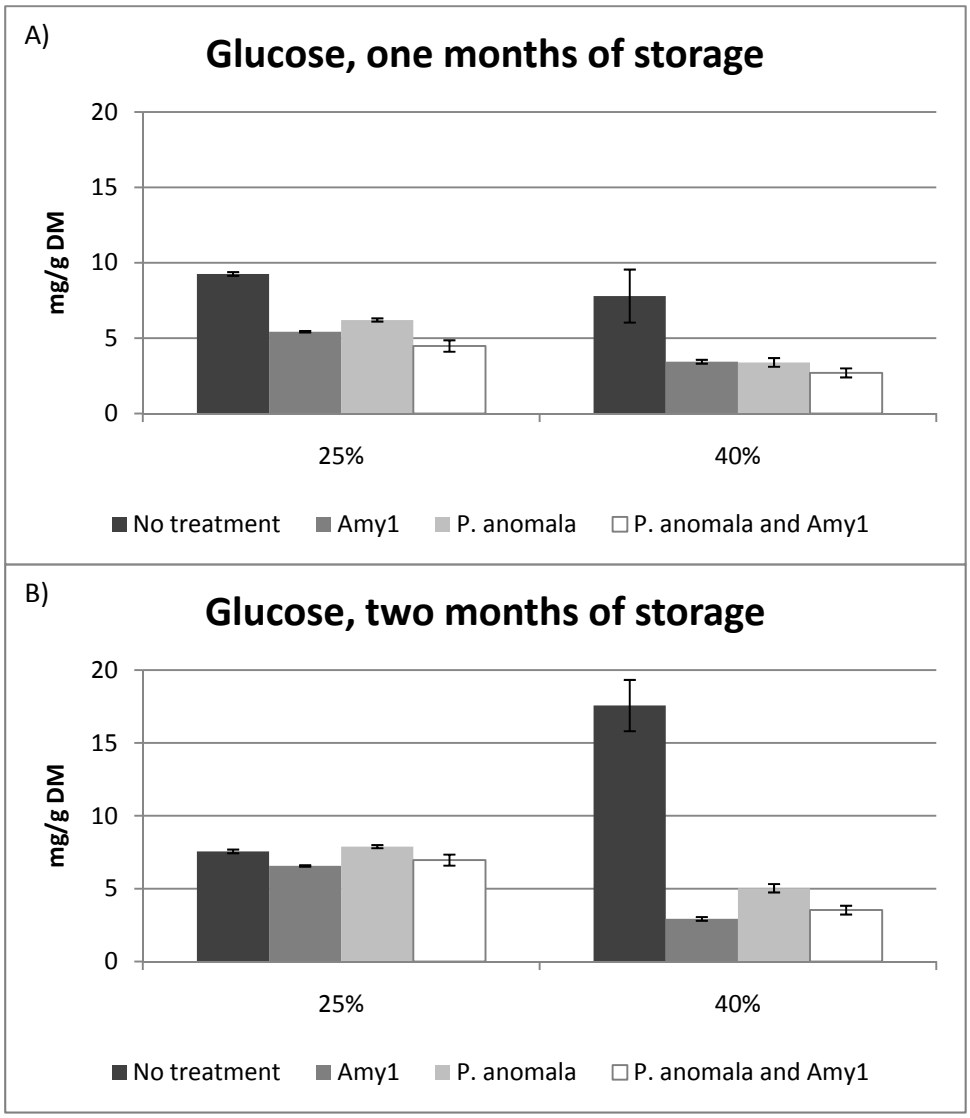


Figure 9 Comparison of glucose concentrations between grain with different microbial pretreatment after A) one month of storage and B) two months. (Data presented are mean values where n=2.)

Final glucose concentrations were slightly higher in grain with moisture content 25% compared to 40% except in grain with no microbial treatment (Figure 9). This might be due to lower microbial growth in grain of the lower moisture content.

In Figure 9, the different pretreatments are compared regarding glucose levels in the grain after storage and observed variations were very small except for grain with no microbial treatment, 40%. Why the recorded glucose concentration for that treatment was high might be explained by lower microbial growth since the grain was not inoculated with any microorganisms. However, elevated glucose concentration was not recorded for no microbial treatment, 25% where the concentrations were similar to the other treatments. To

conclude, pre-treating grain with *L. plantarum* Amy 1 did not increase glucose concentrations, levels are decreased or at the same level.

4.4.3 Glucose concentrations after enzymatic pretreatment

Glucose concentrations after enzymatic pretreatment with Stargen 001 are presented as g/g DM in Figure 10 where the different microbial pre-treatments during storage are compared to each other. The grain was stored for two months and non-stored rehydrated grain was also included in the comparison (denoted 0h).

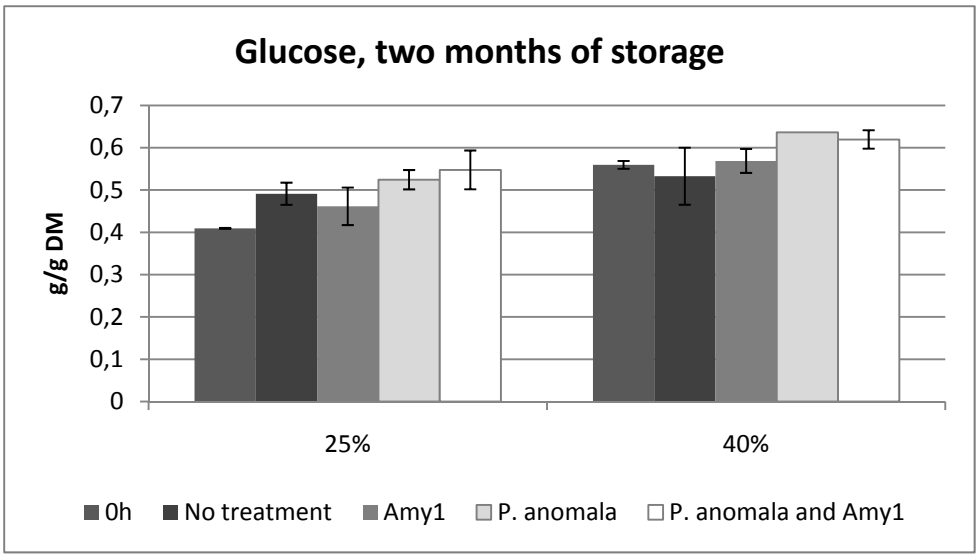


Figure 10 Glucose concentration comparisons between the different microbial pre-treatments after two months of storage. Glucose concentration was measured after enzymatic pre-treatment prior ethanol fermentation. Non-stored grain was also included in the comparison (Data presented are mean values where n=2, except for treatment *P. anomala* J121, 40% where n=1).

Pre-treating grain with *L. plantarum* Amy 1 did not seem to affect glucose concentrations after enzymatic pretreatment in a positive way (Figure 10). There was no obvious difference in glucose level in grain between no microbial treatment and grain inoculated with *L. plantarum* Amy 1, neither in pretreatment with *P. anomala* J121 and *L. plantarum* Amy 1 compared to grain treated with *P. anomala* J121 alone.

Free glucose in the grain (section 4.4.1) constitutes only a small portion of the glucose released when starch is enzymatically degraded, about 3 to 18 mg/g DM compared to 400 to 600 mg/g DM. It seems that the small difference in free glucose before starch is degraded is not noticeable after enzymatic pretreatment.

The result in Figure 10 partially confirms earlier findings that higher glucose concentrations were recorded after enzymatic pre-treatment of moist grain compared to dry grain (Passoth *et al.* 2009). Non-stored grain (0h) was rehydrated for two days at +4°C to ensure an even distribution of the moisture and for a moisture content of 25%, recorded glucose levels were lower compared to grain stored for two months. Data for moisture content 40% show no real differences in glucose concentrations between 0h, no microbial treatment and *L. plantarum* Amy 1 but inoculation with *P. anomala* J121 and *L. plantarum* Amy 1 resulted in a higher concentration. One of the samples pre-treatment *P. anomala* J121, 40% was unfortunately lost because the bottle broke during enzymatic pretreatment and therefore no standard deviation is shown for this data in Figure 10. Additional replicates for all the microbial treatments analyzed would give a more reliable result and is of course to prefer but was not possible due to limited time.

Glucose concentrations after enzymatic pretreatment in grain stored for one month are presented in Figure 11. For all treatments, concentrations are much lower in these samples than observed in grain stored for two months or grain with no storage time (Figure 10). Ethanol fermentations were performed in two batches starting with grain stored for one month. The preparations were slightly modified for the second batch to increase mixing of gelatinized starch and added water before incubating the samples with Stargen 001. Increased mixing of the gelatinized grain material with the water seems to have made the starch more accessible to the enzyme and the result more reliable. Thus, results from ethanol fermentation on grain stored for two months are the main focus in this discussion. There was also an accident with the rotary shaker during the first 24 hours of ethanol fermentation of grain stored for one month, the fermentation was interrupted and fluid was lost.

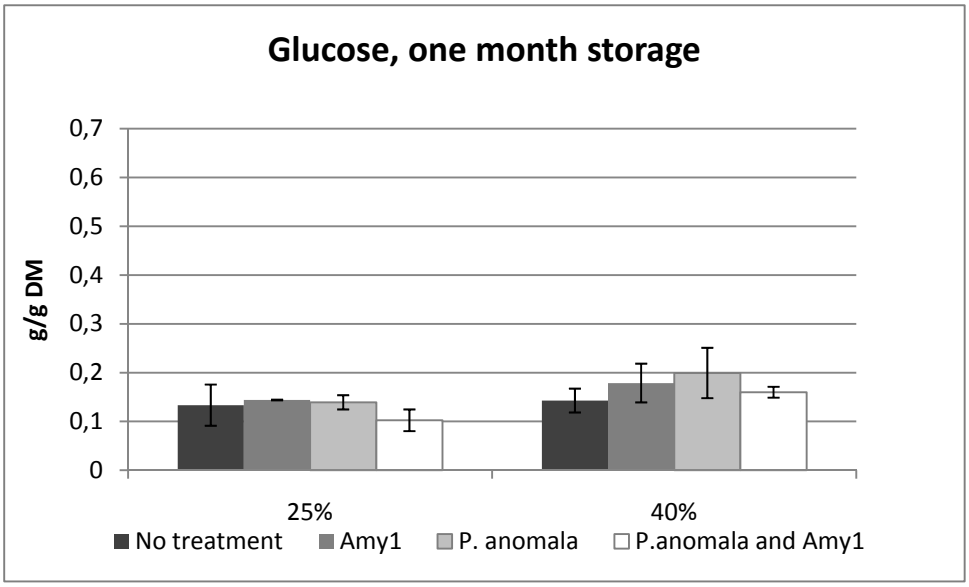
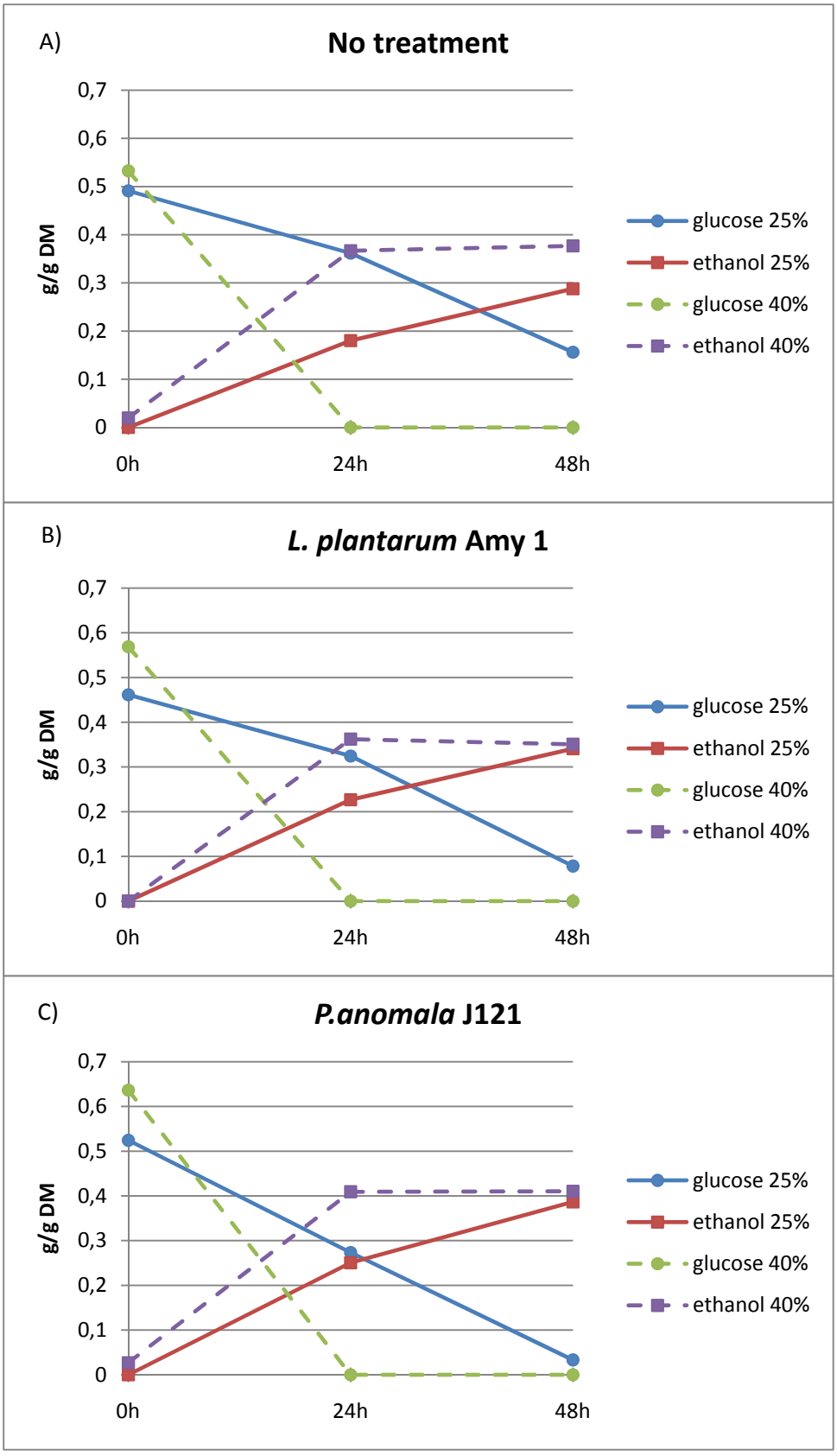


Figure 11 Glucose concentration comparisons between the different microbial pre-treatments after one month of storage. Glucose concentration was measured after enzymatic pretreatment prior ethanol fermentation. (Data presented are mean values where n=2.)

4.4.4 Ethanol and glucose concentrations during ethanol fermentation

Glucose, maltose and ethanol concentrations were measured at 24h and 48h during the fermentation. No maltose could be detected in any of the samples. Figure 12 illustrates how glucose concentrations decreased when ethanol was produced.



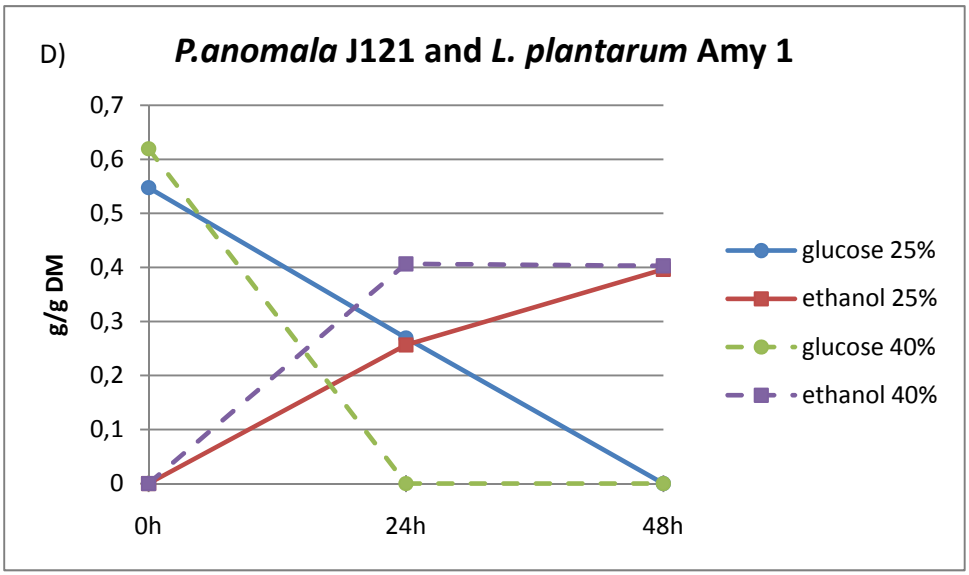


Figure 12 Glucose and ethanol concentrations during fermentation on grain of moisture content 25% and 40% stored for two months and pretreated with A) no microbial treatment, B) *L. plantarum* Amy 1, C) *P. anomala* J121 and D) *P. anomala* J121 and *L. plantarum* Amy 1, moisture content 25% and 40%. (Data presented are mean values where n=2, except for treatment *P. anomala* J121, 40% where n=1.)

Results from all four different pretreatments show the same pattern in glucose consumption and ethanol production when comparing the different treatments at the same moisture content level. In samples with moisture content 40%, no glucose was detected at 24h and ethanol had reached its maximum level. Glucose concentrations for moisture content 25% had only decreased to about half of its initial value at 24h and in pretreatment *L. plantarum* Amy 1 and no microbial treatment was glucose still present at 48h.

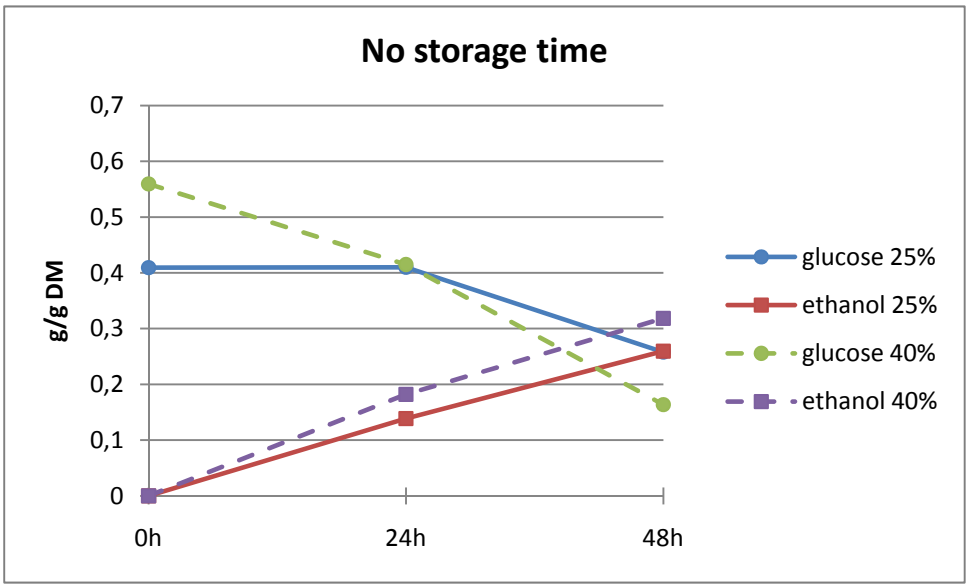


Figure 13 Glucose and ethanol concentrations during fermentation of grain with no storage time in mini-silos, moisture content 25% and 40%. (Data presented are mean values where n=2)

Rehydrated but non-stored grain did not follow the same pattern as stored grain (Figure 13). Ethanol was produced in the same amount and at the same rate regardless moisture content and the amounts of ethanol produced were lower compared to stored grain.

It seems that glucose and/or other nutrients become more accessible to the yeast during fermentation when moist grain has been stored for some time. Higher moisture content resulted in higher ethanol production rate but ethanol yields were not higher in the long run. At 48h ethanol concentrations are similar in fermentates derived from grain with different moisture content.

4.4.5 Ethanol yields

The aim of this study was to investigate if amylase producing bacteria can increase ethanol yields and therefore are ethanol yields for the different pretreatments at 24 and 48 hours of fermentation compared in Figure 14.

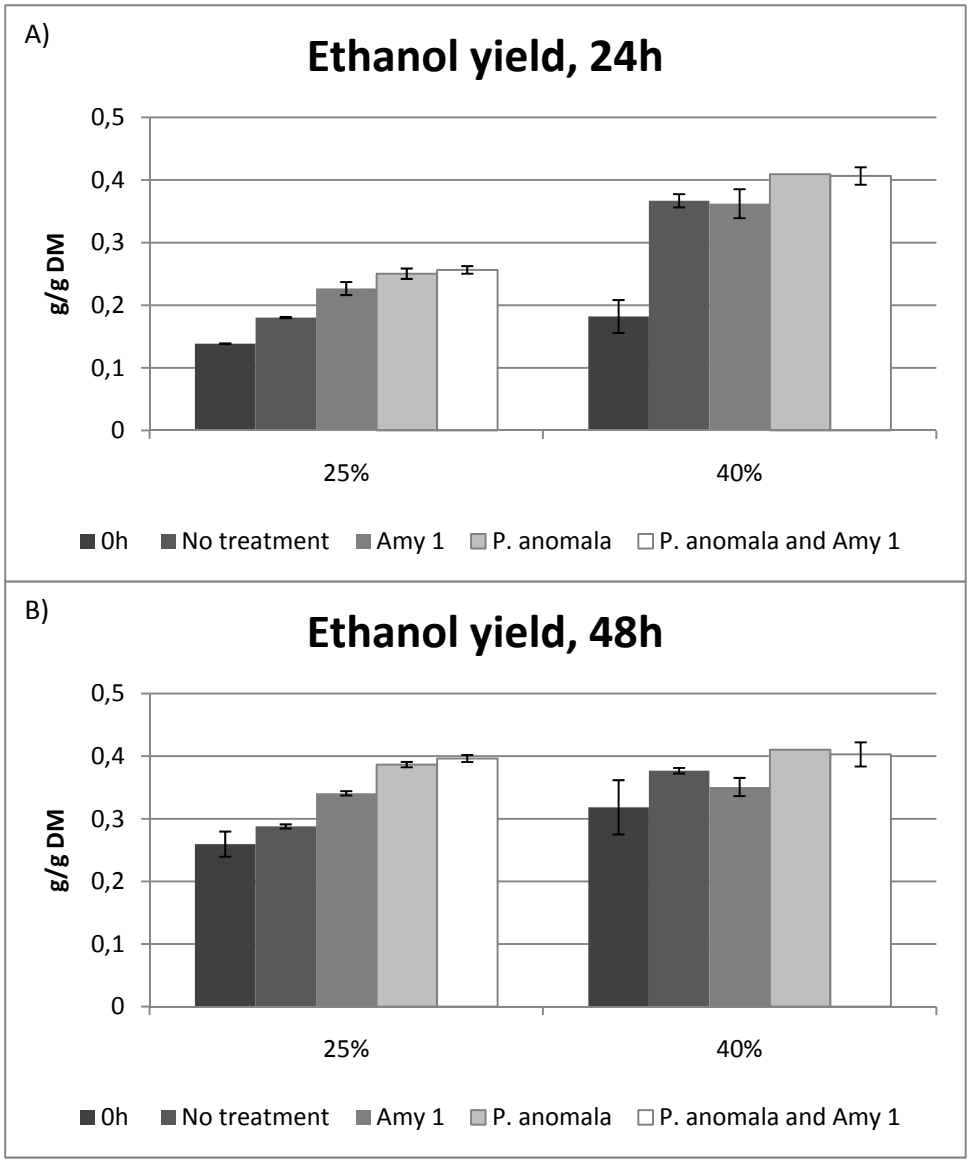


Figure 14 Ethanol yield comparisons between the different microbial pre-treatments after two months of storage. Non-stored grain was also included in the comparison. Samples were taken after A) 24 hours and B) 48 hours of fermentation. (Data presented are mean values where n=2, except for treatment *P. anomala* 40% where n=1.)

After 24h, ethanol concentrations from fermentations of grain with moisture content of 40% were higher than in grain with 25% for all four pretreatments (Figure 14 A). It is also clear that storing moist grain for some time increases ethanol yields. Grain pretreated with

L. plantarum Amy 1 had higher ethanol concentration than no microbial treatment for moisture content 25% but not for 40%. Pretreatment with *P. anomala* J121 has a larger effect on ethanol yields since treatment *P. anomala* J121 and treatment *P. anomala* J121 and *L. plantarum* Amy 1 had the highest yields.

Pretreatment with *P. anomala* J121 in combination with *L. plantarum* Amy 1 or alone still had the highest yields after 48h (Figure 14 B). Comparing ethanol concentrations of moisture content 25% and 40% for the same pretreatment, no real difference was observed. The ethanol production rate was higher in fermentations of grain with the wetter grain but in the long run about the same amount ethanol was produced from the drier grain. Corresponding figure for ethanol yields after 48h of fermentation of grain stored for one month is presented in appendix 2 (Figure 15).

In summary, treating grain *L. plantarum* Amy 1 seems not to affect the ethanol yields while pretreatment with pretreatment with *P. anomala* J121 seems to have a positive effect on ethanol yields.

4.4.6 Microbial population in fermentations

Concentrations of *S. cerevisiae* J672 after 48 hours of fermentation were determined by viable count on YPD-C (Table 10-12). Contaminating microorganisms can interfere with the ethanol fermentation and therefore was general growth on TSA-D studied. Growth of unidentified microorganisms was observed on TSA-D for most of the fermentations (Table 10-12).

Table 10 Concentrations of *S. cerevisiae* J672 (cfu/ml), unknown microorganism (cfu/ml), and ethanol (g/l) in fermentations at 48h. Grain used in the fermentations had moisture content 25% and was stored for two months

Treatment	***S. cerevisiae* (cfu/ml)**	**Unidentified microorganism (cfu/ml)**	**Ethanol (g/l)**
No microbial treatment	3.7×10^7	2.4×10^7	20.1
No microbial treatment	3.1×10^7	2.0×10^7	19.8
P. anomala	2.9×10^7	6.7×10^7	27.0
P. anomala	6.3×10^7	3.0×10^7	26.5
P. anomala and Amy1	8.3×10^7	n. d.	26.9
P. anomala and Amy1	4.7×10^7	n. d.	27.5
Amy1	3.4×10^7	1.3×10^6	23.4
Amy1	3.6×10^7	n. d.	23.7

Table 11 Concentrations of *S. cerevisiae* J672 (cfu/ml), unknown microorganism (cfu/ml), and ethanol (g/l) in fermentations at 48h. Grain used in the fermentations had moisture content 40% and was stored for two months

Treatment	***S. cerevisiae* (cfu/ml)**	**Unidentified microorganism (cfu/ml)**	**Ethanol (g/l)**
No microbial treatment	7.3×10^7	6.6×10^7	20.9
No microbial treatment	3.6×10^7	1.0×10^8	21.2
P. anomala	6.4×10^7	n. d.	22.8
*P. anomala**			
P. anomala and Amy1	8.1×10^7	4.9×10^7	23.9
P. anomala and Amy1	7.5×10^7	2.8×10^7	22.3
Amy1	9.2×10^7	n. d.	20.1
Amy1	9.2×10^7	n. d.	18.9

*Sample lost

Table 12 Concentrations of *S. cerevisiae* J672 (cfu/ml), unknown microorganism (cfu/ml), and ethanol (g/l) at 48h in fermentations of non-stored grain

Moisture content	***S. cerevisiae* (cfu/ml)**	**Unidentified microorganism (cfu/ml)**	**Ethanol (g/l)**
25%	3.7×10^7	2.3×10^6	17.0
25%	2.7×10^7	2.5×10^7	19.0
40%	4.6×10^7	3.7×10^7	19.5
40%	1.6×10^7	n. d.	16.1

S. cerevisiae J672 was detected in similar concentrations in all the fermentations of grain stored for two months (Table 10-11) and fermentations of non-stored grain (Table 12). Corresponding data for grain stored for one month are presented in appendix 2 (Table 19-20).

Unidentified microorganisms were detected on TSA-D in most of the fermentations. PCR-fingerprinting was used to investigate if the microorganisms could be identified as *P. anomala* J121, *L. plantarum* Amy 1 or *S. cerevisiae* J672 but the results were inconclusive. Randomly picked microorganisms were studied in a microscope and by the size of the organism it was assumed to be yeast. Naturally occurring microorganisms or added microorganisms in the pretreatment have probably survived the gelatinization step in heated water bath (100°C) during enzymatic pretreatment. It is also possible that the YPD-broth with *S. cerevisiae* J672 was contaminated and that contaminating microorganisms were transferred to all fermentations when the samples were inoculating with *S. cerevisiae* J672. Observed growth in fermentations on grain with no microbial treatment indicates that it was not *P. anomala* J121 or *L. plantarum* Amy 1. When comparing ethanol production in fermentations of grain with the same treatment and moisture content, presence of the unidentified yeast did not seem to affect yields negatively.

4.5 Inhibition of Penicillium roqueforti *J9*

The aim of this study was to ensure that any starch degrading capacity of *L. plantarum* Amy 1 did not increase mould growth during storage. Therefore, *P. roqueforti* J9 was added as a spoilage mould. A heterofermentative lactic acid bacterium, *L. fermentum* T14, was included in one treatment in an attempt to increase acetic acid concentration since *L. plantarum* Amy 1 alone did not inhibit growth of *P. roqueforti* J9 in the overlay assay (section 4.2).

Initial concentrations of *P. roqueforti* J9 in the grain (MC 40%) were confirmed to be between 4.0×10^3 and 9.0×10^3 cfu/g grain and after 15 days of storage concentrations had decreased to 200 cfu/g grain or less (data not shown). After additional storage for 11 days under exposure to air, *P. roqueforti* J9 was not detected at all. Thus, no conclusions about the inhibitory effect of the different biopreservation microorganisms can be drawn from this study. However, starch degrading activity in grains treated with *L. plantarum* Amy 1did not seem to increase mould growth.

5. Conclusions

Amylase producing *L. plantarum* Amy 1 was able to grow on grain but the pretreatment did not result in any increased glucose concentrations. Sugar concentrations tend to be higher in grain stored for two months than one month which might be due to that enzymatic activity by the added microorganisms benefit from the more accessible starch in rehydrated grain. Furthermore, glucose concentrations after enzymatic degradation of starch prior to ethanol fermentation were not positively affected by pretreatment with *L. plantarum* Amy 1 during storage.

Ethanol production rates were higher in fermentations on grain of moisture content 40% than 25%. Ethanol concentrations reached their maximum levels after 24h in samples of the wetter grain, while in grain with the lower moisture content, only about half of the final amount ethanol had been produced at 24h. It seems that sugar or/and other nutrients becomes more accessible if the grain is stored at a high moisture content resulting in higher ethanol production rates. Ethanol yields are not increased in the long run, after 48h ethanol concentrations are similar in fermentations of grain of moisture content 25% and 40%. Previous studies that ethanol yields are increased by storing grain moist was confirmed but pre-treating grain with *L. plantarum* Amy 1 did not increase ethanol yields any further. Slightly increased ethanol yields were observed in fermentations of grain pretreated with *P. anomala* J121.

No inhibiting activity against *P. anomala* J121 or *P. roqueforti* J9 was observed for *L. plantarum* Amy 1 to 7 but all seven strains showed antifungal activity against *F. culmorium* J617. No conclusions about increased storage stability could be drawn in this study but pre-treating grain with *L. plantarum* Amy 1did not seem to increase mould growth.

The aim of this study was to investigate if ethanol yields can be increased by pre-treating grain with a combination of amylase producing strains of *L. plantarum* and *P. anomala* J121 during airtight storage. *L. plantarum* Amy 1 did not increase ethanol yields so this microbial pretreatment do not show any promise about improved ethanol production processes when fermenting grain into bioethanol.

6. Acknowledgements

Thanks to members of the Department of Microbiology at Swedish University of Agriculture Science in Uppsala. A special thanks to my supervisor Karin Jacobsson for all the help and support during this last part of my education. I learned a lot and I really enjoyed my time at SLU. I would also like to thank Katarina, Emily and David for great company this spring.

7. References

Adinarayana Reddy C and Abouzied MM 1986. Glucose feedback inhibition of amylase activity in *Aspergillus* sp. and release of this inhibition when cocultured with *Saccharomyces cerevisiae. Enzyme and Microbial Technology* 8: 659-664
beans and chocolate products. *Journal of Food science* 45: 555-563

Björnberg A and Schnürer J. 1993. Inhibition of the growth og grain-storage moulds in vitro by the yeast *Pichia anomala* (Hansen) Kurtzman. *Canadian Journal of Microbiology* 36: 623-628

Boysen ME, Björneholm S and Schnürer J 2000. Effect of the biocontrol yeast *Pichia anomala* on interactions between *Penicillium roqueforti, Penicillium carneum,* and *Penicillium paneum* in moist grain under restricted air supply. *Postharvest Biology and Technology* 19: 173-179

Börjesson P 2004. Energianalys av drivmedel från spannmål och vall. Rapport 54, Miljö- och energisystem, Lunds Universitet, Lund

Börjesson P 2006. Energibalans för bioetanol – En kunskapsöversikt. Report No. 59, Department of Technology and Society Environmental and Energy Systems Studies, Lund University, Lund

Cardona CA and Sánchez ÓJ 2007. Fuel ethanol production: Process design trends and integration opportunities. *Bioresource Technology* 98: 2415-2457

De Man JD, Rogosa M and Sharpe ME 1960. A Medium for the Cultivation of Lactobacilli. *Journal of Applied Microbiology* 23: 130-135

Fredlund E, Druvefors U, Boysen ME, Lingsten KJ and Schnürer J 2002. Physiological characteristics of the biocontrol yeast Pichia anomala J121. *FEMS Yeast Research* 2:395-402

Geiger DR and Servaites JC 2001. Starch and starch grains In: *Encyclopedia of Life Science.* John Wiley & Sons Ltd, Chichester. http://www.els.net/ [doi: 10.1038/npg.els.0000700]

Hill J, Nelson E, Tilman D, Polasky S and Tiffany D 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *PNAS* 103: 11206-11210

Kheshgi HS, Prince RC and Marland G 2000. The potential of biomass fuels in the context of global climatic change: focus on transportation fuel. *Annual Review of Energy and Environment* 25: 199-244.

Labuza TP and Altunakar B 2008. Water activity prediction and moisture sorption isotherms. In Barbosa-Cánovas GV, Schmidt S. and Fontana A eds. *Water activity in foods: Fundamentals and applications* Ames: Wiley-Blackwell. Ch 2.

Lacey J 1989. Pre-and post-harvest ecology of fungi causing spoilage of foods and other stored products. *Journal of Applied Bacteriology - Symposium Supplement* 67: 11–25

Lineback DR Starch In: AccessScience@McGraw-Hill. The McGraw-Hill Companies, http://www.accessscience.com [doi: 10.1036/1097-8542.651700]

Liu ZL, Saha BC and Slininger PJ 2008. Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In Wall J., Harwood C. S., Demain A. eds. *Bioenergy*. Washington, DC: ASM Press. Ch 2.

Madigan MT, Martinko JM and Parker J 2003. *Brock biology of microorganisms*. 10th ed. Upper Saddler River: Pearson Education. pp. 400-401

Magnusson J and Schürer J 2001. Lactobacillus coryniformis subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Applied and environmental microbiology* 67: 1-5

Markeberg R, Carlsen M and Nielsen J 1995. Induction and repression of a-amylase production in batch and continuous cultures of *Aspergillus oryzae*. *Microbiology* 141:2449-2454

McEniry J, O´Kiely PO, Clipson NJW, Forristal PD and Doyle EM 2010. Assessing the impact of various ensilage factors on the fermentation of grass silage using conventional culture and bacterial community analysis techniques. *Journal of Applied Microbiology* 108: 1584-1593

Müller V 2008. Bacterial fermentation In: *Encyclopedia of Life Science*. John Wiley & Sons Ltd, Chichester. http://www.els.net/ [doi: 10.1002/9780470015902.a0001415.pub2]

Nichols NN, Monceaux DA, Dien BS and Bothast RJ 2008. Production of ethanol from corn and sugarcane. In Wall J., Harwood C. S., Demain A. eds. *Bioenergy*. Washington, DC: ASM Press. Ch 1.

Nicholson P 2009. *Fusarium* and *Fusarium*- Cereal Interactions In: *Encyclopedia of Life Science*. John Wiley & Sons Ltd, Chichester. http://www.els.net/ [doi: 10.1002/9780470015902.a0021266]

Nigam P and Singh D 1995. Enzymes and microbial systems involved in starch processing. *Enzymes and Microbial Technology* 17: 770-778

Olstorpe M, Borling J, Schnürer J and Passoth V 2010. *Pichia anomala* yeast improves feed hygiene during storage of moist crimped grain under Swedish farm conditions. *Animal Feed Science and Technology* 156: 47-56

Passoth V, Eriksson A, Sandgren M, Ståhlberg J, Piens K and Schnürer J 2009. Airtight storage of moist wheat grain improves bioethanol yields. *Biotechnology for Biofuels* 2: 16

Petersson S and Schnürer J 1995. Biocontrol of mould growth in high-moisture wheat stored under airtight conditions by Pichia anomala, Pichia guilliermondii, and Saccharomyces cerevisiae. *Applied and Environmental Microbiology* 61: 1027–1032

Petersson S, Jonsson N and Schnürer J 1999. *Pichia anomala* as a biocontrol agent during storage of high-moisture feed grain under airtight conditions. *Postharvest Biology and Technology* 15: 175-184

Pimentel D 2003. Ethanol fuels: energy balance, economics and environmental impact are negative. *Natural Resources Research* 12: 127-134

Pimentel D and Patzek T 2005. Ethanol production using corn, switchgrass, and wood; Biodiesel production using soybean and sunflower. *Natural resources researches* 14: 65-76

Postma E, Verduyn C , Scheffers WA and van Dijken JP 1989. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of saccharomyces cerevisiae. *Applied and environmental microbiology* 55: 468-477

Reid F 2008. Water activity: Fundamentals and relationships. In Barbosa-Cánovas GV, Schmidt S. and Fontana A eds. *Water activity in foods: Fundamentals and applications* Ames: Wiley-Blackwell. Ch 2.

Reijnders L and Huijbregts MAJ 2007. Life cycle greenhouse gas emissions, fossil fuel demand and solar energy conversion efficiency in European bioethanol production for automotive purposes. *Journal of Cleaner Production* 15: 1806-1812

Schemieder RL and Keeney PG 1980. Characterization and quantification of starch in cocoa

Stratford M and Eklund T 2003. Organic acids and esters. In Russell N. J. and Gould G. W. eds. *Food preservative*. New York: Kluwer Academic/Plenum Publishers. Ch 4.

Versalovic J, Koeuth T and Lupski JR 1991. Distribution of Repetitive DNA-Sequences in Eubacteria and Application to Fingerprinting of Bacterial Genomes. *Nucleic Acids Research* 19: 6823-6831

Weiss C 1990. Ethyl alcohol as a motor fuel in Brazil. A case study in industrial policy. *Technology in Society* 12: 255-282

Ädel Druvefors U, Passoth V and Schnürer J 2005. Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during airtight storage of wheat. *Applied and environmental microbiology* 71: 1865-1869

Appendix 1

Growth media

Table 13 Medium composition of YPD medium

YPD medium	**(g/l)**
Yeast extract (Oxoid)	10
Bacterial peptone	20
Glucose (Duchefa Biochemie, Haarlem, the Netherlands)	20
Agar	16

Table 14 Medium composition of YPD broth

YPD broth	**(g/l)**
Yeast extract	10
Bacterial peptone	20
Glucose	20

Table 15 Medium composition of MRS agar with starch and no glucose

MRS agar with starch and no glucose	**(g/l)**
Bacterial peptone	10
Lab-Lemco Powder (Oxoid)	8.0
Yeast extract (Oxoid)	4.0
Starch of different origin	20
Tween 80	1 ml/l
Di-potassium hydrogen phosphate (Merck)	2.0
Sodium acetate $\times 3H_2O$ (Merck)	5.0
Tri-ammonium citrate (VWR International, Poole, UK)	2.0
Magnesium sulphate $\times 7H_2O$ (Merck)	0.2
Manganese sulphate $\times 4H_2O$ (Sigma Chemical CO, St. Louis, US)	0.05
Agar	10

Appendix 2

Degradation of starch by Lactobacillus plantarum *Amy 1-7*

Table 16 Seven strains of *L. plantarum* Amy 1-7 were ranked by their ability to produce amylase when the bacteria was grown on potato or pea starch, starting from the top with the best strain

Potato starch		**Pea starch**	
***L. plantarum* strain**	**Comment**	***L. plantarum* strain**	**Comment**
Amy 5		Amy 5	
Amy 1		Amy 4	
Amy 7		Amy 1, Amy 2, Amy 3 and Amy 7	Clear zone equal in size
Amy 2 and Amy 3	Clear zone equal in size		
Amy 6	Poor bacterial growth		
Amy 4	Poor bacterial growth	Amy 6	Poor bacterial growth

Table 17 Seven strains of *L. plantarum* Amy 1-7 were ranked by their ability to produce amylase when the bacteria was grown on starch from Merck, starting from the top with the best strain

Starch from Merck, type one		**Starch from Merck, type 2**	
***L. plantarum* strain**	**Comment**	***L. plantarum* strain**	**Comment**
Amy 1		Amy 3	
Amy 2 and Amy 5	Clear zones are equal in size	Amy 5	
Amy 3 and Amy 4	Clear zones are equal in size	Amy 1	
Amy 7		Amy 7	
Amy 6	Poor bacterial growth	Amy 2 and Amy 4	Clear zone equal in size
		Amy 6	Poor bacterial growth

Table 18 Seven strains of *L. plantarum* Amy 1-7 were ranked by their ability to produce amylase when the bacteria was grown on starch from Merck, starting from the top with the best strain

Starch from Merck, type 3		**Starch from Merck, type 4**	
***L. plantarum* strain**	**Comment**	***L. plantarum* strain**	**Comment**
Amy 1, Amy 2, Amy 3, Amy 5 and Amy 7	Clear zone equal in size	Amy 3, Amy 4 and Amy 5	Clear zone equal in size
Amy 4	Partially poor bacterial growth	Amy 1, Amy 2 and Amy 7	Clear zone equal in size
Amy 6	Poor bacterial growth	Amy 6	Poor bacterial growth

Ethanol yields

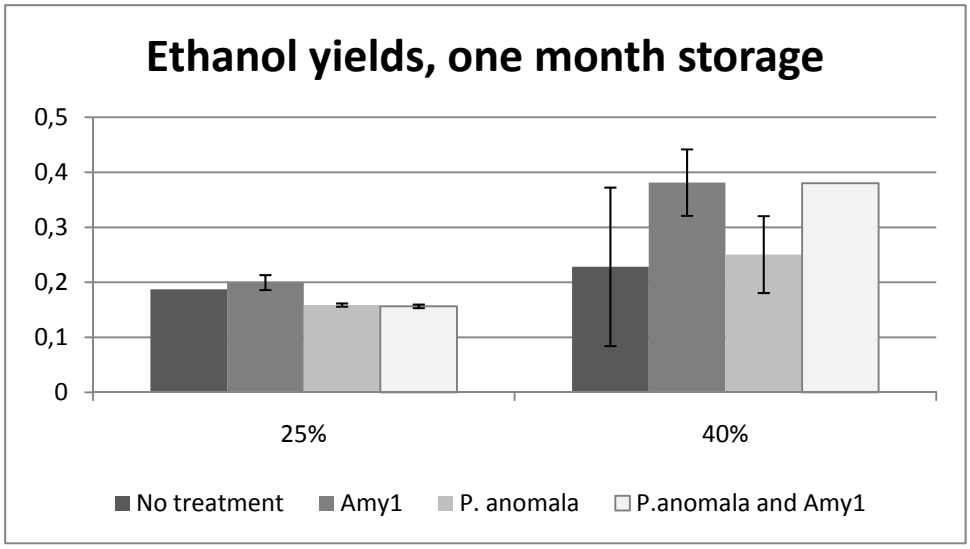


Figure 15 Ethanol yields after 48h in fermentations on grain stored for one month. (Data presented are mean values where n=2 except for no treatment 25% and *P. anomala* J121 and *L. plantarum* Amy 1, 40%.)

Microbial population in fermentation

Table 19 Concentrations of *S. cerevisiae* J672 (cfu/ml), unknown microorganism (cfu/ml), and ethanol (g/l) at 48h in fermentations on grain of moisture content 25% stored for one month

Treatment	***S. cerevisiae* (cfu/ml)**	**Unidentified microorganism (cfu/ml)**	**Ethanol (g/l)**
No microbial treatment	3.9×10^{7}	3.7×10^{7}	14.2
No microbial treatment*			
P. anomala	8.3×10^{7}	n.d.	12.3
P. anomala	8.3×10^{7}	1.7×10^{7}	11.7
P. anomala and Amy1	3.0×10^{7}	1.7×10^{7}	8.4
P. anomala and Amy1	5.2×10^{7}	2.9×10^{7}	8.3
Amy1	2.4×10^{7}	2.2×10^{7}	11.0
Amy1	1.0×10^{8}	8.0×10^{7}	14.3

*Sample lost

Table 20 Concentrations of *S. cerevisiae* J672 (cfu/ml), unknown microorganism (cfu/ml), and ethanol (g/l) at 48h in fermentations on grain of moisture content 25% stored for one month

Treatment	***S. cerevisiae* (cfu/ml)**	**Unidentified microorganism (cfu/ml)**	**Ethanol (g/l)**
No microbial treatment	2.5×10^{8}	8.6×10^{7}	19.6
No microbial treatment	2.0×10^{8}	2.3×10^{6}	8.1
P. anomala	2.5×10^{8}	5.1×10^{7}	13.6
P. anomala	2.6×10^{8}	7.6×10^{7}	12.9
P. anomala and Amy1*			
P. anomala and Amy1	1.6×10^{8}	1.2×10^{8}	24.4
Amy1	1.5×10^{8}	8.3×10^{7}	14.4
Amy1	1.2×10^{8}	9.6×10^{7}	18.5

*Sample lost