Storage of sugar beets for biofuel production using biocontrol microorganisms

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Independent project 2010:11

ISSN 1101-8151
ISRN SLU-MIKRO-EX–10/11–SE

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Keywords: **biocontrol, biogas, *Candida oleophila*, ethanol, *Pseudomonas fluoresence*, sugar beet**

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

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Preface

This report is my final thesis for my degree in Master of Science in Engineering Biology with the profile Industrial biotechnology and production at Linköping Institute of Technology, Linköping University. The work has been performed at the Department of Microbiology, Swedish University of Agricultural Sciences, within the MicroDrivE research program.

Abstract

The new sugar reform in EU has lead to a reduced price on sugar, and consequently on sugar beets produced for sugar production in Sweden. The high content of convertible carbohydrates in sugar beets make them suitable for bioenergy production and the price on sugar beets would not change if they are used in a bioenergy purpose. Thus, the sugar beet production in Sweden would still be profitable. The limiting factor is the short storage capability of sugar beets, and to be able to use them for a longer period, the storage time needs to be prolonged.

Biogas and bioethanol are both renewable energy sources and seen as promising alternative fuels. Biogas is produced through anaerobic digestion of a wide range of organic materials, while bioethanol mainly is produced from different sugar crops and grains by fermentation.

Previously the biopreservation organisms *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3 had been isolated from sugar beets and shown to inhibit various fungal pathogens on agar medium. Hoping to prolong the storage time for sugar beets, a model scale storage study was set up with the biocontrol organisms *Candida oleophila* JÄ3 and *Pseudomonas fluoresence* KJ36. Small cubes of sugar beets were stored for one and four months at 10°C and 15°C. Microbial growth during storage was determined trough viable count and the effect of the biocontrol organisms was then analysed with regard to biogas and ethanol production through anaerobic digestion and fermentation experiments. To evaluate the effect of the biocontrol microorganisms on sugar content during storage, sucrose, glucose and fructose concentrations were determined through HPAEC-PAD analysis.

From this study, it can be concluded that the biopreservation organisms *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3 did not to inhibit various fungal pathogens. The endogenous bacteria increased in number and consequently do not seem to be affected by biopreservation organisms on the surface of the sugar beet cubes. Neither was mould growth on the surface inhibited. Consequently, the effect on sugar content and therefore also on the ethanol production potential was negative. The biocontrol and spoilage organisms have decreased the yield by decreasing the sugar content and subsequent ethanol yield from samples stored for four months. The biogas production from the beet samples decreased with storage time due to the consumption of organic material by the biocontrol and spoilage organisms. The storage time of sugar beets cannot be prolonged using the biocontrol organisms *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3.

Keywords: biocontrol, biogas, *Candida oleophila* JÄ3, ethanol, *Pseudomonas fluoresence* KJ36, sugar beet

Sammanfattning

Den nya sockerreformen i EU har lett till sänkta priser på socker och därmed även på sockerbetor odlade för sockerproduktion. Tack vare den höga andelen tillgänglig sukros i sockerbetor är de även lämpliga för produktion av bioenergi. Priset på sockerbetorna skulle inte ändras om de istället användes till bioenergi och på så sätt skulle sockerbetsproduktionen kunna fortsätta att vara lönsam i Sverige. Nackdelen med sockerbetor är dock deras korta lagringstid och för att kunna använda dem under en längre tid måste lagringstiden förlängas.

Biogas och bioetanol är båda förnyelsebara energikällor som anses vara lovande alternativa bränslen. Biogas produceras genom rötning av en mängd olika organiska material som substrat, medan etanol främst produceras genom fermentering av olika sockergrödor och spannmål.

I tidigare studier har de biopreserverande organismerna *Pseudomonas fluoresence* KJ36 och *Candida oleophila* JÄ3 isolerats från sockerbetor och visat en bra svamphämmande förmåga på agarmedium. I hopp om att kunna förlänga lagringstiden för sockerbetor startades en lagringsstudie i labbskala med *Pseudomonas fluoresence* KJ36 och *Candida oleophila* JÄ3 som biokontrollorganismer. Kuber av sockerbetor lagrades i en och fyra månader vid 10°C och 15°C. Den mikrobiella tillväxten under lagringen bestämdes genom levandehaltsbestämningar och effekten av biokontrollorganismerna på utbytet vid biogas- och etanolproduktion undersöktes genom utrötnings- och fermenteringsförsök. För att utvärdera biokontrollernas effekt på sockerinnehållet under lagringen analyserades sukros-, glukos- och frukoskoncentrationerna med HPAEC-PAD- analys.

Från den här lagringsstudien kunde det konstateras att *Pseudomonas fluoresence* KJ36 och *Candida oleophila* JÄ3 inte hämmade svamptillväxten på sockerbetorna under lagringen. Det endogena bakterieantalet ökade under lagringen och verkade inte påverkas av biokontrollorganismerna på sockerbetkubens ytor. Följaktligen var effekten på sockerinnehållet och därmed också etanol produktionspotentialen negativ. Både biokontroll- och förskämmelseorganismerna hade minskat utbytet genom minskat sockerinnehåll och därmed efterföljande etanolutbyte för proverna lagrade fyra månader. Biogasproduktionen från sockerbetorna minskade med lagringstiden på grund av biokontroll- och förskämmelseorganismernas konsumtion av organiskt material. Lagringstiden för sockerbetor kan ej förlängas med hjälp av biokontroll organismerna *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3.

Nyckelord: biogas, biokontroll, *Candida oleophila* JÄ3, etanol, *Pseudomonas fluoresence* KJ36, sockerbetor

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1 Introduction

The European Union's new sugar reform has lead to decreasing sugar beet prices due to a reduced price of sugar (Jordbruksverket 2009). The reform only affects sugar beets if they are used for sugar production. If the sugar beets instead are used for bioenergy production their price would not change. (Nilsson 2006)

It is now generally accepted that the accelerated release of fossil entombed CO_2 due to human activity is the major factor contributing to the global warming (Antoni et al. 2007). This environmental factor together with the fear that the global crude oil reserve is finite has put the hope to biomass and bioenergy (Bai et al. 2008). Biogas and bioethanol are both renewable and seen as a promising alternative fuels (Lantz et al. 2007, Bai et al. 2007).

Sugar beets have a high yield of convertible carbohydrates which make them suitable for renewable energy production (Cooke and Scott 1993). The problem with sugar beets is the short storage capability (Nilsson 2006). To be able to use sugar beets for a longer period during the year the storage time needs to be extended. Biological control, the use of microorganisms to protect against postharvest pathogens (Sharma et al. 2009) might be one way to prolong the storage of sugar beets.

2 Background

2.1 Biogas

Biogas is a combination of methane (55-75 vol %) and carbon dioxide (25-45 vol %) (de Mes et al. 2003) and is the end product when organic material is degraded without oxygen, a process called anaerobic digestion. In nature, biogas is formed in anaerobic ecosystems such as the rumen, sediments, rice fields and water-logged soils where anaerobic digestions occur naturally. (Ahring 2003)

Biogas can be produced from a wide range of organic materials; manure, energy crops, crop residues and municipal and industrial organic waste. Biogas is a renewable fuel and can be used in different energy purposes such as a vehicle fuel, heat or combined heat and power. (Lantz et al. 2007) A disadvantage is that biogas is a gaseous fuel under normal conditions (temperature and pressure) which makes it harder to distribute and store than liquid fuels (Åhman 2010).

The residue, digestate, from the anaerobic digestion can be used as fertilizer in agriculture. Plant nutrients are non-degradable substances and therefore remain in the biogas residue. The fertilization efficiency increases since the digestion process increases the plant availability of the nitrogen in the digested material compared to the undigested. (Lantz et al. 2007) More environmental benefits from utilisation of biogas residue as fertilisers are reduced use of mineral-based fertilisers, the production of which is very energy consuming (Svenska Biogasföreningen et al. 2010).

2.1.1 The biogas process

The biogas process can be divided into four steps which depend on different groups of microorganisms (Ahring 2003, Gavala et al. 2003). The first step is the *hydrolysis* in which organic polymers decompose to monomers or dimers, which can pass the cell membrane (Gavala et al. 2003). The biodegradation of organic polymers is an

enzymatic process carried out by extracellular enzymes excreted by the acidogenic bacteria. *Acidogenesis* is the next step in the biogas process in which the dissolved organic matter is biodegraded to volatile fatty acids, alcohols and carbon dioxide by the acidogenic bacteria. (Gavala et al. 2003, de Mes et al. 2003) The volatile fatty acids and alcohols are then converted into acetate or hydrogen and carbon dioxide by the acetogenic bacteria in the phase called *acetogenesis* (de Mes et al. 2003). The last step in the biogas process is the *methanogenesis* in which two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide respectively (Ahring 2003, de Mes et al. 2003). Methanogenesis is the most pH sensitive step in anaerobic digestion and only proceeds when the pH is neutral (6.5 -7.5). The three first steps of the biogas production can function at a wide range of pH values. It is essential that the various steps in the production remain coupled during the process in order to have a stable digestion and prevent accumulation of intermediate compounds. (de Mes et al. 2003)

Anaerobic digestion is classified into three groups depending on at what temperature the process is run at; pshychrophilic (10-20°C), mesophilic (20-40°C) and thermophilic (50-60°C) digestion (de Mes et al. 2003). A higher temperature results in higher rates of bacterial growth and digestion of the organic material, allowing a high loading rate. Psychrophilic digestion therefore requires long retention times and consequently is not so common. (Nordberg 2006, de Mes et al. 2003) The process is although more sensitive at thermophilic conditions due to faster ammonium inhibition i.a. (Schnürer and Jarvis 2009).

2.2 Bioethanol

Ethanol can be produced either synthetically or by fermentation. Bioethanol is ethanol produced by fermentation of raw material from agriculture and forestry. (Nilsson 2006) Production of bioethanol is today the largest industrial microbial process (Bai et al. 2008). Bioethanol is mainly produced from starch feedstocks and sugar (Antoni et al. 2007) by batch fermentations with yeast *Saccharomyces cerevisiae* (Bai et a. 2008). Out of the total world production of bioethanol 57% is from sugar crops, 42% from grains and 1% from forest products (Nilsson 2006).

Ethanol can be used as a fuel and is by being both renewable and environmentally friendly believed to be one of the best alternatives to fossil fuels (Bai et al. 2008). One advantage for bioethanol compared to biogas is that it is a liquid at atmospheric pressure. It is therefore easier to distribute, store and use as a fuel than biogas which requires a new distribution infrastructure. (Antoni et al. 2007) Disadvantages of bioethanol is its corrosiveness, low vapour pressure which makes cold starts difficult and its lower energy density than gasoline (bioethanol has 66% the energy that gasoline has). The raw material is one of the major problems for the production of bioethanol. The availability of the raw material differs from season to season and is dependent on geographic locations. These are some of the reasons to why the price of the raw material is highly volatile and therefore strongly affect the production costs of bioethanol. (Balat et al. 2008).

2.2.1 The ethanol fermentation

The glycolysis, also called Embden-Meyerhof-Parnas pathway, is the main metabolic pathway utilized in ethanol fermentation. Through glycolysis one molecule of glucose is converted to two molecules of pyruvate. The pyruvate is then under anaerobic

conditions reduced to ethanol and carbon dioxide. (Bai et al. 2008) From one mole glucose two moles of carbon dioxide and two mole ethanol is obtained;

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2\ C_2H_5OH \qquad \text{(Antoni et al. 2007, Nilsson 2006).}$$

The fermentation temperature can both be mesophilic (25-37°C) or thermophilic (45-55°C). The mesophilic fermentation is the most common one. However, the thermophilic process is more productive but also more sensitive, and are therefore focus for more and more research and developing. (Antoni et al. 2007)

Ethanol production is tightly coupled to the growth of the yeast cell. In the glycolysis two ATPs are produced which are used to drive the biosynthesis of yeast cells. If the ATPs are not consumed by the growth of yeast cells, the intracellular accumulation of ATP will inhibit the glycolysis and no more ethanol will be produced. There is a 30 folds slower ethanol fermentation rate for non growing yeast cells than for growing yeast. (Bai et al. 2008)

2.3 Sugar beet

Sugar beet, *Beta vulgaris*, is together with sugar cane one of the two most important sources of sugar (sucrose). Sugar cane can only be grown in tropical and subtropical regions whereas sugar beet is grown almost all over the world, but mostly in temperate regions. (Cooke and Scott 1993)

Sugar beets are grown from seeds and the first period of the plants growth is a period of leaf initiation. During these first six weeks the root grows very little but the plant's 8-10 leaves have developed. Onward from this stage the root and leaf grow simultaneously and the root becomes an increasing part of the total plant dry weight (Cooke and Scott 1993) Crops are usually sown in April and harvested in the autumn starting in October (Erlandsson et al. 2010).

All sugar beet farming in Sweden is situated in the southern parts; Skåne, Halland, Småland, Öland, Blekinge and Gotland. It is only cultivated in the southern parts because of the requirement of a long growing period of sun and warmth. The farming is both capital- and labour intensive due to requirements of time, knowledge and special equipments of the farmer. Nordic Sugar former Danisco Sugar AB buys all produced sugar beets in Sweden, and the farming is based on contracts that regulate price and delivery time. (Nilsson 2006)

There is only one sugar factory in Sweden so the process intensity is very high during the harvest period. The factory can therefore not store all harvested sugar beets so the different farmers store their own sugar beets if necessary. (Nilsson 2006) The beets are stored in piles on the farm. During the harvest, transportation and piling the sugar beets are inevitably wounded. Bruising, root breakage, cuts and surface abrasions are usual injuries for the sugar beet. The different injuries induce cell division, respiration and production of different compounds for repairing of damaged tissue, sealing wounds and defending against opportunistic pathogens. All this is done on the expense of sucrose. (Klotz et al. 2006)

Sugar beets have a high content of convertible carbohydrates which make them suitable for renewable energy production (Cooke & Scott 1993). To be able to use them for

bioenergy, the sugar beets need to be stored in a good way with minimum losses of sucrose during the whole year. Biological control might be the solution of the storage problem.

2.4 Biological control

Today, one major reason for postharvest losses during transportation and storage on fruits and vegetables is postharvest diseases. Approximately 20-25 % of the harvested fruits and vegetables are estimated to be decayed by different pathogens during the postharvest handling. (Sharma et al. 2009) The primary method to control these postharvest diseases has been synthetic fungicide treatments (Lima et al. 1997, Sharma et al. 2009). Fungicides kill or inhibit fungi but can also in some cases control bacterial growth (McGrath 2009). A strong public and scientific concern with fungicides, due to fungicide residues and fungicide resistant pathogens, have increased the search for a more environmentally friendly and safe alternative to reduce the postharvest decay (Lima et al. 1997, Sharma et al. 2009).

Biological control by microbial antagonists is seen as a promising alternative to fungicide methods (Lima et al. 1997, Sharma et al. 2009). Microbial antagonists can be used in two ways to control postharvest diseases; either use the already existing microorganisms on the fruits and vegetables or introduce new ones (Sharma et al. 2009). The biocontrol activity of microbial antagonists is a complex process and several interactions are involved (Mikani et al. 2008, Sharma et al. 2008) and the mechanisms behind have not yet been fully understood (Sharma et al. 2009). Understanding the mechanisms will help to select more effective antagonists. Nutrient competition, production of antibiotics, direct parasitism, induced resistance and competition for space are some of the actions with which the biocontrol organisms suppress the pathogens. The competition of space and nutrients is considered as the most important mode of action with which the microbial agents control the pathogens causing the postharvest decay. The microbial antagonists should be more efficient to adapt to various environments and nutritional conditions than the pathogen to be able to compete successfully in the wounded sites. (Sharma et al. 2009)

The biocontrol organisms can either be applied before the harvest, pre-harvest application, or after the fruit and vegetables been harvested, postharvest application. Decays after harvest are often results of latent infections from pathogens that infested the fruit or vegetables in the field. The purpose of the pre-harvest application of the microbial agent, is to pre-colonize the surface immediately before the harvest in order for the wounds caused during the harvest to be colonized by the antagonist and not the pathogens. However, this has been difficult to accomplish because of low survival of the microbial agent in the field. Postharvest application have therefore been more successful and described as a better, more practical and useful way to control postharvest diseases. The biocontrol organisms are in this method applied as sprays or as solutions dips after the harvest. (Sharma et al. 2009)

2.4.1 *Candida oleophila*

Candida oleophila is a yeast which has been shown to suppress blue, green and gray moulds (Sharma et al. 2009). *Candida oleophila* Montrocher (strain 182) (Bar-Shimon et al. 2004) is the microbial agent in the biocontrol product Aspire produced by Ecogen Inc., USA (Sharma et al. 2009, Mikani et al. 2008). Aspire has been registered for postharvest use on apple, pear and citrus (Sharma et al. 2009) but has been withdrawn (Mikani et al. 2008).

2.4.2 *Pseudomonas fluorescence*

Pseudomonas fluorescence is a gram negative bacterium which can suppress both fungal and bacterial plant pathogens (Mikani et al. 2008). The strain A506 is a microbial pesticide (Johnson et al. 2004), registered under the product name Blight Ban A 506, produced by Nu Farm Inc., USA (Sharma et al. 2009, Mikani et al. 2008, Johnson et al. 2004). It can be used to control fire blight (disease killing different tissues of the tree (Kuflick et al. 2008) and soft rots on apple, pear, strawberries and potatoes (Sharma et al. 2009). In studies *Pseudomonas fluorescence* has also been shown to protect sugar beet against the fungus *Pythium ultimum* causing the "damping off" in seedlings (Dunne et al. 1996). Damping off, also called blackleg is the term to describe the collapse of sugar beet seddlings caused by infections from different fungi (Cooke and Scott 1993).

3 Aim

The aim of the study was to evaluate if the storage time of sugar beets can be prolonged if the biocontrol organisms *Candida oleophila* JÄ3 or *Pseudomonas fluoresence* KJ36 are used.

The effect of the biocontrol organisms on sugar content, growth of spoilage organisms and yields of biogas and ethanol production was studied to evaluate the effect of the organisms.

The model scale storage used in this project was set up before this project started. Only the analyses to evaluate the effect of the biocontrol organisms were made in this project.

4 Materials and methods

A storage study was set up with two different biocontrol organisms, *Candida oleophila* JÄ3 and *Pseudomonas fluoresence* KJ36. The biopreservation organisms *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3 had previously been isolated from sugar beets and shown to inhibit various fungal pathogens on agar medium (K Jacobsson, unpublished results). The sugar beets were also inoculated with two different spoilage organisms, *Fusarium culmorum* J617 and *Cladosporium cladosporioides* J308, and incubated at two temperatures, 10°C and 15°C, for one or four months. The microbial growth during storage was determined trough viable count. The effect of the biocontrol organisms was then analysed in regard to biogas and ethanol production through anaerobic digestion and fermentation experiments, respectively. To evaluate the effect of the biocontrol organisms on sugar content during storage, sucrose, glucose and fructose was determined through HPAEC-PAD analyses.

4.1 Material

4.1.1 Sugar beets

The sugar beets used in the study were supplied from Syngenta Seed AB, Landskrona, Sweden. After harvest, the beets were stored at +4 °C and used within one week.

4.1.2 Biogas inoculum

The biogas residues used were supplied from the biogas plant in Västerås. The plant is operated on source-sorted organic household waste, sludge from grease separators and ensiled ley crops, at a mesophilic temperature (Svenska Biogasföreningen et al. 2010).

The inoculums, biogas residues, had a VS-content of 2.2-2.7 % and were incubated, for 1-2 weeks before the batch start, to reduce the background production of biogas from the remaining organic material. The residue was also filtered to remove all undigested grass silage residues to obtain a more homogenous inoculum.

4.1.3 Microorganisms and growth medium

The microorganisms used in the study were:

- Biocontrol organisms: *Candida oleophila* JÄ3 and *Pseudomonas fluoresence* KJ36
- Spoilage organisms: *Fusarium culmorum* J617 and *Cladosporium cladosporioides* J308
- Fermentation yeast: *Saccharomyces cerevisiae* J672

All are from the culture collection at the Department of Microbiology, SLU

Bacteria were grown on TSA/D (Tryptic Soy Agar (OXOID LTD, Basingstoke, England) containing 0.1 g/l delvocid (Gist-brocades, Delft, The Netherlands) to prevent fungal growth). All fungi expect *S. cerevisiae* J672 were grown on MEA/C (Malt Extract Agar (OXOID LTD, Basingstoke, England) containing 0.1 g/l chloramphenicol (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.1 g/l streptomycin (Duchefa Biochemical, Haarlem, The Netherlands), to prevent bacterial growth). *S. cerevisiae* J672 was grown in YPD-medium (40 g/l D-glucose (Duchefa Biochemical, Haarlem, The Netherlands), 10 g/l peptone (OXOID LTD, Basingstoke, England), 5 g/l yeast extract (OXOID LTD, Basingstoke, England)). *S. cerevisiae* J672 were grown on YPD/C (same composition as YPD-medium but with the 12 g/l agar (OXOID LTD,

Basingstoke, England) and 0.1 g/l chloramphenicol to prevent bacterial growth). *C. oleophila* JÄ3 was grown on YPD/C/Cyc (same composition as YPD/C but with the addition of 10 µg/l cycloheximide (Sigma Chemical CO, St. Louis, USA) to prevent growth of *S. cerevisiae* J672). All incubations were at 25 °C unless stated otherwise.

4.2 Methods

The model scale storage used in this project was set up before this project started, but the method and results are still presented in this report.

4.2.1 The storage study

The sugar beets were cut into small cubes, 2x2x2 cm, which were surface sterilised with 1.5 % sodium hypochlorite, NaClO, thoroughly rinsed in distilled water and sterile air dried. Thereafter they were inoculated with $5 \cdot 10^7$ cells/ml (peptone water) of the biocontrol organisms, *C. oleophila* JÄ3 or *P. fluoresence* KJ36, in peptone water or treated with peptone water alone. After sterile air drying, each treatment was divided into three portions which were treated with peptone water, 10^5 spores/ml (peptone water) of the spoilage organisms *Fusarium culmorum* J617 and *Cladosporium cladosporioides* J308 (see Figure 2 for the inoculating scheme). After inoculation the samples were sterile air dried and all treatments divided into eight portions of approximately 100 g (11 cubes) in polystyrene jars, Figure 1, and sealed with lids with a gas permeable filter allowing 13 gas exchanges per day (COMBINESSnv, Eke, Belgium). The exact weight of each sample was noted. Four jars from each treatment were incubated at two temperatures, 10 °C and 15 °C, for one and four months. Untreated cubes were chopped in a kitchen mixer and frozen for later use as controls (called "SB prestorage"). The number of endogenous bacteria was determined as described below. To ensure that the surface sterilization was satisfactory, 8 cubes chosen at random were stamped on MEA/C and TSA/D.

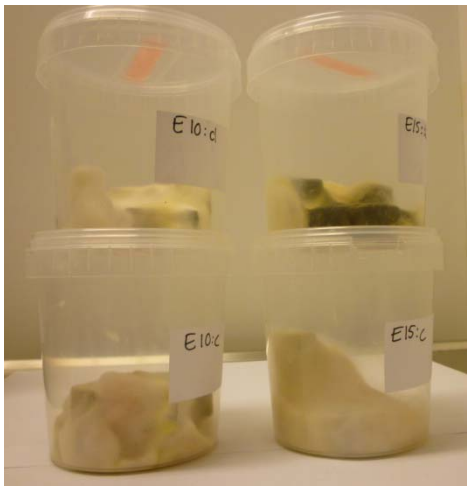

Figure 1: Polystyrene jars for storage of sugar beets.

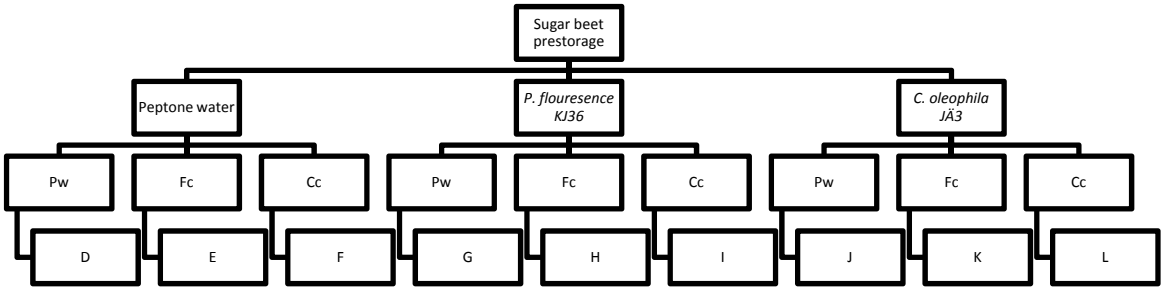

Figure 2: Inoculating scheme over how the different samples were inoculated in different combinations. Pw = peptone water, Fc = *Fusarium culmorum* J617 and Cc = *Cladosporium cladosporioides* J308.

Each treatment consists of two samples and every result presented in the report is an average of the two.

4.2.2 Termination of the storage study

After one and four months two jars from each treatment and temperature were opened and the beet cubes were weighed to measure any weight lost during storage. The sugar beet cubes were then finely chopped in a mixer. Ten g of the finely chopped material was mixed with 90 ml peptone water, treated for 2 minutes in a Stomacher at normal speed and the total amount of bacteria, mould and yeast were quantified through viable count. The remaining material was frozen at -20 °C for later analysis.

4.3 Analyses

4.3.1 Total and volatile solids

In order to be able to load the biogas batches correctly, i.e. not to overload the system, the organic content of the different sugar beets samples was determined. The amount of total solids, TS, or dry matter, DM, in the samples was analysed by drying the sample in a 105 °C oven over night. By weighing the sample before and after drying DM, i.e. the inorganic and organic matter, in the sample could be calculated. When the TS-content was determined the volatile solids, VS, i.e. the organic matter in the sample, could be measured. The sample was burned in a 550 °C oven over night. The oven was first heated to a temperature of 300 °C, which was held for an hour, in order to prevent the samples from burning too violently causing lose of sample and cross contamination. The temperature was then set to 550 °C for the rest of the time. By weighing the remaining ash after the burning and subtract it from the weight before the burning the VS-content was calculated.

4.3.2 Extraction of volatile fatty acids and sugars

To be able to determine the concentration of volatile fatty acids and sugars in the samples an extraction was preformed. Approximately 5 g of chopped sugar beets was weighed into a stomacher bag. Deionised water ten times the weight was added before the suspension was processed in the Stomacher machine for 120 s in normal speed. Around 10 ml of the suspension was transferred to a 15 ml test tube and frozen. The samples were then sterile filtered with a pore size of 0.45 µm (Sarstedt, Nümbrecht, Germany) before the HPLC analyses of VFA and HPAEC-PAD analyses of sugar.

4.3.3 Determination of volatile fatty acids by HPLC

If the samples have a high concentration of volatile fatty acids (VFA) it will affect the result from the total and volatile solid determination. The TS- and VS-values will be too low because VFA:s are not measurable as they will evaporate during heating in the detection method. Therefore the VFA concentrations were determined with HPLC to allow correction of the TS- and VS-values from the volatile solid determination.

The extracted samples were sterile filtered with a pore size of 0.45 µm. The analysis was performed by HPLC Aglient 1100 system (Agilent Technologies, Stockholm, Sweden) with a refractive index detector, at 40 bar and 60 °C. The mobile phase was 5 mM H_2SO_4 with a flow at 0.6 ml/min. The column used was Rezex-ROA-Organic Acid H+ column (Skandinaviska Genetec, Västra Frölunda, Sweden).

Mixtures of glucose, lactate, acetate, propionate, I-butyrate, butyrate, I-valerate, I-kapronate and kapronate at concentrations 0.250 g/l, 0.5 g/l, 1 g/l and 2 g/l were included as standard solutions. The resulting data was analyzed in ChemStation for LC systems (Agilent Technologies, Stockholm, Sweden).

4.3.4 Determination of sugar content by HPAEC-PAD

The sugar content in the sugar beets was determined through High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection ICS3000 system (Dionex, Sunnyvale, USA) with a Carbopac PA10 4mm column (Dionex, Sunnyvale, USA). The programme Chromelion 6.80 (Dionex, Sunnyvale, USA) was used for control and data analysing.

For standard solutions mixtures of different concentrations of glucose, sucrose and fructose were used. The eluents used was 100% water and 0.2 M NaOH with a flow of 1 ml/min. The programme used for separation can be viewed in Appendix II.

4.3.5 Potential ethanol production

The potential ethanol production of the stored sugar beets was investigated by test fermentations. An overnight culture of *S. cerevisiae* J672 was grown in YPD medium under shaking (130 rpm/minute) at 30 °C. Approximately 2.5 g chopped sugar beet was added to 50 ml testmedium, Difco Yeast Nitrogen Base, according to manufacturers protocol (Difco Laboratories Inc., Detroit, USA), in an 80 ml serum bottle. One ml of the overnight culture was added to each bottle. The serum bottles were sealed with a rubber plug equipped with a syringe to release the produced gas and thereby avoid overpressure. The cultures were incubated under shaking (130 rpm/minute) at 30 °C.

After 24 h and 42 h (when the fermentation was ended) samples were taken for HPLC analysis to measure the amount ethanol produced. The samples were sterile filtered with a pore size of 0.45 µm (Sarstedt, Nümbrecht, Germany). The analysis was performed by Agilent 1100 system (Agilent Technologies, Stockholm, Sweden) with a refractive index detector, at 40 bar and 60 °C. The mobile phase was 5 mM H_2SO_4 with a flow at 0.6 ml/min. The column used was Rezex-ROA-Organic Acid H+ column (Skandinaviska Genetec, Västra Frölunda, Sweden).

Mixtures of glucose, glycerol, acetate and ethanol at concentrations 0.1 g/l, 1 g/l, 5 g/l, 10 g/l, 15 g/l and 30 g/l were included as standard solutions. The resulting data was analyzed in ChemStation for LC systems (Agilent Technologies, Stockholm, Sweden).

From the 42 h samples, the number of the yeast and bacteria were also analysed by viable count on YPD/C and TSA/D-medium at 25 °C for 48 h. One set of YPD- plates also contained cycloheximide to prevent growth of *S. cerevisiae* J672 and thus be able to count the colonies of the biocontrol yeast *C. oleophila* JÄ3 and any other cycloheximide resistant yeast from the sugar beets.

4.3.6 DNA-fingerprinting

To check if the yeast growing on some TSA-plates with delvocid added was *C. oleophila* JÄ3 a DNA-fingerprinting technique was used. Colonies from the plates were suspended in sterilized deionised water and boiled in water for 2-3 minutes in order to lyse the cells. The samples were then centrifuged at 8 000 *g* for 2 minutes to remove the cell residues. One µl of the supernatant was mixed with 9 µl of a premix made of four PURE-taq PCR beads (GE Healthcare UK, Buckinghamshire, UK) dissolved in 80 µl sterilized deionised water and 10 µl REP-primer (5´-GTG GTG GTG GTG GTG-3´) (Versalovic et al. 1991). The PCR analyse was run with a MiniCycler (MJ Research, Waltham, USA) with the program in Table 1.

Table 1: PCR-programme

Step	Temperature	Time
1	95	7 min
2	90	30 s
3	95	1 min
4	40	1 min
5	65	8 min
6 Go to step 2, 29 more times		
7	65	16 min
8	4	92 h

The amplified PCR-products were analysed with gelelectrophorese. The DNA-fragments were separated on 1% agarose (Abgene, New York, USA) gel in TBE-buffer at 50 V for 30 minutes and then 70 V for 3 h. For detection a BIORAD Gel Doc 2000 (Bio-Rad Laboratories Inc., UK) was used.

4.3.7 Potential gas production

The potential gas production of the differently treated sugar beets was determined by a biogas batch test. The reactors were of 309 ml glass bottles. The organic loading rate, OLR, used was 9 g VS/L. In order to have a stable process a relation of 2:1 of the inoculum and substrate was used. The chopped sugar beets were added at an amount corresponding to that the sample with the highest VS-content had an OLR of 3 g VS/L (~ 2.6-3.5 g), just to be sure to not overload the system. The inoculum (~ 42-52 g) was transferred to the vials during flushing with mixed nitrogen and carbon dioxide gas to reduce exposure to oxygen. Water was added to give a total volume, working volume, of 190 ml. Three controls were made without the addition of sugar beets to be able to determine the background production of methane from the inoculum. The bottles were sealed with a rubber plug and metal cap, and incubated on a 130 rpm rotary shaker at 37°C.

By measuring the pressure in the vials at different incubation times the total gas production could be determined. The pressure was measured using GMH 3110 Digital pressure meter (Greisinger electronic GmbH, Regenstauf, Germany). The amount methane produced was determined by gas chromatography from a gas sample taken at the same time as measuring the pressures. Two ml gas was injected to 30 ml bottle with a syringe. By connecting the batch vials to a gas bag after sampling, the pressure in the vials were dropped to atmospheric pressure and thereby zeroed to next measurement.

4.3.7 Determination of methane concentration by gas chromatography

To measure the methane produced, the gas sample taken at the sampling point was analysed by gas chromatography (Perkin Elmer Instruments, Waltham, USA). A Clarus 500 column (7’ HayeSep N 60/80, 1/8 SF) and FID detector was used. Headspacesampler Turbo Matrix 110 was used as an aid for injection of the samples. The following temperatures were used; injection 60°C; oven 125°C; detector 250°C. Helium was used as a carrier gas at a flow of 31 ml/min. Methane at different concentrations was included as external standards.

5 Results and discussion

5.1 Storage study

Using $5 \cdot 10^7$ cells/ml of the biocontrol strains corresponded to $3.5 \cdot 10^5$ CFU/cm^2 of *P. fluoresence* KJ36 and $5.3 \cdot 10^6$ CFU/cm^2 of *C. oleophila* JÄ3 immediately after inoculation. The infection doses of the spoilage organisms were $1.3 \cdot 10^3$ and $2.2 \cdot 10^3$ CFU/cm^2 of *F. culmorum* J617 and *C. cladosporioides* J308 respectively. The surface sterilization had worked as no organisms grew on the stamps from the sugar beets after sterilization. However, the endogenous amount of bacteria was determined to 550 CFU/g which corresponds to approximately $5 \cdot 10^3$ CFU/ sugar beet cube. The endogenous bacteria are the bacteria that exist naturally in plant tissue including roots of sugar beets (Jacobs et al. 1985).

5.1.1 Weight loss during storage

Losses in weight increased with time and were higher for samples stored at 15 °C than at 10 °C (see Table 2). Weight loss seems not to have been affected by inoculation of biocontrol and/or spoilage organisms.

Table 2: Weight loss in percent calculated as an average with n=2.

Sample	Weight loss %			
	1 month		4 months	
	10°C	15°C	10°C	15°C
D	1.8	2.5	9.4	14.7
E	2.3	3.9	11.1	14.4
F	1.9	4.0	10.6	13.5
G	1.8	3.2	14.4	17.4
H	2.5	3.5	12.1	16.3
I	2.0	3.2	13.5	13.9
J	1.8	2.0	9.7	15.4
K	2.0	4.4	12.4	15.5
L	1.7	2.8	10.4	16.9
Average	2.0	3.3	11.5	15.3
Stdev	0.3	0.8	1.7	1.3

The weight loss can be explained by the cell respiration of the sugar beets. During respiration carbon compounds, primarily sucrose for sugar beets, are oxidised to provide the cells with metabolic energy and substrates needed for maintenance (Klotz et al. 2008). During this process CO_2 is released (Klotz et al. 2008) and the sugar beet loses weight. For both temperatures, weight loss seams to increase with storage time, hence weight losses are larger after four months than after one. The weight loss has been taken into consideration in all subsequent calculations.

5.1.2 Microbial growth during storage

The results from the viable count made after one month are presented in Table 12 and after four months in Table 13 in Appendix I.

In summary, the conclusion from those results is that the number of endogenous bacteria has increased and consequently does not seem to be affected by biopreservation organisms on the surface of the sugar beet cubes. The amount of endogenous bacteria increased from 550 CFU/g to approximately 10^6 CFU/g in all samples. From the photos taken before opening the jars (Figure 1) it can be concluded that the visible mould

growth does not correspond to the results from the viable counts. This is likely explained by two factors; mostly by that *C. oleophila* inhibits mould growth on the plates but also by how much the fungi had sporulated on the cubes. Several fungal inhibitory compounds had earlier been investigated; none of which inhibited growth of *C.oleophila* JÄ3 (K Jacobsson, unpublished data). Therefore mould growth could not be reliably quantified.

Overall the conclusion is that the biopreservation did not work as the biocontrol organisms did not inhibit the growth of spoilage organisms. The treated samples, G-L, had as much growth as the untreated samples, D-F.

5.2 Volatile fatty acids content

The concentrations of volatile fatty acids in the sugar beets samples after storage are presented in Table 3. From those results it seems like samples treated with *P. fluoresence* KJ36 have a higher amount of volatile fatty acids in them.

Table 3: VFA concentrations. The values presented are mean values with n=2 and in the calculations weight loss have been regarded, i.e. the results are based on the initial weight of the sugar beet. Samples with no detectable concentration are not presented in the table.

Sample	VFA	Concentration g VFA/g SB
One month		
G (15°C)	Butyrate	0.03
I (15°C)	Butyrate	0.04
Four months		
G (10°C)	Butyrate	0.04
H (10°C)	Butyrate and valerate	0.04; 0.02
I (10°C)	Butyrate	0.06

5.3 Total and volatile solids

The amounts of total solids and volatile solids after adjusting for the volatile fatty acids concentration, as determinated by HPLC-analysis, is shown in Table 4.

The SB prestorage samples have the highest TS- and VS-values and the samples stored at 15˚ C for four months the lowest. The SB prestorage samples have in this experiment slightly lower values than found in literature; total solids (TS) content of 25% and volatile solid content of 23% (Carlsson and Uldal 2009).

The TS-value decreased for all treatments during storage. A lower TS-value means a higher content of water, less dry sample. During the respiration, carbon compounds are oxidised to CO_2 and H_2O, which explains the decreased TS-values. The VS-values also decreased for all samples during storage probably due to respiration by the sugar beets cells and growth of biocontrol and spoilage organisms which consumes carbon compounds; consequently this also affected the TS-value. The decreased TS- and VS-values are confirmed by the sugar analysis, which showed that the total amount of sugar decreased during the storage (Figures 3 and 4). The total sugar content decreased both in comparison to the SB prestorage samples and between the one month and four months samples.

Table 4: Total and volatile solids for the different samples and storage time as a mean value with n=2. The calculations have been made in regard to the weight loss for each sample, i.e. the results are based on the initial weight of the sugar beet.

Sample	Total solids %				Volatile solids %			
	1 month		4 months		1 month		4 months	
	10°C	15°C	10°C	15°C	10°C	15°C	10°C	15°C
D	19.0	18.7	14.9	11.9	18.8	18.4	14.6	11.4
E	17.9	17.1	11.1	8.0	17.8	16.6	10.8	7.7
F	19.0	18.8	13.7	8.8	18.7	18.7	13.4	8.4
G	19.1	19.9	13.4	6.8	18.9	19.4	11.8	6.4
H	18.4	16.7	17.4	7.0	18.1	16.5	15.5	6.8
I	18.3	20.8	16.2	6.7	17.9	20.3	14.3	6.5
J	19.8	19.0	13.3	7.0	19.6	18.6	13.6	6.8
K	17.8	15.8	7.4	6.1	17.4	15.5	7.1	5.8
L	19.7	18.7	12.5	6.8	19.5	18.4	12.2	6.4
Average	18.8	18.4	13.3	7.7	18.5	18.0	12.6	7.3
SB prestorage	21.4				21.1			

Temperature and storage time had a big impact on the TS- and VS-values. More organic material is utilised at a higher temperature and at a longer storage times.

5.4 Sugar content

5.4.1 Samples stored for one month

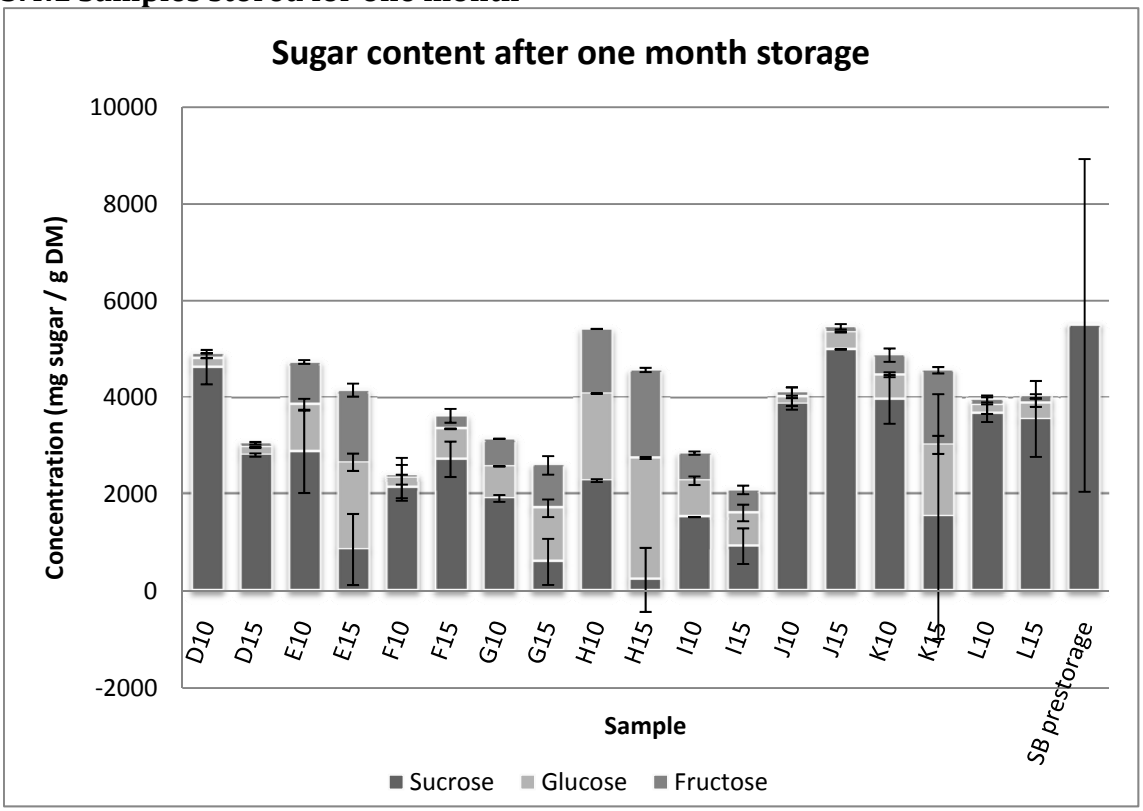

Figure 3: Sugar content after one month storage. The values presented are a mean value with n=2 and in the calculations weight loss have been regarded, i.e. the results are based on the initial weight of the sugar beet.

The total sugar content decreased in all samples compared to the SB prestorage (Figure 3). The SB prestorage sample mainly consisted of sucrose. The amount glucose and fructose have increased in the treated samples which indicate that the sucrose in the samples has been converted to glucose and fructose. In samples inoculated with

F. culmorum J617 (E, H and K) more sucrose has been converted compared to the other treatments. It seems like *C.cladosporioides* J308 does not have as strong effect on conversion of sucrose to glucose and fructose in the samples. This conversion is the main reason why sugar beets used for sugar production cannot be stored, but for bioenergy production, the conversion into monosaccharides it is likely less a problem.

5.4.2 Samples stored for four months

The sugar content is much lower for all samples stored four months compared to one month (Figures 3 and 4). Respiration and growth of spoilage organisms have decreased the total amount of sugar. Only the samples not treated with a biocontrol, D-F, contained detectable amounts of sugar after storage at 15°C. D, the totally untreated sample, is the only one among the 15°C samples with a significant amount of sugar. It therefore seems that both the addition of spoilage and biocontrol organisms decrease the amount of total sugar. The biocontrol agents have had no protecting effect on the sugar content or the conversion of sucrose into glucose and fructose. However, it cannot be excluded that sucrose has been converted to other sugars than glucose and fructose. During storage is e.g. raffinose formed from sucrose (Kenter and Hoffman 2009).

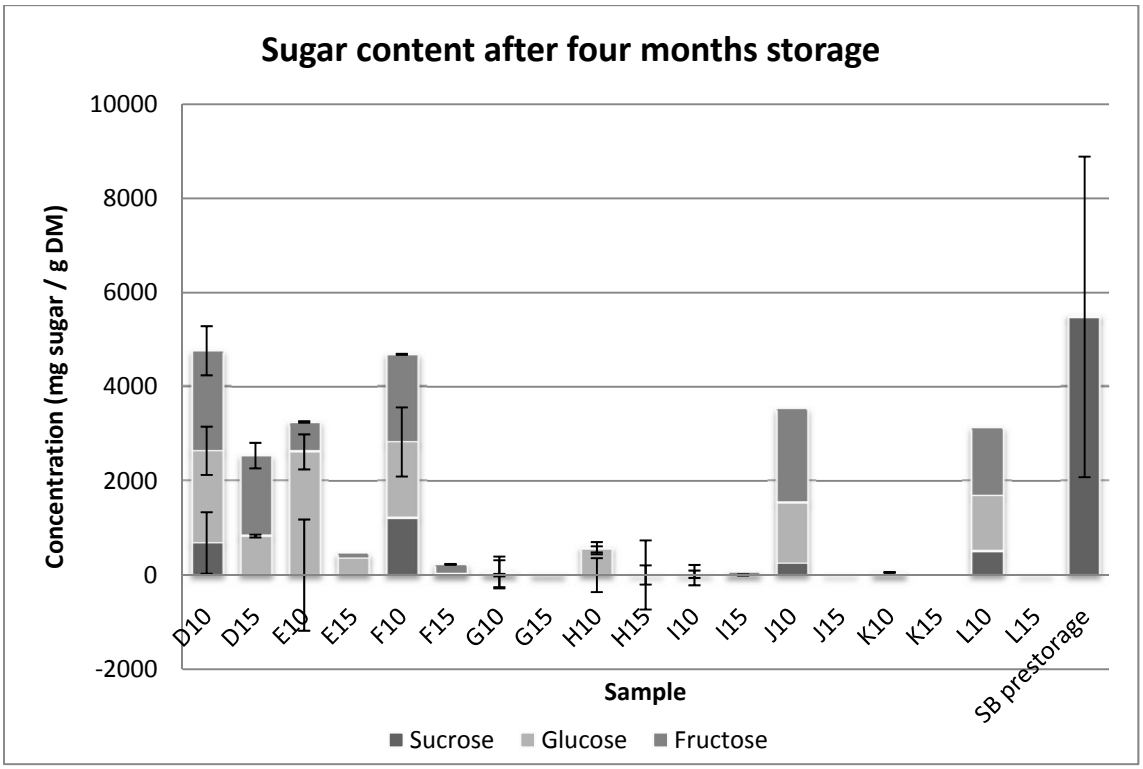

Figure 4: Sugar content after four months storage. The values presented are a mean value with n=2 and in the calculations weight loss have been regarded, i.e. the results are based on the initial weight of the sugar beet.

5.5 Ethanol production

5.5.1 Ethanol yields

The ethanol yield per g dry matter is presented in Table 5. The same SB prestorage material was used in all fermentations. Still, the ethanol yield per gram dry matter was lower in the first fermentation (10°C, one month) than in the other fermentations (Table 5). The reason for this in unknown and therefore the yields are also presented as percent of the SB prestorage sample in all fermentations to be able to compare the different treatments (Figure 5).

The ethanol yield per dry matter gives more information about how much bioethanol can be obtained after the different treatments of the sugar beet.

Table 5: Ethanol yield (g ethanol per g dry matter) for the different samples and storage time. The yields are presented as a mean value with n=2. The weight loss has been regarded in the calculations, i.e. the results are based on the initial weight of the sugar beet.

Sample	Ethanol yield (g ethanol per g dry matter)			
	1 month		4 months	
	10°C	15°C	10°C	15°C
D	0.27	0.31	0.23	0.08
E	0.27	0.27	0.15	0.00
F	0.27	0.29	0.19	0.00
G	0.24	0.20	0.02	0.00
H	0.25	0.28	0.05	0.00
I	0.25	0.22	0.04	0.00
J	0.27	0.30	0.19	0.00
K	0.26	0.25	0.00	0.00
L	0.26	0.30	0.19	0.00
Average	0.26	0.27	0.12	0.01
SB prestorage	0.28	0.35	0.34	0.32

When comparing the ethanol yield per gram dry matter, there was not a big difference between the different treatments after storage for one month (Figure 5). Yields are usually higher from samples stored at 10°C than from those stored at 15°C. There was a more significant difference between the samples stored one and four months (Figure 5). The samples treated with *P. fluoresence* KJ36, G-H, have the lowest yields for both temperatures after storage for four months. The ethanol yield obtained from samples treated with the biocontrol organism *C. oleophila* JÄ3, J-L, was not lower than for the untreated samples, D-F, for the 10°C samples except for sample K treated with *F. culmorum* J617. At four months, in all treated samples, E-L, stored at 15°C no ethanol production was observed. This corresponded to the sugar analyses (Figure 4), where the corresponding samples contained almost no sugar, thus no substrate from which the fermentation yeast could produce ethanol.

The conclusion from this is that during storage for one month, the storage temperature had little effect. No treatment was better than the untreated samples. The biocontrol organisms have rather decreased the yield in form of decreased sugar content and subsequent ethanol yield in samples stored for four months.

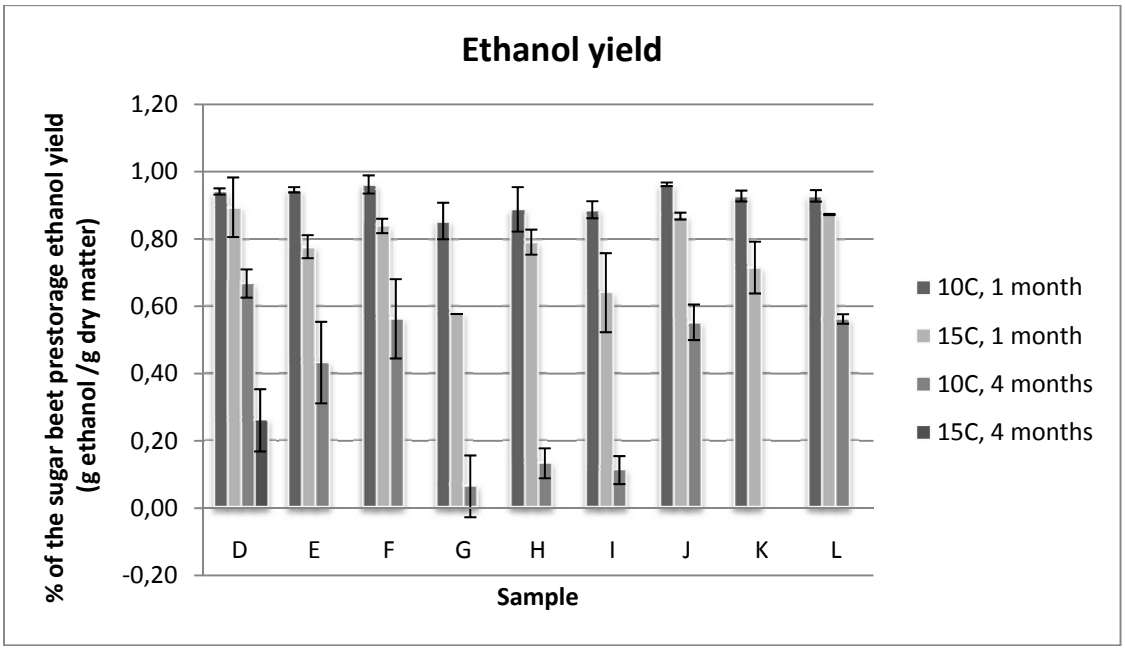

Figure 5: Ethanol yield (g ethanol / g dry matter) presented as percent of the SB prestorage sample. The yields are calculated as mean values with n=2 and the weight loss have been regarded in the calculations, i.e. the results are based on the initial weight of the sugar beet.

5.5.2 Viable count

5.5.2.1 One month storage at 10°C

Table 6: Viable count for the fermentation of the 10°C and 1 month storage samples after 42 h.

Sample	CFU/ml		
	Bacteria	Cycloheximide resistant yeast*	*S. cerevisiae*
D	<10	<10	3.6E+07
E	<10	7.0E+01	3.5E+07
F	<10	<10	2.8E+07
G	1.0E+07	<10	2.9E+07
H	<10	<10	3.6E+07
I	<10	7.5E+01	3.6E+07
J	<10	8.2E+04	2.7E+07
K	<10	2.2E+04	3.8E+07
L	<10	8.8E+04	2.1E+07
SB prestorage	<10	<10	4.6E+07

*****Cycloheximide resistant yeast is analysed to investigate if biocontrol organism *C. oleophila* JÄ3 is effecting the fermentation.

The amount of bacteria and yeasts in the fermentation samples is presented in Table 6. Overall, the amount of bacteria in fermentations of samples stored for one month at 10°C was very low. Only one sample, G treated with *P. fluoresence* KJ36 but no spoilage organisms, contained high numbers of bacteria $1.0 \cdot 10^7$ per ml. Thus the endogenous bacteria detected after storage does not seem to be a problem during fermentation. The samples with high growth of cycloheximide resistant yeasts were the samples with *C. oleophila* JÄ3 added as a biocontrol organism. The growth of *S.*

cerevisiae J672 was more or less the same for all samples, 2.1 – 3.8·10^7 CFU/ml for the stored samples and highest on 4.6·10^7 CFU/ml for the SB prestorage sample. The initial inoculum at the start of the fermentation was 2.2·10^7 CFU/ml.

5.5.2.2 One month storage at 15°C

The amount of bacteria and yeasts in the fermentation samples is presented in Table 7. These samples were stored at a higher temperature and had a higher bacterial growth than the samples stored at 10°C. The SB prestorage sample also showed growth of bacteria after the fermentation thus it cannot be excluded that the bacterial growth detected is the result of a contamination. In samples, D and H, cycloheximide resistant yeast was detected even though they are not treated with *C. oleophila* JÄ3. This suggests that there was *C. oleophila* naturally in the sugar beets or some other cycloheximide resistant yeast. Also in this fermentation the growth of *S. cerevisiae* J672 was in the same range for all samples suggesting that the bacterial contamination did not affect growth of the fermentation yeast. The initial inoculum at the start of the fermentation was 2.7 ·10^7 CFU/ml.

Table 7: Viable count for the fermentation of the 15°C and 1 month storage samples after 42 h.

Sample	CFU/ml		
	Bacteria	Cycloheximide resistant yeast	*S. cerevisiae*
D	<10	1.1E+02	5.8E+07
E	2.0E+07	<10	5.8E+07
F	5.4E+06	<10	6.8E+07
G	2.5E+05	<10	4.8E+07
H	1.5E+05	1.1E+03	4.8E+07
I	1.0E+05	<10	5.0E+07
J	<10	1.9E+05	6.5E+07
K	<10	2.5E+02	5.1E+07
L	<10	5.5E+04	5.1E+07
SB prestorage	5.1E+06	3.4E+02	6.1E+07

*****Cycloheximide resistant yeast is analysed to investigate if biocontrol organism *C. oleophila* JÄ3 is effecting the fermentation.

5.5.2.3 Four months storage at 10°C

The amount of bacteria and yeasts for the four months stored samples at 10°C is presented in Table 8. As different from the fermentations made from samples stored for one month, high amounts of moulds were detected (data not shown). The moulds thus have survived both the freezing and the fermentation. The number of cycloheximide resistant yeast was also slightly higher in this fermentation (Table 8) indicating a higher number of *C. oleophila* JÄ3 or some other cycloheximide resistant yeast naturally occurring in the sugar beets. The initial inoculum at the start of the fermentation was 2.2 ·10^7 CFU/ml. The growth of *S. cerevisiae* J672 was also the in the same range as previous fermentations.

Table 8: Viable count for the fermentation of the 10°C and 4 months storage samples after 42 h.

Sample	CFU/ml		
	Bacteria	Cycloheximide resistant yeast	*S. cerevisiae*
D	5.0E+05	9.9E+02	5.3E+07
E	<10	2.7E+03	2.1E+07
F	1.0E+01	<10	1.4E+07
G	1.0E+05	9.5E+05	1.4E+07
H	4.1E+06	2.5E+01	1.2E+07
I	<10	6.4E+05	2.3E+07
J	<10	4.4E+05	5.4E+07
K	6.9E+08	5.3E+06	4.2E+07
L	3.5E+05	2.3E+06	4.1E+07
SB prestorage	<10	<10	8.3E+07

*****Cycloheximide resistant yeast is analysed to investigate if biocontrol organism *C. oleophila* JÄ3 is effecting the fermentation.

5.5.2.4 Four months storage at 15°C

Samples stored for four months at 15 °C contained the highest levels of bacteria and moulds after fermentation (Table 9). The amount of cycloheximide resistant yeast was even higher than in fermentations of the samples stored at 10°C for four months. The higher storage temperature has favoured growth of yeast, bacteria and mould. The growth of *S. cerevisiae* J672 might therefore have been suppressed in some samples where the amount detected have decreased from the amount that was inoculated, $2 \cdot 10^7$ CFU/ml.

Table 9: Viable count for the fermentation of the 15°C and 4 months storage samples after 42 h.

Sample	CFU/ml		
	Bacteria	Cycloheximide resistant yeast	*S. cerevisiae*
D	<10	7.0E+03	3.0E+07
E	<10	1.9E+07	3.4E+07
F	5.6E+08	9.5E+05	1.5E+07
G	5.8E+08	2.1E+07	1.0E+05
H	7.0E+08	5.1E+06	2.5E+07
I	9.0E+08	1.6E+07	2.8E+07
J	5.8E+08	1.2E+07	7.7E+06
K	4.5E+08	1.2E+07	2.8E+07
L	5.5E+08	4.1E+06	3.2E+07
SB prestorage	<10	<10	7.4E+07

*****Cycloheximide resistant yeast is analysed to investigate if biocontrol organism *C. oleophila* JÄ3 is effecting the fermentation.

5.5.2.5 Conclusions from the viable count of the fermentation samples

Both a longer storage period and a higher storage temperature favoured the growth of endogenous bacteria, yeasts and moulds. The addition of biocontrol organisms did not inhibit growth of endogenous or spoilage organisms in samples stored for four months; it rather increased compared to the untreated samples, D-F. The primary reason for no ethanol production was the absence of sugar in the stored samples, but growth of spoilage organisms did not improve the situation. The growth of *S. cerevisiae* was

highest for the samples with high ethanol production. This correlate to that the production of ethanol is tightly coupled with the growth of yeast cells (Bai et al. 2008). The high growth of cycloheximide resistant yeasts in samples not inoculated with *C. oleophila* JÄ3 suggests that *C. oleophila* or some other cycloheximide resistant yeast occur naturally in the sugar beets used in this study.

5.5.3 DNA-fingerprinting

Unfortunately the results from these analyses were inconclusive.

5.6 Gas production

The graph over the accumulated methane production gives information of methane production over time. The final value gives information about the total methane potential for each treatment; i.e. how much methane that can be obtained from the substrate.

5.6.1 One month storage

5.6.1.1 Storage temperature 10°C

From Figure 6, a clear difference in production rate is shown. Treatment with the biocontrol organism *P. fluoresence* KJ36, samples G-I, seems to slow down the initial production rate. Instead, the production seems to continue for a longer time, 36 days, and for some of them give a higher methane potential than the other treatments (Table 10). The VFA-concentrations for sample G-I were below the detection limit see Table 3. High concentrations of VFA could otherwise have been an explanation to the inhibition of the production rate, hence high levels of VFA inhibits the methanogenesis (Gavala et al. 2003). It therefore seems like treatment with *P. fluoresence* KJ36 slowed down the initial methane production, suggesting that biomass from *P. fluoresence* KJ36 is slower to degrade.

The sudden increase in production at day 30 for sample D-F, J-K and the SB prestorage can be explained by the background production. Sugar beets are easily digestable for the biogas organisms and it seems like the digestion of the endogenous material from the inoculum does not start until the sugar beets have been digested. Several batch experiments with sugar beets have shown this behaviour, (A Schnürer, unpublished data). Therefore the methane potential for sample D-F, J-K and the SB prestorage was determined at day 27. The methane potential for samples G-I was determined at day 36.

5.6.1.2 Storage temperature 15°C

Treatment with *P. fluoresence* KJ36 effected the methane production for the samples stored for one month at 15°C (Figure 7). Also in this batch experiment the production was delayed at the start and throughout the experiment for samples G-I. Here the methane potential is lower compared to the other samples (Table 10). The gas production from sample H is delayed just as much for the samples with the same treatment stored at 10°C, while G and I are even more delayed than the corresponding samples stored at 10°C. Sample G and I had higher amounts of VFA, Table 3, suggesting a possible inhibition to the methanogenesis due to lower pH caused by the acids (Schnürer and Jarvis 2009). The methane potential was determined at day 29 for the SB prestorage, D-F and J-K samples and at day 37 for G-I.

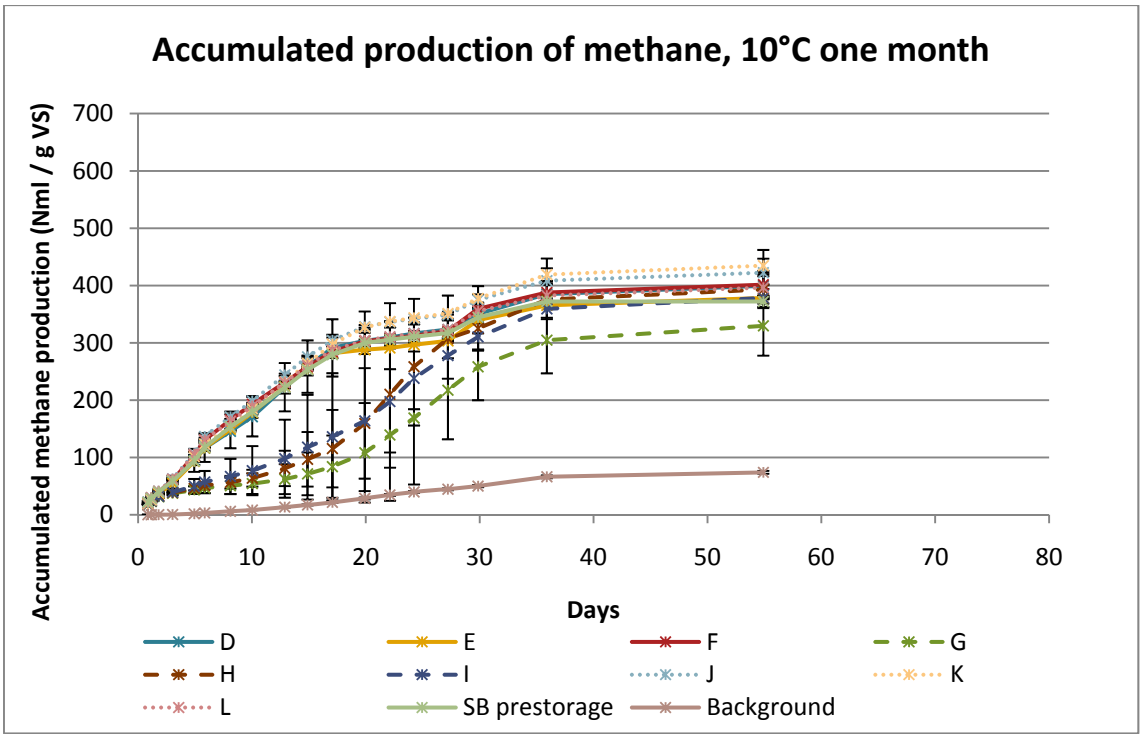

Figure 6: Accumulated production of methane in Nml / g VS, background production is withdrawn, for the one month storage at 10°C. Each treatment is an average between duplet samples and the accumulated production is standardised to 0°C and 1 atm.

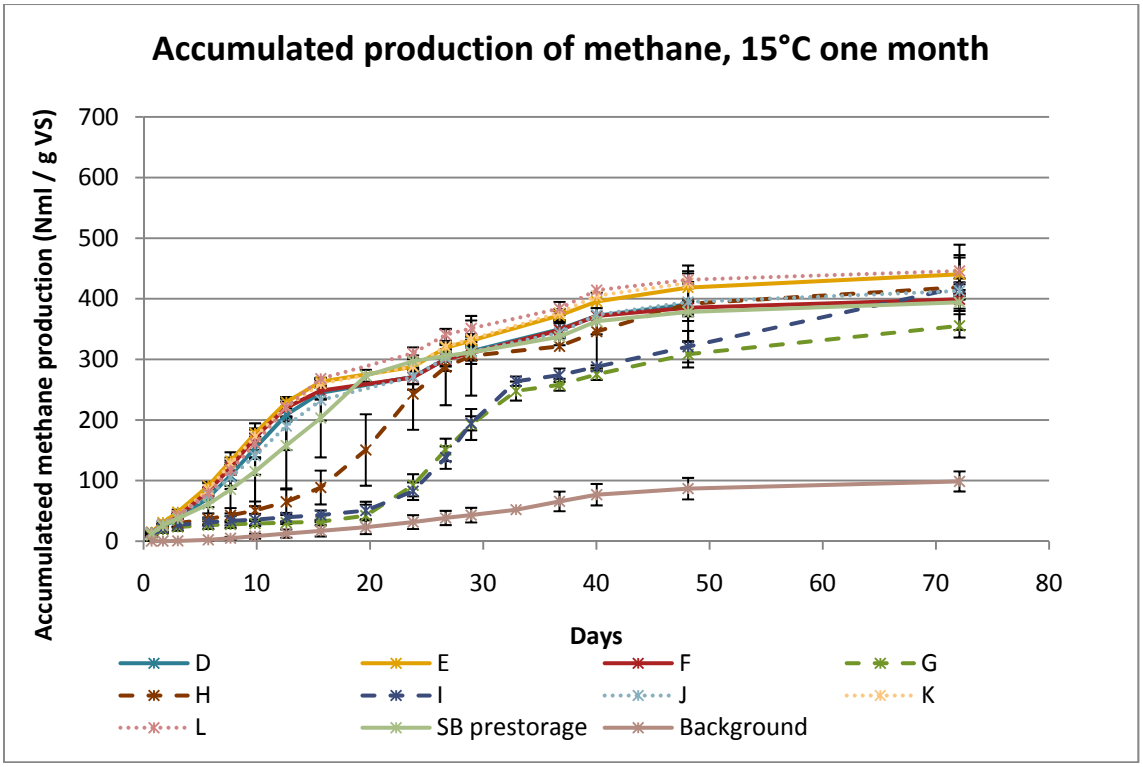

Figure 7: Accumulated production of methane in Nml / g VS, background production is withdrawn, for the one month storage at 15°C. Each treatment is an average between duplet samples and the accumulated production is standardised to 0°C and 1 atm.

5.6.2 Four months storage

5.6.2.1 Storage temperature 10°C

The samples with addition of *P. fluoresence* KJ36 appear to be much more delayed than digestions of the corresponding samples stored for one month (Figure 8). These samples also had a higher concentration of VFA (Table 3) suggesting a possible inhibition of the methanogenesis due to lower pH caused by the acids (Schnürer and Jarvis 2009). There was also a much bigger difference between the other samples (D-F, J-L and SB prestorage) compared to the previous two batch experiments. The methane potentials for samples D-F, J, L and the SB prestorage were determined at day 29 as previous digestions. Sample G-I were determined at day 45 and sample K at day 33. The methane potential is presented in Table 10.

5.6.2.2 Storage temperature 15°C

The methane potential for the sugar beet samples stored four months at 15°C is shown in Table 10. The anaerobic digestion with the sugar beet samples stored four months at 15°C is still in progress for sample G and H (Figure 9). The difference between the *P. fluoresence* treated samples, G-I, to the others is not as big as in the previous anaerobic digestions. Overall, methane production from all samples is slower than in the previous anaerobic digestions. The methane potential for sample D-F, J, L and the SB prestorage were therefore determined at day 35. Sample I was determined at day 44 while sample G and H still digesting at the latest day 57.

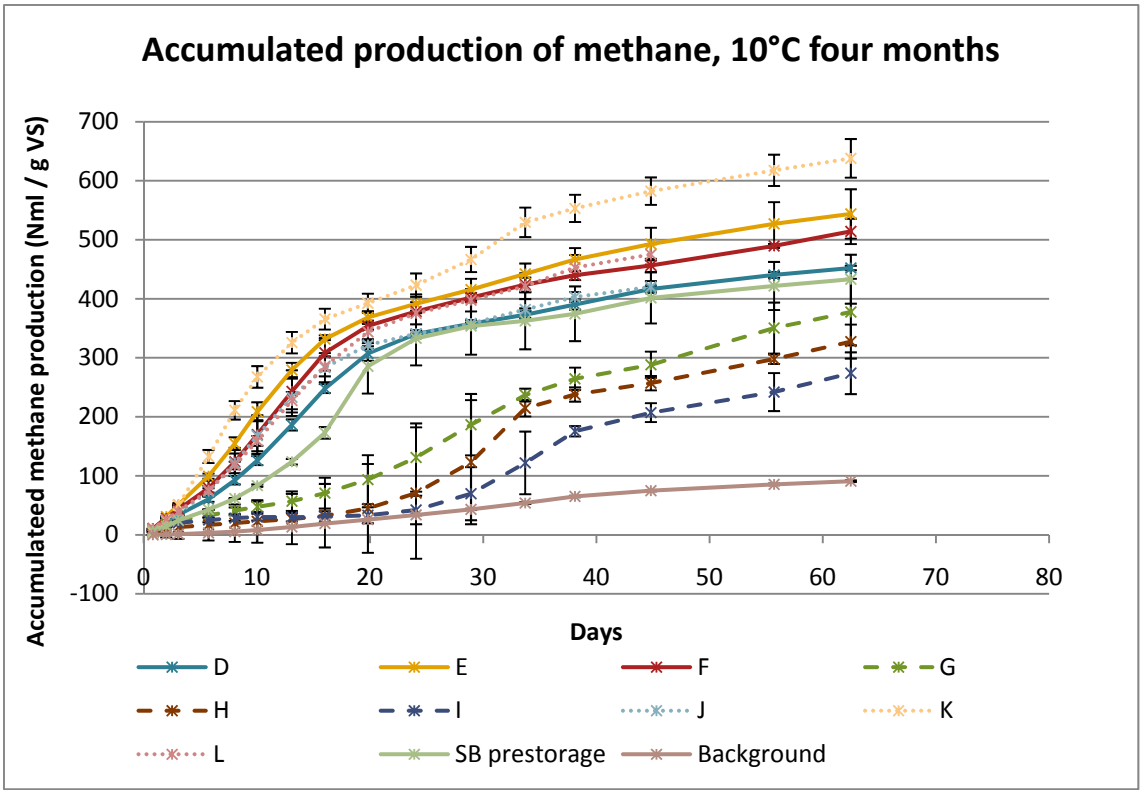

Figure 8: Accumulated production of methane in Nml / g VS, background production is withdrawn, for the four months storage at 10°C. Each treatment is an average between duplet samples and the accumulated production is standardised to 0°C and 1 atm.

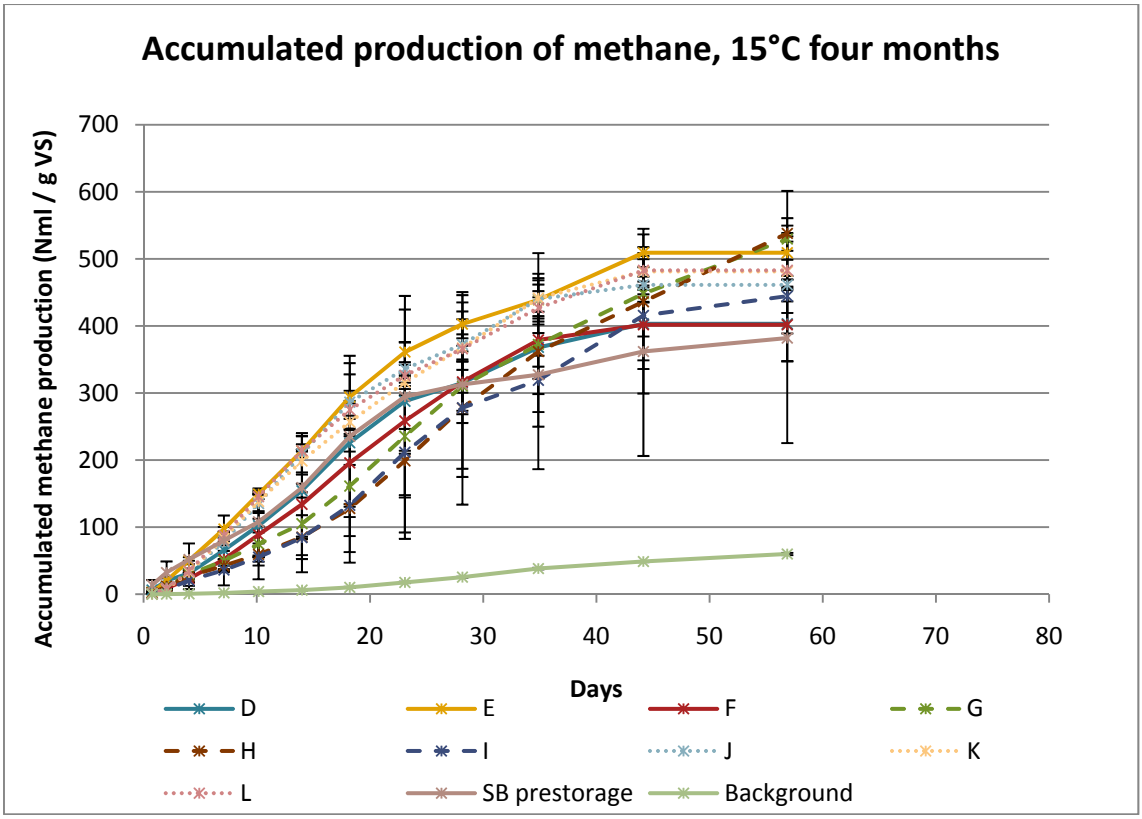

Figure 9: Accumulated production of methane in Nml / g VS, background production is withdrawn, for the four months storage at 15°C. Each treatment is an average between duplet samples and the accumulated production is standardised to 0°C and 1 atm.

5.6.3 Accumulated methane production

The methane production per g VS gives information about the methane potential of the samples compared to the methane production per wet weight which gives information if there had been some losses in methane potential during the storage. It is important to look at both these figures; otherwise if just methane production per g VS is analysed a sample stored for four months seems to be better than the SB prestorage sample.

The methane yield for the SB prestorage samples is slightly lower than 413 Nml/g VS and 80 Nml/g wet weight found in literature (Carlsson and Uldal 2009).

Table 10: Accumulated methane production per g VS and g wet weight, WW, standardised to 0°C and 1 atm. Background production is withdrawn.

Sample	Acc. methane production (Nml/g VS)				Acc. methane production (Nml/g WW)			
	1 month		4 months		1 month		4 months	
	10°C	15°C	10°C	15°C	10°C	15°C	10°C	15°C
D	333	313	356	368	55	50	42	28
E	303	331	416	439	49	46	33	22
F	330	310	402	379	55	49	43	19
G	345	258	288	530	54	37	18	17
H	401	322	257	538	61	36	27	20
I	367	274	207	416	54	43	17	14
J	359	313	358	440	63	51	39	17
K	351	334	530	441	54	42	23	13
L	332	351	398	427	58	56	38	15
Average	347	312	357	442	56	46	34	18
SB prestorage	328	311	354	327	62	58	58	37

Higher temperature and longer storage time decreased the production per wet weight, Table 10. This corresponded to the decrease in volatile solid contents during storage (Table 4). A lower VS content means lower carbon content and consequently less carbon available for methane production. This was also in accordance with decreasing sugar content during storage. The carbon compounds have been consumed by sugar beets' cell respiration and by the growth of biocontrol and spoilage organisms.

Of the biocontrol organisms *C. oleophila* JÄ3, J-L, appears to be better than *P. fluoresence* KJ36, G-I. Less methane is produced from sample G-I than J-L.

Another difference between samples treated with *P. fluoresence* KJ36, G-I and the others, was that the methane content in the produced biogas did not reach 50-60% until day 20 compared to day 10 for the other samples (not presented data). However, this was not the case for samples stored at 15 °C for four months where none of the samples reached the methane concentration 50-60% until day 20 (not presented data). This is also shown in the accumulated methane curves in Figures 6 to 9, where sample G-I are delayed in the first three digestions while accumulation is slower from all treatments in the last experiment.

5.6.4 Accumulated biogas production

The accumulated biogas production per g VS and wet weight is presented in Table 11 and illustrated for the one month 10°C samples in Figure 10.

The accumulated biogas production, was as the methane production, slightly lower for the SB prestorage samples than found in literature 787 Nml/g VS and 150 Nml/g wet weight (Carlsson and Uldal 2009). The biogas production per wet weight gives, just like methane production per wet weight, a more accurate value to analyse than the production per g VS.

The accumulated biogas production follows the accumulated methane production which is illustrated in Figure 10.

Table 11: Accumulated biogas production without background per g VS and wet weight, WW, standardised to 0°C and 1 atm.

Sample	Acc. biogas production (Nml/g VS)				Acc. biogas production (Nml/g WW)			
	1 month		4 months		1 month		4 months	
	10°C	15°C	10°C	15°C	10°C	15°C	10°C	15°C
D	833	760	760	776	132	114	85	59
E	715	780	876	997	103	100	64	45
F	790	753	837	872	127	114	84	43
G	770	471	657	1192	115	64	39	34
H	895	653	550	1205	130	75	54	40
I	860	473	480	1003	122	68	36	30
J	825	739	741	992	139	113	74	35
K	793	790	1107	1048	116	92	41	29
L	781	804	833	1028	131	122	73	34
Average	807	691	760	1013	124	96	67	39
SB prestorage	776	643	687	463	142	116	121	71

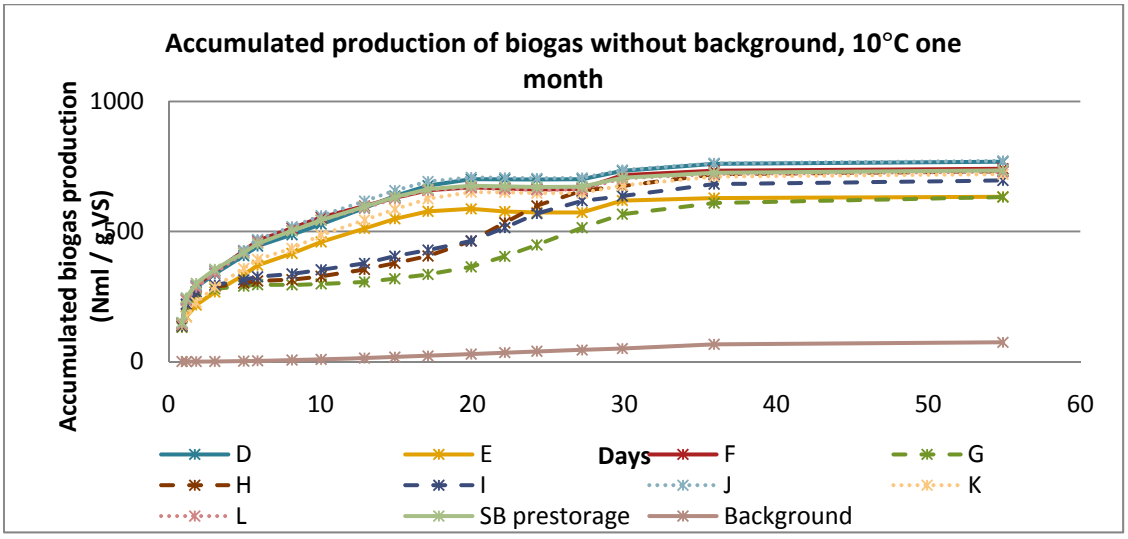

Figure 10: Accumulated production of biogas without background for the one month storage at 10°C. Each treatment is an average between duplet samples and the accumulated production is standardised to 0°C and 1 atm.

6 Conclusions

The aim of the study was to evaluate if the storage time of sugar beets could be prolonged if the biocontrol organisms *Candida oleophila* JÄ3 or *Pseudomonas fluoresence* KJ36 were used. The effect of the biocontrol organisms on sugar content, growth of spoilage organisms and yields of biogas and ethanol was studied to evaluate the effect of the organisms.

The biopreservation did not work as the biocontrol organisms did not inhibit the growth of spoilage organisms. The endogenous bacteria increased in number and consequently do not seem to be affected by biopreservation organisms on the surface of the sugar beet cubes. Neither was mould growth on the surface inhibited.

The biocontrol agents did not prevent the decrease in sugar content or the conversion of sucrose into glucose and fructose during storage; as the sugar content decreased and the fraction of glucose and fructose increased with time. However, it cannot be excluded that sucrose has been converted to sugars other than glucose and fructose. The temperature and storage time had a large impact on sugar content as it decreased in all treatments, especially between one and four months of storage.

The decreasing sugar content corresponds to decreasing TS- and VS-values. More organic material was utilised at 15 °C than at 10 °C. Carbon compounds have most probably been consumed by the cell respiration of the sugar beets and also by the growth of biocontrol and spoilage organisms.

The decreasing VS-value indicates that less carbon was available for methane production and therefore the production per wet weight decreased with time. Further, less methane was produced from samples treated with either of the two biocontrol organisms than from untreated samples The methane accumulation was slower for samples treated with *P. fluoresence* KJ36 in all experiments except in the 15°C four months trial, where accumulation was slow for all treatments.

The temperature had little effect on the ethanol yield after storage for one month. No treatment was better than the untreated samples. The biocontrol organisms have rather decreased the ethanol yields by decreasing the sugar content in samples stored for four months. The primary reason for no ethanol production is the absence of sugar in the stored samples, but growth of spoilage organisms did not improve the situation.

The biogas production was not as much affected by storage as the ethanol production. Hence the biogas process is less dependent on sucrose, glucose and fructose concentrations than the ethanol fermentation.

From this study it can be concluded that the biopreservation organisms, *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3, did not to inhibit the various fungal pathogens as intended. Consequently, the effect on sugar content, and therefore also on the ethanol production was negative. The biogas production was decreased by the consumption of organic material by the biocontrol and spoilage organisms. The storage time of sugar beets cannot be prolonged using the biocontrol organisms *Pseudomonas fluoresence* KJ36 and *Candida oleophila* JÄ3.

7 Acknowledgement

I would like to send a big thank you to my supervisor Karin Jacobsson for her guidance and support during the project. I also thank Anna Schnürer and Maria Erikson for all their help with the biogas experiments, Elisabeth Börjesson for all her help with the HPLC-analyses and Henrik Hansson for the help with HPAEC-PAD-analysis. The department of Microbiology, SLU, and MicroDrivE research program for giving me the opportunity of doing my thesis work at SLU. Finally, I would like to thank Emily, David and Ingrid for the company, especially Ingrid for all our times in the little "skrubb" and for discussing this master thesis with me during its progress.

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Appendix I

Microbial growth during storage

Table 12: Viable counts after one month.

Sample	CFU/ml					
	Bacteria		Yeast		Mould	
	10°C	15°C	10°C	15°C	10°C	15°C
D	1.1E+08	8.7E+07	5.4E+05	3.6E+05	1.3 E+04	7.0E+04
E	6.0E+07	1.6E+08	9.1E+05	3.4E+06	5.0E+04	4E+04
F	1.8E+08	3.2E+08	1.0E+05	2.0E+05	2.4 E+07	1.1E+07
G	1.0E+08	1.0E+08	9.7E+06	1.2E+08	<1.0E+04	5.0E+05
H	4.0E+07	4.0E+07	4.7E+06	2.0E+07	6.5E+04	1.9E+06
I	7.5E+07	7.5E+07	2.0E+07	1.1E+08	1.3E+07	2.0E+07
J	2.4E+07	2.4E+07	1.6E+08	2.1E+08	<1.0E+04	0<1.0E+04
K	1.9E+07	1.9E+07	1.0E+08	4.1E+08	<1.0E+04	<1.0E+04
L	5.7E+07	5.7E+07	1.1E+08	1.9E+08	1.1E+06	<1.0E+04

Table 13: Viable counts after four months.

Sample	CFU/ml					
	Bacteria		Yeast		Mould	
	10°C	15°C	10°C	15°C	10°C	15°C
D	4.6E+07	1.3E+07	3.3E+07	2.5E+06	1.1E+07	2.5E+06
E	9.0E+05	2.0E+06	1.2E+07	8.9E+07	2.8E+05	2.2E+06
F	4.2E+06	<1.0e+06	2.6E+06	3.0E+06	2.9E+07	2.1E+07
G	<1.0E+05	4.0E+06	3.8E+08	1.3E+09	1.4E+07	1.0E+07
H	<1.0E+05	6.9E+06	1.8E+08	5.7E+08	3.6E+07	1.0E+07
I	8.0E+04	1.1E+08	6.8E+08	7.9E+08	7.5E+06	1.1E+07
J	3.6E+06	3.6E+06	6.7E+08	7.3E+08	<1E+06	6.0E+06
K	9.0E+05	9.0E+05	1.4E+09	1.4E+09	<1E+06	2.0E+06
L	1.5E+07	1.5E+07	3.8E+08	5.8E+08	6.5E+06	3.5E+06

Appendix II

HPAEC-PAD

The method is based on that negatively charged OH- groups are delayed different in the column. The amount of electricity needed to oxidise the sample in the detector are compared to the standard solutions used and a measure of the amount of the specific OH-group in the sample.

Eluents used was;
A: 100% H2O
B: 0.2 M NaOH

The flow was 1 ml/min.

The samples were separated after following programme;

Before injection of the samples the column was equilibrated:
-9 min -> -7 min: isocratic elution with 50% A + 50% B (Washing/regeneration of the column)
-7 min -> -6 min: fast gradient to 100% A
-6 min -> 0: isocratic elution, 100% A (Preparation of separation for the sample)

Injection:
Time: 0 min

Separation:
-0 -> 17.5 min: isocratic elution, 100% A (Separation part one, most monosaccharides are eluted here)
-17.5 min - 28 min: gradient till 50% B (Separation part two, remaining monosaccharides and disaccharides are eluted)

Detection:
To have a good signal at detection a post column is used, PA10-coulmn (Dionex, Sunnyvale, USA), with a flow of 75 mM NaOH at 0.25 ml/min.