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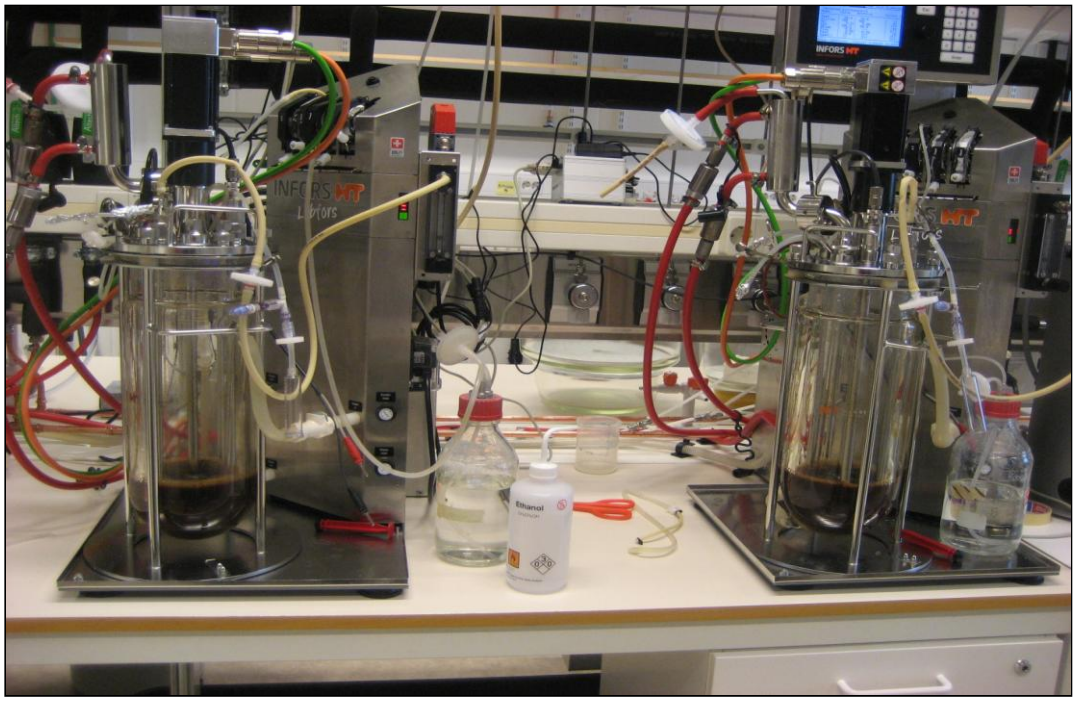

Effects of nutrients supplementation on fermentability of lignocellulosic hydrolysates under high gravity conditions.

Master of Science Thesis

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Preface

This project was performed as a partial fulfilment for Master of Science in Engineering Biology at The Institute of Technology at Linköping University. The work was carried out from February until June 2012 at the Industrial Biotechnology group, Department of Chemical and Biological Engineering, in Chalmers University of Technology, Göteborg, Sweden.

Abstract

Bioethanol produced from lignocellulosic materials is emerging as a promising alternative to fossil fuels. Fermentation of these substrates results in low yields due to the presence of inhibitory compounds and non-fermentable sugars. The main advantage of biomass bioconversion under HG (initial dry mater concentrations above 10% w/w) or very high gravity (VHG, above 30% w/w) conditions is the generation of high final ethanol concentrations (above 5% v/v), decreasing the cost of the distillation step, which is considered one of the main constraints in the bioethanol industry. However, many challenges are associated with the increase in initial dry mater. With this the generation of inhibitors during the pre-treatment step is increased, which in turn dramatically reduces the fermentability of the material.

In this project, the effects of nutrients on the fermentative performance of *Saccharomyces cerevisiae* on toxic spruce hydrolysates were evaluated. Fermentations were performed with the addition of selected nutrients to evaluate their effects on inhibitor tolerance and fermentation rates. Yeast extract was found to have positive effects on the fermentation rate and biomass growth in comparison to other nutrients at fermentations in 78% of hydrolysate. The effect was evaluated with smaller inoculum sizes, 1 and 0.5 gL^{-1}. For 1 gL^{-1} of initial cell dw, and less, the nutrient addition was crucial for biomass growth and ethanol formation. Cultivations performed with an initial cell density of 1 gL^{-1} and with yeast extract supplementation resulted in an ethanol concentration of 19.2 gL^{-1}. In comparison, the unsupplemented cultivation produced an ethanol concentration of 1.5 gL^{-1}. The conversion of inhibitory compounds was also affected by the supplementation. At 1 gL^{-1} of inoculums, HMF and furfural was not converted to less toxic compounds without the addition of yeast extract. The positive effects were found to be independent from the concentration starting at 0.5% w/v of yeast extract. The results were used for scaling-up experiments in bioreactors. In these controlled conditions the addition of yeast extract resulted in higher production rates and yields. Spruce hydrolysate was found to be fermentable at a concentration of 90%. However, the high concentration resulted in lower ethanol yield. A yield of 0.16 was reached after 36 hours for the fermentation at 90% of hydrolysate, which is considerably lower than the theoretical yield of 0.5. Fermentations at 80 % of hydrolysate reached yield of 0.35

Key words: Lignocellulosic hydrolysate, High gravity fermentation, yeast extract, inhibitors

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1. Introduction

The interest for alternative fuels is not new. With the increases in oil prices in the 1970s the need for alternative fuels increased. The goal then was to be less dependent on oil and on the oil producing countries, today the depletion of oil and the increasing emission of greenhouse gases is the main focus and it has driven the need for new fuels further. Bioethanol and other fuels produced from renewable and natural resources can become a competitive alternative to petroleum based fuels.

The raw material for bioethanol production today mainly originates from sugar canes or maize, the production is therefore primarily located in Brazil and USA respectively. Bioethanol produced from these or other sucrose and starch rich materials are referred to as 1st generation bioethanol. This process is well known and optimized for an efficient ethanol production (Almeida et al. 2007). Using these feedstocks is however not perfect solution as the price of the raw material itself can reach up to 40 % of the bioethanol cost as reviewed by Zaldivar et al (2001) and there is also an ethical dilemma in growing crops for fuel production when land is needed for the cultivation of food or feed.

An interesting resource that can be utilized for ethanol production is lignocellulosic materials, such as residues from the forest industry and agricultural, municipal or industrial wastes. The ethanol produced from these raw materials can be referred to as 2nd generation bioethanol (Almeida et al. 2007). Lignocellulosic feedstocks are an abundant and cheap source of sugars for ethanol production and therefore serve as a good alternative to sucrose and starch rich materials. However the production of ethanol from lignocellulose is connected to a number of difficulties. When using these materials additional steps have to be added, to the ethanol production process, to achieve a fermentable substrate. These process steps lead to increased costs and introduce inhibitory compounds to the fermentation broth. To have an efficient production there is a need for further knowledge about the process steps, how to optimise them and about how the material affects the fermenting organism (Olsson and Hanh-Hägerdahl 1996).

For an economically favourable production of ethanol, the yield of ethanol and the time needed are crucial parameters. Higher ethanol concentrations lead to lowered recovery and distillation costs. By using high gravity conditions higher ethanol concentrations can be achieved. This means that a high concentration of fermentable sugars is present at the start of fermentation. This process however, imposes stresses on the fermenting organism both in terms of higher osmotic stress on the cells as well as an elevated concentration of the inhibitors formed during pre-treatment and hydrolysis (Jørgensen et al. 2007).

1.1 Problem formulation

Cultivations performed with lignocellulosic hydrolysates at high gravity are nutrient limited while the concentration of inhibitors is high. It is therefore crucial that the microorganism used for fermentation can withstand these harsh conditions. With the nutrient supplementation the cells viability and growth is positively affected and thereby also their ability to convert and tolerate the inhibitors present in the hydrolysate. Such nutrients will be identified and tested to improve the overall fermentative performance of *S .cerevisiae*

1.2 Aim of the thesis

The aim of this master thesis was to improve the overall ethanol production process by nutrient supplementation of spruce hydrolysate. Moreover, the effects of specific nutrients on the fermentative performance of *S. cerevisiae* were investigated with regards to tolerance of the inhibitors found in the hydrolysate.

Distinguish a nutrient that has positive effects on the fermentative performance of *S. cerevisiae* in spruce hydrolysate, in a screening performed in 15-50 mL scale (falcon tubes and shakeflasks)

Evaluate the effects of the found nutrients in relation to ethanol fermentation and inhibitor tolerance and if the effects are influenced by the concentration used for supplementation

Use the findings and evaluate these in a larger (1.5L) with a controlled environment (constant temperature, pH and stirring).

2. Theoretical background

2.1 Bioethanol as fuel

Ethanol was used as a transport fuel as early as 1905 and Henry Ford was at this time planning for it to be the main fuel of the T-ford. Unfortunately, the price of ethanol soon became higher than that of gasoline and the interest for ethanol came to a halt, as reviewed by Mousdale (2008) However the interest for this fuel was not lost and Brazil as one of few countries has actively utilised and produced ethanol as a fuel source since 1925 (Balat 2009) and is today the largest and together with the US the main producers of bioethanol (Demirbas et al. 2009).

Today's regained interest for bioethanol and other biofuels is due to multiple factors. Among these the main motives are the need to be less dependent on oil, due to higher oil prices and oil depletion, and the will to lower our emission of greenhouse gases

Bioethanol produced today is mainly produced from feedstocks rich in sugar and starch, such as grains, maize or sugarcanes (Tian et al. 2009). If renewable energy is used during the cultivation the net emission of carbon dioxide for the fuel is zero. This since the same amount of carbon dioxide produced during combustion will be absorbed during the growth of new plants (Galbe et al. 2005).

Bioethanol has a number of advantages apart from its low emission of CO_2, but in comparison to gasoline there are also a number of weaknesses. These advantages and disadvantages are presented in table 1.

Table 1 Advantages and disadvantages of ethanol as a fuel. (Balat 2009)(Wheals et al. 1999)

Advantages	**Effects**
Higher octane rating compared to gasoline	Can therefore replace octane enhancers in gasoline, this will lead to reduced emission of particles , a higher octane number will also result in a higher efficiency of the fuel
Can be used as a oxygenated fuel additive	Reduced emission of CO, NO_x, and hydrocarbons
Renewable	No risk of depletion
Can be used in blends with gasoline in modern cars	Reduced CO_2 emission
Disadvantages	
Lower energy density	Higher fuel consumption compared to gasoline
Corrosiveness	
Lower vapour pressure	Harder to start the engine when cold

2.2 Second generation bioethanol

To use lignocellulosic material as a feedstock is an attractive alternative to growing crops designated for fuel production. This since woody biomass is an abundant feedstock widespread throughout many continents and the cost of the raw material is lower than that of designated crops, it may therefore serve as an economically favourable material source for ethanol production (Olsson and Hahn-Hägerdal 1996).

Lignocellulosic feedstocks are non-food biomass sources, such as woody biomass or waste products. The waste used may come from agricultural and forest residues as well as form

industrial or municipal waste (Galbe and Zacchi 2002). The difference between using these materials and using starch or sugar rich crops for bioethanol production is the chemical and physical structure of lignocellulose, making it resistant to enzymatic hydrolysis, leading to a need for additional process steps in order to have fermentable substrates for the ethanologenic microorganisms (Klinke et al. 2004).

Using lignocellulose as a feedstock have a number of advantages, as earlier stated the price of the raw material is lower than the material used for 1st generation bioethanol and apart from this, the price of storage can also be lower since the material can be harvested year around and has a higher density (Zhu and Pan 2010). However, the production of ethanol using lignocellulose cannot compete with the production from sucrose or starch or that of gasoline, due to higher costs of the production process and lower ethanol yields compared to 1st generation bioethanol. The lower yield can be explained by degradation and loss of sugars during the pre-treatment, inhibition from inhibitory substances in the fermentation broth and that all sugars in the material are not fermentable by microorganisms. To achieve a favourable production there is a need for optimisation at all stages of the production (Olsson and Hahn-Hägerdal 1996). When looking at other aspects there is other advantages, agricultural land can be used for food or feed production instead of for fuel, when crops are not used there is less need for pesticides, fertilisers and the soil degradation connected to intensive cultivation is limited. This will in the long run have an economically favourable effect (Wheals et al. 1999).

The production of ethanol from lignocellulosic materials can be made more profitable if all parts of the material are used. In this biorefinery concept – an integrated process that converts biomass-derived streams to a selection of diverse product streams (Otero et al. 2007) - no part of the material is lost as wastes, thereby reducing the need for waste handling and will instead contribute economically to the process. An example of this is to use the effluents from the process, that can contain residual organic matter, for biogas production; leftovers from this process can be sold and used as fertilisers as reviewed by Kaparaju et al. (2009). Another important aspect is to be able to use the parts not fermentable, such as the lignin portion of the material and non-fermentable sugars. The lignin part is a high quality source of solid fuel; the heat generated from lignin can then be used for example during distillation an otherwise energy demanding process step. The non fermentable sugars can also contribute; these can be sold as feed for livestock (Larsen et al. 2008).

2.3 Lignocellulosic materials for ethanol production

The main components of plant biomass are cellulose, hemicelluloses, and lignin. These components form a complex that is linked via both non-covalent forces and covalent linkages. The composition of the material differs depending on the source of the raw material (table 2) (Olsson and Hahn-Hägerdal 1996). Apart from these main components, that form 90% of the dry matter, extractives (soluble in organic solvents or water) and ash will be found in the remaining dry matter as reviewed by Klinke et al. (2004) Cellulose and hemicelluloses have together a total carbohydrate level of up to 70%. However not all sugars that originate from hemicelluloses are fermentable by *S. cerevisiae* (Klinke et al. 2004).

Table 2 Carbohydrates content of different wood feedstocks. Values are w/w % of the woods dry weight. Adapted from Taherzadeh et al. (1997)

Material	Hexoses[a]	Pentoses[b]	lignin	Extract and ash
Spruce	55.1	4.6	25.7	5.7
Pine	56.1	4.7	24.7	4.9
Aspen	45.9	15.1	16.0	5
Alder	42.8	16.1	20.8	9.4

[a] - glucose, mannose and galactose, [b] - xylose

2.3.1 Cellulose

Cellulose is the main component of lignocellulose, 36-61% of the total dry matter. It is a long polymer consisting of D-Glucose molecules; these are connected via a β-1, 4 linkages in pairs forming cellobiose units (figure 1). These units form long linear polymers that are arranged in a crystalline structure (Olsson and Hahn-Hägerdal 1996). Hydrogen bonds and van der Waals forces are formed between these polymers. The rigid and resistant traits of these polymers are due to these bonds, the orientation and the crystalline structure that the polymers form. With the surrounding environment consisting of hemicelluloses and lignin a though and stable material is formed (Pérez et al. 2002).

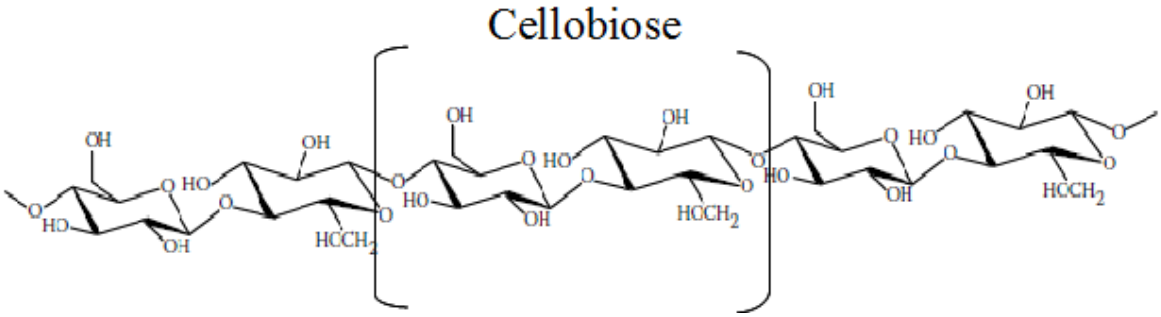

Figure 1 The chemical structure of cellulose: Several hundred to over five thousand Cellobiose units are connected with *β*-1, 4 linkages forming a linear chain-polymer

2.3.2 Hemicelluloses

The other carbohydrate structure of lignocellulose, hemicellulose, is a polymer based on different sugars. The hetropolymers are composed of pentoses and hexoses and are highly branched. Which sugars that is predominant is dependent on the source of the material used, such as the type of wood. Woody biomass is generally divided in to hard- and softwoods, hardwoods are wood derived from angiosperm trees, such as aspen and birch, while softwoods are gymnosperms (evergreen trees). Sugars that are found in hemicelluloses are: D-glucose, D-mannose, D-arabinose, D-galactose and D-xylose. Hemicellulose differs from cellulose in that its polymers are more easily hydrolysed in to monomeric sugars (Pérez et al. 2002). Hemicelluloses, also called polyoses, can be categorised based on their content. These are named after the backbone and side chains. In softwood for example the main type of hemicelluloses is acetylated galactoglucomannans where the backbone comprises of linked glucose and mannose molecules, while in the side chains are acetyl and galactose molecules. Other hemicelluloses that are found in softwoods are arabinogalactan, and xyloglucan. In hardwood other hemicelluloses are found as reviewed by (Peng et al. 2012). As a result of this

diversity the amount and type of sugars that will be present after hydrolysis is dependent on the material used.

2.3.3 Lignin

Lignin is an aromatic polymer consisting of phenylpropane units and is not soluble in water. Lignin can for these reasons not be used in the purpose of fermentation (Almeida et al. 2007). This molecule provides the cell wall with structural support and protection against microbes (Pérez et al. 2002).

2.3.4 Spruce

The material used during this master thesis project was spruce, pre-treated with dilute sulphurous acid and thereafter enzymatically hydrolysed.

Spruce belongs to the group of softwood. This material contains a relatively small amount of pentoses, which can be said to be low in comparison to other substrates, while the lignin content is higher see table 1 (Taherzadeh et al. 1997). The higher amount of lignin will result in a more rigid material, with the need for harsher pre-treatment conditions, because of the low pentose levels no pentose fermenting microorganism is needed (Galbe and Zacchi 2002).

2.4 Ethanol production from lignocellulosic materials

When producing ethanol from lignocellulose a number of steps are needed to have fermentable sugars available in the fermentation broth, steps that are not needed for 1st generation bioethanol production. This is due to the structure and composition of the material which resists enzymatic hydrolysis and therefore also fermentation of the carbohydrates. To have a successful enzymatic hydrolysis, break down of the crystalline structure of and exposure of the sugar polymers are needed.

The process steps involved in ethanol production from lignocellulose are pre-treatment, hydrolysis and fermentation followed by product recovery (Olsson and Hahn-Hägerdal 1996). The process flow is presented in figure 2. There are a number of methods to be used for pre-treatment and two methods normally used for the hydrolysis.

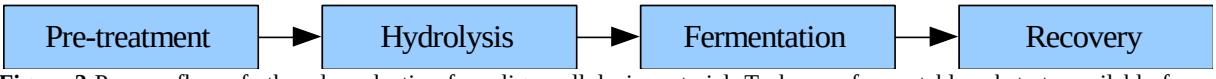

Figure 2 Process flow of ethanol production from lignocellulosic material. To have a fermentable substrate available from lignocellulosic materials pre-treatment and hydrolysis is essential.

The hydrolysis and fermentation step can be performed either separately or simultaneously. When performed separately the process is referred to as separate hydrolysis and fermentation (SHF) when using this method each step can be performed at optimal conditions, but the hydrolysis will not be total because of product inhibition from glucose and cellobiose on the enzymes. With this method there will also be a high concentration of sugars at the start of fermentation which can inhibit the yeast. These problems can be avoided by using simultaneous saccharification and fermentation (SSF) where, as the name indicates, both the hydrolysis and the fermentation are performed at the same time. The released sugars are fermented directly and there will be no build up of sugars in the broth and less waste is also produced through the combining of two separate process steps. However there are also downsides to this processing technique. The two different steps do not have the same optimal conditions, therefore the process will be performed under conditions that are suitable for both but not optimal. Performing these simultaneously reduces the possibility of reusing the yeast as it is mixed with the solid residues in the fermentation broth (Galbe et al. 2005). SSF has

despite this been found superior to SHF when comparing the overall ethanol yields (Hoyer et al. 2010).

2.4.1 Pre-treatment

The object of the pre-treatment step is to render the carbohydrates in the cellulose accessible to the enzymes of the following step, this should be done while minimizing the destruction of fermentable sugars. In lignocellulose the cellulose forms a rigid structure with hemicelluloses and lignin and enzymes cannot access the sugar polymers of cellulose for hydrolysis. Thus the result of the pre-treatment is either a more porous material or a decomposed material where cellulose is exposed (Galbe and Zacchi 2002). In most cases lignin and hemicelluloses are solubilised during the pre-treatment whereas cellulose remains in solid form (Klinke et al. 2004). Hemicellulose is also hydrolysed by this steps resulting in free solubilised sugar molecules (Olsson and Hahn-Hägerdal 1996). Without the pre-treatment step the sugar yield after hydrolysis will be less than 20% of the theoretical value. With the use of pre-treatment the yields are raised to above 90% (Lynd 1996).

During pre-treatment there is a physical step which reduces the size of the material particles, increasing the surface area for enzymatic hydrolysis and a physico-chemical or chemical step which solubilise or remove the hemicelluloses and lignin components (Zhu et al. 2009).

The different methods used for pre-treatment have different mechanisms and the end result will differ. Therefore some methods are more suitable than other for specific materials. Following is a short description of some of these methods that are used before hydrolysis.

Chemical pre-treatments

Dilute acid pre-treatment is a method that utilises mineral acids such as sulphuric acid at low concentrations (less than 4% of the weight) at a temperature between 160 and 200°C. All constituents of the material is affected by this treatment, the acid cleaves the polymers of the material randomly (Mosier et al. 2005). The treatment will cause solubilisation of the hemicelluloses, but also a formation of volatile degradation products (Hendriks and Zeeman 2009). Concentrated acids is a powerful tool for pre-treatment but is associated with high toxicity, corrosiveness and a need for recovery of the used acids, leading to increased costs(Sun and Cheng 2002). Alkaline pre-treatment can be performed at a lower temperature and with less pressure than when using dilute acids. Sodium, potassium or ammonium hydroxide is used in the treatment which will result in a breakage of the bonds between lignin and the sugar polymers. Organic solvents such as methanol, ethanol and acetone are used in the pre-treatment method “organosolv”. The solvents are used together with a small amount of a mineral acid and solubilise the lignin (Galbe and Zacchi 2012).

Physico-chemical methods

Steam explosion is a method where chipped biomass is treated with a high-pressure steam; the pressure is then swiftly reduced resulting in an explosive decompression. The treatment will release acids from the material; these acids will then hydrolyse the hemicelluloses. Moreover, steam explosion might lead to production of HMF, furfural and phenolic compounds. The treatment is performed at a temperature between 160 and 260°C for a time period ranging from between several seconds up to a couple of minutes. Time needed is depending on the moisture content of the material. The result will dependent on time and the size of the chipped biomass. Addition of an acid such as sulphuric acid is beneficial for the following enzymatic hydrolysis and will also lead to less inhibitory compounds being formed. A similar method is ammonia fibre explosion (AFEX) which is performed at similar conditions but with the addition of liquid ammonia instead of steam (Sun and Cheng 2002).

Biological methods
Microorganisms such as fungi can also be used for the breakdown of lignocellulosic materials (Balat 2011)

For the pre-treatment of softwoods the alternatives for pre-treatment are fewer because of the higher recalcitrance in softwoods compared to other materials. Therefore acid hydrolysis and steam pre-treatment is the most appropriate and used methods (Galbe and Zacchi 2012).

2.4.2 Hydrolysis

Hydrolysis is the conversion of carbohydrate polymers to fermentable monomeric sugars.
There are two main ways of hydrolysis of the remaining cellulose fraction, enzymatic hydrolysis or hydrolysis performed with the use of acids, concentrated or weak (Taherzadeh et al. 1997).

Acid hydrolysis
When utilising acids the pre-treatment step and hydrolysis are performed simultaneously. Using concentrated acids will result in high yields at low operating temperature, but the acid recovery is energy demanding and equipment corrosion might be a problem. The use of dilute acids will reduce these problems, but instead high temperatures are needed. The increased temperature will increase the amount of hemicelluloses sugars that are degraded into by-products (Galbe and Zacchi 2002).

Enzymatic hydrolysis
Cellulose can be hydrolysed enzymatically by cellulases. The enzymatic hydrolysis results in higher glucose yields, fewer sugars are decomposed and fewer by-products are formed when sugars degrade than during acid hydrolysis (Taherzadeh et al. 1997).

Cellulase is a class of enzymes that simultaneously and synergistically can hydrolyse cellulose in to glucose. Endoglucanase, Exoglucanase and β-glucosidase are involved in the mechanism of this process. Endoglucanase will randomly cleave the intramolecular β-1.4-glucoside bonds; by this action new chain ends are produced. This ends can thereafter be digested in to cellobiose or glucose molecules by the action of Exoglucanase. Finally β-glucanase will cleave the cellobiose molecules into single glucose molecules. By doing this the product inhibition of cellobiose on exoglucanase is decreased. However, there is still product inhibition of glucose on β-glucosidase (Percival Zhang et al. 2006).

When comparing acid and enzymatical hydrolysos neither of them was found to be a less economical process than the other (von Sivers and Zacchi 1995).

2.4.3 Degradation products formed during pre-treatment and hydrolysis

The addition of process steps will apart from increasing the cost also introduce a number of inhibitory compounds to the hydrolysate. During the harsh conditions of the pre-treatment lignin and sugar are degraded to some extent, which products that are formed therefore depend on the type of sugar and lignin that is present (figure 3). Therefore the inhibitors found in the hydrolysate are depending on which source of material that is used, the sugar content of different materials is presented in table 1. The composition of lignin also differs depending on the material and thereby different inhibitors are formed when lignin is broken down (Palmqvist and Hahn-Hägerdal 2000).

Also affecting the amount of inhibitors formed is what pre-treatment methods and condition that have been used. The temperature, time and pH used during this process step will affect the inhibitor concentration. In having more harsh conditions more of the hemicelluloses will be hydrolysed however more severe conditions will also lead to a higher degree of sugar degradation and by that more inhibitors formed as reviewed by Palmqvist and Hahn-Hägerdal (2000).

The majority of the inhibitors formed during pre-treatment and the following hydrolysis belong in three chemical groups: furan derivatives, weak acids and phenolic compounds. The most usual furan derivatives, furfural and 5 hydroxymethyl-2-furaldehyde (HMF), are formed when sugars are dehydrated. Furfural is the product of pentose degradation while HMF originates from hexoses. The weak acids include acetic-, formic-, and levulinic acid. Acetic acid is produced as a result of de-acetylation of hemicelluloses, while levulinic and formic acid are formed from when HMF degrades. Formic acid can also be produced from furfural under acidic conditions. The third group, phenolic compounds, are produced during lignin degradation (Almeida et al. 2007).

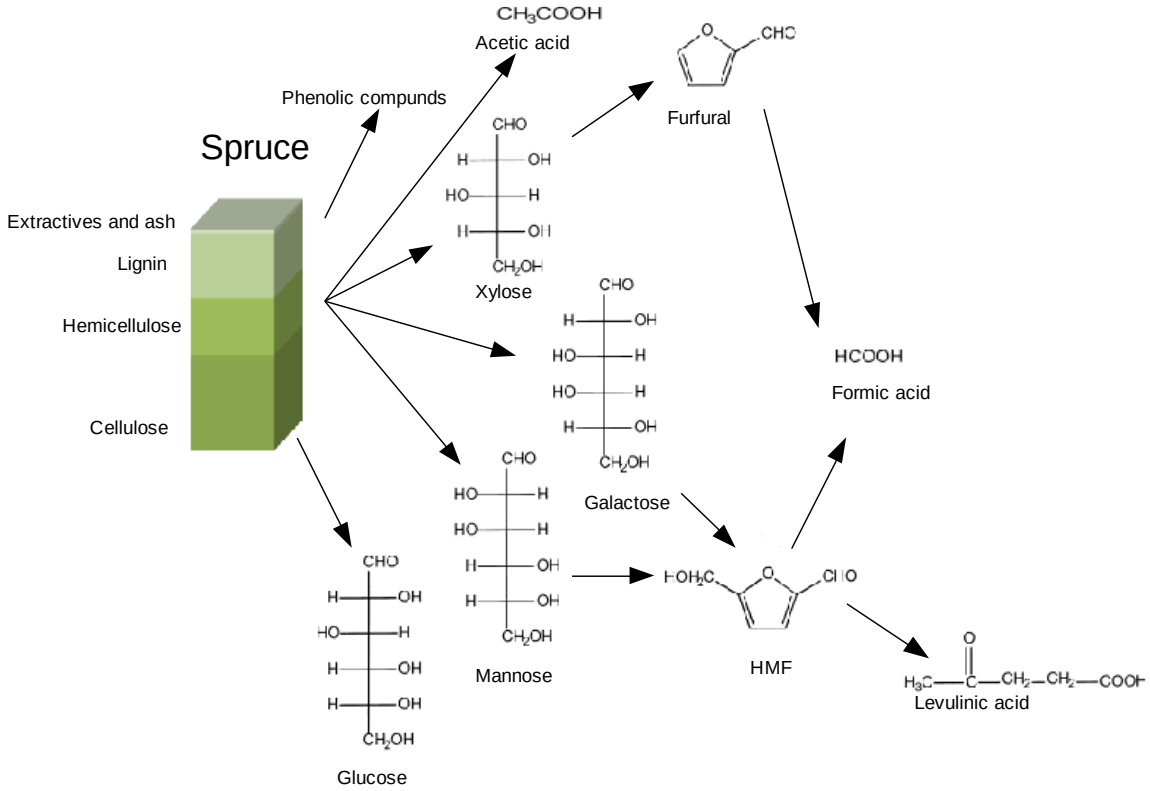

Figure 3 Inhibitors formed during pre-treatment and hydrolysis of spruce. Furan derivatives are formed from the degradation of sugars, while phenolic compounds are lignin degradation products. Adapted from Palmqvist and Hahn-Hägerdal (2000).

2.4.4 Fermentation

Fermentation is the process of converting monomeric sugars in to ethanol by microorganisms. In this thesis the ethanol producing microorganism *Saccharomyces cerevisiae* has been used.

The wild-type of *S. cerevisiae* can ferment glucose, mannose and galactose via different pathways. The fermentation of glucose and mannose follows the Embden-Meyerhof pathway. While galactose is fermented via a combination of the Embden-Meyerhof and Leloir pathway (van Maris et al. 2006). These pathways are displayed in figure 4.

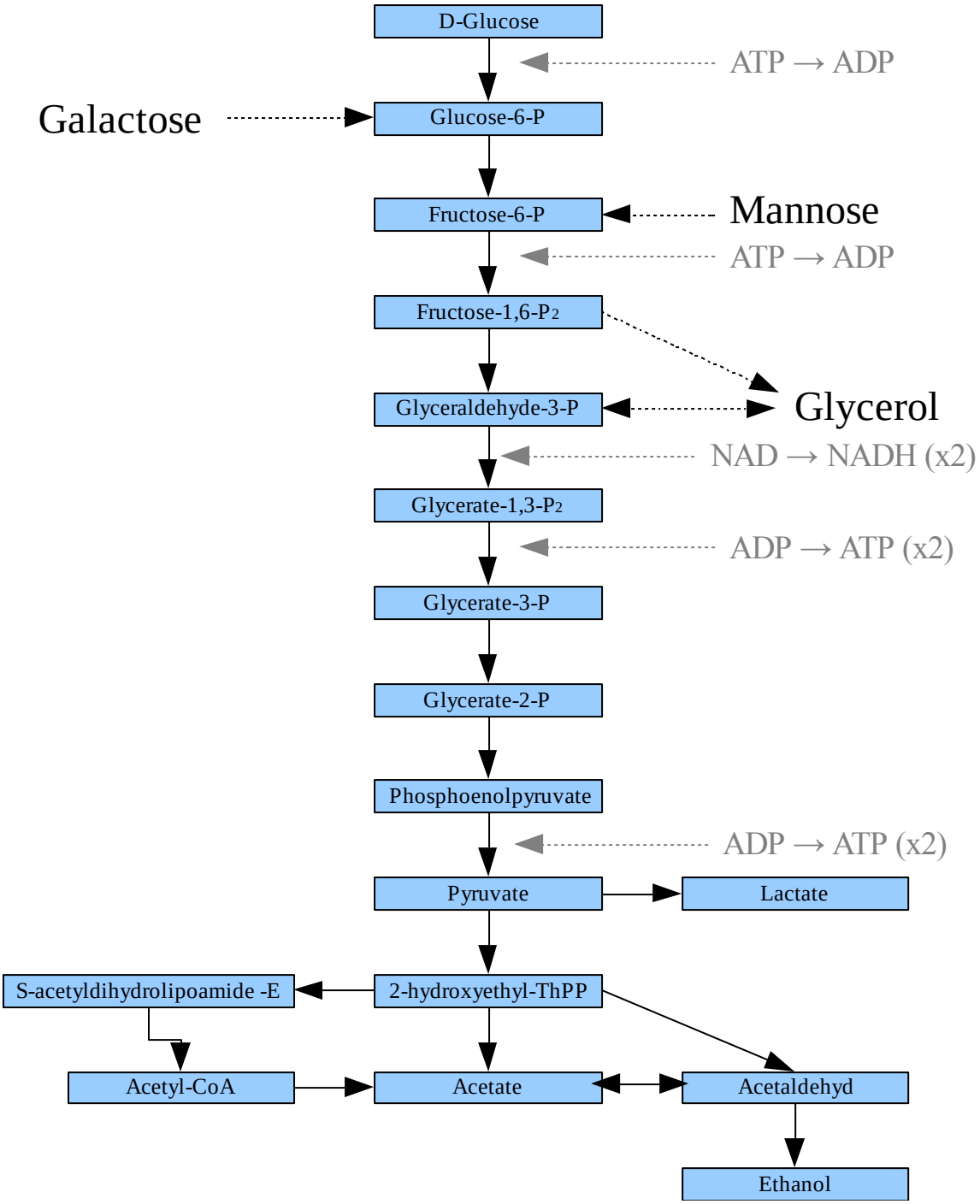

Figure 4 Sugar metabolisms in *S. cerevisiae* under anaerobic conditions. Sugars are metabolized through the glycolytic pathway and ethanol is generated from pyruvate through acetaldehyde. Although ethanol is the main product, in some cases, depending on the process and conditions of fermentation, by-products such as glycerol and acetic acid may be found in the fermentation medium (van Maris et al. 2006; Taherzadeh et al. 1996).

The fermentation of galactose cannot take place in the presence of glucose. This since the presence of glucose represses the genes regulating the galactose fermentation pathway. Galactose is therefore fermented after glucose resulting in longer fermentation times (van Maris et al. 2006).

The most common microorganism used during fermentation is *S. cerevisiae*. This yeast species has been used for ethanol production throughout history and has thereby adapted to the environment and conditions of ethanol fermentation. The adaptation has resulted in high ethanol yields and an increased resistance to high ethanol concentrations (Olsson and Hahn-Hägerdahl 1996). Fermentation of glucose to ethanol can take place even in strictly anaerobic conditions and the ethanol formation is directly related to the cell growth as stated by Albers et al. (1996). Theoretically the fermentation process will convert one glucose molecule into two ethanol molecules and two molecules of carbon dioxide according to the condition:

$$C_6H_{12}O_6 + 2P_i + ADP + 2H^+ \rightarrow 2CO_2 + 2C_2H_5OH + 2ATP + 2H_2O$$

This equation provides the theoretical ethanol yield where glucose is not used for biomass growth, maintenance or by-product formation. Resulting in a theoretical yield of 51.1grams of ethanol per 100 grams of glucose (van Maris et al. 2006), fermentation of galactose and mannose results in the same yield as they enter the same fermentation pathway as glucose (see figure 4).

Pentoses originating from hemicelluloses will be present during fermentation. The amount and sort will depend on the raw material used. These sugars are not metabolised by *S. cerevisiae* resulting in lower total ethanol yield. To be able to utilise all carbon sources present in the fermentation broth there is either a need for other microorganisms or to introduce this ability through metabolic engineering (Hahn-Hägerdal et al 2007).

By-products formed during fermentation are glycerol, acetic and lactic acid. Formation of these will lead to lower ethanol yield since carbon and energy is used during the formation of these and they can also be inhibitory to the fermenting organism. Glycerol is formed during anaerobic conditions to reoxidise NADH, to $NAD^{+,}$ that has been formed during biomass synthesis. However for the ethanol yield this process is negative since it consumes up to 4% of the available carbon during the formation of glycerol. Glycerol is also produced and accumulated in the cell under osmotic stress, under these conditions the glycerol functions as an osmolyte (Nissen et al. 2000).

2.4.5 *Saccharomyces cerevisiae*

S. cerevisiae is traditionally used for baking, wine making and brewing. Apart from these traditional uses, *S. cerevisiae* is the microorganism that has been well studied as the fermenting microorganism in bioethanol production processes. In comparison with other ethanol fermenting microorganisms, such as *Escherichia coli* and *Zymomonas mobilis*, *S. cerevisiae* has been shown to be very robust during fermentation in lignocellulosic materials. *S. cerevisiae* consumed the present sugars at a higher rate at a low pH and thereby also had an efficient ethanol production. The fermentative performance of *S. cerevisiae* is a result of being used as an ethanol producer throughout history and thereby adapting to such conditions (Olsson and Hahn-Hägerdal 1993).

S. cerevisiae is the most studied eukaryotic microorganism. It has also been used as a model organism and aided the research and understanding of other organism and cells. The yeast *S. cerevisiae* is non-pathogenic and with a long history of usage within food and beverage production, it is classified as a GRAS microorganism (Generally regarded as safe) (Ostergaard et al. 2000).

For the cultivation of yeast cells there are some required nutrients and elements to have growing and viable cells. These elements are presented in short in table 3. They are needed in the formation of macro molecules such as proteins, glycoproteins, polysaccharides, lipids and nucleic acids that form the cell structure and function. According to analysis of *S. cerevisiae* the elementary composition is $C_{47}H_{6.3}O_{33}N_8P_{1.2}Salts_{4.5}$ (depending on growth conditions and strain). This may give an indication of how the cell is built up and what is crucial for its viability. The main source of carbon is sugars that both actively and by diffusion are transported in to the cell, not all sugars can be utilised by *S.cerevisiae*. Nitrogen is found in amino acids, peptides and purines. Amino acids that are taken up by the cell can either by incorporated into proteins or catabolised and used as pure elements. *S.cerevisiae* is capable of taking up both di- and tripeptides without hydrolysis. Phosphorus is essential for yeast as it is found in nucleic acid and phospholipids and the elements make up 3 to 5% of the cells dry weight. The minerals that are needed by yeast are similar to that of other living cells. Such minerals are potassium, magnesium and trace elements are crucial for yeast growth.

To have a growing cell there is also a need for growth factors. These are substances that the cell more or less cannot produce by itself and thereby need to be present in the growth medium. Growth factors may belong to a variety of molecules such as vitamins, fatty acids or amino acids. For *S.cerevisiae* such vitamins are biotin, phantothenic acid, inositol and thiamine as reviewed by Walker (1998).

Table 3 Required elements for yeast growth, where they are found and function. As described by Walker (1998)

Element	**Source**	**Function**
Carbon	Sugars	Major structural element of yeast cells in combination with hydrogen, oxygen and nitrogen. Energy source
Hydrogen	Protons from acidic environments	Trans membrane proton-motive force vital for yeast nutrition. Intracellular acidic pH necessary for yeast metabolism
Oxygen	air, O_2	Substrate for respiratory and other mixed-function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4+, Salts, urea, amino acids	Structurally and functionally as organic amino nitrogen in protein and enzymes
Phosphorus	Phosphates	Energy transduction, nucleic acid and membrane structure
Potassium	Salts	Ionic balance, enzyme activity
Magnesium	Salts	Enzyme activity, cell and organelle structure
Sulphur	Sulphates, methionine	Amino acids and vitamins
Calcium	Salts	Possible second messenger in signal transduction
Copper	Salts	Redox pigments
Iron	Salts	Cytochromes
Manganese	Salts	Enzyme activity
Zinc	Salts	Enzyme activity
Nickel	Salts	Urease activity
Molybdenum	Na_2MoO_4	Nitrate metabolism, vitamin B_{12}

2.4.6 The effect of inhibitors on growth and ethanol production

The inhibitors found in the lignocellulosic hydrolysate have different effects on fermentation and cell growth as well as different mechanisms for these effects. Furfural and HMF are furan derivatives formed by the degradation of pentoses and hexoses, respectively. Presence of these substances leads to inhibition of growth and an extended lag phase (Almeida et al. 2007). However, the ethanol yield is not affected when using *S. cerevisiae* as fermenting organism (Klinke et al. 2004). Furan derivatives can be metabolised by *S. cerevisiae* into less inhibitory compounds. Conversion of these substances can take place in aerobic and anaerobic environments. Alcohol dehydrogenase reduces furfural to furfuryl alcohol. During the reduction NAD^+ is regenerated, therefore less glycerol is formed during the fermentation. HMF is also reduced into its alcoholic form, the route is however not determined. The reduced inhibitors were found not to be inhibitory to the cell (Palmqvist and Hahn-Hägerdal 2000). HMF and furfural also have a synergistic effect; in the presence of both, yeast metabolises the substances slower and HMF is converted after the uptake of furfural. In a study by Taherzadeh et al (2000) it was also found that there was no growth during the presence of both these substances. Nevertheless both substances can be taken up and converted into less inhibitory compounds by yeast and the inhibition is therefore only present until the substances are converted. HMF is converted at a slower rate than furfural due to lower membrane permeability (Palmqvist and Hahn-Hägerdal. 2000).

The inhibitory effect of furfural and HMF is due to the damages to the cells metabolism. They reduce enzymatic activities, break down DNA and thereby reduce RNA and protein synthesis. These effects are dependent on the initial concentration; with longer lag-phases corresponding to higher concentrations (Liu et al. 2004). For spruce hydrolysate (treated with dilute acids) the concentration of HMF might vary from 2 gL^{-1} to 5.9 gL^{-1} while furfural ranges from 0.5 gL^{-1} up to 1gL^{-1}. The effects of furfural and HMF can to some extent be explain by the fact that the yeasts energy reserves are used for repairing damages caused by the inhibitors. The intracellular amounts of ATP and NAD(P)H are thereby reduced leading to less available energy for growth and cell maintenance (Almeida et al. 2007).

Furfural will also reduce the glycerol formation under anaerobic conditions. This since glycerol is not needed for reoxidisation of NADH in the presence of furfural. NAD^+ will instead be regenerated during the conversion of furfural (Palmqvist and Hahn-Hägerdal. 2000). Therefore furfural at low concentrations might have a stimulatory effect on the ethanol yield, since carbon sources are not used for glycerol formation but instead for ethanol production (Palmqvist et al. 1999).

The effects of the weak acids are dependent on the pH of the fermentation broth. Weak acids will be found in an undissociated form if the pH is below the acids pKa. In this form they are liposoluble and can diffuse over the cell membrane into the cell. When inside, the acid will dissociate due to the higher pH of the environment and this will lead to a decrease in the internal pH (Pampulha and Loureiro-Dias 1989). The lowered pH is compensated by ATPase which uses ATP to pump protons out of the cell (Verduyn et al. 1992). Less ATP is consequently available for production of biomass, and at high concentrations of acids there might be a depletion of ATP leading to acidification inside the cell. A lowered internal pH will effect and inhibit the metabolic activity during fermentation (Pampulha and Loureiro-Dias 1990). Acetic acid might also interact with other inhibitors causing a more severe effect. This has been seen for acetic acid and furfural; they cause an additional decrease in cell growth when present simultaneously during fermentation (Palmqvist et al. 1999). On the other

hand, low concentrations of the acids might stimulate the ATP production and give an increase in the ethanol yield (Larsson et al. 1999). Since the weak acids in the hydrolysate are most inhibitory at a pH below their respective pKa raising the pH of the fermentation broth might limit the inhibitory effect. However raising the pH leads to higher risk of bacterial contamination (Kádár et al. 2007)

Other inhibitors found in the fermentation broth are; by products from the fermentation, such as glycerol, acetic and lactic acid and metals released from equipment and additives (Olsson, Hahn-Hägerdal 1996). This will be a problem when the process streams are recirculated and thereby a build up of these substances are formed. Lactic acid is less inhibitory then acetic acid, this since it is less soluble due to an extra hydroxyl group, therefore it cannot cross the lipid membrane as easily and a larger concentration is needed to get an inhibitory effect. Ethanol will also exert an inhibitory effect on the yeast due to end-product inhibition of the ethanol production pathway. This inhibition will begin at a concentration of 25 gL^{-1} of ethanol. The inhibition of the ethanol formation will also affect the cell growth and thereby reduce it. A high ethanol concentration will also cause an osmotic pressure on the cells (Maiorella et al. 1983).

The amount of inhibitors in the process stream will be increased due to accumulation as a need for recirculation of the liquids has been proposed. In such a system all non metabolised compounds will accumulate over time, so also inhibitors (Olsson and Hahn-Hägerdal 1996).

With an increasing knowledge on the inhibitors, and their effect on the fermentation process, their effects can be minimized. This is even more crucial if the liquids from the process are to be recirculated leading to accumulation of inhibitors and other solubilised substances or if the process is performed at high gravity. To have an economically favourable process there is need for a reduction of the inhibitors and their effects (Klinke et al. 2004). This can be accomplished with the use of detoxification. Specific detoxification methods are used for each type of inhibitors. The utilization of detoxification methods will elevate the production costs, not reduce all inhibitors and might lead to a loss of sugar. It is therefore not a feasible alternative in large scale production. Instead other methods can be used to reduce or resist the inhibitor effect. Nutrients added to the fermentation might aid cell growth and thus decrease the effect from inhibitors (Olsson and Hahn-Hägerdal 1996). Another alternative is to make the cell itself more tolerant to the conditions, by adapting them to the content of the hydrolysate by evolutionary engineering. When using this method the yeast is exposed to the inhibitory material in increasing concentrations. By this the evolutionary pressure is used to find and evolve more tolerant cells (Tian et al. 2010)

2.4.7 Fermentation under high gravity (HG) conditions

For bioethanol produced from lignocellulose to be economically competitive alternative for fossil fuel the cost of production needs to be decreased. A large part of the cost for the production is due to the energy demand of the recovery process. By having a higher ethanol concentration less energy is needed and the production economics will be improved (Bai et al. 2004).

The higher final ethanol concentration needed can be reached by high gravity (HG) conditions. This is performed with a high initial substrate concentration, initial dry mater concentrations above 10% w/w for HG or above 30% w/w for very high gravity (VHG). Apart from the higher ethanol concentrations other benefits are also connected to this technique. Among these are an increased fermentation rate, reduced capital cost (the size of

the equipment needed is reduced) and a lowered risk for bacterial contamination (Tao et al. 2012).

HG fermentation is as stated performed at high sugar concentrations, to accomplish this high dry weight of lignocellulosic material is needed already during the pre-treatment (Thomas et al. 1996). The high concentration of solids will result in higher viscosity and problems in stirring during hydrolysis, with this there is also a need for more enzymes. Similar problems will also arise during fermentation which can be stuck and sluggish due to the higher substrate content and with lower ethanol yields as result (Matano et al 2012).

The stress on the yeast is higher during HG than in conventional fermentations. The stress is related to high osmotic pressure, due to high sugar concentrations at the beginning of the fermentation stage and high ethanol concentrations by the end of the fermentation stage. Stress can lead to loss of cell viability and cell lysis. If SSF is used under these conditions the osmotic stress caused by the high sugar concentration will be avoided. When using lignocellulosic materials the stress will also be associated with an increased concentration of inhibitory compounds in the fermentation broth which is a result of the higher substrate concentrations used (Zhao and Bai 2009). To uphold the redox and osmotic balance glycerol is produced at higher levels during HG conditions. As a result less carbon is available for ethanol formation (Tao et al. 2012).

In the severe conditions of lignocellulosic HG fermentations the stress will not only affect yeast but also other contaminating organisms. Therefore the risk of bacterial contamination is reduced (Huang et al. 2011). Because of this reduced risk of contamination the fermentation can be performed at a higher pH, a low pH is often used in regular cultures to reduce the risk of contamination (Olsson and Hahn-Hägerdal 1993). With this raise in pH the inhibitory effect of the weak acid will decrease and a more optimum pH can be used for the yeast (Pampulha and Loureiro-Dias 1990).

During ethanol production from lignocellulosic materials a substantial amount of waste will be produced. When using high gravity conditions the materials are used more efficiently and less waste will be produced. This will result in cost savings on the expenses connected to waste handling (Zaldivar, et al. 2001).

The stress of fermentation under high gravity can be reduced by several methods. As earlier stated the amount of initial sugar can be reduced with SSF or by feeding the substrate. Ethanol inhibition can be reduced by removing the ethanol formed during the fermentation process and by detoxifying the material inhibitors are removed. As the nutrients in the hydrolysate is limited addition of nutrients will be beneficial to the growth and viability of the cells and thereby also their ability to withstand the inhibitory compounds. The addition of nutrients is explored during this master thesis.

2.4.8 Recovery

In the last process step the ethanol will be separated from the fermentation broth, if possible the yeast cells can also be recovered for reuse. The recovery of ethanol from the fermentation broth in most bioethanol processes is performed by distillation. In this way ethanol can be separated and concentrated (Balat 2011).

2.5 Nutrient supplementation of lignocellulosic hydrolysates

Lignocellulosic hydrolysates have a limited nutrient supply; the dominating nutrient found is sugars that can be utilized by the cells as a carbon and energy supply. However other nutrients that are needed for cell growth often limited, such as nitrogen (Jorgensen 2009). For example in pretreated wheat straw the nitrogen content is only about 0.4% (dry weight basis). However the amount of nitrogen present will allow total fermentation although at a slow pace (Jones and Ingledew 1994). Adding nutrients to this material can therefore be beneficial to growth of cells and thereby also the inhibitory tolerance and fermentation. Nutrients can also be added that are beneficial against the effects of the inhibitors.

At high gravity fermentation the need for certain nutrients is increased. This is due to increased stress from both high sugar and ethanol concentrations. In this case the yeast is in need of osmoprotective substances. Glycine has shown to improve the ethanol yield and cell growth under such conditions (Sankh et al. 2011). The presence of unsaturated fatty acid can also improve the cells tolerance to high ethanol concentrations.

An important nutrient for cell proliferation and biomass growth during anaerobic fermentations is nitrogen (Devantier et al. 2005). Therefore it is essential that enough nitrogen is present during fermentation. However, in lignocellulosic materials nutrients are limited and therefore the material often lacks adequate amounts of nitrogen. With a low level of nitrogen the reduced biomass growth will negatively affect the conversion of inhibitory compounds as this is dependent on the cell density (Taherzadeh et al. 2000). It has also been stated that at HG and especially at VHG the presence of free amino nitrogen (FAN) is of more importance for the result of the fermentation (Laopaiboon et al 2009), and with an increasing FAN concentration present in the growth media the final ethanol concentration is increased (Bafrncova et al. 1999). A further benefit of the presence of FAN is that less glycerol is produced. This since less amino acids need to be produced when FAN is found in the growth media and therefore less glycerol needs to be produced to uphold the redox balance.

Yeast extract and peptone is two nitrogen rich materials that are often used in a variety of growth media. Yeast extract is autolysed yeast cells. This extract is a mixture of amino acids, peptides, carbohydrates and vitamins. Peptone is enzymatically digested proteins, which can have a range of different origins. Peptone is a very god nitrogen source as it mainly constitutes of peptides and amino acids. The content of these two substances is described in table 4. The nutrient content of both yeast extract and peptone might be different depending on brand since the yeast strain and cultivation conditions and the proteins used might differ.

Yeast extract and peptone is however expensive and complex media where the exact composition varies between different sources and companies. Therefore it can be economically favourable to find other nutrient sources that have the same influence on the fermentation. Attempts have been made to combine crucial vitamins and a nitrogen source to see if the combination will give a good result on fermentation. When compared to a complex media such as yeast extract it was found to be less effective (Jörgensen 2009).

In this thesis, supplementation of the lignocellulosic material to improve fermentability was the main goal. As earlier mentioned the aim was to find a nutrient with a positive influence and thereafter determine the concentration needed of this nutrient. Supplementation has previously been performed mainly at high gravity conditions but fewer reports are found on the effect in lignocellulosic materials.

Table 4 Composition of yeast extract and peptone. The composition of yeast extract was analysed by Eden et al (2002), they analysed yeast extract provided by Sigma. The peptone composition was provided by BD biosciences user manual for bacto peptone. Yeast extract from Sigma and Bacto peptone from BD biosciences were used during the experiments.

Yeast extract	**%**	**Peptone**	**%**
Fat	0.21	Total nitrogen	15.4
Carbohydrate	13.04	Total carbohydrate	0.629
Nitrogen	5.33	Ash	3.8
Nucleotides	14.6	Loss on drying	2.7
Moisture	2.74	NaCl	1.7
Calcium	2.34	Calcium	$3.00*10^{-5}$
Chromium	$5.10*10^{-4}$	Iron	$7.80*10^{-6}$
Potassium	9.4	Magnesium	$1.70*10^{-5}$
Molybdenum	$2.57*10^{-4}$	Potassium	$2.48*10^{-3}$
Sodium	7.54	Chloride	0.90
Phosphorus	4.27	Sulphate	0.32
Chloride	0.071	Phosphate	0.40
Amino acids	14.757	Amino acids	71.8

3 Materials and methods

3.1 Raw material

The lignocellulose material used during the experiments was hydrolysed spruce (pre-treated at SEKAB, Örnsköldsvik, Sweden). The spruce was pre-treated with dilute sulphuric acid treatment and thereafter hydrolysed enzymatically. Two batches were used during the experiments. Batch A was used for the initial experiments, while batch B was used during the cultivations performed in bioreactors. The sugar and inhibitor composition of these materials are showed in table 5 and 6 respectively.

Table 5 Sugar composition of the spruce hydrolysate used during the experiments. Analysis was performed with the use of IC

Material	Glucose	Mannose	Galactose
A	43.3g/L	25.5g/L	5.1g/L
B	84.6g/L	32.7g/L	5.6g/L

Table 6 inhibitor concentrations in the spruce hydrolysate used during the experiment. Analysis was performed with HPLC

Material	Acetic acid	Levulinic acid	HMF	Furfural
A	3.5g/L	0.5g/L	1.2g/L	0.6g/L
B	5.9g/L	1.7g/L	4.8g/L	3.1g/L

3.2 Yeast strain

An industrial strain of *S. cerevisiae,* Thermosacc (Lallemand Ethanol Technology, Montreal, Canada) was used as the fermenting organism during all experiments performed in this thesis. The strain is produced for use in high-gravity conditions, e.g. it can withstand high temperatures and a high concentration of both sugar and alcohols. It readily ferments hexoses but not pentoses such as xylose. Prior to use the yeast was stored in a glycerol stock with an OD of 1.7-1.8 in -80°C

3.3 Aerobic growth (inoculum production process)

For each experiment the aerobic growth phase was executed in a uniform manner. The composition of the defined media, trace element and vitamin solutions used are displayed in table 7,8 and 9 (modified from Verduyn et al. (1992)). Prior to inoculation, the media was pH adjusted to 5.4 and sterilised through filtration. To each volume, of 50mL of defined media, 100µL of yeast cells were inoculated. All cultures were grown in 500 mL shake flasks in 30°C at 170 rpm.

Table 7 Composition of defined media for use during aerobic growth during all experiments

Compound	Concentration
Glucose	20 g/L
$(NH_4)_2SO_4$	7.5 g/L
KH_2PO_4	3.5 g/L
$MgSO_4$	0.75 g/L
Vitamin solution	1 mL/L
Trace element solution	500 µL/L

Table 8 Composition of vitamin solution used for aerobic growth and nutrient supplementation, modified from Verduyn et al. (1992)

Compound	Concentration
D-Biotin	0.05 g/L
Ca D(+) pantothenate	1.0 g/L
Nicotinic acid	1.0 g/L
Myo-Inositol	25.0 g/L
Thiamine hydrochloride	1.0 g/L
Pyridoxal hydrochloride	1.0 g/L
p-aminobenzoic acid	0.2 g/L

Table 9 Composition of trace element solution used for aerobic growth and nutrient supplementation, modified from Verduyn et al. (1992)

Compound	Concentration
EDTA	15.0 g/L
$ZnSO_4 \cdot 7H_2O$	4.5 g/L
$MnCl_2 \cdot 2H_2O$	0.8 g/L
$CoCl \cdot 6H_2O$	0.3 g/L
$CuSO_4 \cdot 5H_2O$	0.3 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.4 g/L
$CaCl_2 \cdot 2H_2O$	4.5 g/L
$FeSO_4 \cdot 7H_2O$	3.0 g/L
H_3BO_3	1.0 g/L
KI	0.1 g/L

Harvesting was performed after ~22 hours for experiments performed in defined media. The cell culture was centrifuged for 10 minutes at 1000*g and thereafter resuspended in a 0.9% NaCl solution. Cell concentration was determined based on the dry weight of the cell culture.

Cells that were to be used in hydrolysate media needed adaptation to the material prior to cultivation. This was achieved with an adaptation step during the aerobic growth. Hydrolysate (pH 5.4) was added to the defined media after 22 hours, the final volume of hydrolysate was 25%. After an additional 22 hours the yeast cells were harvested as previously stated.

3.4 Experimental setup

Six different experimental series were performed within the master thesis project. All conditions were performed in duplicates and pH was initially set to 5.4 in all experimental setups. Samples were taken after inoculation and at regular intervals to be used for OD measurements and the supernatant were thereafter stored in -20°C up to analysis. All media and hydrolysates were sterilised through filtration instead of autoclaving to avoid browning reactions and increased amounts of inhibitors due to the harsh autoclave conditions.

3.4.1. Inhibitors effect on fermentation in defined medium

The cultivations were performed at a volume of 15 mL, in 50 mL falcon tubes, with an initial concentration of 3 gL^{-1} of cells. Cells were cultured in a defined media with addition of either acetic acid, levulinic acid, furfural, HMF or a mixture of these. The composition of the defined media and the amounts of inhibitor added to the different cultures are found in table 10 and 11 respectively. Concentration of inhibitors added was based on analysis of pre-treated spruce.

Table 10 Composition for cultures performed in defined media, the high sugar concentration created conditions similar to those of high gravity.

Compound	Concentration
Glucose	75 g/L
Mannose	25 g/L
$(NH_4)_2SO_4$	7.5 g/L
KH_2PO_4	3.5 g/L
$MgSO_4$	0.75 g/L
Vitamin solution	1 mL/L
Trace element solution	500 µL/L
Inhibitor	See table 10

Table 11 Conditions and concentration of inhibitors used during the screening. The concentration of inhibitors was based on analysis of pre-treated spruce.

Condition	Concentration
Acetic acid	7.2 g/L
Levulinic acid	0.9 g/L
Furfural	1.8 g/L
HMF	3.5 g/L
Mix of inhibitors	

3.4.2 Fermentation with the addition of acetic acid at different pH, in defined medium

Cultivations were performed in a defined media (table10), in falcon tubes at a volume of 15mL with an addition of 7.2 gL^{-1} of acetic acid (setup see figure 5). The cultivations were set to pH 4, 5 and 6. Initially the amount of cells was set to 3 gL^{-1}.

Figure 5 Photo of the setup of fermentations in falcon tubes and shake flasks: The falcon tubes (50 mL) were immobilized in a tilted position on a rotary shaker at 30 ^{o}C. The shaking speed varied depending on the experimental setup while the working volume was 15 mL for the falcon tubes. When shake flasks equipped with glycerol loops were used (shown on the right side of the photo), the working volume was 50 mL.

3.4.3. Screening for nutrients with a beneficial effect on fermentation in inhibitory hydrolysate

Cultivations were performed in a fermentation broth containing spruce hydrolysate (78%) and Milli-Q water, with 3 gL^{-1} cells from start, at a volume of 15 mL in falcon tubes. The fermentation broth was supplemented with various nutrients (table 12).

Table 12 Cultivation conditions used to evaluate the effect of the addition of nutrients. Salt, trace and vitamin composition and concentrations are found in table 6,7 and 8. Enzymatically hydrolysed spruce was used as the main carbon source. The pH was adjusted to 5.4 and cultivations were kept on a rotary shaker at 30 ^{o}C.

Condition	Amount of supplement	pH adjusted with
A. Control	-	NaOH
B. Control NH_3	-	NH_3
C .Yeast extract	1%	NH_3
D. Peptone	1%	NH_3
E. Yeast extract and peptone	1% of each supplement	NH_3
F. Salts, trace and vitamins	Concentrations as in defined media	NH_3
G. Salts, trace, vitamins, yeast extract and peptone	1% of peptone and yeast extract, others as in defined media	NH_3

3.4.4 Supplementation with different amounts of yeast extract

In 50 mL falcon tubes, fermentation was performed in 15 mL of liquid consisting of 80% of hydrolysate and a supplementation of different amounts of yeast extract. The conditions used are displayed in table 13

Table 13 Cultivation conditions for the evaluation of the effects of the yeast extract concentration on the fermentation of spruce hydrolysate. Cultivations were performed in 80 % of spruce hydrolysate.

Condition	Supplementation
A. 2 g/L initial cell dw	pH adjusted with NaOH
B. 2 g /L initial cell dw	0.5% yeast extract, pH adjusted with NaOH
C. 2 g/L initial cell dw	1% yeast extract, pH adjusted with NaOH
D. 2 g/L initial cell dw	2% yeast extract, pH adjusted with NaOH

3.4.5 Effect of yeast extract at different inoculum sizes, in semi and anaerobic environments

The experiment was performed in parallel both in falcon tubes (semi anaerobic condition) and under anaerobic condition in shake flasks with the same fermentation conditions in both. The conditions used are presented in table14, the total volume in the falcon tubes were 15 mL and 25 mL in the flasks, with 77 % of hydrolysate and 2 gL^{-1} of cells in both (figure 5).

Table 14 Conditions used to evaluate the effect of yeast extract at different inoculums. The cultivations were performed under the same conditions in both shake flasks and falcon tubes.

Condition	Supplementation
A. Control, 2 g/L initial cell dw	pH adjusted with NaOH
B. Control, 1 g/L initial cell dw	pH adjusted with NaOH
C. Control, 0.5 g/L initial cell dw	pH adjusted with NaOH
D. Yeast extract, 2 g/L initial cell dw	1% yeast extract, pH adjusted with NH_3
E. Yeast extract, 1 g/L initial cell dw	1% yeast extract, pH adjusted with NH_3
F. Yeast extract, 0.5 g/L initial cell dw	1% yeast extract, pH adjusted with NH_3

3.4.6 Up-scaling in bioreactors

Aerobic growth was performed in a 3.6 L bioreactor, labfors, (Infors HT, Basel, Switzerland). The growth phase was performed at 300 rpm, 1vvm of airflow, pH 5.4 and at 30°C The media composition was the same as for prior experiment (table 7). The cultivations were monitored using BCpreFerm gas analyser (BlueSens, Herten, Germany). By following the CO_2 concentration an estimation of glucose, acetic acid and ethanol consumption could be done, as the production of CO_2 is related to the metabolism of the cells. During the ethanol consumption face, after 14 hours of cultivation 200mL of hydrolysate, adjusted to pH 4.5 with ammonia and with 10 gL^{-1} of glucose, was added to adapt the cells to the inhibitory substances.. Harvest was performed with a sampling tube equipped with a super safe sampler (INFORS HT, Basel, Switzerland), the broth was centrifuged in 50 mL falcon tubes at 1000*G for 10 minutes. The cells were thereafter resuspended in a 0.9% NaCl solution and the cell density was determined measuring the cell dry weight of the culture broth.

The fermentations were carried out in the same type of bioreactors as for the aerobic growth, however under strictly anaerobic condition (figure 6). The bioreactors were stirred at 200 rpm, with a nitrogen flow of 1 vvm and the temperature was kept at 30 °C. The system was held at pH 5.4 with addition of 2 M NaOH that was pumped automatically in response to a decrease in pH. Inoculation size was based on dry weight and set to 2 gL^{-1} for conditions A and B, and 3 gL^{-1} for condition C. The conditions are presented in table 15

Table 15 Conditions used to evaluate prior findings in a larger and more controlled environment. Fermentation was performed in bioreactors at a constant ph (5.4), temperature (30°C) and stirring (200 rpm). Condition A and B were performed in duplicates while C was performed in a single bioreactor.

Condition	Supplementation
A. 2 g/L initial cell dw, 80% hydrolysate	1% yeast extract, pH adjusted with NaOH
B. 2 g/L initial cell dw, 80 %hydrolysate	pH adjusted with NaOH
C. 3g/L initial cell dw, 90% hydrolysate	pH adjusted with NaOH

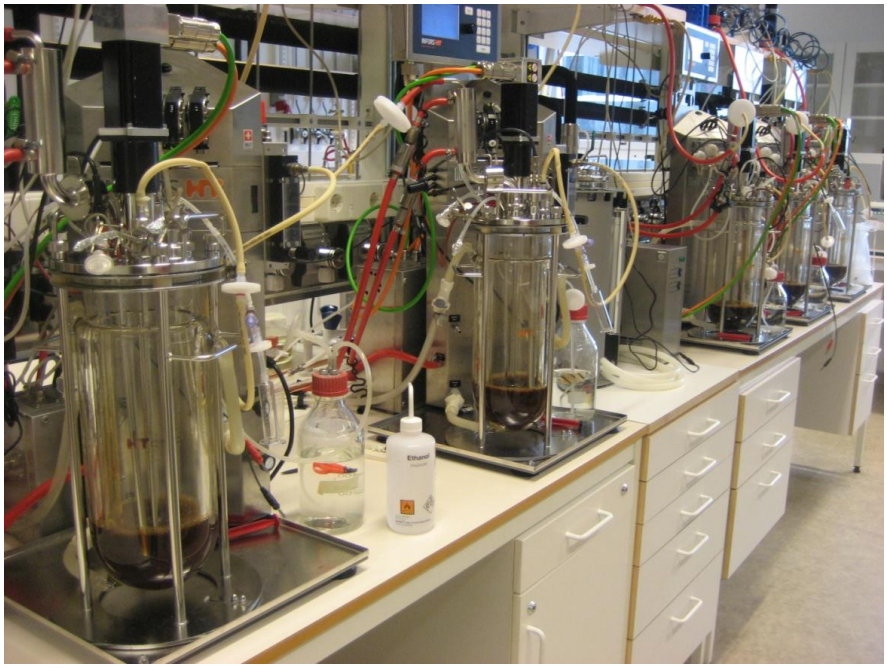

Figure 6 Photo of bioreactors setup during scale-up experiment performed in Labfors bioreactors (Infors HT, Basel, Switzerland). Fermentations were initially monitored with BCpreFerm gas analyser (BlueSens, Herten, Germany), (not seen in the photo). The temperature, stirring and pH was kept constant during the cultivations (30 ^{o}C, 200rpm, pH 5.4).

Samples were taken through a sampling tube equipped with a super safe sampler (INFORS) during the cultivation. Samples were measured using dry weight and OD and the supernatant of the samples were stored in -20 for later analyses using IC and HPLC.

3.5 Analytical methods

Samples taken during cultivation were analysed to follow growth, sugar consumption, inhibitor consumption, by-product formation and ethanol production.

3.5.1 OD $_{600\ nm}$

Samples from the cultivations were analysed directly after sampling with optical density to follow the increase in biomass. The samples were diluted with Milli-Q-water to reach an absorbance within the linear region (< 0.6). Water was used as a blank for the cultivations in controlled media while when measuring cultivations with hydrolysate the filtered supernatant of each sample was used as blank. The measurements where performed in a Thermo Scientific Genesys 20 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), the absorbance was measured through a 1cm quvett at λ=600 nm

3.5.2 IC

The sugar content of the samples collected during the cultivations were analysed by high performance anion chromatography using a CarboPac PA1 column (Thermo Fisher Scientific) Glucose, mannose and galactose amounts were quantified to follow the yeast cells sugar consumption. The supernatant was diluted and filtered with a 0.2µm filter before analysis.

3.5.3 HPLC

The extra cellular metabolites and inhibitors were analyzed by high performance liquid chromatography using an AminexHPX-87H column with 30 x 4.6 mm Cation-H Biorad micro guard column maintained at 45°C. The components were detected using the RI-detector maintained at 35°C and UV detector at 210 nm. The HPLC was performed in order to determine ethanol production, inhibitor consumption (Acetic acid, HMF, furfural, levulinic acid) and by-product formation (glycerol). The sample was diluted and filtered using a 0.2µm filter prior to analysis.

4. Results

4.1 Inhibitors effect on fermentation in defined medium

The aim of the initial experiment was to determine the inhibitory effect of known inhibitory compounds that are found in lignocellulosic material. Fermentations were performed according to table 10 and 11. The result showed a lower ethanol yield when furfural was added (appendix 9.2). (Calculation methods are found in appendix 9.1). To confirm these results a new cultivation was performed where furfural was compared to the mixture of inhibitors, acetic acid and a control. The results are presented in figure 7

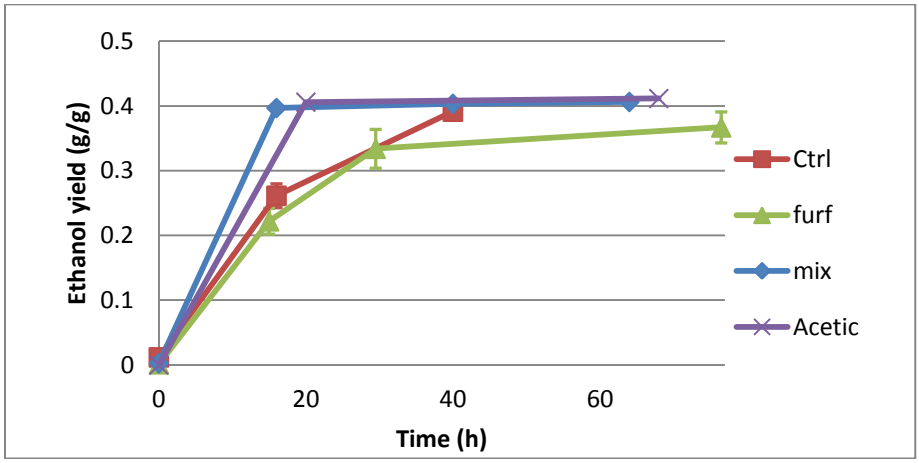

Figure 7 Ethanol yield on available sugars during the cultivation. Cultivations were performed in defined media with the addition of inhibitors. A slower fermentation is seen for the cultivations without any inhibitors and the cultivation with furfural. For the cultivation with furfural the final yield is also 0.03 lower than that of the others.

For cultures with the addition acetic acid and a mixture, no inhibition was detected while furfural addition resulted in a lower ethanol yield. The lower yield caused by furfural could however by explained by a decrease in pH during the fermentation, this decrease is present in the control and furfural fermentations (table16). These differences in pH can therefore give an explanation to the differences seen between the cultivations with furfural and the mix. The decrease in pH will not only affect the uptake of weak acids but also increase the passive proton uptake across the plasma membrane. With this increase there is a negative effect on cell viability and the fermentative performance (Verduyn et al. 1990). Since no inhibition was seen for the mixture of inhibitors, it can be concluded that neither of the inhibitors were inhibitory at the concentrations used combined with the amount of yeast cells used as inoculum. These results are in accordance with reports stating that the inhibitory effect on *S. cerevisiae* is a function of the inoculum size. Furfural was found to extend the lag phase at a concentration of 2 gL^{-1} at an inoculum size of 0.1 gL^{-1}, an increase of the inoculum size to 9 gL^{-1} reduced the lag phase (Boyer et al 1992).

Table 16 Set pH and pH after fermentation. A large decrease in pH takes place during the control cultivation and the cultivation with furfural. The decrease starts directly after inoculation and is not seen in the same degree when the mixture of inhibitors is used.

Condition	Set pH	Final pH
Mix	5.4	4.39
Ctrl	5.4	2.46
Furfural	~5.4	2.6

4.2 Fermentation with the addition of acetic acid at different pH, in defined medium

As stated by Pampulha and Loureiro-Dias (1989) the effect of acetic acid is dependent on the pH in the fermentation broth. Therefore an experiment was performed to determine the effect of acetic acid at different pH, ranging from 4-6. Cell growth was monitored by OD measurements (figure 8) A difference between the conditions is evident but there is still growth in all condition. When measuring glucose and mannose consumption the sugars were consumed in 20 hours for pH 5 and 6, for pH 4 the time needed for sugar consumption was more than 44 hours. When comparing the ethanol production of the different conditions the final ethanol concentration is the same for all three conditions, however fermentative performance at a pH 4 is significantly slower, this is presented in table 21 in appendix 9.3. At pH 5 and 6 no inhibition is visible and in the experiments following this, pH was set to 5.4, which is an optimal pH for yeast (Verduyn, Postma et al. 1990).

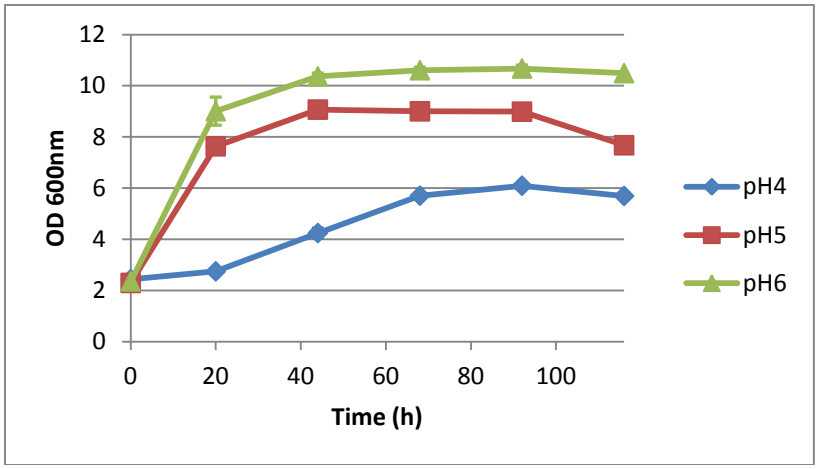

Figure 8 Absorbance of the culture broth during fermentation as a function of time. Fermentation was performed in defined medium with the addition of 7.2 $gL^{-1}$of acetic acid, pH was set to 4, 5 and 6 respectively. At pH 4 a increased lag phase and a decrease in biomass formation was seen in comparison to the other conditions.

4.3 Screening for nutrients with a beneficial effect on fermentation in inhibitory hydrolysate

The effects of several nutrients supplementations on ethanol production by *S. cerevisiae* were investigated during fermentation experiments under semi aerobic conditions. Results for all conditions are displayed in appendix section 9.4; the conditions with the most effect are presented in this section.

The fermentation rates were elevated with the addition of yeast extract when compared to the control fermentations. The effects of peptone was not clear due to a large standard error (figure 9 and table 17), however, it appears as if that the addition of peptone has no direct positive influence on the fermentation process. The addition of salts, trace and vitamins led to a lower production rate of ethanol. Biotin, which has been found to be a crucial vitamin for the cell, as it acts as a coenzyme (Brandberg et al 2007), was added as a part of the vitamin solution added to this condition (table 8). Laopaiboon et al (2009) found that the addition of $(NH_4)_2SO_4$ during HG and VHG fermentations resulted in a lower ethanol concentration in comparison to cultivations performed without the addition of a nitrogen source. Addition of ammonium in to large quantities might, according to Laopaiboon et al (2009), lead to a higher formation of by-products and thereby a loss of carbon sources that can be utilized for ethanol fermentation. During these experiments no increased by-product formation was detected due to the addition of $(NH_4)_2SO_4$ (glycerol and acetic acid was evaluated). A similar observation of the negative effect of $(NH_4)_2SO_4$ was done by Jörgensen (2009), the report concluded that fermentation of pre-treated wheat straw was not improved by the addition of $(NH_4)_2SO_4$. The addition was performed separately or in combination with either urea $MgSO_4$ or vitamins.

After 22 hours the ethanol yield when using yeast extract was two times higher than the ethanol yield for the condition with salts, trace and vitamin (figure 9). The ethanol concentrations are presented in table 17 at three time points during the fermentation, the remaining conditions are found in appendix 9.4. The final ethanol concentration was found to be approximately 20g/L for all conditions used. The results obtained here are in accordance with earlier reports about the effects of various supplementations on ethanol productivity and yield. The use of oilseed meal extract at 4%, as a supplementation during VHG fermentation experiments was found to enhance ethanol production by almost 50% and enhanced sugar tolerance from 8% to 16% (Sankh et al 2011). The ethanol production rates were also affected beneficially during high gravity fermentation of sugarcane molasses by the addition of nutrients. The fermentation period was reduced to 48 h and the by-products obtained were less in concentration, upon supplementation with nutrients (or osmoprotectants) like soy flour/wheat bran to the fermentation medium. The improved yeast cell viability during fermentation was found to be one of the major reasons (Pradeep and Reddy 2009).

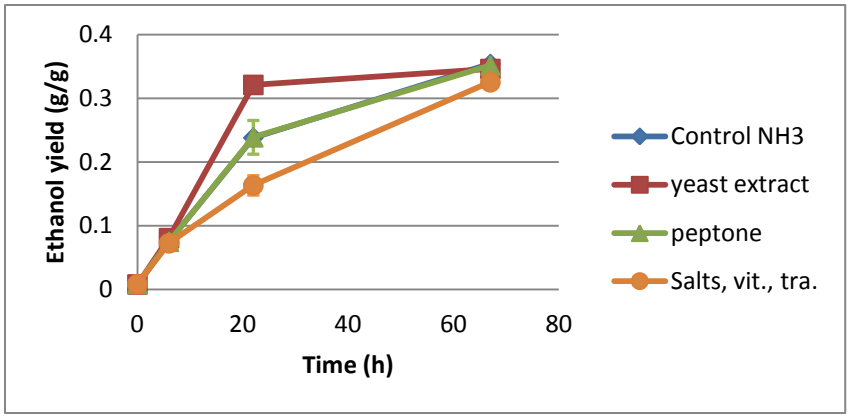

Figure 9 Ethanol yield on available sugars plotted against time. Fermentation was performed in78% of hydrolysate with 3 gL^{-1} of inoculum. All of the displayed conditions were pH adjusted with NH3. The curve displaying the control fermentation is covered by the curve of the fermentation with peptone, as these two fermentations were similar. Yeast extract addition led to a decrease in fermentation time. Addition of salt, trace and vitamins resulted in a slower fermentation. No significant effect on the final ethanol yield due to addition of nutrients is clear.

Table 17 Ethanol concentration at different times during fermentation. After 22 hours the addition of yeast extract is clear. The effect caused by the addition of peptone is not clear due to a large standard error at 22 hours.

	6 h	**22 h**	**67 h**
Ctrl NH3	4.3 ±0	13.7 ±0	20.4 ±0.1
Yeast exract	4.6 ±0.1	18.5 ±0.1	19.9 ±0.2
Peptone	4.3 ±0.2	13.7±1.5	19.5 ±0.3
Salts, trace, vit.	4.2 ±0.3	9.4 ±0.9	18.7 ±0.5

The effects seen in figure 9 were confirmed by the sugar consumption of the different conditions (figure 10 a, b, c). The addition of yeast extract increases the rate of which the sugars are consumed while the addition of salts, trace and vitamin causes a decreased consumption rate. For the culture supplemented with yeast extract, glucose and mannose are consumed within 22 hours. As the sugars are consumed the cell needs other carbon sources and starts utilizing acetic acid, glycerol and ethanol as carbon sources, if there is a supply of oxygen. This could explain why the concentration of ethanol is lower in the culture supplemented with yeast extract in comparison with the control, as is seen at 67 hours (table 17). Addition of yeast extract gives a fermentation time of approximately 22 hours (at this time point glucose and mannose are consumed). For the control cultivation there are still a significant amount of glucose and mannose at this time and the ethanol concentration is two thirds of the maximum value. These results are in agreement with reports concerning the effect of yeast extract and other free amino nitrogen (FAN) supplements. Bafrncova et al. (1999) reduced the fermentation time to half the time needed for the control, at VHG fermentation of glucose, with the addition of yeast extract, cell walls, glycine and soya flour. According to Jörgensen (2009) the addition of yeast extract resulted in a faster fermentation, with a fermentation time within 24 hours. In this report peptone was also evaluated separately or together with other nitrogen rich nutrient. The positive from addition of peptone was however not as clear as that from yeast extract.

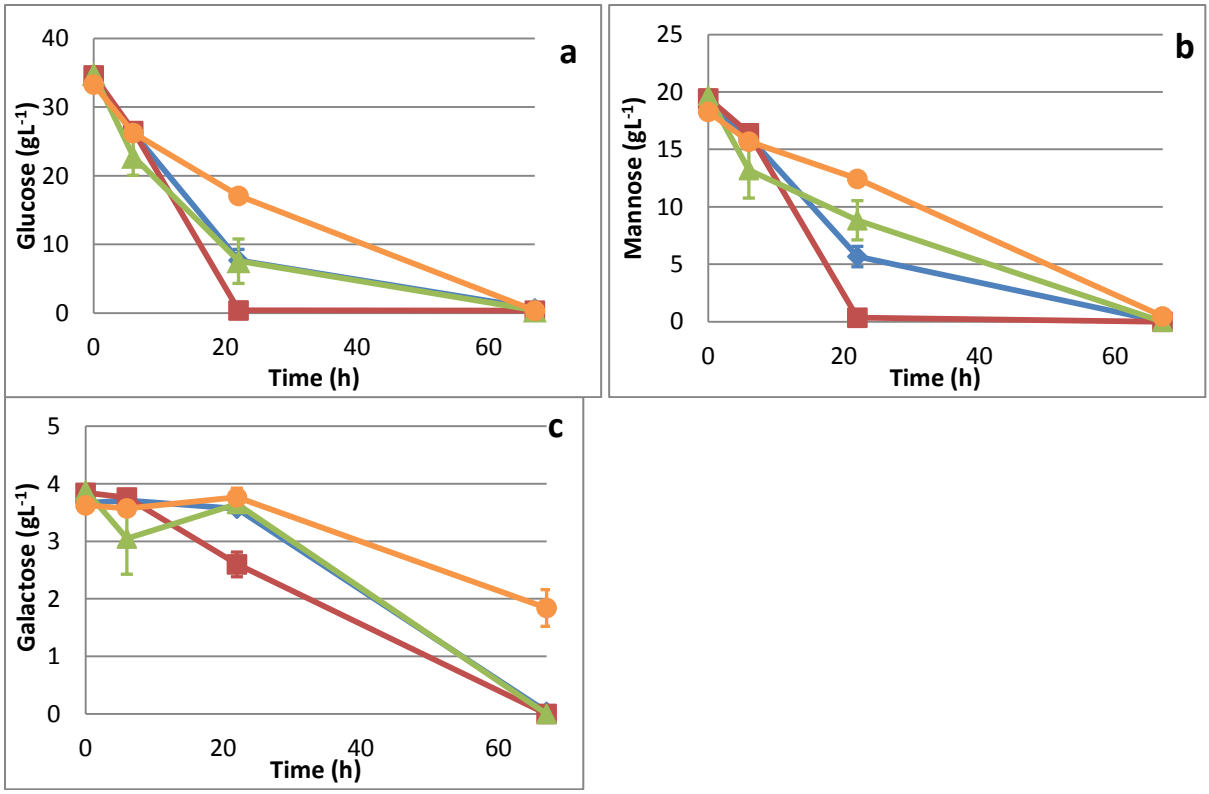

Figure 10 a,b,c Sugar consumption. A-glucose consumption, B- mannose consumption, C-galactose consumption (blue - ctrl, red – yeast extract, green – peptone, orange- salt trace and vitamin). For cultures supplemented with yeast extract there is a clear effect on sugar consumption causing glucose and mannose to be depleted within 22 hours.

In figure 10 c the galactose consumption is presented. Consumption of galactose is first initiated as the glucose concentration approaches zero. According to van Marris et al (2006) this is due to the presence of glucose, which represses the genes regulation galactose fermentation.

The consumption of inhibitors was not significantly affected by addition of yeast extract or peptone in comparison with the control. However the addition of salts, trace and vitamins reduced the rate of HMF consumption. The formation of glycerol and acetic acid was the same independent on the conditions used. Levulinic acid was constant during the fermentation for all conditions.

4.4 Supplementation with different amounts of yeast extract

To determine if the positive effect of yeast extract is based on the concentration, different amounts of yeast extract were added to the cultivations. The experiment was performed with a smaller inoculum size (2 gL^{-1}) than previous experiments so that the effects could be seen more clearly. The positive effect on growth caused by yeast extract is evident; the different amounts of yeast extract gave similar results, as seen in the OD graph (figure 11).

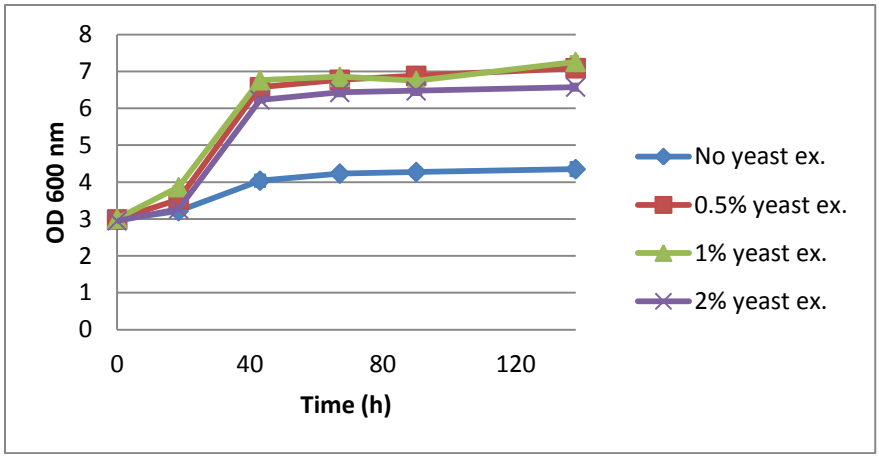

Figure 11 Absorbance as a function of time, fermentations were performed with 80% hydrolysate and 2 gL^{-1} of cells. The effect of yeast extract on biomass formation is independent of the concentration used. The largest OD was 7.3±0.1 and caused by the cultivation supplemented with 1% of yeast extract.

The ethanol yield displays the same pattern as that of the absorbance (figure 12 and table 18). The rate of ethanol production is increased by the addition of yeast extract independent of the concentration used. After 90 hours the ethanol concentration is decreased due to consumption; however the decrease is lower for the culture without yeast extract in comparison with the others. According to Jörgensen (2009) the effect of yeast extract is dose dependent. In the experiments presented in the paper, an increasing amount of yeast extract will lead to an increased fermentation rate and a decrease in glycerol production. Both of these statements were not confirmed by the cultivations performed here.

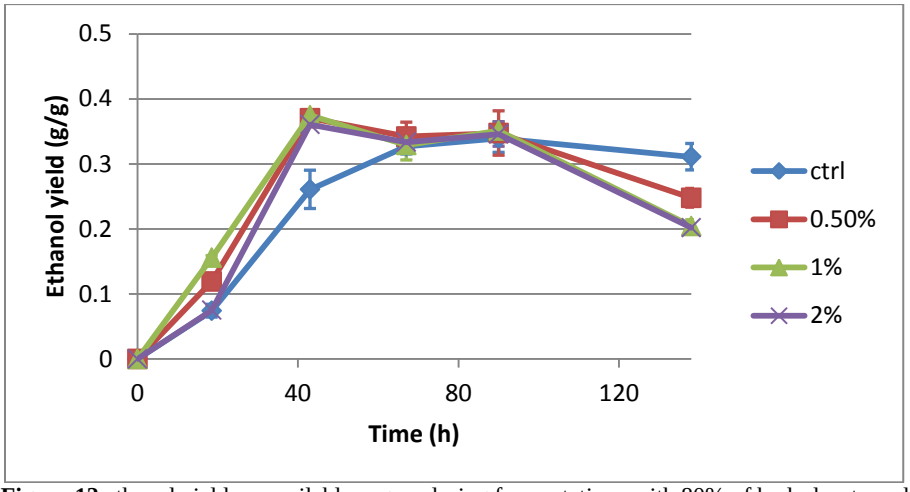

Figure 12 ethanol yield on available sugars during fermentations with 80% of hydrolysate and an initial cell dw of $2gL^{-1}$. A maximum yield is reached after 40 hours if yeast extract is added. After that the ethanol is consumed as an available carbon source.

Table 18 Ethanol concentration (gL^{-1}) for different times during the cultivation. The addition of yeast extract result in a faster fermentation. After 43 hours the ethanol concentration in all cultures with yeast extract are decreased.

	18.5 h	**43 h**	**67 h**
Ctrl	4.4 ±0.6	15.4 ±1.7	19.3 ±0.7
0.5%	7.0 ±0.4	21.9 ±0.2	20.2 ±1.3
1%	9.2 ±0.2	22.2 ±0.1	19.4 ±1.4
2%	4.4 ±0.3	21.3 ±01	19.7

Acetate and glycerol concentrations decrease after 90 hours. In these cases the decrease is smaller for the cultivation performed without yeast extract.

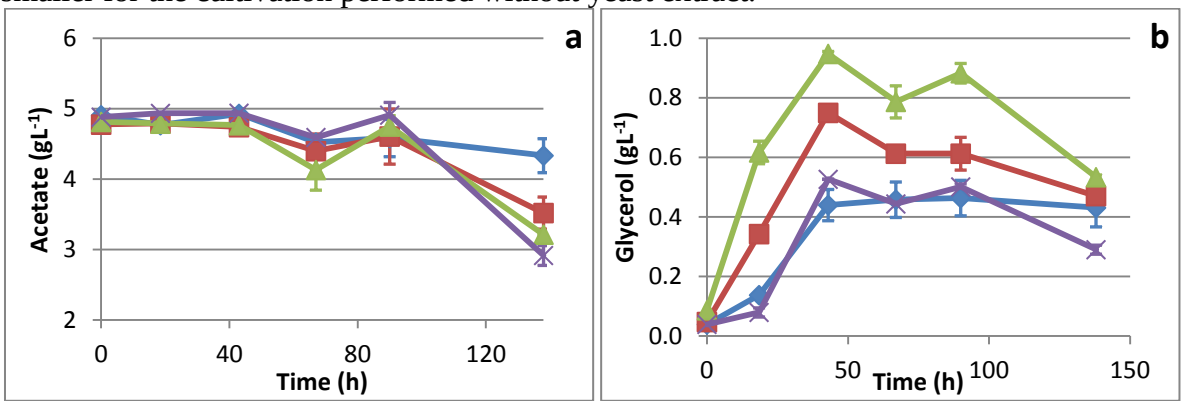

Figure 13 a,b Acetic and glycerol concentrations during the fermentation. Blue – no supplementation of yeast extract, red – supplementation with 0.5% of yeast extract, green – supplemented with 1% of YE, purple – 2% supplemented with 2% of YE. The addition of yeast extract have no apparent effect on the formation of acetic acid. However the addition seems to affect the formation of glycerol.

The consumption of ethanol, acetic acid and glycerol is initiated due to depletion of sugars in the fermentation broth and the semi anaerobic environment in the falcon tubes (figure 12 and 13). The cells starts to utilize other available carbon sources as no sugars are available, this does not occur under totally anaerobic conditions.

The consumption of sugars for the condition with 1% of yeast extract and the control fermentation is presented in figure 14. When yeast extract is present glucose and mannose is consumed within 46 hours while galactose is consumed within 90 hours. For the control fermentation, the slower consumption of sugars reduces the need for utilizing other available carbon sources, therefore acetic acid, glycerol and ethanol was not consumed in the same extent as for the other conditions.

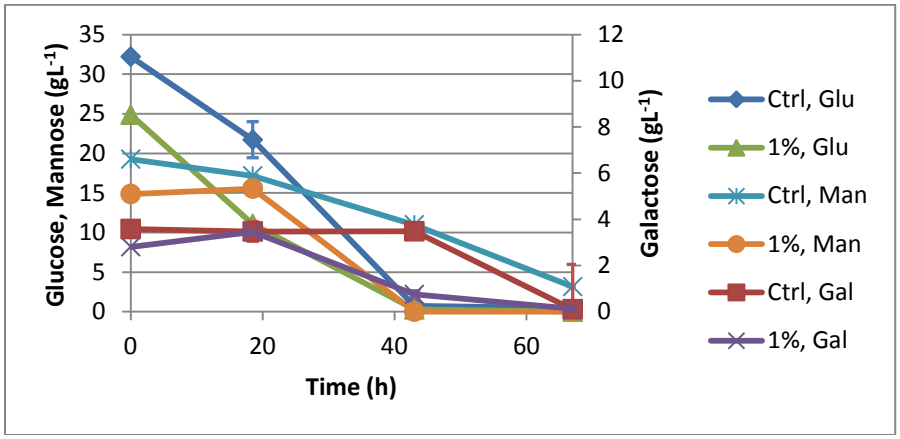

Figure 14 Glucose consumption as a function of time. The fermentation performed with the addition of 1% yeast extract and without are compared. The rate of sugar consumption is faster when YE was added in comparison to the control.

Furfural was consumed in all cultivations regardless of the conditions used within 18 hours. For HMF the time needed for conversion was longer, after 43 hours residual amounts of HMF were still present in the fermentation broth (0.084 gL^{-1}) for the control cultivation. At the end of cultivation HMF was entirely consumed for the conditions with addition of yeast extract. However, there was still residual amounts of HMF present in the control fermentation.

4.5 Effect of yeast extract at different inoculum sizes, in semi and anaerobic environments

At an inoculuom size of 3 and 2gL^{-1}, dry weight, inhibition was unclear and the effect of yeast extract was primarily on the rate of fermentation. With a decreased initial cell density the inhibitory substances found in the hydrolysate are converted at a slower pace (Taherzadeh et al. 2000) and the addition of yeast extract will thereby be more crucial. The conditions of this experiment was performed both in the semi anaerobic environment of falcon tubes and in an anaerobic environment in shake flasks. With his experimental setup the culture in falcon tubes could be compared to that of a more anaerobic environment and thereby evaluate the effects of the aeration that is present during sampling from the falcon tubes.

Yeast extract addition stimulated the biomass production at 3, 2 and 1 gL^{-1} (figure 15). The largest effect was seen at 1 gL^{-1} where the addition of yeast caused a threefold increase in biomass in comparison to cultivation performed without the addition of yeast extract. For the cultivations performed with 0.5 gL^{-1} of cells there was no clear effect on biomass growth caused by the nutrient supplementation.

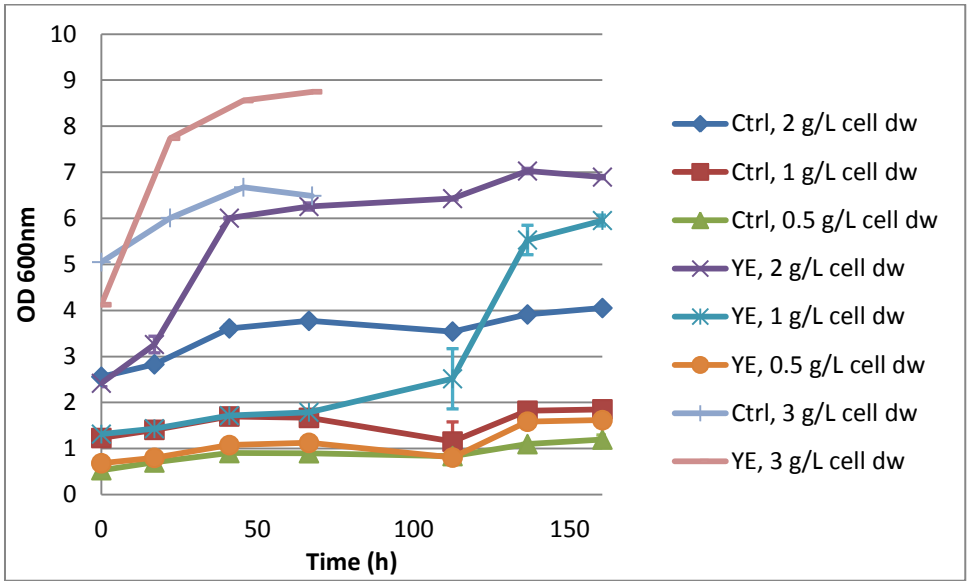

Figure 15 Absorbance measured during cultivation performed in falcon tubes with 77% of hydrolysate. For the supplemented cultivations 1% of yeast extract was used. For 3,2 and 1 gL^{-1} of initial cell dw the addition of yeast extract has a positive effect on biomass formation. For 1gL^{-1} the addition appears to be crucial for biomass growth.

The ethanol yield on available sugars is presented in figure 16 a and b. These two graphs represent cultivations performed in shake flasks and falcon tubes respectively. There are no apparent differences between the ethanol yields in the different environments. The final ethanol concentrations for both cultivations are presented in appendix 9.5.

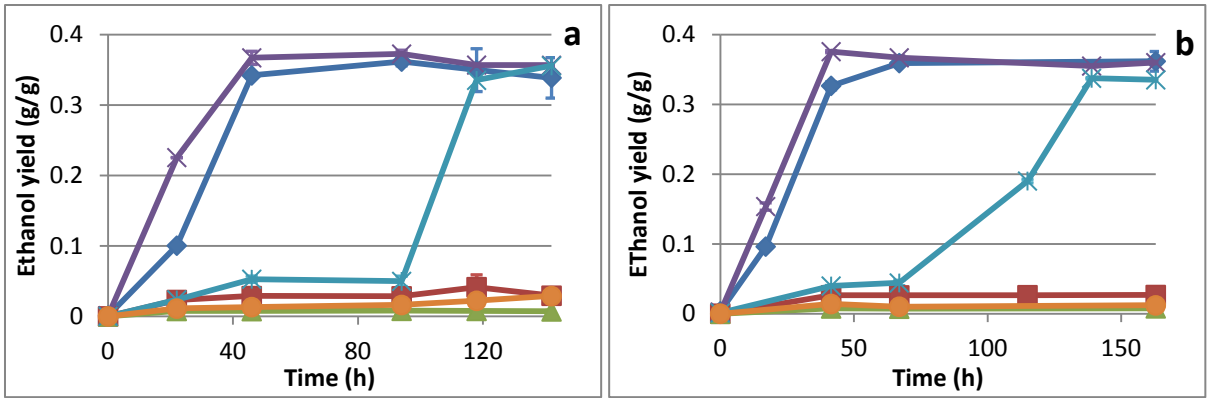

Figure 16 a(shake flasks) b(falcon) Yield on available sugars as a function of time, fermentation was performed in anaerobic shake flasks and falcon tubes with 77% of hydrolysate. Purple – 2 gL^{-1} with yeast extract, dark blue – 2 gL^{-1} ctrl, light blue – 1 g/L with yeast extract, red – 1 gL^{-1} ctrl, orange - 0.5 gL^{-1} with yeast extract, green – 0.5 gL^{-1} ctrl. For 1gL^{-1} of yeast extract the addition of yeast extract is essential for the fermentation of spruce hydrolysate. However, in comparison to 2gL^{-1} the fermentation time was increased..

Sugar consumption for the cultures in shake flasks and falcon tubes were compared. There was no significant difference between these two environments when the inoculum was 2 and 1 gL^{-1}. Sugar consumption for the cultivation performed in falcon tubes are presented in figure 17 a and b. When using 1 gL^{-1} of cells addition of yeast extract is crucial for the sugar consumption, without the addition only a small amount if glucose is consumed while the concentration of the other sugars are unchanged. The positive effect of yeast extract was also confirmed by Jorgensen (2009).

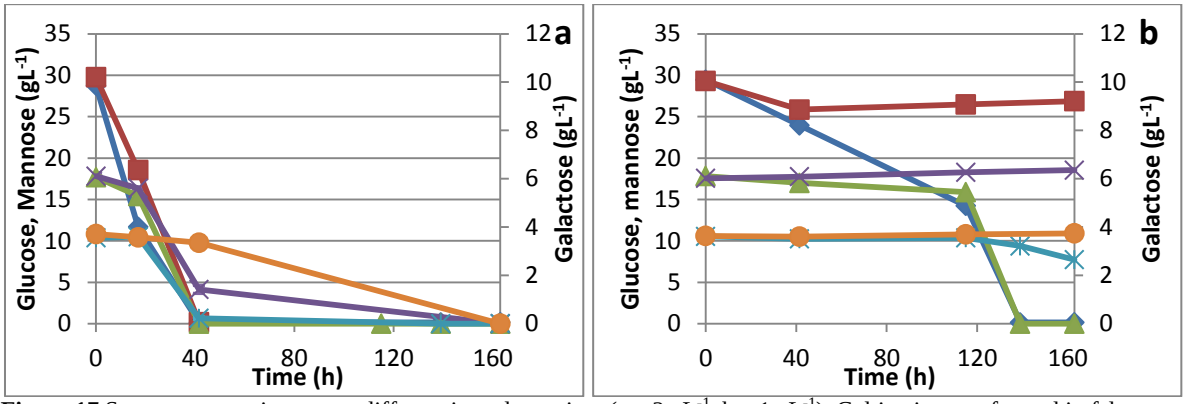

Figure 17 Sugar consumption at two different inoculums sizes (a – 2 gL^{-1}, b – 1 gL^{-1}). Cultivations performed in falcon tubes. Glucose consumption for ctrl (red) and with yeast extract (dark blue), mannose consumption for ctrl (purple) and with yeast extract (green), galactose consumption for ctrl (orange) and with yeast extract (light blue). In b (1gL^{-1}) it is evident that the addidtion of yeast extract is fundamental for the sugar consumption

However for 0.5 gL^{-1} no consumption was noticeable in the falcon tubes, while for the cultivation in shake flasks a minor consumption of glucose and galactose were detectable. This is also confirmed by a higher ethanol production in the shake flasks compared to cultivations in falcon tubes (figure 18). In the shake flasks the increase in ethanol concentration is four times higher as a result of the addition of yeast extract. In the falcon tubes however the increase is 50%.

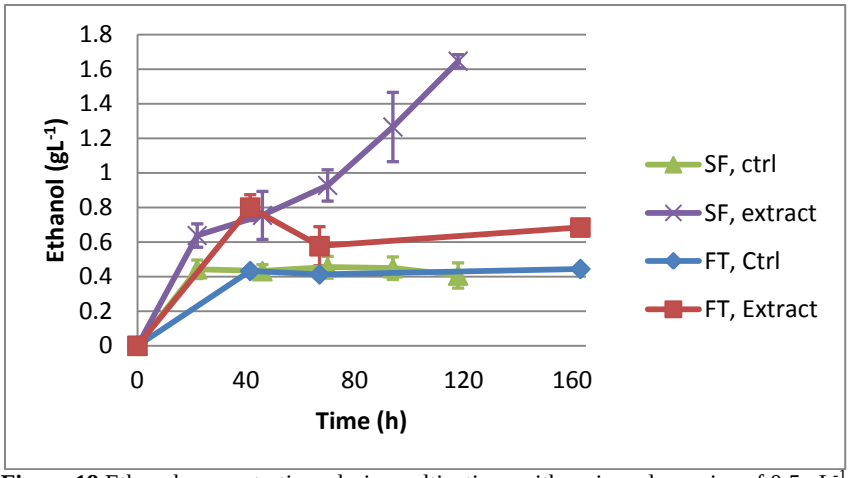

Figure 18 Ethanol concentrations during cultivations with an inoculums size of 0.5 gL^{-1} in shake flasks (SF) and falcon tubes (FT). Fermentations were performed in77% hydrolysate, conditions used were addition of yeast extract and a control without any additions. For the cultivation shake flasks the addition of yeast extract also has an effect on the fermentation at 0.5gL^{-1} of cell dry weight

The glycerol formation was not directly affected by the addition of yeast extract. Glycerol however is formed to reoxidise the NADP that has been formed during biosynthesis (Nissen et al. 2000). A larger biomass growth will therefore increase the amount of glycerol produced. The concentrations of acetic acid and levulinic acid were not affected by either the inoculum size or the addition of acetic acid.

The rate of conversion of HMF and furfural is correlated to the amount of cells and the amount of these that are growing (Taherzadeh et al. 2000) For the smaller inoculums sizes the addition of yeast is necessary for biomass growth and thereby conversion of the inhibitory compounds .Therefore the cell growth caused by the addition of yeast extract is crucial, the smaller inoculums sizes for the conversion of these inhibitory substances. The consumption of furfural and HMF are presented in figure 19. HMF was consumed within 46 hours at 2 $gL^{-1}$and with the addition of yeast, while furfural was consumed within 22 hours regardless of the addition of yeast extract or not. The effect of the cell density on the consumption of furfural was reported by Boyer et al. (1992). In this report an increase of the inoculum size from 0.1$gL^{-1}$to 2gL^{-1}Led to a decrease in the time needed for conversion of furfural from 15-20 hours to 5 hours.

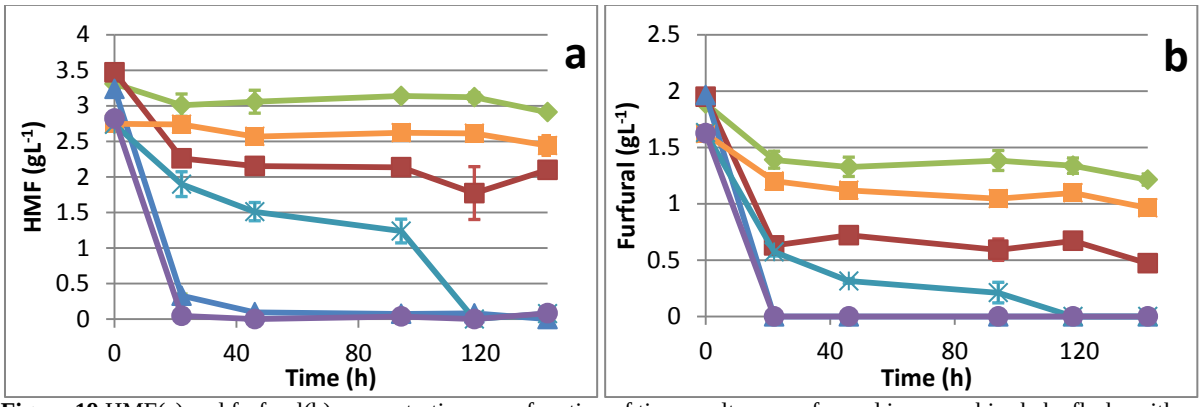

Figure 19 HMF(a) and furfural(b) concentrations as a function of time , cultures performed in anaerobic shake flasks with different inoculum sizes. Purple - 2 gL^{-1} initial cell dw and the addition of 1% of yeast extract, dark blue – 2gL^{-1} cultivation without supplementation, light blue – 1$gL^{-1}$with YE, red – 1gL^{-1} control, orange – 0.5gL^{-1} with YE, green – 0.5gL^{-1} control. The addition of yeast extract has a positive effect on the conversion of HMF and furfural to less inhibitory compounds.

4.6 Up-Scaling in bioreactors

Fermentation experiments in bioreactors were performed in order to ensure totally anaerobic conditions and also to control the pH and temperature during the fermentation. It was also performed to monitor the growth and fermentation in a larger scale than previous attempts and to investigate if the beneficial effects on the fermentative performance seen by the addition of yeast extract in the fermentation medium, would be similar under these conditions. The amount of yeast extract added was chosen to 1%, despite that the previous experiment showed a positive effect already at 0.5%, this was done not to change parameters in comparison with earlier experiments. By not changing parameters an evaluation of the controlled environment could be performed.

The biomass increase of the condition with yeast extract was more than double that of the fermentation performed without yeast extract during the first 11 hours (figure 20). During microscopy of the cells a difference in size was detectable between the two conditions with larger and more budding cells in the culture supplemented with yeast extract.

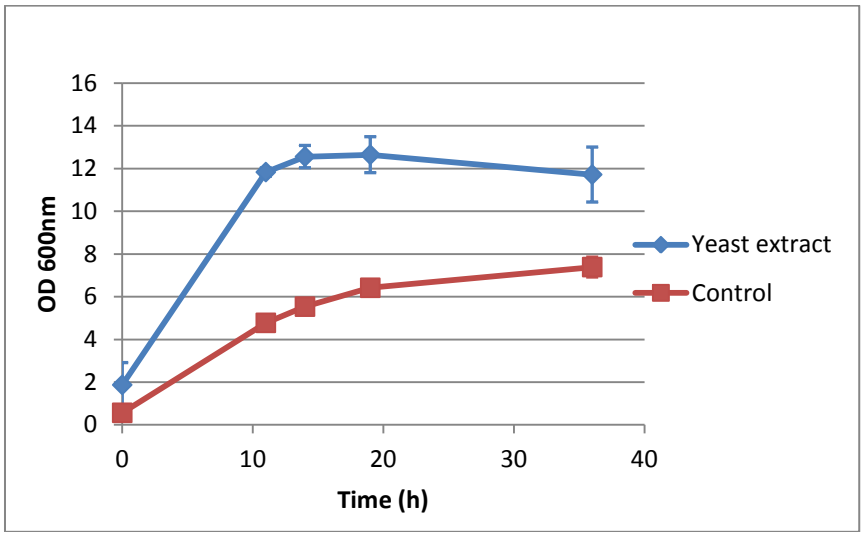

Figure 20 Absorbance as a function of time, measured at 600 nm. Cultures were grown in 80% of hydrolysate and with 2 gL^{-1} of inoculum.. Two conditions were used, with and without the addition of 1% of yeast extract. With the addition of YE the formation of biomass is increased. After 17 hours the addition of yeast extract caused a biomass increase of more than 100%.

The ethanol yield on available sugars during the cultivation is displayed in figure 21 and the ethanol concentration during the first 20 hours is presented in table19. The rate and yield of the fermentation was increased by the addition of yeast extract.

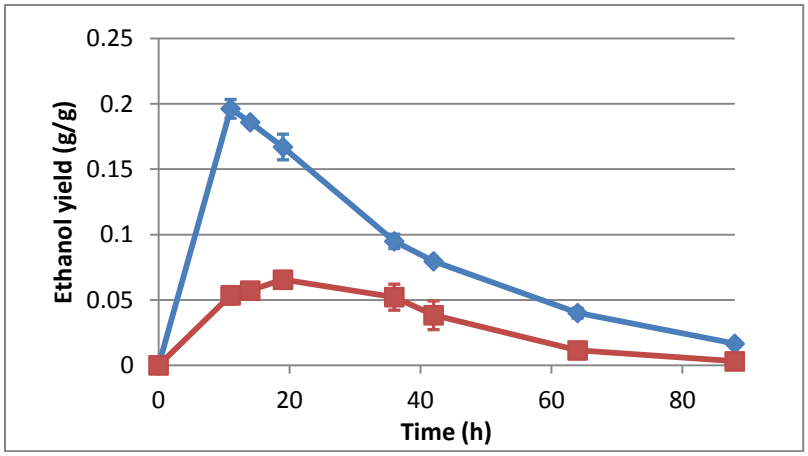

Figure 21 Ethanol yield from available sugars as a function of time. Blue – Cultivation with the addition of yeast extract, red – Control cultivation performed without the addition of yeast extract. Fermentation was performed in 80% of hydrolysate with an inoculum size of 2 gL^{-1}. The addition of yeast extract increased the rate of fermentation substantially, however due to a high evaporation rate it is not possible to make any conclusions about the final ethanol yield.

Table 19 Ethanol concentration.at time points during the start of fermentation. The maximum ethanol concentration for the cultivation with yeast extract is reached after 11 hours. Thereafter a fast decrease of ethanol concentration is present due to evaporation.

	11h	**14h**	**19h**
Yeast extract	19.3±0.7	18.3±0.2	16.4±1.0
Control	5.3±0.7	6.4±1.1	7.3±0.8

In an anaerobic environment ethanol is not consumed after the depletion of sugars. Therefore the decline in yield seen on both figure 21 and table 19 is not due to this. Instead the decline is due to evaporation during fermentation which has been aided by the nitrogen flow of 1 vvm in the bioreactor. With the evaporation the amount of ethanol in the samples will not correspond to the actual amount of ethanol produced. Previous experiments have resulted in yields at approximately 0.35 g of ethanol per gram of sugar available in the fermentation broth. A yield of that magnitude would result in an ethanol concentration of 43.0 g for the cultivation with yeast extract. It is not possible to say if the control cultivation would reach a similar yield under these conditions. However, in these experiments the amount of biomass produced is higher than in previous experiments which could have resulted in a lower final yield due to loss of carbon sources in the biomass production

The consumption glucose, mannose and galactose for the two fermentation conditions are presented in figure 22. With the addition of yeast extract the consumption rate is increased significantly in comparison to the control and all sugars are consumed within 20 hours. For the control cultivation the time needed for consumption of glucose and mannose is the double, 40 hours, while galactose is hardly consumed. This in compliance with the results from Bafrncova et al. (2009) that reported that the addition of yeast extract, cell walls, glycine and soya flour resulted in halving of the time needed for fermentation.

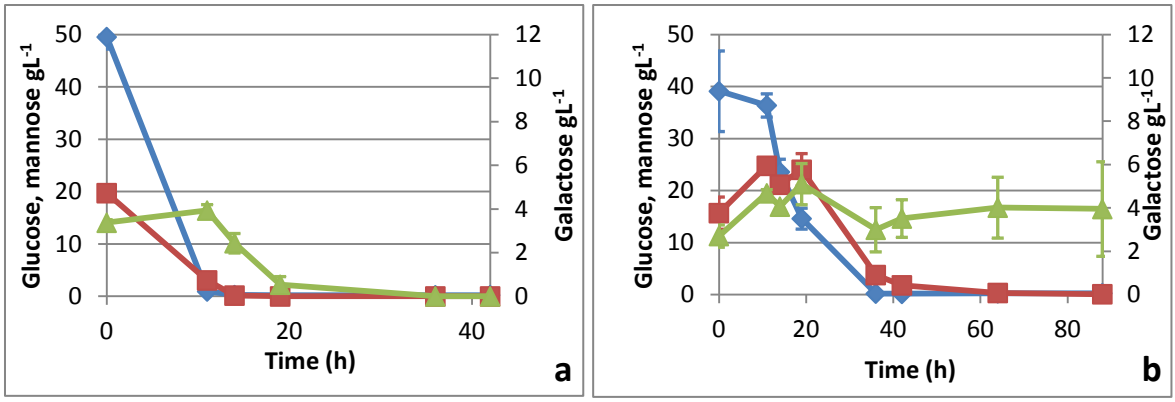

Figure 22 Sugar consumption as a function of time A- cultivation with yeast extract, B- Cultivation performed without yeast extract. Blue – Glucose, Red – Mannose, Green – Galactose) Without any supplementation the fermentation time is more than doubled and galactose has not decreased significantly.

The conversion of inhibitors and the formation of by products were also analysed. After 11 hours furfural was not detected in the fermentation broth, HMF however was present in small amounts at this time and consumed within 14 hours.

A culture was also performed in 90 % of hydrolysate to evaluate the inhibitory grade of the material. In figure 23, OD for the cultivations performed in bioreactor is presented. At 90% we have an increase in biomass. The biomass increase is similar to that of the control with 80% of hydrolysate and 2 gL^{-1}. If no inhibition was present the biomass amount with 3 gL^{-1} should be larger than that of 2 gL^{-1}

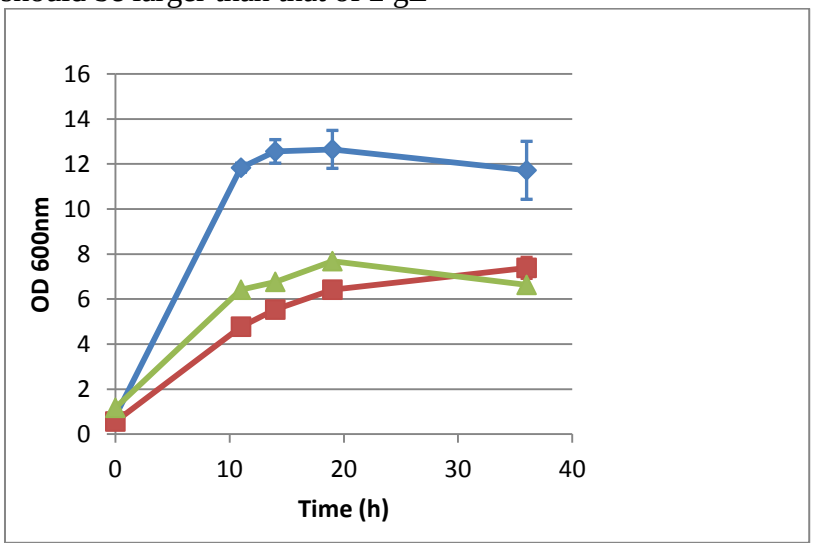

Figure 23 Absorbance for the cultivations performed in bioreactors. An evaluation of the material was performed with a higher amount of hydrolysate (green). The amount of inoculum was raised to 3gL^{-1} to withstand the higher degree of toxicity. This cultivation was compared to the two other cultivations performed in bioreactors. Blue- Fermentation of 80% of hydrolysate with the addition of 1% of yeast extract with an inoculum size of 2gL^{-1}. Red – control performed with 80% of hydrolysate and 2gL^{-1} of inoculum

The bioreactor containing the culture with 90% of hydrolysate had a lower degree of nitrogen flowing through the fermentation broth due to blockage of the sparge. Therefore a comparison is best made through analysing the sugar consumption. In figure 24 the sugar consumption for this experiment and control cultivation performed in 80 % are displayed. Despite the higher inoculum in the experiment with 90% the consumption is similar to that of the control. If the toxicity of the material did not affect the fermentation the sugar consumption should be higher where the inoculum is higher.

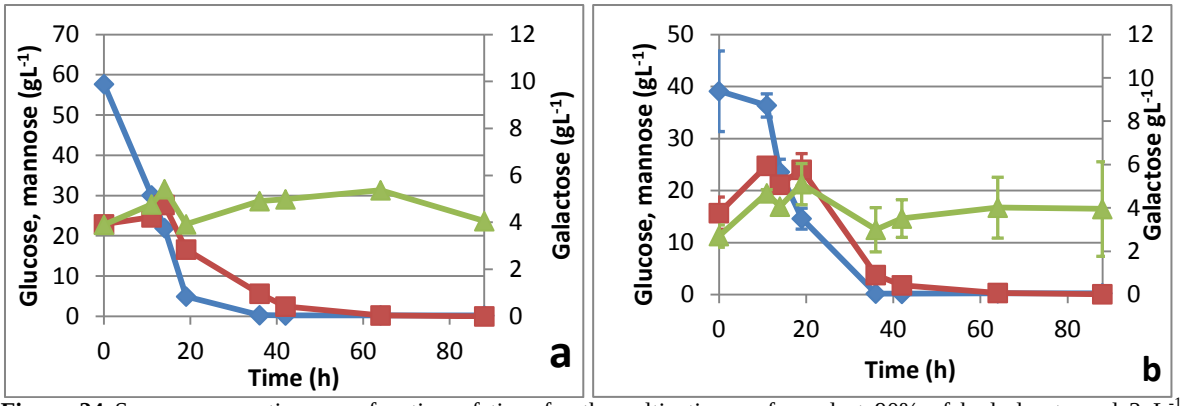

Figure 24 Sugar consumption as a function of time for the cultivation performed at 90% of hydrolysate and 3gL^{-1} of inoculum (a) and the control cultivation performed at 80% of hydrolysate and with 2gL^{-1} of inoculum (b).The grafs are quite similar for both cultivations with the same time needed for consumption of glucose and mannose, while galactose is not consumed. This despite the higher inoculum level in a.

In comparison to earlier experiments the yield was also in this experiments low, despite a smaller evaporation than in the other bioreactor experiements. The yield after 36 hours was 0.16 indicating that the high concentration of hydrolysate, and therefore a high concentration of inhibitory compounds, affects the fermentability of the material. However the extended lag phases inhibition of growth presented by Almeida et al. (2007) cannot be seen from these results. The highest concentration of ethanol was 17.9 gL^{-1} (figure 25). However a certain degree of evaporation was also present here as can be seen by the decrease in ethanol concentration after 36 hours.

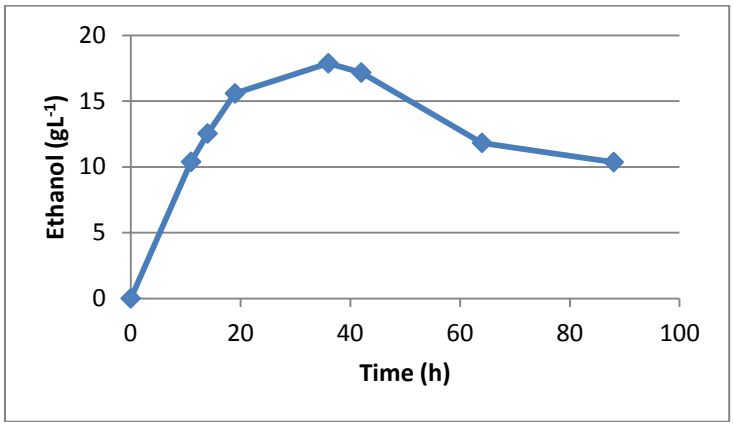

Figure 25 Ethanol concentration during the cultivation performed at 90% hydrolysate with an inoculum of 3gL^{-1}. Due to evaporation the ethanol concentration is decreased after 40 hours.

The inhibitors HMF and furfural was converted to less inhibitory substances at the same rate as for the experiments performed in 80% hydrolysate, despite the higher concentrations. The first sample was however taken after 11 hours and at this time most of the inhibitors were consumed. However it cannot be said when during this 11 hours the most part of the conversion was done for any of the cultivations.

An experiment at 93% of hydrolysate was performed to evaluate the effects of yeast extract at a high hydrolysate concentration (in falcon tubes). The outcome of this experiment was however not satisfactory. Results and experimental design of this experiment is found in the appendix 9.6

5. Discussion

5.1 The inhibitory compounds

No inhibition was demonstrated in the cultivation with all inhibitors present in defined media (section 4.1), at the concentrations and inoculum size that were used. However a slightly lower ethanol yield on available sugars caused by a decrease in pH during the fermentation was seen for the cultivation performed with the addition of furfural and control. For the cultivation performed with all inhibitors acetic acid was present resulting in a low pH when adjusting this before cultivation a buffer was produced and the cultivation could therefore withstand the decrease in pH normally caused by the formation of acetic acid during fermentation. For the cultivation with furfural and the control the pH adjustment was smaller and therefore no buffering qualities were present. Therefore the lower yield of furfural and the control cultivation was linked to the decreasing pH, which will have affected the fermentative performance as stated by Verduyn et al. (1990). Using a mixture of these inhibitors is usually connected to synergistic effects causing more inhibition (Palmqvist et al. 1997; Taherzadeh et al. 2000) and as no reports of synergistic effects that have a positive influence on the fermentation were found this is probably not the cause of the higher yields in this cultivation.

The absence of inhibition was a result of amount of inhibitors used in combination with a relatively high inoculums size. A high cell density will lead to a fast conversion of the inhibitory compounds and thus no inhibiton will be noticable (Palmqvist et al. 1999). The presence of furfural and HMF in inhibitory levels would cause an extended lag phase and inhibition of growth if present at inhibitory levels (Almeida et al. 2007). For cultivations performed no inhibition can be seen. Thus no extended lag phases or significant differences in growth when comparing the cultures containing HMF or furfural to the control.

In all cultivations performed during this thesis, with $3gL^{-1}$ of inoculum no extended lag phases were seen. This was however seen when lowering the inoculum size. These findings were therefore in accordance with the findings of Palmqvist et al. (1999) and Almeida et al, (2007)

5.2 Effects of different nutrients

Yeast extract was found to have a positive effect on the time needed of ethanol production and sugar consumption. This was seen during the screening of nutrients and the subsequent experiments. In previous studies similar results have been presented (Bafrncova et al. 1999; Thomas and Ingledew 1990; Jorgensen 2009). According to (Thomas and Ingledew 1990) the nitrogen source of yeast extract is the main cause of this positive effect. Nitrogen is especially essential during anaerobic cultivations since it is needed for proliferation and biomass growth, and therefore also for ethanol production (Devantier et al. 2005). With limited amounts of nitrogen growth will be affected, the amount of intracellular proteins formed will be decreased and with this the capacity of fermentation is also reduced.(Jørgensen, Olsson et al. 2002). Barfncova et al. (1999) also states that a lack of a sufficient nitrogen supply will result in lower final ethanol yields. During the experiments performed during this thesis the final ethanol yield is independent of the supplementation performed. Also without the addition of a nitrogen source the final ethanol yield is the same as for when yeast extract is present. This indicates that the nitrogen is not the cause of yeast extracts positive effect and that the amount of nitrogen present in the material is sufficient for the fermentation. However (Thomas and

Ingledew 1990) also states that the effect of the nitrogen depends on the form it is given in (what kind of nutrient source that is used).

Supplementation with the salts, trace elements and vitamins that are typically used in culture media seemed to have a negative effect on the fermentation rate. This was also seen by Laopaiboon et al. (2009). This negative effect was proposed to be caused by an increased by product formation which was the result of a higher concentration of ammonium. This was however not seen during this project. In the analysis of by-product formation (glycerol and acetic acid), no increase could be seen in comparison with the other conditions at all time points measured. But during the fermentation, less ethanol was produced initially by the salt, trace and vitamin supplemented culture, resulting in a higher amount of glycerol produced per amount of ethanol. Thereby confirming the findings of Laopaiboon et al (2009).

Peptone was found to be less effective as yeast extract. In comparison to the control pH-adjusted with ammonia, no difference was detectable. When comparing yeast extract and peptone it can be seen that yeast extract is a more complex additive with more fat and carbohydrates present and with a more optimal composition of growth factors (table 4). However, many of these important growth factors are also present in the mixture of salts, vitamins and trace elements that was added as a supplementation condition. Therefore the success of yeast extract is more complex. Fatty acids present in the yeast extract can work as osmoprotectants which protects the cell against the high concentrations of sugar and ethanol. . Sankh et al. (2011) found the addition of lipids and fatty acids to be beneficial for the fermentative performance. The same report also stated that fatty acids can have a role as osmoprotectants against ethanol stress. During HG the presence of osmoprotectans is crucial due to high sugar and ethanol concentrations during the fermentation, as these exert a high osmotic pressure on the yeast cells. The addition of nitrogen as amino acids can also be beneficial since these can be used directly and with that less glycerol is produced to withhold the redox balance.

The most important effect of nutrient supplementation during fermentation of lignocellulosic materials is the biomass increase. With a higher density of cells the conversion rate of the inhibitory substances is increased and their negative effect on fermentation is diminished (Boyer et al. 2009; Taherzadeh et al 2000). The addition of yeast extract cause a substantial biomass increase and thereby is a good nutrient in such an environment.

Yeast extract is an expensive additive and therefore it might not be a suitable alternative in large scale processes (Thomas and Ingledew 1990). Thus it is important to know the mechanism behind the effect of yeast extract and how this can be mimicked with less expensive additives. In cultures with lignocellulosic materials the concentration of nutrients are low and if HG is used the amount of inhibitory compounds will be higher. Therefore the need of nutrients is greatly increased. Their positive effect on biomass growth will result in a more tolerant fermentation.

For cultures were yeast extract was added there was an increase in biomass growth. Resulting in a faster consumption of inhibitors than in the cultivations with limited nitrogen where the growth is slower. At higher cell densities furfurals inhibitory effect is decreased due to a rapid bioconversion (Boyer et al. 1992) Thus will the inhibition of furfural and HMF is higher in the control condition leading to a lower growth and therefore less ethanol production.

5.3 Amount of yeast extract needed

Since yeast extract is an expensive additive, much can be gained in optimizing the concentration used for supplementation. Initially $1gL^{-1}$ of yeast extract was found to be beneficial. Therefore an experiment with a lower and higher concentration was also performed to see if there was any difference in the effects. No real difference could be seen, on the fermentative performance, between these different concentrations. However, Jorgensen (2009) found that the amount of yeast extract used had an effect on the fermentation of pretreated wheat straw at high gravity. In the same article it is also stated that the glycerol production is lowered with the addition of yeast extract.

In the experiment performed in this thesis the amount of glycerol produced seems to increase with an increasing amount of yeast extract (for 0.5 and 1 gL^{-1}). This, despite that the addition of free amino nitrogen reduces the glycerol formation according to Jorgensen (2009). However, Laopaiboon et al. (2009) found that an excessive amount of ammonium leads to a larger by product formation. Therefore it can be reasonably to assume, that already $0.5gL^{-1}$ of yeast extract is too much for supplementation of the lignocellulosic material used during this experiment.

5.4 Increasing need of nutrients with a decreasing amount of cells

With a decreased inoculum the inhibitory effects of the lignocellulosic material becomes clearer. Extended lag phases and inhibition of growth is present when the cell density decreases (figure 15). At these conditions the addition of nutrients is crucial for a successful fermentation. This finding was confirmed by the report of Boyer et al. (1992). In this results it is also clear that the conversion of HMF and furfural is dependent on the cell density and that the presence of both inhibitors effectively inhibit growth and fermentation.

5.5 Bioreactors

For the fermentations performed in bioreactors the ethanol yield was substantially lower than for previous experiments. All sugars are consumed during the fermentation and no contamination was found to be present in the broth, this indicates that the yields should be substantially higher. As stated in the result section the flow of nitrogen through the fermentation broth aided the evaporation on ethanol from the culture. This is also confirmed by the decrease in ethanol after approximately 20 hours. Apart from this the lower yield is also a result of an increased glycerol production. The glycerol production of these cultivations was about 3 times higher than the amounts produced during the inoculum experiment. By the elevated glycerol formation less carbon is available for ethanol production. According to Albers et al. 1996 it is important to decrease the production of glycerol not to lose raw material that can be used for ethanol formation. The higher formation of glycerol could be connected to increased osmotic stress or a larger need for reoxidization of NADH (Nissen et al. 2000)

In all the fermentations with hydrolysate the sugar consumption pattern was similar. Galactose was consumed after the depletion of glucose in all cases or not consumed at all. This is according to van Marris et al (2006) an effect of glucose inhibiting the genes of the galactose fermenting pathway. This will lead to longer fermentation times if all sugars are to be utilized. Nevertheless it can be seen that the addition of yeast extract will reduce the time needed, this since the consumption rate of sugars are increased.

5.6 Future perspective

An aspect that is not taken into account during these experiments was the cell viability. The cell density was measured by OD which will give no information about the status of the cells. It would therefore be interesting to see the effect of the addition of nutrients on the viability of the cells and to evaluate the effect of the inhibitors on cell viability. This could for example be performed through measuring the amount of colony forming units

In all experiments performed the cells were adapted to the hydrolysate during the aerobic growth phase. It can be interesting to analyse the seen effects on none adaptive cells. This to see if the beneficial effects found can aid the biomass growth in such conditions.

During this project the effect of yeast extract was independent on the amount added from 0.5%. Therefore it can be interesting to evaluate if the amount can be decreased further with maintained results and if the glycerol formation thereby is decreased. Further optimization of the amount of yeast extract needed should also be correlated to the amount of inhibitors present and the gravity of the material so that a variation in the material used can be meet by a correct dose of additives.

In a larger context there are more research needed to have a large scale production of bioethanol from lignocellulose. The process of High Gravity is a step in the right direction but more investigations are essential. Addition of nutrients can be a way thus a cheaper alternative to yeast extract will be needed. Alternatively the effects of yeast extract can be further studied so that they can be mimicked by other nutrient or genomic engineering.

6. Conclusions

The poor fermentative performance of yeast cells in the presence of inhibitors is often attributed to the decreased viability of the yeast cells. The experiments performed under high gravity conditions (30% w/w) showed that the addition of nutrients supplementations to the medium can enhance significantly the fermentability of spruce hydrolysate.

Analysis of the material revealed considerable amounts of potentially inhibitory compounds such as levulinic acid, acetic acid, HMF, furfural. Fermentations in the presence of these compounds in the actual concentrations in spruce hydrolysate showed that none of the above mentioned compounds alone, neither the mixture of them had significant inhibitory effects neither on yeast growth nor in ethanol production. This was due to the concentration used and the high concentration of cells.

The effect of yeast extract has a positive effect on the ethanol production rate during HG conditions. Here the need for nutrient supplementation is substantially higher due to more inhibitors and a nutrient limited environment. A nitrogen source is needed to raise the growth rate and thereby minimize the effect of certain inhibitors. Yeast extract was found to have a positive effect not only on the production rate but also on biomass growth. However it was concluded that the beneficial effects of yeast extract addition were not proportional to the amount of yeast extract added. The effect of yeast extract was seen in both the semi anaerobic environment of falcon tubes and in a more anaerobic environment both in a smaller and larger scale.

The process parameters were found to be crucial for the visibility of the results. Using smaller amount of inoculum the effect of yeast extract became clearer. Below a certain amount of

inoculum, the addition of yeast extract is crucial for ethanol and biomass production. The addition of yeast was also crucial for the conversion of HMF and furfural.

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9.Appendix

9.1 Calculations

At the different experiments there were differences in the sugar concentrations due to difference is materials and conditions. To be able to compare the different experiments were normalised so that all are presented as ethanol yields on available and fermentable sugars. By doing this the graphs are comparable.

The ethanol yield at different times was calculated by this method:

$$Yield = \frac{ethanol\ gL^{-1}}{glucose\ +\ mannose\ +\ galactose\ gL^{-1}\ (initial)}$$

Ethanol yield on consumed sugars was calculated as follows:

$$Yield = \frac{ethanol\ gL^{-1}}{Initial\ sugar\ conc.\ gL^{-1} - remaining\ conc\ of(\ glucose + mannose + galactose)gL^{-1}}$$

9.2 Inhibitors effect on fermentation in defined medium – extended results

In section 4.1 all cultivations are not presented. In figure 26 and table 20 the initial evaluation of inhibitors is presented. Here a negative effect on fermentation is seen by furfural. These results were confirmed by the results presented in section 4.1. The corresponding sugar consumption is displayed in figure 27 and presents a similar observation.

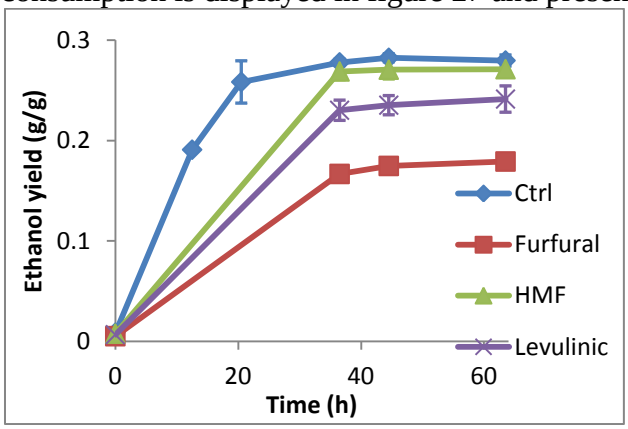

Figure 26. Ethanol yield on available sugars, fermentation was performed in defined medium with the addition of inhibitory compounds. A relatively low yield (in comparison to later experiments in this thesis) was achieved, the reasons for this are not clear. The cultivation with furfural has a clearly lower yield in comparison to the other conditions. This was re-evaluated in a repeated experiment presented in section 4.1

Table 10 Ethanol concentration (gL^{-1}) at different times after inoculation (h). A clearly lower concentration is evident for the cultivation containing furfural.

	36.5(h)	44.5(h)	63.5(h))
Controll	23.0	23.4	23.2
Furfural	**15.5**	**16.2**	**16.6**
HMF	25.0	25.2	25.2
Levulinic acid	21.4	21.9	22.4

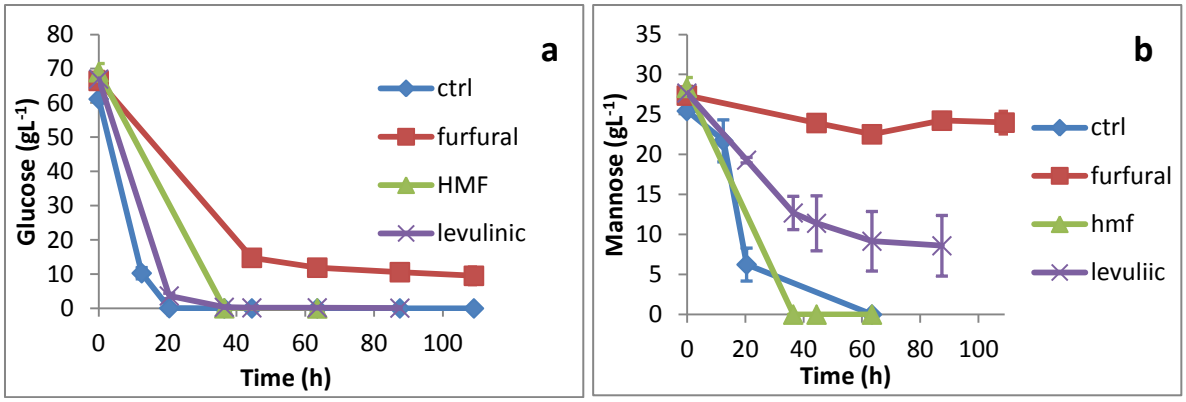

Figure 27 Sugar consumption, a- glucose consumption b- mannose consumption shows a similar result as figure 25 and table 19. When furfural has been added to the cultivation (performed in defined media) not all sugars are consumed and arrest of fermentation can be seen after approximately 40 hours.

9.3 Fermentation with the addition of acetic acid at different pH, in defined medium – extended results

Table 21 Ethanol concentrations during cultivations in defined media with the addition of acetic acid (7.2 gL^{-1}) at different pH. The result shows that inhibition of acetic acid is dependent on the pH of the fermentation broth.

	20h	**68h**
pH 4	3.1	34.8
pH 5	35.0	35.5
pH 6	33.8	32.8

9.4 Screening for nutrients with a beneficial effect on fermentation in inhibitory hydrolysate - all conditions

In figure 28 all conditions used during the screening experiment are displayed as ethanol yield during cultivation. In table 22 the ethanol concentration of all conditions are displayed.

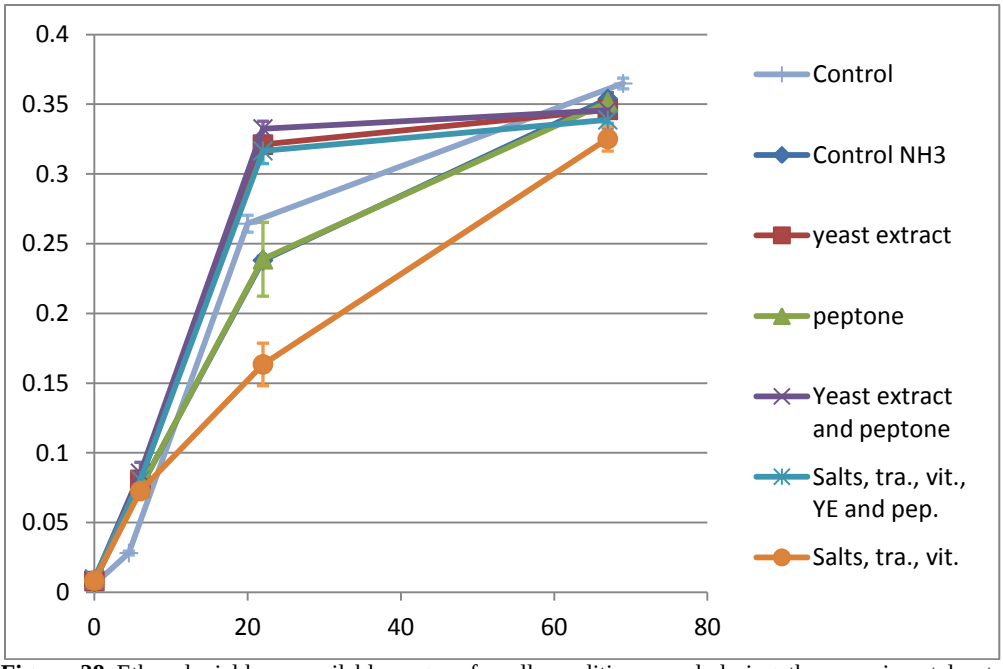

Figure 28 Ethanol yield on available sugars for all conditions used during the experimental setup. Cultivations were performed in a hydrolysate broth (78% spruce hydrolysate). Yeast extract has a beneficial effect on fermentation while the addition of salts, trace and vitamins has a negative effect. Peptone and Control NH_3 show approximately the same values.

Table 22 Ethanol concentration at different time points during fermentation. A screening was performed to compare the effect of different supplementations. After 22 hours it can be seen that the addition of yeast extract is beneficial to the fermentation of lignocellulosic materials. The addition of salts, trace and vitamins caused a decrease in ethanol concentration; this is seen after 22 hours of cultivation. At 67 hours ethanol evaporation can affect the results.

	4.5h	6h	20h	22h	67h
Control	1.6±0.1		15.2±0.3		21.0±0.2
Control + ammonia		4.3±0.2		13.7±0.2	20.4±0.3
Yeast extract		4.6±0.1		18.5±0.1	19.9±0.2
Peptone		4.3±0.2		13.7±1.5	19.5±0.3
Yeast extract + peptone		4.9±0.4		19.3±0.3	19.9±0.7
Salts, vitamins, trace, YE, peptone		4.6±0.1		18.2±0.5	19.5±0.1
Salts, vitamins and trace		4.2±0.3		9.4±0.9	18.7±0.5

9.5 Effect of yeast extract at different inoculum sizes, in semi and anaerobic environments

The ethanol concentration during cultivation in shake flasks and falcon tubes are presented in table 23 and 24.

Table 23 Ethanol concentration during the cultivation performed in shake flasks with different inoculum sizes and with and without the addition of yeast extract. The fermentation was performed in 77% lignocellulosic hydrolysate at 30° C on a rotary shaker. The effect of yeast extract is clear at an inoculum size of $1gL^{-1}$, after 100 hours the concentration of ethanol in the cultivation with YE is 8 times higher than the control cultivation without supplementation of YE.

Shake flasks	22h	46h	100+h
2 gL^{-1}	5.7±0.1	19.5±0.1	20.0±1.2
1 gL^{-1}	1.3±0.0	1.6±0.0	2.4±0.1
0.5 gL^{-1}	0.4±0.0	0.4±0.0	0.4±0.1

2 gL^{-1}+YE	12.9±0.0	21.0±0.4	20. 4±0.0
1 gL^{-1}+YE	1.3±0.0	3.0±0.2	19.1±0.0
0.5 gL^{-1} +YE	0.6±0.1	0.8±0.1	1.3±0.0

Table 22 Ethanol concentrations during the cultivation performed in falcon tubes. Lignocellulosic hydrolysate was fermented with different inoculum sizes, with and without the addition of yeast extract. The falcon tubes were immobilized on a rotary shaker at 30° C during the fermentation. The effect of yeast extract was similar to the cultivations performed in shake flasks.

Falcon tubes	**41.5h**	**67h**	**100 +h**
2 g/L	18.7±0.3	20.5±0.2	20.7±0.8
1 g/L	1.5±0.0	1.5±0.1	1.5±0.0
0.5 g/L	0.4±0.0	0.4±0.0	0.4±0.0
2 g/L +YE	21.5±0.1	21.0±0.2	20.6±0.2
1 g/L +YE	2.3±0.0	2.5±0.2	19.1±0.1
0.5 g/L +YE	0.8±0.1	0.6±0.1	0.7±0.0

9.6Evaluation of fermentation in high hydrolysate concentration

Cultivations with 93% of hydrolysate were performed in four different conditions (table 25). The fermentation was carried out in 50mL falcon tubes with a fermentation volume of 15 mL.

Table 25 conditions used in 93% hydrolysate

Condition	**Amount of supplement**	**pH adjusted with**
A. Control	-	$NaOH_4$
B. Yeast extract	1%	NH_3
C. Salts, trace and vitamins	Concentrations as in controlled media	NH_3
D. Salts, trace, vitamins and yeast extract	1% of peptone and yeast extract, others as in controlled media	NH_3

In 77.9% of hydrolysate there is no clear inhibitory effect from the material. Therefore an experiment was performed at a higher concentration of hydrolysate. The condition used was chosen from the results of previous experiments.

For this experiment biomass was only increased with an OD of 1 which in comparison to previous experiment was noteworthy. The amount of ethanol produced during this experiment was also considerably lower than for previous experiment. Ethanol production and metabolite consumption comes to an arrest already after 22 hours (figure 29).

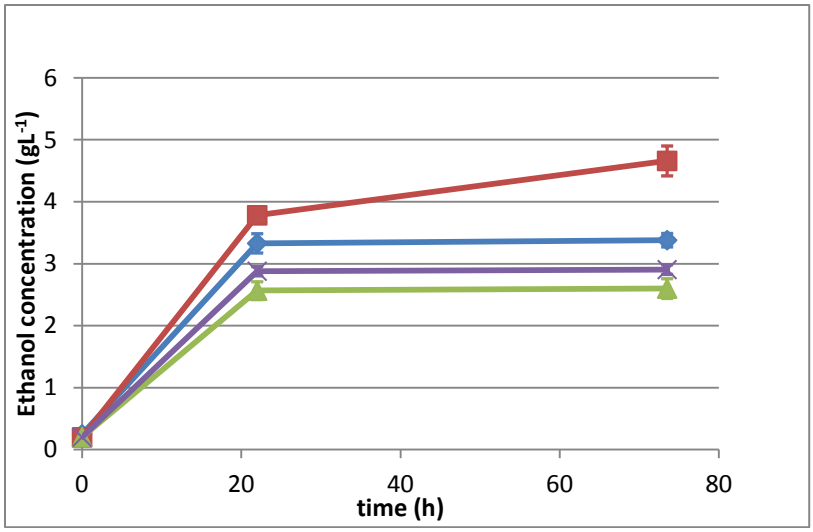

Figure 29 Ethanol production as a function of time at 93% of hydrolysate. Red – yeast extract, blue – ctrl, purple – salt, trace, vitamins and yeast extract, green – salts, trace and vitamins

The low amounts of ethanol produced, the consumption arrest and the low biomass growth implicated that this was not only caused by the high hydrolysate levels.