Master Thesis

Evaluation of Different Enzymes and Yeasts, and Their Impact on Bioethanol Production Based on Debranned Wheat

Lina Lindberg

LITH-IFM-A-EX--09/2103—SE

Spring 2009

Department of Physics, Chemistry and Biology
Linköping University
SE-581 83 Linköping, Sweden
Master Thesis

Evaluation of Different Enzymes and Yeasts, and Their Impact on Bioethanol Production Based on Debranned Wheat

Lina Lindberg

Spring 2009

Performed at Chematur Engineering AB
Karlskoga, Sweden

Supervisor
Anna Eriksson

Examiner
Uno Carlsson
Abstract

Bioethanol is a fuel of tomorrow, and progress in the use of enzymes and reduction of non-fermentable materials by debranning will probably be a part to make it more economical with low environmental impact.

Ethanol production based on debranned wheat was optimized in this study by batch experiments as well as continuous experiments in laboratory scale. Enzymes from Novozymes and Genencor were compared and no significant differences were discovered between the different set of enzymes. The yeast strains Ethanol Red and AmyloFerm were compared with traditional baker’s yeast and baker’s yeast were surprisingly the fastest to ferment, but Ethanol Red had higher viability during fermentation. Protease addition during saccharification does not seem to improve fermentation with baker’s yeast. Prolonged liquefaction and saccharification time does probably not have any large impact on glucose yield. The continuous lab-scale process has a potential to be a realistic model but the stirring has to be improved and the pipe diameter increased.

Keywords: Bioethanol, debranned wheat, α-amylase, glucoamylase, protease, baker’s yeast, Ethanol Red, AmyloFerm, HPLC, GC, viscosity
# Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>DP3</td>
<td>Saccharide of three glucose units, also called maltotriose</td>
</tr>
<tr>
<td>DP4+</td>
<td>Saccharide of four or more glucose units</td>
</tr>
<tr>
<td>DS</td>
<td>Dissolved solids</td>
</tr>
<tr>
<td>FS</td>
<td>Fiber solids</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>SS</td>
<td>Suspended solids</td>
</tr>
<tr>
<td>TDS</td>
<td>Total dry solids</td>
</tr>
</tbody>
</table>
# Table of Contents

1 INTRODUCTION ............................................................................................................................ 1-1
   1.1 BACKGROUND .............................................................................................................................. 1.1-1
   1.2 AIM OF THE STUDY ....................................................................................................................... 1.2-2

2 THEORETICAL BACKGROUND ........................................................................................................ 2-3
   2.1 GRAINS ...................................................................................................................................... 2.1-3
       2.1.1 Wheat ................................................................................................................................. 2.1.1-3
       2.1.2 Debranned Grains .............................................................................................................. 2.1.2-3
       2.1.3 Starch ............................................................................................................................... 2.1.3-4
       2.1.4 Sugars .............................................................................................................................. 2.1.4-4
   2.2 YEAST ....................................................................................................................................... 2.2-4
       2.2.1 Stress Factors ...................................................................................................................... 2.2.1-5
       2.2.2 Wild Yeast .......................................................................................................................... 2.2.2-6
       2.2.3 Baker’s yeast ...................................................................................................................... 2.2.3-6
       2.2.4 Ethanol Red ........................................................................................................................ 2.2.4-6
       2.2.5 AmyloFerm .......................................................................................................................... 2.2.5-6
   2.3 ENZYMES .................................................................................................................................. 2.3-7
   2.4 THE PROCESS .............................................................................................................................. 2.4-7
       2.4.1 Milling ................................................................................................................................. 2.4.1-7
       2.4.2 Mixing ............................................................................................................................... 2.4.2-8
       2.4.3 Liquefaction ...................................................................................................................... 2.4.3-8
       2.4.4 Saccharification .................................................................................................................. 2.4.4-8
       2.4.5 Yeast Propagation .............................................................................................................. 2.4.5-8
       2.4.6 Fermentation ...................................................................................................................... 2.4.6-8
   2.5 PROCESS PROBLEMS ................................................................................................................. 2.5-9
       2.5.1 Nonsolubilized Starch ........................................................................................................... 2.5.1-9
       2.5.2 Retrogradation ..................................................................................................................... 2.5.2-9
       2.5.3 Reversion ............................................................................................................................. 2.5.3-9
       2.5.4 Maillard Reactions .............................................................................................................. 2.5.4-10
       2.5.5 Bacterial Contamination ..................................................................................................... 2.5.5-10
   2.6 BY-PRODUCTS ............................................................................................................................ 2.6-10
       2.6.1 Glycerol ............................................................................................................................... 2.6.1-10
       2.6.2 Aldehydes ........................................................................................................................... 2.6.2-11
       2.6.3 Acids .................................................................................................................................... 2.6.3-11
       2.6.4 Esters .................................................................................................................................... 2.6.4-11
       2.6.5 Fusel Oils ............................................................................................................................. 2.6.5-11

3 MATERIALS AND METHODS ......................................................................................................... 3-12
   3.1 MATERIALS ................................................................................................................................. 3.1-12
       3.1.1 Yeast .................................................................................................................................... 3.1.1-12
       3.1.2 Enzymes ............................................................................................................................ 3.1.2-12
       3.1.3 Reagents and Chemicals ...................................................................................................... 3.1.3-12
   3.2 STANDARD EXPERIMENTAL SETUP ....................................................................................... 3.2-12
       3.2.1 Mixing .................................................................................................................................. 3.2.1-12
       3.2.2 Liquefaction ....................................................................................................................... 3.2.2-12
       3.2.3 Saccharification ................................................................................................................... 3.2.3-12
       3.2.4 Yeast Propagation .............................................................................................................. 3.2.4-13
       3.2.5 Fermentation ....................................................................................................................... 3.2.5-13
   3.3 ANALYTICAL METHODS ............................................................................................................ 3.3-13
       3.3.1 Sieve Analysis ....................................................................................................................... 3.3.1-13
       3.3.2 Dry Solids ............................................................................................................................ 3.3.2-13
       3.3.3 Starch Analysis ..................................................................................................................... 3.3.3-14
1 Introduction

The extensive use of fossil fuels is no longer sustainable and alternative energy sources are necessary. The intergovernmental panel on climate change stated 2007 a sharp warning that global warming is a fact and that actions has to be taken immediately (IPCC, 2007). Another fact that will force the world to use alternative fuels are peak oil and the people must be considered living in the post oil era (ASPO, 2005). What the world needs now is clean and secure transportation fuels (European Commission, 2006). The European Union has a vision that by 2030, 25% of the road transport fuels should be clean and CO$_2$-efficient biofuels. The European Commission stated that it should be biofuels compatible with today’s fuels, so that it in a transition period is possible to use the current infrastructure and engine technology.

Ethanol is a renewable CO$_2$ efficient biofuel that can be produced completely green and works well in the world’s current car park. Brazil started to produce ethanol in 1975 (Wheals et al., 1995). Today, ethanol production is spread around the world and had 2007 a net production of 50 billion liters worldwide (Licht, 2007). To reach the EU vision, domestic biofuel production within EU needs to be considerably increased and in balance with international biofuel trade (European Commission, 2006). The European Commission stated three parallel paths for ethanol production. First, traditional grain based production, secondly, production based on agricultural waste and the third path is to produce ethanol from energy crops. Grain based ethanol production is the technology that works today, and hence the focus should lay on optimizing the industrial production. In parallel, focus should be on research and development for making the other two technologies ready for industrial production. In conclusion, increase the grain based production in short term and ethanol production from agricultural waste and energy crops are a long term goal. The increased costs for using biofuels instead of fossil fuels has to be put into relation with enhanced security of supply, reduced green house gas emissions and employment opportunities.

Ethanol is mainly produced from sugar cane, sugar beet and corn, but in Europe, wheat based ethanol production is the most common (European commission, 2006). Some voices are raised against ethanol production claiming it drives up the grain prices while other see it as an opportunity for the farmers to get more out of every harvest. Large amounts of grains get spoiled every year due to weather conditions (Suresh et al., 1999). These spoiled grains are cheap and suitable for ethanol production.

1.1 Background

This thesis was performed at Chematur Engineering AB in Karlskoga, Sweden. The strategy of the company is to construct and sell chemical production plants around the world. Ethanol production is one of their technologies and they have a patented way to continuously produce ethanol, called Biostil 2000.
1.2 Aim of the Study

The aim of this study was to further develop grain based ethanol production, with the use of debranned wheat as raw material. The main tasks were:

- To compare enzymes from two different manufacturers to find enzymes that improve the Biostil 2000 process.
- To compare yeast strains available for ethanol production to find yeasts that improve the Biostil 2000 process.
- To evaluate if an increased duration time during liquefaction and saccharification negatively affects the yield of glucose.
- To develop a lab scale process for continuous accomplishment of liquefaction and saccharification.
2 Theoretical Background

2.1 Grains
Many different kinds of cereal grains can be used in ethanol production. The important thing is that they have a high starch content since starch is converted to glucose and glucose is the source for yeast to produce ethanol. Corn, potatoes, wheat, barley, sorghum and cassava root are some examples of starch rich materials used in industrial ethanol production. (Nigam and Singh, 1995; Power, 2003)

2.1.1 Wheat
Wheat is a cereal grain with approximately 60-74% starch content (Åman, 1987). It is composed of germ and endosperm that is covered by many layers of bran (Figure 1).

![Figure 1: Structure of wheat grain (The Baker, no date).](image)

2.1.2 Debranned Grains
Debranning is a process that removes the outer layer of grains sequentially by friction and abrasion (Dexter and Wood, 1996). This process is done prior to milling. How much of the bran that is removed depends on the number of times the process repeats. Debranning increases the amount of starch and decreases the amount of by-products in the flour. However, wheat looses approximately 13-14% starch and 22-26% proteins during the debranning process (Wang et al., 1997). Grains used in Biostil 2000 are not debranned so much and only loose a few percents of the total starch content (Eriksson, 2009).
Debranning of triticale increased fermentation efficiency from 88% with whole grains to 91%, despite the loss of starch (Wang et al., 1997). No difference in fermentation efficiency between whole wheat and debranned wheat could be seen. That result can be explained by the fact that the wheat bran does not contain fermentation inhibitors in the same way as the bran in triticale and other grains.
2.1.3 Starch
Most of the energy cereal grains produced during photosynthesis is stored in long chains of glucose units called starch (Robyt, 2001). Two different forms of starch are present in the cereal grain (Figure 2). Amylose is a form of starch with glucose units linked by α-(1,4) linkages creating a straight starch molecule. The second form of starch, amylopectin consists of glucose units liked by α-(1,4) linkages and α-(1,6) branch linkages creating a branched molecule.

Figure 2: Structural formulas of the two starch forms. a) Amylose and b) amylopectin (Robyt, 2001).

Starch is stored in granules inside the cereal grain (Robyt, 2001). Size and shape of granules depend on the botanic origin but granules in wheat and rye are disk shaped with 10 µm thickness and 20-30 µm in diameter. Granules resist water and enzyme penetration at room temperature but get more permeable at higher temperatures (Power, 2003).

2.1.4 Sugars
Starch broken down to polysaccharides is called dextrin. DP4+ is the term for saccharides with four glucose units or more. DP3, also called maltotriose, consists of three glucose units linked together by α-(1,4) linkages and maltose is two glucose units linked together with an α-(1,4) linkage. Isomaltose and maltulose are isomers of maltose linked with α-(1,6) linkages. (Power, 2003)

2.2 Yeast
Yeast is a unique microorganism because of its ability to switch between respiration and fermentation. Respiration occurs during aerobic conditions and is a process that gives the cell large amounts of energy, so it can grow and divide. In anaerobic conditions, the cell produces ethanol and less amounts of energy by fermentation. Substrate for yeast is sugars with low molecular weight e.g. glucose. If the concentration of glucose is high,
above 1 g/L, ethanol is produced by fermentation even during aerobic conditions. This phenomenon is called the Crabtree effect. Thus, an ethanol fermentor does not have to be anaerobic. In fermentation, glucose is converted to ethanol by a reaction cycle called the glycolytic pathway or Embden-Meyerhof-Parnas (EMP) pathway. In this reaction one glucose molecule yields two molecules of ethanol, two molecules of carbon dioxide and a small amount of energy (Equation 1). (Russell, 2003)

\[ C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy} \quad (1) \]

Yeast cells in the exponential growth phase can produce over 30 times as much ethanol as non-growing cells (Kelsall and Lyons, 2003b). The aim with a continuous process is therefore to spoon feed the yeast with sugar so that it stays in the exponential growth phase. Too high glucose concentrations can also inhibit yeast growth. Yeast cells multiply through budding (Russell, 2003). Lifespan of yeast is measured by the number of times it replicates. A cell can replicate 10-33 times, depending on yeast strain before it reaches the limit, called Hayflick limit and enters the next stage called senescence, which leads to cell death.

Glucose is the yeast’s favorite substrate because it enters the cell by diffusion (Russell, 2003). Maltose and maltotriose pass the cell membrane by an active process and is broken down to glucose inside the cell. Yeast can not utilize polysaccharides longer than three units. Isomaltose and maltulose can not be utilized either because of their α-(1,6) linkage instead of α-(1,4) linkage (Power, 2003).

Nitrogen is important for yeast growth and the yeast can only utilize small nitrogenous molecules. Nitrogen shortage can therefore become a problem during ethanol production. Phosphorus is important to the yeast to build structural molecules such as phospholipids and nucleic acids (Russell, 2003).

### 2.2.1 Stress Factors

There are numerous of stress factors that affect yeast growth and fermentation (Russell, 2003). Yeast tolerates temperatures up to 35-43 °C and pH down to 3-4 depending on strain. Ethanol yield of 180 g/L is possible for yeast to tolerate but stress factors act synergistically so it tolerates less of one parameter if another parameter is high (Kelsall and Lyons, 2003a). Infections are another stress factor for yeast and is further described in section 2.5.5.
2.2.2 Wild Yeast
Wild yeast is a common problem in ethanol production (Campbell, 2003). The fermentor can be contaminated with wild yeast that grows faster than cultured yeast and will therefore gradually take over the fermentor. This may lead to decreased productivity or other process problems. One study followed yeast population in an industrial distillery (Gosselin, 2009). At the beginning of fermentation, 100% of the yeast was culture yeast. After 3-5 days, 70% of the yeast population was culture yeast while 30% was wild yeast.

2.2.3 Baker’s yeast
*Saccharomyces cerevisiae* is generally called baker’s yeast and has been used for a long time in baking industries (Cedeño Cruz, 2003). It is a cheap commercially available yeast strain with the properties as described in section 2.2. A problem with baker’s yeast is the relatively high levels of wild yeast and bacteria contamination (Suihoko and Mäkinen, 1981).

2.2.4 Ethanol Red
Ethanol Red is a selected strain of *Saccharomyces cerevisiae* that is developed especially for the ethanol industry. The strain has high ethanol tolerance and high cell viability also in highly concentrated mash. Optimal fermentation temperature is 30-40 °C. Ethanol concentrations as high as 142 g/L have been reported during 35 °C fermentation (Fermentis, no date).

2.2.5 AmyloFerm
SIHA AmyloFerm is a wild yeast strain called *Saccharomyces diastaticus*. Unique with this strain is that it can ferment liquid starch, dextrin, maltose and melibiose and this yeast can therefore ferment directly after liquefaction without need for saccharification. However, this yeast is also good for use in a process with saccharification. Optimal temperature is 30-38 °C but it can ferment up to 40 °C. AmyloFerm tolerates up to 142 g/L ethanol (Bergerow GmbH & Co, 2000).
2.3 Enzymes

Yeast cannot utilize starch present in cereal grains, so hydrolysis of starch is necessary for a high yield of ethanol (Power, 2003). Enzymatic hydrolysis can be done in different ways depending on the process. In general, α-amylase is used to break down the two forms of starch called amylase and amylopectin. α-amylase is an endoenzyme that hydrolyses randomly within the chain of glucose units, creating smaller pieces of starch called dextrins. Dextrins are then converted to glucose units by glycoamylase, which is an exoenzyme that cleaves off single glucose molecules by hydrolyzing the α-(1,4) linkage from the non-reducing end of dextrins. α-(1,6) linkages are also hydrolyzed but very slowly. Pullulanase is a debranching enzyme that much faster hydrolyses the α-(1,6) linkage in amylopectin. Cellulase and xylanase are enzymes that modify and digest non-starch carbohydrates and are used to lower the mash viscosity during saccharification (Kesall and Lyons, 2003b). Protease is an enzyme that hydrolyzes proteins to amino acids which the yeast can utilize as a nitrogen source. The enzyme is used during saccharification if the mash nitrogen levels are too low. However, amino acids can react with sugar and cause process problems (Section 2.5.4) so amino acid levels should not be too high.

Novozymes is the manufacturer for the enzymes used in Biostil 2000. In this thesis enzymes from a manufacturer called Genencor are also evaluated. For specific information about the enzymes used, see Table I.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme name</th>
<th>Manufacturer</th>
<th>pH range</th>
<th>Temp. range (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-amylase</td>
<td>Liquozyme SC DS</td>
<td>Novozymes</td>
<td>5.7-6.0</td>
<td>82-86</td>
</tr>
<tr>
<td></td>
<td>GC 420</td>
<td>Genencor</td>
<td>5.5-6.5</td>
<td>85-95</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>Spirizyme Fuel</td>
<td>Novozymes</td>
<td>3.5-4.5</td>
<td>65-70</td>
</tr>
<tr>
<td>Glucoamylase and Pullulanase</td>
<td>Distillase VHP</td>
<td>Genencor</td>
<td>4.0-4.5</td>
<td>60</td>
</tr>
<tr>
<td>Cellulase and Xylanase</td>
<td>Viscozyme Wheat</td>
<td>Novozymes</td>
<td>4.5-5.5</td>
<td>50-55</td>
</tr>
<tr>
<td></td>
<td>Optimash BG</td>
<td>Genencor</td>
<td>3.5-5.5</td>
<td>55-75</td>
</tr>
<tr>
<td>Protease</td>
<td>Fermgen</td>
<td>Genencor</td>
<td>3.5-5.0</td>
<td>28-65</td>
</tr>
</tbody>
</table>

2.4 The Process

Ethanol production can be performed as a batch process in which only one vessel is used, or as a continuous process with at least one vessel for each process step. Biostil 2000 is a way to continuously produce ethanol at steady state, which means that all process parameters in the fermentor are constant all the time (Chematur, no date).

2.4.1 Milling

Milling is the first step in ethanol production and the purpose is to break up cereal grains into smaller particles that better adsorb water (Dexter and Wood, 1996). The particles have to be small enough to yield high water access, but they should not be too small so that they cause problems in the recovery of co-products. The most common mill used in distilleries is a hammer mill, with hammers that rotate at high speed, crushing the grains.
in the grinding chamber. A retention screen is used to hold back the largest milling particles. Debranned grain meal is produced by bran removal before milling. Particle size distribution after milling can be checked by sieve analysis described in section 3.3.1 (Kelsall and Lyons, 2003b). Desired particle size distribution in Biostil 2000 is normally distributed between 200-1000 µm (Figure 6; Stenmark, 2009).

2.4.2 Mixing
Each process based on cereal grains starts with a mixing stage where grain meal and water are mixed under heat (Kelsall and Lyons, 2003b). The temperature and duration time vary but temperatures around 40-60 °C and duration time between a few minutes to 30 minutes are common. Viscosity reducing enzymes can sometimes be used to decrease the mash viscosity (Koops, 2009).

2.4.3 Liquefaction
The mash is moved to a liquefaction tank where it is heated to 90 °C for 2 h (Kelsall and Lyons, 2003b). The enzyme α-amylase is added to break down starch into dextrins. At approximately 65 °C, starch granules have adsorbed so much water that they swell into large gelfilled sacs with lost crystalline structure. That phase is called gelatinization, where the mash has a very high viscosity. The swollen granules are now permeable for α-amylase, allowing it to penetrate and break down starch, which markedly reduce mash viscosity. The high liquefaction temperature is also used to minimize bacterial contamination. In large continuous processes it is common to use two liquefaction tanks in serie (Eriksson, 2009).

2.4.4 Saccharification
The next process step is saccharification where dextrins are broken down to glucose by the enzyme glycoamylase (Kelsall and Lyons, 2003b). The mash is kept at around 60 °C and the pH is adjusted to 4.0-4.5 (Power, 2003). The extent of this step varies between different processes. In Biostil 2000, a saccharification time of 21-24 h is used to give a complete saccharified mash (Eriksson, 2009). However, it is nowadays common to do simultaneous saccharification and fermentation (SSF) or to just use a short saccharification step prior SSF (Kelsall and Lyons, 2003b). During saccharification the viscosity is reduced with cellulase and xylanase (Novozymes, 2005). In the Biostil 2000 process more than one saccharification tank are used in serie (Eriksson, 2009).

2.4.5 Yeast Propagation
Yeast used during fermentation is first propagated in conditions optimal for yeast growth (Heist, 2008). Small amounts of pitching yeast are added to the propagation tank and after around 10 h, propagation is completed and a large amount of cells in growth phase are available for fermentation. Instead of propagation, large quantities of purchased yeast can be used in fermentation (Russell, 2003). Activated dry yeast is most common and this yeast has to be rehydrated in water before inoculation of the fermentor.

2.4.6 Fermentation
Fermentation is carried out with the addition of yeast that converts sugar produced during pretreatment to ethanol (Russell, 2003). Baker’s yeast or modified strains are commonly
used and a suitable fermentation temperature is 32-35 °C. The optimal pH for yeast is around 5.0-5.2, but it is very common to ferment at a lower pH to decrease the bacterial growth. pH does also decrease during fermentation as a result of yeast excretion of H\(^+\) ions. The residence time in the fermentor varies with yeast concentration and the extent of yeast stress factors, but 10-60 h of fermentation is common (Kelsall and Lyons, 2003a). Unique with Biostil 2000 is that fiber and yeast are separated from the fermented mash and yeast is recycled back to the fermentor (Ingledew, 2003). Yeast concentration in a Biostil 2000 fermentor is high, up to 1000 million cells/ml which is five to ten times more than in a regular continuous fermentation.

After separation of fibers and yeast from the fermented mash, ethanol is extracted by distillation (Madson, 2003).

### 2.5 Process Problems

Many process problems can arise if the process parameters are not kept at the right values, so maintaining the process in balance is very important.

#### 2.5.1 Nonsolubilized Starch

After gelatinization some starch granules stay non soluble despite their lost structural organization. These granules are commonly referred to as ghosts since they are still visible particles but with some lost content. The formation of ghosts is not well understood but it seems like starch is containing some kind of specific stabilizing factor. Ghosts decrease the starch yield and thereby the ethanol yield. Mash with ghosts has a short texture instead of the usual long viscous texture (Debet and Gidley, 2007).

#### 2.5.2 Retrogradation

Retrogradation is a phenomenon when starch molecules released from granules, reorganize forming helices with intermolecular hydrogen bonds. These helices are resistant to degradation by \(\alpha\)-amylase, and will thereby decrease the yield of degraded starch. The straight form of starch called amyloase, forms much stronger helices than amylopectin, which is the branched starch form. Retrograded amyloase is therefore particularly resistant to \(\alpha\)-amylase digestion. Retrogradation occurs when mash is heated above gelatinization and then cooled down to lower temperature. Greater differences between heating and cooling temperatures increase formation of helices. Retrogradation has been observed in wheat starch, heated to 90 °C and then cooled to 30 °C. However, retrogradation was slower for wheat than for maize and potato starch (Fechner et al., 2005).

#### 2.5.3 Reversion

A problem that can arise when glucose concentrations are high is that glycoamylase catalyzes repolymerization of glucose forming maltose, isomaltose and other glucose by-products (Crabb and Shetty, 1999). It is the formation of saccharides with \(\alpha\)-(1,6) linkage, for example isomaltose, that usually is referred to as reversion products, because of the utilization inability of the yeast. If no branches are present, reversion can not occur. Therefore, addition of pullulanase that breaks \(\alpha\)-(1,6) linkages in amylopectin can limit
this problem. Too large dosing of glycoamylase has been one factor known to cause this problem (Power, 2003).

2.5.4 Maillard Reactions
Maillard reaction, also called non-enzymatic browning, occurs when reducing sugars such as glucose, maltose, fructose or ribose react with amino acids forming a complex with lower solubility called maillard reaction product (MRP) (Tauer et al., 2004). Different amino acids and different sugars form various kinds of MRP. The formation of MRP is temperature and pH dependent, with increased formation at high temperatures and low pH. The MPR is resistant to α-amylase degradation which leaves sugar unavailable for fermentation (Kesall and Lyons, 2003b). The MRP has also an inhibiting effect on yeast, causing decreased ethanol formation (Tauer et al., 2004). The inhibiting effect is pH dependent with a weak inhibiting effect at pH 5, and a large inhibiting effect at pH 7 and pH 8. Since ethanol production is carried out below pH 5, the MRP will probably not inhibit yeast in a noticeable way.

2.5.5 Bacterial Contamination
Bacterial contamination is a problem in ethanol production (Narendranath, 2003). The bacteria compete with yeast for the available sugar resulting in less ethanol yield and the bacteria do also produce by-products that inhibit the yeast. Proper cleaning and sanitizing of the equipment can significantly reduce bacterial contamination, but it is difficult to completely get rid of the problem. One difficulty is that bacteria multiply every 20-30 min while yeast multiplies every third hour (Kelsall and Lyons, 2003a). One single bacterium in the start can result in approximately 30 million bacteria after 24 h.

Lactic acid bacteria are the most problematic bacteria, since they tolerate high temperature and low pH (Narendranath, 2003). These bacteria produce lactic acid and acetic acid which slows down or stop yeast fermentation. Acetic acid bacteria such as Acetobacter and Gluconobacter could be harmful in the yeast propagation tank. The bacteria are aerobic so they usually die in the fermentor tank, but they may have produced enough acetic acid to slow down or inhibit the yeast growth in the fermentor. Concentrations higher than 8 g/L lactic acid and 0.5 g/L acetic acid decrease yeast growth (Russell, 2003).

2.6 By-products
Ethanol is a product from yeast fermentation but there is also many other substances that yeast produces during fermentation and oxidative growth. The level of by-products produced depends on the yeast environment (Russell, 2003).

2.6.1 Glycerol
Glycerol is a very common fermentation product. It is important in order to maintain the cells redox balance by reoxidation of NADH. During anaerobic conditions, glycerol fermentation is the only way the cell can get rid of NADH. Glycerol has also a role in protecting the cell against high osmotic pressure and heat shock (Russell, 2003).
2.6.2 Aldehydes

Acetaldehyde is a middle product in the glucose to ethanol pathway. During fermentation acetaldehyde is converted to ethanol while in oxidative conditions it is converted to acetic acid (Russell, 2003). Oxidation of ethanol can also generate acetaldehyde, which occurs when glucose levels decline (Cedeño Cruz, 2003).

Acrolein is an aldehyde that can be produced from glycerol. Lactic acid bacteria can degrade glycerol into 3-hydroxy propionaldehyde that spontaneously and slowly is converted to acrolein.

2.6.3 Acids

Succinic acid is produced by an incomplete citric acid cycle or by reductive pathways that contain citric acid cycle enzymes (Russell, 2003). Acetic acid can be produced from acetaldehyde, that is produced during oxidation of ethanol (Cedeño Cruz, 2003). This occurs when glucose levels decrease and the oxidative process takes over fermentation. Yeast can also produce acetic acid during hyperosmotic stress when the sugar levels are too high (Erasmus, 2004).

2.6.4 Esters

It is not well understood why yeast produces esters but it might be a way to reduce the toxic effects of fatty acids. Increased yeast growth and increased oxygenation seems to decrease the amount of produced esters. Ethyl acetate, the most common ester, is synthesized from acetic acid and ethanol (Cedeño Cruz, 2003).

2.6.5 Fusel Oils

The most commonly produced higher alcohols, called fusel oils are 1-propanol, amyl alcohol (methyl-2-butanol and methyl-3-butanol), isoamyl alcohol and phenethyl alcohol. Production of isoamyl alcohol and isobutanol begin with decreased sugar levels. High temperatures of around 38 °C instead of 32 °C increase the production. Many of the fusel oils are produced when yeast use amino acids as a nitrogen source (Cedeño Cruz, 2003).
3 Materials and Methods

3.1 Materials

3.1.1 Yeast
Three different yeast strains were used, all of them in the form of dry yeast. Baker’s yeast from Jästbolaget AB, Sweden, Ethanol Red from Fermentis, France and AmyloFerm from SIHA, Germany.

3.1.2 Enzymes
Seven enzymes were used in the process. Liquozyme SC DS, Spirizyme Fuel and Viscozyme Wheat were a kind gift from Novozymes (Novozymes A/S, Bagsvaerd, Denmark). GC 420, Distillase VHP, Optimash BG and Fermgen were a kind gift from Genencor (Danisco US Inc., Genencor division, Rochester, USA).

3.1.3 Reagents and Chemicals
(NH₄)₂SO₄, KH₂PO₄, KI, agar and succinic acid were purchased from Merck. MgSO₄ came from Kebo AB, Stockholm, Sweden, yeast extract was produced at Biolife, Milan, Italy and Rhodamine B from Alfa Aesar, Karlsruhe, Germany.

3.2 Standard Experimental Setup
The batch process and the continuous process experiments were preformed in small laboratory scale with a working volume between 200-600 ml. All experiments followed a standard experimental setup which is discussed in this section. Variation from the setup is described together with each experiment. Debranned wheat grains (Procop, Czech Republic) was used as raw material and heating was obtained by a temperature controlled hot plate stirrer (IKA RCT Basic) with magnetic stirring bar.

3.2.1 Mixing
Milled grain was mixed with water to obtain a mash with 33% solids (w/w). The mash was heated to 50 °C during 30 min.

3.2.2 Liquefaction
The α-amylase Liquozyme SC DS (0.266 g/kg wheat) was added to the mash and it was heated to 90 °C for two hours. Timekeeping started when the mash reached 85 °C.

3.2.3 Saccharification
The temperature was reduced to 60 °C and the pH was adjusted to 4.1-4.2 using H₂SO₄ (18 M). Spirizyme (0.398 g/kg wheat) which contains glucoamylase, and Viscozyme Wheat (0.149 g/kg wheat) which contains cellulase and xylanase were added to the mash. Saccharification was carried out at 60 °C during 21 h.
3.2.4 Yeast Propagation

Yeast was propagated in yeast sucrose media containing sucrose (50 g/L), (NH$_4$)$_2$SO$_4$ (5 g/L), KH$_2$PO$_4$ (2 g/L), MgSO$_4$ (0.5 g/L), yeast extract (5 g/L) and succinic acid (3 g/L). Yeast was stored on solid agar (16 g/L) containing yeast sucrose medium but without succinic acid to prevent agar degradation. The medium and the agar were sterilized by autoclavation.

Dry yeast of AmyloFerm was cultured and stored on a solid agar plate. Yeast from the agar plate was then propagated in the yeast sucrose medium at 32 °C during 26 h. Centrifugation (980 g, 10 min) separated the medium from yeast before fermentation. In the experiments with baker’s yeast and Ethanol Red, enough dry yeast was available so propagation was not necessary. The dry yeast was instead rehydrated at 32 °C during 30 min in five times its weight of sterilized water.

3.2.5 Fermentation

Saccharified mash was diluted with distilled water (228 ml/L mash) before addition of approximately 500×10$^6$ yeast cells/ml. The temperature was held at 32 °C in roughly about 42-66 h for complete fermentation. The fermentation was regarded as complete when the glucose concentration decreased below 1 g/L. Antifoam (1 ml/L mash) was added after around 30 min of the fermentation to prevent large amounts of foam. The pH was adjusted to 4.2-4.3 using NaOH (11 M) after 18 h of fermentation or to pH 4.5 after a fermentation time of 2 h.

3.3 Analytical Methods

3.3.1 Sieve Analysis

Size distribution of particles in wheat flour, was determined with a set of nine test sieves (Retsch, Haan, Germany) in the range of 71-1400 µm. 100 g of flour was added and the sieves were shaken by hand for 2 min. The collected flour in each sieve was then weighed.

3.3.2 Dry Solids

Total dry solids (TDS), suspended solids (SS), dissolved solids (DS) and fiber solids (FS) were determined with measurements and calculations. An infrared dryer (Mettler PM 100 or Mettler PM 460) measured TDS and dry solids in the supernatant achieved after centrifugation. SS was then calculated with equation 2 and DS was calculated with equation 3.

\[
SS = 1 - \frac{TDS - 1}{Supernatant \ dry \ solids - 1} \tag{2}
\]

\[
DS = TDS - SS \tag{3}
\]
FS was measured by rinsing 10 g of mash with water through a tea-strainer. Fibers in the tea-strainer were oven dried at 120 °C over night before weighing and a fiber content calculation.

The percentage of solids and fibers was also measured by a spin test. 10 ml of mash was centrifuged (980 g, 10 min) in a graded test tube. The supernatant was discarded and the test tube was refilled with water and centrifuged again (980 g, 10 min). The percentage of solids and fibers in the mash could then be read from the test tube.

### 3.3.3 Starch Analysis

A starch iodine test was used to determine if the depolymerization of starch during liquefaction was complete. A liquefaction sample was centrifuged (980 g, 10 min) and the supernatant was diluted 2.5 times. Small amounts of KI-I₂ solution were added until a permanent color change was obtained. The degree of color indicated the degree of starch polymerization (DP) (Table II).

<table>
<thead>
<tr>
<th>Degree of Polymerization (DP)</th>
<th>Color of the carbohydrate-iodine complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-8</td>
<td>None (yellow)</td>
</tr>
<tr>
<td>9-14</td>
<td>Brown</td>
</tr>
<tr>
<td>15-29</td>
<td>Red</td>
</tr>
<tr>
<td>30-38</td>
<td>Red-violet</td>
</tr>
<tr>
<td>39-46</td>
<td>Blue-violet</td>
</tr>
<tr>
<td>&gt; 47</td>
<td>Blue</td>
</tr>
</tbody>
</table>

### 3.3.4 Viscosity

Viscosity, centipoise (cP) of the sample after every process step was measured by a viscometer (Brookfield Viscometer DVII+ Pro) with a small sample adapter and spindle SC4-34. A program with a speed between 1-160 rpm was used, which corresponds to a shear rate between 0.28-44.8 s⁻¹. Measurements were performed at the specific temperature for that process step.

### 3.3.5 HPLC

High performance liquid chromatography (HPLC) (Shimadzu 20 systems, Shimadzu corporation) was used to determine the concentration of different sugars and acids in saccharification and fermentation samples. Detection was achieved by a refractive index detector. A Rezex RPM-monosaccharide Pb²⁺ column (Phenomenex, USA) was used to measure the amount of different sugars during saccharification. Ultrapure water with a flow rate of 0.6 ml/min was used as a mobile phase and the column temperature was 75 °C. This column was able to detect DP4⁺, DP3, isomaltose, maltose, maltulose and glucose but the present method was not fully optimized so it was not possible to determine the exact concentration of the substances. A Rezex ROA-organic acid H⁺ column (Phenomenex, USA) was used to determine concentrations of ethanol, sugar, glycerol and acids in fermentation samples and to find out the exact concentration of glucose in saccharification samples. The column temperature was 65 °C and 2.5 mM H₂SO₄ with a flow rate of 0.6 ml/min was used as mobile phase. Compounds which the
column could detect were DP4+, DP3, maltose, glucose, fructose, succinic acid, lactic acid, glycerol, acetic acid and ethanol. This column was used as a standard column for the HPLC analysis, so if nothing is written, this column was used.

The samples was centrifuged (980 g, 10 min), the supernatant was filtered with 0.45 µm filter (Phenomenex) and diluted to appropriate concentrations before analysis. The results were compared with known standards of each substance. Centrifugation and filtration of the supernatant gave somewhat higher concentrations than in reality, since the pellet was taken away. To get a more correct concentration determination, the mash was diluted and filtrated without any centrifugation.

3.3.6 GC

Gas chromatography (GC) (Shimadzu, GC 2010) with an autoinjector (Shimadzu, AOC-20i) was used to determine the formation of alcohols and other by-products in the fermentation. A Zebron ZB-WAX plus column with a phase of polyethylene glycol was used and detection was carried out with a flame ionization detector (FID) at 230 °C. Helium was used as both carrier gas and makeup gas. The temperature was programmed according to Figure 4. The pressure was constant at 100 kPa.

![Figure 4: Temperature profile of the GC analysis.](image)

The samples were prepared in the same way as for the HPLC analysis (section 3.3.5) with the exception that they were not diluted and that every sample was mixed with allyl alcohol, which was used as an internal standard. The GC analysis could detect diethyl ether, acetaldehyde, acetone, acrolein, ethyl acetate, methanol, isopropanol, ethanol, diacetyl, 2-butanol, 1-propanol, isobutanol, 1-butanol, methyl-2-butanol and methyl-3-butanol.
3.3.7 Yeast Quantification and Viability
The number of yeast cells in the fermentor was counted using a hemocytometer (Kemolab, Stockholm, Sweden). The cells were stained with rhodamine B (1 g/L) which stains dead cells pink while living cells break down rhodamine B and remain uncolored.

3.3.8 Determination of Enzyme Concentration
Enzyme concentration was determined spectrophotometrically (UV-1601 UV visible spectrophotometer SHIMADZU) by the Lowry-Kalckar formula (Equation 4; Li, 2008)

\[
\text{Enzyme concentration (mg/ml) = } 1.45 \times A_{280} - 0.74 \times A_{260}
\] (4)

3.4 Batch Experiments
Different experiments were performed to find better enzymes and yeast strains to use in the process. Experiments were also done to get a better understanding of parameters that affect the yield of glucose from starch.

3.4.1 Enzyme Comparison
\(\alpha\)-amylase, glucoamylase and viscosity reducing enzymes from Genencor was compared with the enzymes from Novozymes that are usually used in the Biostil 2000 process. The concentrations of the enzymes from Genencor’s were the following; GC 420 0.266 g/kg wheat, Distillase VHP 0.550 g/kg wheat and Optimash BG 0.03 g/kg wheat. Samples were taken after 0, 3, 22 and 26 h of fermentation and analyzed by HPLC and GC. Initial yeast count was performed and dry solids and viscosity were measured after every process step.

3.4.2 Yeast Comparison
The fermentation ability of baker’s yeast, Ethanol Red and AmyloFerm was compared by setting up a trial with three different batches. Rehydrated dry yeast of baker’s yeast and Ethanol Red was used while AmyloFerm was stored on solid agar and propagated prior to fermentation. Samples were taken after 5, 19, 23 and 27 h of fermentation and analyzed by HPLC and GC. Yeast count and viability were preformed after 5 and 24 h of fermentation. Viscosity and dry solid measurements were done after completed fermentation.

3.4.3 Evaluation of Protease Usage
The effect of protease addition during saccharification to improve fermentation was tested using the protease Fermgen from Genencor. The experiment was carried out according to the standard setup but with the addition of Fermgen (0.035 g/kg wheat) during saccharification. A batch without Fermgen was used as a reference. Samples were taken after 4, 21, 25, 29 and 45 h of fermentation and analyzed by HPLC and GC. Yeast count, viscosity, and dry solid measurements were done after completed fermentation.
3.4.4 Fermentation Directly After Liquefaction
The ability of AmyloFerm to break down dextrins and liquid starch was examined by fermentation directly after liquefaction. A batch was liquefied according to the standard setup and divided into two batches prior to fermentation. AmyloFerm from solid agar was propagated and added to one batch while dry baker’s yeast was rehydrated and added to the second batch to use as reference. Samples were taken after 0, 3, 19, 23, 27, 43, 47, 51, 67 and 71 h of fermentation and analyzed by HPLC and GC. Viscosity and dry solid measurements were done after completed fermentation.

3.4.5 Prolonged Liquefaction Time
The consequences of letting the duration time for liquefaction be longer than the standard setup time were evaluated. Four separate 200 ml batches were liquefied for 2, 4, 6 and 7 h respectively. After liquefaction the batches were saccharified for 21 h according to the standard setup. The samples were then analyzed by HPLC using Rezex RPM-monosaccharide Pb\(^{2+}\) column.

3.4.6 Prolonged Saccharification Time
The consequences of letting the duration time for saccharification be longer than the standard setup time were evaluated. Liquefaction and saccharification were performed according to the standard setup with the exception that the saccharification went on for 93 h. A sample was taken after 21, 26, 31, 44, 69 and 93 h of saccharification and analyzed by HPLC using Rezex ROA-organic acid H\(^{+}\) column (standard column) and Rezex RPM-monosaccharide Pb\(^{2+}\) column.

3.4.7 Mixing at 60 °C
A mixing step at 60 °C, with the addition of Optimash BG (0.03 g/kg wheat) for viscosity reduction was compared with 50 °C mixing without enzyme which is regularly used in the Biostil 2000 process. The experiment was first performed in an E-flask heated by a temperature controlled hot plate stirrer as in the standard setup but was then tested in a larger reactor of 1 L that could better control the temperature.

3.5 Continuous Experiments
Since Biostil 2000 is a continuous process, laboratory trials in batch form are not always a good model for the industrial process. Therefore, a continuous laboratory process for liquefaction and saccharification was developed (Figure 5). Liquefaction and saccharification were performed continuously but the mash was cooled and stored between the steps. One mixing tank, two liquefaction tanks and three saccharification tanks were used. Heating was obtained by a water bath on the hot plate stirrer. Liquefaction enzymes were added directly to the mixing tank and mash was pumped into the bottom of the tank and processed mash left the tank from the tank top. Mash that passed from the first tank was with a small pipe led to the bottom of the next tank. In the startup of liquefaction, the first tank was filled to 20 % with mash and heated to 80 °C before the pumping and time keeping started. The liquefaction time was 4 h to assure
complete liquefaction, while the saccharification time was 21 h, similar to the batch process.

![Figure 5: Continuous experimental setup for liquefaction and saccharification. Two tanks were used in liquefaction and three tanks in saccharification.](image)

### 3.5.1 Optimizing Continuous Process

Two different sets of tanks were available for continuous experiments. Large tanks, each with a volume of 565 ml and small tanks, each with a volume of 300 ml. Liquefaction in two tanks was carried out with the two different sets of tanks.

Continuous saccharification was tested with a set of three tanks á 300 ml since this mash has lower viscosity and was therefore expected to be stirred easier than liquefied mash.

### 3.5.2 Liquefaction in One or Two Tanks

The difference between using one or two tanks during liquefaction was evaluated with the larger 565 ml tanks. The testing material was a grain meal and water mix from Lantmännen Reppe AB in Lidköping and the material was kept frozen before usage. Starch analysis and viscosity measurements were performed on samples from each batch.

### 3.5.3 Enzyme Comparison; Continuous Process

A comparison between enzymes from Novozymes and Genencor was done similar to Experiment 1 in section 3.4.1, but as a continuous process instead of a batch process. Liquefaction and saccharification were done but not fermentation.
4 Results

4.1 General Measurements

General measurements were performed to get more information of the wheat, enzymes and measurements used.

4.1.1 Physical Properties of Wheat Flour

Debranned wheat flour (Prokop, Czech Republic) was used in all experiments. The sieve analysis (Figure 6) showed that the majority of particles were between 125-250 µm and that almost no particles were larger than 710 µm. The particles were too small for Biostil 2000 and the blue curve illustrates the desired particle distribution.

![Figure 6: The particle size distribution obtained by sieve analysis. The red curve shows result with debranned wheat flour (Prokop, Czech Republic) and the blue curve indicates the desired particle distribution in Biostil 2000.](image)

A nutrition analysis of the flour was performed by Eurofins laboratory in Lidköping (Table III).
Table III: Nutrition analysis of debranned wheat flour (Prokop, Czech Republic)

<table>
<thead>
<tr>
<th>Name of analysis</th>
<th>Result (%)</th>
<th>Margin of error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry substance</td>
<td>85,6</td>
<td>±10</td>
</tr>
<tr>
<td>Raw protein content</td>
<td>11,8</td>
<td>±7</td>
</tr>
<tr>
<td>Raw fat content</td>
<td>1,7</td>
<td>±9</td>
</tr>
<tr>
<td>Glucose</td>
<td>0,06</td>
<td>±25</td>
</tr>
<tr>
<td>Fructose</td>
<td>0,04</td>
<td>±25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0,46</td>
<td>±15</td>
</tr>
<tr>
<td>Maltose</td>
<td>1,2</td>
<td>±15</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>1,5</td>
<td>±15</td>
</tr>
<tr>
<td>Starch</td>
<td>68,8</td>
<td>±10</td>
</tr>
</tbody>
</table>

Concentrations Measured by HPLC

To correct for the higher concentrations achieved when the mash samples were centrifuged before analysis, measurements were done on mash samples that only have been diluted and filtered. In this way the following relationship (Equation 5) was obtained:

Concentration diluted sample = 0.86 × concentration centifuged sample  

(5)

All the results measured by HPLC have been recalculated with this relationship to achieve more accurate concentration results.

4.1.2 Determination of Enzyme Concentration

Concentration of the enzymes used, was determined by absorbance measurements at 260 nm and 280 nm. Liquozyme SC DS and Viscozyme wheat had a higher optical density at 260 nm than at 280 nm, which may seem strange, since an absorbance maximum at 280 nm is specific for proteins. This indicates that the enzymes are not pure enough for spectrophotometric concentration determination.

4.2 Batch Experiments

4.2.1 Enzyme Comparison

α-amylase, glucoamylase and viscosity reducing enzymes from Genencor were compared with the enzymes from Novozymes which is usually used in the Biostil 2000 process. Both batches were completely liquefied with DP 4. Similar amounts of glucose were produced during saccharification and equal amounts of ethanol during fermentation (Figure 7).
Carrying out the process with the two different sets of enzymes produced similar concentrations of glycerol and succinic acid while no acetic acid or lactic acid were produced (Figure 8).

GC analysis showed that both batches produced similar amounts of by-products. 1-propanol, isobutanol and 2-methyl-butanol were the by-products with highest concentrations, around 15 ppm in the end of fermentation.
It was slightly lower viscosity during saccharification in the batch with enzymes from Genencor’s compared to the batch with enzymes from Novozymes (Figure 9). Viscosity after the other process steps was similar.

![Figure 9: Viscosity during saccharification in a batch with enzymes from Novozymes compared to a batch with enzymes from Genencor.](image)

TDS, SS, DS, FS and spin results achieved from the batch with enzymes from Novozymes was gathered in a dry solid profile (Figure 10). The profile was similar for the batch with enzymes from Genencor.

![Figure 10: Dry solid measurements from the batch with enzymes from Novozymes. The profile was similar for the batch with enzymes from Genencor.](image)

Initial yeast concentrations in the two batches were approximately $700 \times 10^6$ cells/ml.
4.2.2 Yeast Comparison

Fermentation ability of the three different yeast strains baker’s yeast, Ethanol Red and AmyloFerm were compared. Baker’s yeast carried out the fastest fermentation since it had the lowest glucose concentration and highest ethanol concentration after 5 and 19 h (Figure 11). Ethanol Red and AmyloFerm used glucose with similar speed but AmyloFerm was slightly faster to produce ethanol.

![Graph showing glucose and ethanol concentration during fermentation by baker’s yeast, Ethanol Red and AmyloFerm. Results obtained by HPLC analysis.](image)

Baker’s yeast produced no acetic acid while Ethanol Red and AmyloFerm produced approximately 2.5 g/L (Figure 12). Baker’s yeast and Ethanol Red produced similar amounts of succinic acid while AmyloFerm produced somewhat less. Ethanol Red produced glycerol to a larger extent than the others, which might explain the lower ethanol yield, compared to AmyloFerm, when the similar amounts of glucose were consumed. No lactic acid was present in any of the batches.
Analysis with GC showed that baker’s yeast increased production of acetaldehyde after 19 h fermentation while AmyloFerm started to produce it after 23 h and Ethanol Red did only produce small amounts (Figure 13). Approximately 7000 ppm 3-methyl-butanol and 5000 ppm isobutanol were detected in all batches after 23 h fermentation. Concentrations up to 500 ppm of acetone, ethyl acetate, acetal, methanol, 2-butanol, 1-propanol, 1-butanol and 2-methyl-butanol were also found in all batches.
The yeast count after 5 h of fermentation showed similar concentrations of viable cells in all three batches, but the total count was lower for AmyloFerm (Figure 14). The lower amount of dead cells was probably because of the propagation step that was excluded for the other yeast strains. The yeast count after 24 h of fermentation showed an increase in viable cells for Ethanol Red while it decreased for baker’s yeast and AmyloFerm. This could be an indication of that Ethanol Red tolerates higher ethanol levels than the others.

![Figure 14: Yeast count and viability after 5 h and 24 h of fermentation by baker’s yeast, Ethanol Red and AmyloFerm.](image)

The dry solid profile during fermentation was according to the standard dry solid profile (Figure 10) and similar for all yeast forms.

### 4.2.3 Evaluation of Protease Usage

This experiment examined if addition of protease during saccharification improved the fermentation step. Samples taken during fermentation showed no significant difference in glucose or ethanol concentration in the batch with Fermgen compared to the batch without Fermgen (Figure 15).
Both batches contained similar concentrations of glycerol and succinic acid while no acetic acid or lactic acid were present (Figure 16).

Addition of Fermgen seems to give slightly lower production of acetaldehyde (Figure 17). The result of acetaldehyde concentration in the batch with Fermgen after 4 h of fermentation is probably a measurement error. Both batches contained around 200 pm
isobutanol, 160 ppm 2-methyl-butanol and 80 ppm 1-propanol. Ethyl acetate, methanol and 3-methyl-butanol were in both batches detected in small amounts below 30 ppm.

![Graph showing concentration of acetaldehyde during fermentation](image_url)

**Figure 17:** Formation of acetaldehyde during fermentation by baker’s yeast. A batch with Fermgen added during saccharification was compared to a batch without added Fermgen. Results obtained by GC analysis.

Total yeast count after completed fermentation were $1150 \times 10^6$ cells/ml for the batch with Fermgen and $1260 \times 10^6$ cells/ml for the batch without Fermgen. There were no viable cells in any of the batches.

Addition of Fermgen gave no difference in viscosity during saccharification or fermentation. Dry solids were in both batches similar to the standard dry solid profile from Experiment 1 (Figure 10).

### 4.2.4 Fermentation Directly After Liquefaction

In this experiment, the ability of AmyloFerm to break down dextrins and liquid starch was examined. Baker’s yeast used as a reference, was in the beginning able to produce ethanol, from glucose and maltose, but when those sources were empty, the yeast started to break down ethanol to produce acetic acid (Figures 18-20). AmyloFerm used maltose at a lower rate but was able to utilize DP3 and DP4+. Ethanol production of AmyloFerm was in the beginning slower than for baker’s yeast but the ethanol concentration increased all the time with a maximum concentration of 75 g/L after 71 h of fermentation.
Figure 18: Glucose and ethanol concentration during fermentation directly after liquefaction. Fermentation by baker’s yeast was compared to fermentation by AmyloFerm. Results obtained by HPLC analysis.

Figure 19: Saccharide concentration during fermentation directly after liquefaction. Fermentation by baker’s yeast was compared to fermentation by AmyloFerm. Results obtained by HPLC analysis.

The formation of glycerol and succinic acid was similar between the two batches and none of the batches contained lactic acid (Figure 20).
Figure 20: Acids and glycerol concentration during fermentation directly after liquefaction. Fermentation by baker’s yeast was compared to fermentation by AmyloFerm. Results obtained by HPLC analysis.

Analysis with GC showed that baker’s yeast produced more acetaldehyde while AmyloFerm produced more 2-butanol and 1-propanol (Figure 21). Acetone, ethyl acetate, methanol, diacetyl, isobutanol, 1-butanol, 2-methyl-butanol, 3-methyl-butanol were produced in concentrations below 160 ppm.

Figure 21: By-product formation during fermentation directly after liquefaction. Fermentation by baker’s yeast was compared to fermentation by AmyloFerm. Results obtained by GC analysis.
The dextrin breakdown was also confirmed by the dry solid tests (Figure 22). Fermentation with AmyloFerm gave lower amounts of solids in all measurements.

![Bar chart showing dry solid measurements after completed fermentation. Fermentation carried out directly after liquefaction by baker’s yeast and AmyloFerm.](image)

The batch with AmyloFerm had a lower viscosity after the fermentation compared to the batch with baker’s yeast. This was probably because of the lower amount of solids in the mash with AmyloFerm. Total yeast concentration during fermentation was around $900 \times 10^6$ cells/ml.

### 4.2.5 Prolonged Liquefaction Time

This experiment evaluated the effect of a prolonged liquefaction time. HPLC analysis with Rezex RPM-monosaccharide Pb$^{2+}$ column showed no significant difference in sugar formation between the four batches. This indicates that a prolonged liquefaction time is acceptable and does not affect further process steps.

### 4.2.6 Prolonged Saccharification Time

This experiment evaluated the effect of a prolonged saccharification time. Glucose levels were unaffected of prolonged saccharification time and relatively stable around 275 g/L. The levels of maltose increased from 10 g/L after 21 h to 17 g/L after 96 h of saccharification. Chromatograms from HPLC run with the Rezex RPM-monosaccharide Pb$^{2+}$ column showed an increased peak area for isomaltose with prolonged saccharification time (Figure 23). The increase of maltose detected by the standard HPLC run was probably an increase in isomaltose since the method cannot separate peaks from maltose and isomaltose. However, in Biostil 2000 it is enough glucose available so the yeast does not have to utilize maltose and then it does not matter if isomaltose was produced instead of maltose. The important thing was that the glucose yield remained unaffected.

---

4.2.6-30
4.3.2-31

Figure 23: Selected part of chromatogram from HPLC run with a Rezex RPM-monosaccharide Pb\(^{2+}\) column. Retention time for isomaltose is 11.68 min and retention time for maltose is 12.01 min.

### 4.2.7 Mixing at 60 °C

This experiment evaluated a mixing step at 60 °C with addition of Optimash BG to reduce mash viscosity. The experiment could not be performed in E-flask with temperature controlled hot plate stirrer, because the mash gelatinized during mixing. The experiment was therefore tested in a reactor with good temperature control, but the mash gelatinized nevertheless. It is possible in theory to avoid gelatinization, since the gelatinization temperature for wheat is 65 °C. However, it was too difficult to control the temperature in laboratory scale and it might be too risky to do that mixing in a large process if something goes wrong and it starts to gelatinize.

### 4.3 Continuous Experiments

#### 4.3.1 Optimizing Continuous Process

This experiment was done to examine if it is any difference between using large or small tanks during liquefaction and to see if saccharification works well in small tanks. Liquefaction with 565 ml tanks seemed to be better than 300 ml tanks, since the stirring worked better in larger tanks. However, starch iodine test gave violet samples, indicating a DP of around 38 for both of the experiments. Batch trials gave around DP 8 so the continuous liquefaction did not work perfectly.

Continuous saccharification during 21 h gave 274 g/L glucose which is good because it is in the same range as the concentrations achieved during batch trials.

#### 4.3.2 Liquefaction in One or Two Tanks

Comparison between continuous liquefaction in one tank and in two tanks was done. Starch iodine test gave DP 16 in the setup with two tanks compared with DP 34 in the setup with one tank. This indicates that two tanks are better to depolymerize starch.
Liquefaction in two tanks seemed to give a slightly lower viscosity of the liquefied mash, compared to the system with one tank (Figure 24).

![Graph showing viscosity of mash liquefied in one tank and mash liquefied in two tanks.](image)

**Figure 24:** Viscosity of mash liquefied in one tank and mash liquefied in two tanks.

### 4.3.3 Continuous Process; Enzyme Comparison

The aim of this experiment was to perform the enzyme comparison similar to Experiment 1, but in the continuous process. However, in continuous liquefaction, the pipe that pumped from the mixing tank was too small so the largest fibers stayed in the mixing tank, making the mash more and more viscous until the pump was not able to pump any longer. However, more mash was made and the liquefaction proceeded successfully with DP 15 from the starch iodine test.

The continuous saccharification was disrupted because of a clog in the pipe between tank 1 and tank 2. Thus, more optimization of the continuous process is necessary before a fair comparison can be performed.
5 Discussion

Finding optimal production conditions of bioethanol is tricky, since factors interact and it is not the same to perform tests in laboratory scale compared to large scale production. Different process parameters have been evaluated in this study, but to be able to get more reliable results, testing in large scale is necessary. No process looks completely the same, so every production plant needs to find their optimal process conditions, but laboratory scale testing is a good first step.

The fact that the grain meal used in the experiments contained too much small particles compared to optimal Biostil 2000 conditions, had probably no important effects on the results, since too small particles are mostly a problem for the industrial process equipment (Dexter and Wood, 1996). Lactic acid was not present in any of the experiments, indicating that all batches were relieved from bacterial contamination. That is a condition that hardly ever occurs in a large continuous process (Skinner and Leathers, 2004). Glycerol levels were as high as 15 g/L after fermentation, probably because it was batch experiments with large initial glucose concentrations (Bideauz, et al., 2005). A continuous process with low sugar levels would probably not have resulted in those high glycerol concentrations.

Results obtained by GC analysis varied a lot during the different experiments, which partly can be explained by different fermentation times and environments in the experiments. However, it might also be insecurities in the measurements. The equipment was new and these were the first measurements performed on the equipment, which may have led to adjustment errors that decreased the accuracy.

5.1 Enzymes

New enzymes are introduced to the market all the time, claiming better performance than before. However, to change enzymes in a large production plant involves risks and it is often better to continue with the old enzymes that have been proven to work. This thesis has evaluated the effect of enzymes from Novozymes and Genencor. No large differences could be seen in the performance of enzymes from the different manufacturers, but a small decrease in viscosity during saccharification in the batch with enzymes from Genencor was discovered. High mash viscosity results in large energy consumption because of poor heat exchange, so even the slight viscosity reduction in the batch with enzymes from Genencor might lead to lower energy consumption. The enzyme activity depends on the amount of added enzyme, but the activity units of the enzymes were not comparable and the concentration was unknown so a spectrophotometric determination of concentration was done. However, the enzymes were not pure enough to give satisfying results. Therefore, the dosage had to be according to recommendations from the manufacturer. A quotation of price would have solved the problem of knowing that similar amounts of enzymes were used in the comparison. However, enzyme costs depend on the client, country and bought quantity, so it was not possible to get any
quotation of price in this thesis. Therefore, the recommended dosage of enzymes from the two manufacturers was assumed to have similar costs.

Addition of Fermgen to improve fermentation does not seem to be any better than ordinary fermentation, except for a lower production of acetaldehyde. However, the yeast during this experiment was not as stressed as in a Biostil 2000 process. Thus, the addition of Fermgen might be useful in Biostil, especially if a grain meal with low nitrogen content is used. Fermgen might also have an effect if another yeast than baker’s yeast is used.

5.2 Yeast

No large difference in the yield of ethanol could be noted between baker’s yeast, Ethanol Red and AmyloFerm. Baker’s yeast was surprisingly the fastest to finish fermentation and did not produce any acetic acid. Ethanol Red and AmyloFerm produced around 2.5 g/L of acetic acid which is harsh conditions for yeast, since only 0.5 g/L affects yeast growth (Russell, 2003). However, baker’s yeast produced 370 ppm acetaldehyde compared to AmyloFerm that produced 260 ppm and Ethanol Red 60 ppm. Ethanol Red was able to grow and survive in higher ethanol concentrations than the others, although in Biostil 2000 ethanol is continuously removed from the fermentor so it never reaches high levels. Baker’s yeast has been used for a long time and has a wide group of customers which gives it a low price compared to the others. A problem with baker’s yeast is that the production conditions are not as strict as for the other specialized yeasts which give it a higher level of bacterial contamination (Rejholt, 2009). However, baker’s yeast may differ between suppliers, and in this project, baker’s yeast produced by Jästbolaget AB in Sweden has been used, so trials with baker’s yeast produced in other countries might not give the same results.

A large negative property with Ethanol Red and AmyloFerm was their high level of produced acetic acid. However, production of acetic acid can occur at too high glucose concentrations (Erasmus, 2004). This was the case during this batch experiment, but will not be the situation in Biostil 2000. It might be that Ethanol Red and AmyloFerm are not suitable for non fed-batch processes, but might work well in a continuous process.

Wild yeast contamination is a problem in production plants (section 2.2.2), and in Biostil 2000, the yeast is recycled for a long time, which means that the cultured yeast will to a large extent been replaced with other yeast strains at the end of fermentation. So another fact to consider when choosing cultural yeast is if it is worth to use an expensive yeast, if the high performance will only last in the beginning of fermentation. This is a consideration to make depending on how long time the same fermentor will be used.

When it comes to choosing yeast strains, all three of them work good, but it seems like baker’s yeast is the best, both in performance and cost. However, if the high levels of bacterial contamination are a problem then some of the other yeasts might be better.

AmyloFerm was tested for its claimed ability to ferment dextrins, and an experiment showed that it works, but it takes very long time. Thus, it is not an option to skip
saccharification and ferment with AmyloFerm directly after liquefaction. AmyloFerm’s performance on saccharified mash was relatively similar to that of the other yeast strains.

5.3 Process Problems
In the start up of a new plant or in plants that are disrupted due to some kind of trouble, difficulties to keep to the right holding times for each process often occur. This study discovered that longer holding times during liquefaction and saccharification do not have any significant effect on the final fermentation. However, there is literature support for the notion that some kind of problem can occur, but it might not occur in the conditions for a Biostil 2000 process (section 2.5).

5.4 Continuous Lab-scale Process
The continuous process in lab scale is working, but not optimal. A larger pipe diameter on the pumping pipe would allow passage of the fibers and a larger pipe diameter between the tanks would relieve the passive flow between the tanks, reducing the risk for clogs. Stirring in the tanks is not optimal and the use of a mechanical stirrer might help. Better stirring will probably also to some extent reduce the clogging problem. The problem that water evaporates away from the water bath could be avoided if glycerol was used instead of water.
6 Conclusion

With results from this thesis, it can be concluded that enzymes from both Novozymes and Genencor seem to work in conditions for Biostil 2000, but that it can be worthwhile to further investigate the enzymes to see if a viscosity reduction occurs in the batch with enzymes from Genencor. Addition of Fermgen does not appear to improve the fermentation.

When it comes to yeast, the difference between the examined yeast strains was small, so the decision may rather be based on the level of bacterial contamination, availability and price.

A prolonged liquefaction time and saccharification time do not seem to affect the final glucose yield to any larger extent. The idea with a continuous lab scale process is very good, but it has to be further developed to give results closer to the large scale process.
7 Recommendations

The results and the work with this project have inspired the following recommendations:

- Perform the enzyme comparison in the continuous lab-scale process to see if enzymes from Genencor really give a viscosity reduction during saccharification.

- If a plant has problems caused by lack of nitrogen during fermentation, Fermgen can be tested again under more stressed conditions to see if it has any effect.

- Evaluate baker’s yeast from other countries where Chematur have or will start production plants, to see if they also give the same high performance.

- Test Ethanol Red and AmyloFerm in a continuous or fed-batch process to see if the production of acetic acid decreases.

- Develop the continuous lab-scale process by using glycerol instead of water in the tempered bath, to avoid leakage problems. Use mechanical stirring instead of magnetic stirring bars to improve the tank stirring. Use a larger pipe diameter on the pumping pipe to allow passage of larger fibers and a larger pipe diameter between the tanks to relieve the passive flow, reducing the risk for clogs.
8 Acknowledgements

I would like to thank Chematur Engineering for giving me the opportunity to do this thesis and I want to send special thanks to:

- My supervisor Anna Eriksson for her invaluable help and support during every step of this thesis.
- Gert Wass for technical and mechanical assistance.
- Lars Stenmark for inspiration and problem discussions.
- My examiner Uno Carlsson for his help with the enzyme work and valuable advice in the report writing.
- Åke Strid at Örebro University for his kindness of letting me borrow their spectrophotometer and Johanna Sundin for help with the measurements.
9 References


ASPO The association for the study of peak oil and gas (2005) Newsletter no 58.


