"SORGANOL®": IN-FIELD PRODUCTION OF ETHANOL FROM SWEET SORGHUM

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To my family…

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NOMENCLATURE

α	Statistical level of significance [α=0.05]
Abs	Absorbance
Bu	Bushel
g	Gram
h	Hour
ha	Hectare
Kg	Kilogram
KJ	Kilo joule
L	Liter
m	Meter
MJ	Mega joule
mM	Millimolar
MPa	Megapascal
μ	Specific growth rate
μg	Microgram
ppm	Parts per million
Psi	Pounds per square inch
SCE	Sugar conversion efficiency
w/v	Weight/volume

CHAPTER I

INTRODUCTION

The energy system of the future must be renewable, sustainable, cost-effective, convenient and safe. Given the present demand for fossil fuel, the depletion of the world's petroleum resources is inevitable. In the last 30 years, worldwide efforts toward identifying, developing and commercializing technology for alternative transportation fuel has gained significant momentum. Putsche and Sandor (1996) indicated that petroleum supplies 97% of the energy consumed for transportation, and petroleum imports represent 20% of the growing US trade deficit (Sheehan and Himmel, 1999). Petroleum provides the single largest fraction of the world's energy, accounting for 37% of all global energy (US DOE, 2002). Burning petroleum has become the major mechanism of global climate change primarily due to the emission of carbon dioxide to the atmosphere (Gnansounou et al. 2005). These changes also result in substantial health costs in our society that are not reflected in the cost of gasoline (Sheehan and Himmel, 1999). Further, petroleum is not sustainable and newer forms of energy need to be developed to address the economic and environmental issues and ensure a greater energy security and reliability.

Fueled by the Iran, Libyan and Arab oil embargoes of the 1970's, development of alternate sources of energy became a national priority. This crisis also led to the development of technologies to utilize renewable energy sources such as wind, solar and

biomass. Given the control of Oil Producing and Exporting Countries (OPEC) over the petroleum prices, the development of domestically produced renewable transportation fuel has gained strategic importance (Sheehan and Himmel, 1999). DOE's Energy Information Administration (1996) paints a dismal picture of growing dependence on foreign oil in terms of increased imports, increased reliance on Middle East oil and continued decrease in domestic supplies with an ever increasing domestic demand.

Bioenergy represents about 8% of the total energy used in the US annually (National Research Council, 2000). The National Research Council (1999) indicated several drivers that provide impetus to pursue development of bioenergy and biobased products. The drivers include emerging market opportunities, increased rural development needs, reduced environmental impacts, increased energy diversity and security, reduced fossil carbon emissions and meeting the increasing demand for energy and materials with sustainable technologies. Bioenergy can be produced from a variety of carbohydrates (mono-, di-, polysaccharides) sourced from various agricultural residues such as corn stover (corn cobs and stalks), sugarcane waste, wheat and rice straw, forestry and paper-mill residues, the paper portion of municipal waste, and dedicated energy crops – collectively termed 'biomass'.

The United States produced about 14.8 billion L of ethanol in 2005 with 85% of it coming from corn (Renewable Fuels Association, http://www.ethanolrfa.org/industry/statistics/). Ethanol offers dramatic environmental, economic, strategic and infrastructure advantages that were not appreciated in the past. For countries with limited petroleum resources, production of ethanol is considered economically strategic by reducing the trade deficit, thereby helping grow the economy

(Wyman, 2001). Until the late 1930's, the production of ethanol compared economically with gasoline; however, with the discovery of many cheaper oilfields, ethanol progressively lost its market. During the first worldwide oil crisis in the 1970's, ethanol became cheaper than gasoline, which led to increased investment in developing technologies and projects to scale up ethanol production (Wyman, 2001). Ethanol production in the United States grew from just a few million gallons in the mid-1970's to over 1.7 billion gallons in 2001. The initial impetus for ethanol commercialization was due to the oil embargo in 1973 and the Iranian revolution of 1978, which caused oil prices to rise dramatically and created much concern over the security of national energy supplies. In 1990 ethanol production received a major boost when the Clean Air Act Amendment (CAA) was passed in an effort to control carbon monoxide and ground-level ozone (Shapouri et. al, 2002). Public policies aimed at increasing production of ethanol are largely motivated by the desire to improve air quality and enhance energy security. A study conducted by DOE in 1993 found that, compared with reformulated gasoline (RFG, gasoline containing lower aromatic compounds resulting in lower air pollutants), a 95% ethanol/5% gasoline (E95) blend reduces sulfur dioxide emissions 60 to 80%, volatile organic emissions 13 to 15%, and ozone precursors such as nitrous oxide and carbon monoxide emissions 20 to 30% (Tyson et al. 1993).

Total production of ethanol fuel worldwide in 2004 was 32 billion L with Brazil contributing 47%. Demand will continue to grow to about 65,000 million L by 2015, twice the present demand (Gnansounou and Dauriat, 2005). The demand is fueled by the following factors:

- Ban on methyl tertiary butyl ether (MTBE) in gasoline

- New legislations promoting the increased utilization of domestically produced, renewable, biobased motor vehicle fuel supplies in the US
- Growth in demand for Flexible Fuel Vehicles (FFV) in Brazil and other countries
- European directive on motor bio-fuel
- Introduction of E10 in China, India and other Asian countries.

The major feedstocks for the current large-scale biomass-to-ethanol industry include sugarcane in Brazil, sugar beets and wheat in the EU, corn and milo (sorghum) in the US. Several studies have shown that sufficiently abundant cellulosic biomass is also available to make a sizeable impact in the transportation fuel market (Wyman, 2001). The primary difficulty in commercialization of ethanol is its high production cost compared to gasoline. The recent increase in gasoline prices has helped bridge this gap; however, feedstock and investment costs significantly affect the economies of scale. Hence the primary challenge for ethanol competitiveness is to reduce the cost of biomass processing to convert this low-cost material into a competitive product.

Sweet sorghum has the potential of being the next U.S. energy crop due to its high sugar content and its adaptability of being grown in diverse environmental conditions. The original concept of Sorganol (ethanol from sweet sorghum) production was conceived by Mr. Lee McClune, (President, Sorganol Production Co. Inc, Knoxville, IA., www.sorganol.com). A schematic representation of the process is depicted in Figure 1.1. The proposed ethanol production process involves harvesting and pressing the sweet sorghum stalks using a new mobile field harvester (patent pending) with a multi-roller press and juice collection unit mounted on the harvester. The harvester accomplishes both

harvest and juice expression in a single pass through the field. The unit uses a standard forage chopper/header and feed rollers. After the stalks are pressed, they are expelled back onto the field. Juice is then pumped from the harvester directly into large storage bladders in the field, where fermentation takes place. Since the fermentation is conducted under non sterilized conditions, it will be important to determine whether the addition of acid will help in inactivating the native microflora present in sweet sorghum juice. It is envisioned that bladders of 75,000-100,000 L will be used to ferment the juice when the process is fully optimized and commercialized. After fermentation, the ethanol would be concentrated with a mobile distillation unit. It is also envisioned that spent baled stalks could be used to fuel the mobile distillation unit.

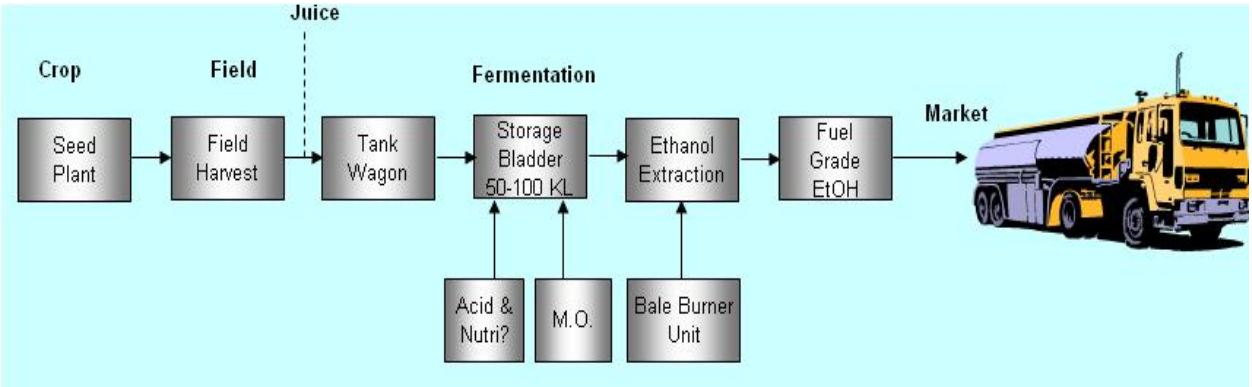


Figure 1.1. Proposed SORGANOL process.

1.1 Research Objectives

Given the background on the Sorganol process, the primary objective of this research was to design a framework and validate the process of in-field production of ethanol from

sweet sorghum, with minimal or no process control. Specific objectives of this research include:

1. Determine the microorganism most suitable for the in-field fermentation process
2. Determine the efficiency of the fermentation process at the ambient temperature extremes
3. Investigate the need for nutrient (urea) addition
4. Determine the effect of pH on the fermentation process
5. Determine the effect of scale-up on the fermentation process
6. Understand the effect of agitation on the fermentation process
7. Determine the compositional stability of sweet sorghum juice at refrigerated conditions

CHAPTER II

REVIEW OF LITERATURE

2.1 Sweet Sorghum

Sweet sorghum (Sorghum bicolor (L) Moench spp *saccharatum*) is a C_4 cereal grass with broad corn like leaves and large clusters of grain atop tall stalks. Sorghum is believed to have originated in Africa and is the world's fifth major cereal crop after wheat, rice, maize and barley. The annual production of grain and sweet sorghum in the world exceeds 140 billion kilograms with a value approaching $30 billion. In the United States, sorghum production ranks third among the cereal crops with about 4 million hectares planted in 2000, exceeded only by corn and wheat (USDA, 2002).

Compared to grain sorghum, sweet sorghum has a greater height and higher sugar content in the juice. The sorghum plant typically grows to a height of about 120 cm to 400 cm depending on the variety and growing conditions and can be either an annual or short perennial crop (Gnansounou et al. 2005). Seeds are typically sown after the rainy season and as soon as the soil temperature remains above 15-18^{o}C. Seed germinates within 24 h in warm and moist soils.

Water and solubles represent about 85% of the total fresh stem weight (Woods, 2000). The sweet sorghum juice composition is known to vary depending on the crop. Mohite and Sivaraman (1984) reported a composition of 60% sucrose, 33% glucose and 7%

fructose in the sweet sorghum juice. Sweet sorghum is harvested seasonally at a high moisture level (70-80%) which leads to rapid deterioration of readily available carbohydrates. Even when sweet sorghum is bundled and stored dry at ambient temperatures (silage) it is known to deteriorate rapidly. Natural microflora of the sweet sorghum includes heterolactic *Leconostoc mesentroides* and *Leuconostoc dextranicum*, coliform bacteria such as *Aerobacter aerogenes* and several yeast genera.

Sweet sorghum is characterized by high photosynthetic efficiency and is one of the most promising crops for ethanol production. It can be grown in diverse temperate zones in both irrigated and non-irrigated environments. It has the C_4 carbon pathway which enables it to bypass the photosynthetic pathway and hence achieve maximum short term growth rates (Loomis and Williams, 1963). C_4 is an adaptive mechanism in response to low atmospheric CO_2 availability, and warm, dry environments. The mechanism helps to increase the water use efficiency and avoids the energetically wasteful photorespiration step. Sweet sorghum is a high biomass yielding crop with a high sucrose content and has the adaptability of being grown in various parts of the United States as an alternate crop for ethanol production. Gosse (1996) found that out of the many 'new crops' currently being investigated as a potential future energy crop, sweet sorghum seems to be the most promising one. It is genetically diverse with over 4000 varieties and is resistant to drought conditions.

Sweet sorghum is considered a versatile and potentially ideal high-energy crop as it offers numerous advantages (Grassi et al. 2004):

- High yielding crop (up to 80 tons/hectare)

- Can be grown in a wide range of latitudes, from tropical to temperate zones
- Can be grown on deficient soils in a pH range of 5.0-8.5
- Nitrogen inputs are low (approximately 100-200 kg/ha per year). This further reduces the risk of water contamination. In areas of restricted land availability, sweet sorghum can be rotated with leguminous crops.
- Low water requirement (approximately 200 kg per kg biomass). This is half the water requirement for corn and about one-third that of sugarcane.
- It has 75% of the biomass distributed in the cane, thus providing ready access of the available carbohydrates
- It has two times the photosynthetic efficiency when compared to other feedstock such as sugar beet and sugarcane.
- Crop is resistant to natural calamities such as drought, floods, soil salinity and alkaline conditions.
- Growing period of sweet sorghum is short (4-5 months), compared to sugarcane, which lasts for about 8-24 months, thus allowing for quicker turnover of the agricultural land.
- Sowing requirements are low, requiring 10-15 kg/ha of seeds, compared to corn which requires 40 kg/ha.
- Sweet sorghum has a high calorific value of 4125 kcal/kg. It also has low sulfur content and a CO_2 balance close to zero.

Sweet sorghum variety, and the location and environment in which it is grown have a significant influence on the fermentable sugar availability in the crop. Currently, most sweet sorghum research focuses on improving yield, disease resistance, stability under

different environmental conditions and improving fermentation performance by improving digestibility of the cellulose fraction. For sweet sorghum to be successfully utilized for ethanol production, several major issues need to be addressed, including carbohydrate storage and accessibility of the lignocellulosic fraction to enzymatic hydrolysis.

Seasonal availability and storability of sweet sorghum are important factors in use of this renewable biomass. Coble et al. (1984) observed that sugar extraction and storability are two serious problems that have limited sweet sorghum as a potential energy crop. Environment has a significant effect on chemical composition and physical properties of the sweet sorghum which in turn significantly affects ethanol yields. Typically juice containing 10 to 15% sugar has been extracted or pressed from sweet sorghum pulp. The low sugar yield is mainly due to low juice press efficiencies, which average between 50 and 60%. The juice can then be fermented directly or evaporated to molasses for storage.

Cost of ethanol is also dependent on the length of the harvesting/processing season because the length of the season determines the amount of fermentable sugar concentrated in the feedstock. The amount of fermentable sugar available is directly related to the ethanol yield and for a given production facility the unit production cost of ethanol will decrease as the production volume increases. Flowering (50% bloom) is considered as the initial stage of harvest as the fermentable sugars tend to accumulate at this point. Collier (1884) observed that delay in harvesting led to a decrease in fermentable sugar in a standing crop. Hence, storage duration between the harvesting and processing of sweet sorghum will dictate the choice of harvest date to avoid a loss in fermentable sugars. Eiland et al. (1983) studied the influence of three different harvesting

methods on loss of fermentable sugars in sweet sorghum, and observed the highest juice brix in hand cut stalks compared to billeted and chopped stalks from a harvester. They also observed that most of the sugar content decreased rapidly in the first 24 h due to continuous respiration by the plant cells, which also caused the temperature in the stored stalks to rise rapidly.

Sweet sorghum is a multipurpose crop serving as a feed, biomass and substrate for crystalline sugar production. The best way to take advantage of this crop is through a flexible conversion facility capable of serving both the sugar and ethanol market demand as influenced by the relative market price of either (Gnansounou et al. 2005).

2.2 Ethanol Production

Ethanol has the potential to ease both natural resource limitations and reduce environmental pollution, and its demand for direct use as a fuel is growing significantly. Around 6 billion L of ethanol were produced in 2002 in the US, mainly from the fermentation of cornstarch (Zhan et al. 2003).

Ethanol (ethyl alcohol, CH_3CH_2OH) is a clear, colorless liquid with a characteristic agreeable odor. It is a chemical compound containing a hydroxyl group, '–OH', attached to a carbon atom. Ethanol can be and is used as an automotive fuel by itself. It can also be mixed with gasoline to produce what is referred to as 'Gasohol'. Gasohol is available in 10% and 85% blends commonly referred to as E10 and E85. One liter of ethanol has an energy equivalent of 20.5 MJ compared to 30.5 MJ from gasoline (Gnansounou and Dauriat, 2005). The ethanol molecule contains an oxygen atom, hence it allows automotive engines to more completely combust the fuel, resulting in fewer emissions of

sulfur oxide and carbon dioxide and ozone precursors such as nitrogen oxide (NOx) and other hydrocarbons. Physical properties of ethanol are given in Table 2.1.

Table 2.1 Physical properties of ethanol

Properties	Values
Chemical Formula	C_2H_5OH
Molecular Weight (g)	46
Carbon (%/w)	52.2
Hydrogen (%/w)	13.1
Melting Point	-114.1^oC
Boiling Point	78.5^oC
Density (at 20^oC)	0.789 g/ml
Latent heat of vaporization (KJ/Kg)	845 kg
Heat of combustion (MJ/kg)	29.7
Stoichiometric air/fuel ratio	9.0
Research octane number	107

Source: http://www.eere.energy.gov/afdc/pdfs/fueltable.pdf

Different methods are known to produce simple alcohols:

- Hydration of ethylene in a three-step process using sulfuric acid (John, 1969) or by direct hydration of ethylene gas combined with water and passed through a fixed bed reactor to form ethanol according to the following reaction (Nelson and Courter, 1954

$$C_2H_4 + H_2O \longrightarrow CH_3CH_2OH$$

- Lignocellulose to Ethanol: Lignocellulose is the principal component of plant cell walls and is composed of 40-60% cellulose, 20-40% hemicellulose and 10-25% lignin fractions (Hamelinck et al. 2005). The lignocellulosic feedstock is first pretreated to dissolve the hemi cellulose fraction and make the cellulose fraction more accessible

during hydrolysis. The pretreatment methods broadly include physical, physico-chemical and biological treatment. Research is currently focused on the development of simultaneous saccharification and fermentation (SSF) of the lignocellulosic fraction. SSF fermentation involves the use of usually exogenous enzymes, capable of hydrolyzing the lignocellulose fraction, to be used in conjunction with principal yeast or bacteria to ferment to ethanol.

- Starch to Ethanol: Starch consists of two major polysaccharides, amylose and amylopectin, which are polymeric chains of α-glucose monomeric units. The conversion of starch to ethanol is a two step process. In the first step, the polymer chain of α-glucose is hydrolyzed into D-glucose, an isomer of glucose, by the action of gluco-amylase and α-amylase enzymes.

$$(C_6H_{10}O_5)n + nH_20 \xrightarrow{\text{gluco-amylase}} nC_6H_{12}O_6$$

In the second step, microbial fermentation of D-glucose yields ethanol, which is then distilled and dehydrated to anhydrous ethanol.

- Cellulose to Ethanol: The cellulose fraction of the biomass is converted to ethanol in a two step process. In the first step, cellulose is hydrolyzed into glucose either by enzymatic hydrolysis using cellulases (endoglucanases, exoglucanases, β-glucosidases and cellobiohydrolases), (Ingram and Doran, 1995; Laymon et al. 1996) or by chemical hydrolysis (dilute or concentrated sulfuric acid). In the second step, glucose is fermented to ethanol by a fermenting yeast or bacteria. Another approach of converting cellulose is termed simultaneous saccharification and fermentation (SSF). During the SSF process either the same microorganism produces cellulase or cellulose is externally added and the glucose is fermented simultaneously.

- Direct fermentation of biomass sugars to ethanol: Common sugars found in biomass include sucrose, glucose, xylose, mannose, galactose and arabinose. The sugars provide a ready source of carbon to be utilized by the yeast and bacteria. Sucrose, for instance is a disaccharide made up of glucose and fructose monosaccharide units and is the most common disaccharide used for ethanol production. Utilization of sucrose during ethanol production is a two step process. First invertase (an enzyme present in the yeast) catalyzes the hydrolysis of sucrose to glucose and fructose.

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{\text{Invertase}} C_6H_{12}O_6 + C_6H_{12}O_6$$

Next the monosaccharide undergoes a series of enzyme catalyzed reactions, called glycolysis to produce ethanol and CO_2.

$$C_6H_{12}O_6 \xrightarrow{\text{Glycolysis}} 2CH_3CH_2OH + 2CO_2$$

2.3 Fermentation

Fermentation is an internally balanced oxidation-reduction reaction in which some atoms donate electrons and become more reduced while other atoms receive electrons and are oxidized. In this process, energy is produced in a step termed phosphorylation. One of the common chemical pathways observed in most fermenting microorganisms utilizing glucose as the carbon source is called glycolysis or the Embden-Meyerhoff-Paranas (EMP) pathway (Figure 2.1).

Glycolysis is divided into three stages involving a series of enzymatic reactions (Madigan et al. 2003). Stage I is a preparatory rearrangement reaction in which no oxidation-reduction reaction takes place. Glucose is the primary six-carbon sugar broken down into two molecules of glyceraldehyde 3-phosphate, a key intermediary compound. No energy

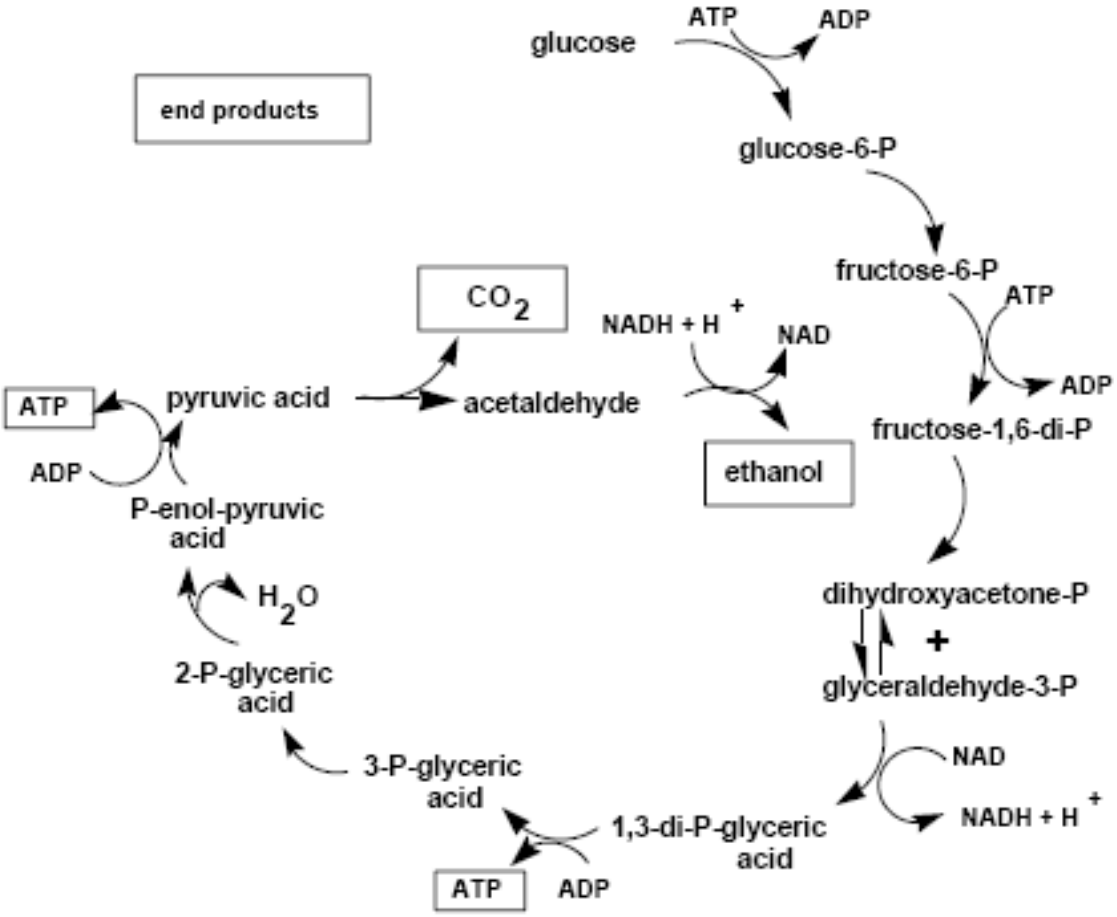


Figure 2.1. Embden-Meyerhoff-Paranas (EMP) pathway for glycolysis exhibited by fermentative microorganism (Ingledew, 1999).

is produced in this stage. Stage II is an oxidation-reduction step wherein energy is conserved in the form of ATP and two molecules of pyruvate are formed. Stage III is a second oxidation-reduction reaction where pyruvate acts as the central hub of glycolysis, and depending upon the organism and the enzyme catalyzing the reaction, different end-products are formed.

The ultimate result of glycolysis is the consumption of glucose resulting in the formation of various fermentation end products under anaerobic conditions and conservation of energy in the form of two ATP's. Common end products of glycolysis include ethanol, lactic acid, alcohols, gaseous substances and a variety of other acids, depending on the organism.

Under aerobic conditions, yeast growth is stimulated, leading to biomass generation. Under anaerobic conditions, carbohydrate sugars are fermented to ethanol (Banat et al. 1996). Yeasts manifest two types of temperature profiles: an associative and a dissociative profile. Yeasts exhibiting a dissociative profile show no significant effect of temperature on exponential growth and are known to be thermotolerant. Yeasts exhibiting associative profiles show a decline in growth yield coefficients above the optimum temperature (van Uden, 1984a). Changes in sugar content will also affect the fermentation process. In general *Saccharomyces cerevisiae* is glucophilic, indicating that the yeast has a clear preference for glucose when a mixed sugar source is available in the media (Cason et al. 1987; Vidrih and Hribar, 1999). The process of sugar uptake is the major control mechanism for the rate of glycolytic flux under anaerobic conditions (Pretorius et al. 2003). Glucose fermentation by *S.cerevisiae* is generally inhibited by oxygen, but micro-aerobic conditions has been shown to enhance the specific alcohol production rate. Hoppe and Hansford (1984) found that a small amount of oxygen does not affect the conversion of sugars to ethanol. However the maintenance coefficient necessary for transport of metabolites across the membrane is greatly reduced.

Inoculum level was also observed to affect ethanol fermentation. An increase in cell inoculum from 10^5 to 10^6 cells/ml improved the fermentation rate but also caused

excessive foaming and higher fermentation temperature caused by too rapid a fermentation rate (Chen, 1981). Normally an inoculum level of 10% corresponding to a population of 10^6-10^7 is recommended for batch fermentation (Strehaiano et al. 1983).

S.cerevisiae is the preferred yeast for ethanol fermentation because of its versatility. Industrial strains are selected with acquired traits such as greater ethanol fermentation capacity, greater tolerance to by-products and greater process hardiness compared to the strains adapted to controlled laboratory conditions. Garay-Arroyo et al. (2004) observed that the industrial *S.cerevisiae* strains grown on Yeast Potato Dextrose (YPD) media, showed different responses to different imposed stress conditions. The cells showed increased survival rates under oxidative stress imposed by adding H_2O_2 to a final concentration of 10 mM and heat shock applied by growing cells at 50^{o}C, but when the cells were subjected to chilling stress imposed by growing cells at -20^{o}C for 2 days and osmotic stress imposed by adding 1.5 M sorbitol, a decreased survival rate was observed.

Constant environmental changes to which present day industrial *S.cerevisiae* strains are commonly exposed to include (Kelsall and Lyons, 1999):

- Wide variety of feedstocks such as cellulosic and lignocellulosic biomass (e.g. wood; agricultural residues such as straws, bagasse and corn stover; grasses such as switch grass and bermuda grass), sugar crops such as sugar cane and beets, starch sources such as corn, and other alternative carbon sources such as whey, spent grains, spoiled and waste food products and syrups.
- Temperature variations from 4 to 50^{o}C.
- Varying medium ethanol concentration of 16 to 23% w/v.
- Varying solute concentration

- Varying medium ionic strength as influenced by the presence of minerals such as phosphates, sulfates and other macro and micro elements.
- Reactive oxygen species (ROS) such as the superoxide anion radical, the hydroxyl radical and hydrogen peroxide that are produced under oxygen limiting conditions that are known to damage DNA, protein and cellular membranes.
- Toxins in culture media such as furans, phenolics and acetic acid.

Saccharomyces cerevisiae

Industrial ethanol production is dependent on the microbial activity particularly of yeasts. Vast literature studies indicate a narrow temperature range, 30 to 35^{o}C, for *S.cerevisiae* growth. Ethanol and temperature tolerance appear to be heavily interrelated in industrial ethanol production. Bioreactor configuration will also have a great impact on yeast performance and ethanol production. Storage viability of *S.cerevisiae* is improved when stored at low temperature and under vacuum or a nitrogen atmosphere.

Zymomonas mobilis

Z.mobilis is a gram negative bacteria and is considered an alternative organism for large scale ethanol production due to its numerous advantages over a variety of yeasts. *Z.mobilis* has been cited in various literatures for higher sugar uptake and ethanol yield, low biomass production, high ethanol tolerance, amenability to genetic manipulation and lack of a micro-aerobic oxygen requirement.

The main limitation of *Z.mobilis* is the fact that the utilizable substrate range is restricted to glucose, fructose and sucrose. It is a facultative anaerobic bacteria utilizing glucose

and fructose via the Entner Deudoroff (E-D) pathway. Ethanol yields as high as 105 g/L in 70 h have been reported by Torres and Barrati (1988) in their batch fermentation studies utilizing wheat starch hydrolysate with an initial sugar concentration of 223 g/L.

Kluyveromyces marxianus

K.marxianus is defined as a respiratory yeast characterized by conversion of low amounts of glucose to ethanol under aerobic conditions and high specific ethanol production rates under anaerobic or micro-aerobic conditions. *K.marxianus* produces ethanol and has a tolerance to pH and osmotic activity comparable to *S.cerevisiae*. Strains of *Kluyveromyces* were reported by Hughes et al. (1984) to produce ethanol above 40^{o}C and to have a maximum growth temperature of 49^{o}C. Others have reported maximum growth temperatures upto 52^{o}C (Banat et al. 1992) in a thermotolerant *K.marxianus* IMB3 strain with high levels of ethanol, however ethanol tolerance was low for the strain compared to *S.cerevisiae*.

2.4 Ethanol Production Scenarios

Use of ethanol as an additive in gasoline has become a billion gallon per year market. It has value as an oxygenate in "CO nonattainment" markets and as a fuel extender and octane booster. Two limitations in the production of ethanol and its use are the availability of biomass and the cost of ethanol. Analysis by Oak Ridge National Laboratory indicates that the maximum amount of agricultural residues that could be collected today is around 144 million dry tons producing 38-53 billion L of ethanol, which is enough to fuel 10% of the light motor vehicles (Walsh et al. 1998). For a long time, the greatest impediment to ethanol's use as an alternative fuel was the availability of ethanol-compatible vehicles in the US. The status has changed considerably, with

automotive manufacturers offering standard automobile models able to run on both E85 and gasoline.

The US Department of Energy's (DOE) research on the development of renewable, domestically produced fuels for transportation is driven by important national issues such as national security, economic competitiveness in the global market, rural economic development, climate change and air pollution (Sheehan and Himmel, 1999).

Process design for ethanol production is based on a fundamental framework comprised of choice of process technology and configuration, choice of feedstock, proposed plant size and dedicated ethanol/electricity versus biorefinery concept (Wooley et al. 1999). The technology platforms available for conversion of biomass to ethanol includes conversion of carbohydrate biopolymers to sugars, fermentation of sugars to ethanol, ethanol recovery and residue utilization.

Each of the biomass to ethanol conversion processes is associated with a preferred feedstock, a conversion rate and certain final and by-products. Assessment of the processing facility also depends on the economies of scale which influences the capital and operating costs. A large bio-energy conversion facility will be cheaper to run per output unit compared to smaller facilities.

2.4.1 Ethanol in Brazil

Brazil is currently the world's largest producer of ethanol. This is due to both widespread availability of sugarcane and heavy governmental subsidies. A National Alcohol Program (PNA) was established in 1975 following the 1973 energy crisis. The PNA has also led to the development of a great number of subsidiary industries for the production of

pesticides and fertilizers, chemicals and equipment required by distilleries and sugar plantations. Cultivation of sugarcane and its subsequent use has contributed significantly to the Brazilian economy. The ethanol industry in Brazil represents 2.3% of the Gross Domestic Product (GDP) (Zanin et al. 2000). The socio-economic impact of the ethanol industry includes creating 1 million production jobs.

One percent of the total cultivated area in Brazil (4.5 million hectares) is presently devoted to sugarcane cultivation. Products created from sugarcane include: sugar, anhydrous ethanol, hydrated ethanol, electricity, biodegradable plastic (e.g. polyhydroxybutyrate, PHB) and acetaldehyde and its derivatives. Currently Brazil consumes 55% of its sugarcane for ethanol production and the remaining 45% is used for sugar processing (da Silveria, 2004).

On average one hectare of land produces 81 to 82 tons of sugarcane and about 7000 L of ethanol (Pessoa-Jr., et al. 2005). The average cost of production is about USD 180/ton of sugar or USD 0.20/L of ethanol. Each ton of sugarcane has an energy equivalent of 1.2 barrels of petroleum. Brazil produced 14.4 billion L of ethanol in 2003-2004. Installed capacity is 18 billion L equivalent to 100 million barrels/yr (http://www/unica.com). The distribution logistics are consolidated by well laid pipelines, railways and highways catering close to 30,000 fuel stations equipped with the necessary infrastructure. (Albuquerque, 1999). In 2003, a fleet of around 18 million light vehicles consumed 27.5 billion L of fuel, 40% if it coming from hydrated or anhydrous ethanol. The vehicles are built with gasoline powered, hydrated ethanol and flex fuel engines, able to run on gasoline and ethanol in any proportion. The flex fuel vehicles are equipped with sensors,

able to detect the proportion of gasoline and ethanol in the mixture and adjust the combustion accordingly.

Three types of juice treatments are employed in the distilleries (Rossell, 1988). The physical treatment uses screens to separate large solid materials and hydrocyclones to separate smaller particles. High contamination, flocculation and gum production are some of the disadvantages associated with physical treatment. The physical and thermal treatment involves the use of screens and hydrocyclones in conjunction with clarification of sugarcane juice at 105^{o}C and then cooling down to the fermentation temperature. In the complete treatment the juice is treated with lime before subjecting it to thermal sedimentation in large clarifier tanks.

Most of the current distilleries use the third generation continuous processes involving multiple stage reactors based on the Melle-Boinot principle of yeast recovery (Zanin et al. 2000). The process uses a centrifuge to separate yeast cream, which is then diluted with fresh water. Suspension pH is reduced to 2.0 to 2.5 by the addition of sulfuric acid and constantly agitated for 1.5 to 4.0 h. Before the start of a new batch, the suspension is aerated and returned to the fermentors. Fed batch and continuous fermentation processes with cell recycling, based on the Melle-Boinot principle, were introduced as advancements to the batch process. In the fed batch process, treated yeast cream is pumped to the fermentor. Wort is continuously pumped into the fermentor, for 4 h while maintaining a brix below 9% (Laluce, 1991). Continuous process involves the use of multiple stage stirred reactors (Zanin et al. 2000). In this process, broth along with yeast cream is fed at the top of the first and emptied from the bottom of each stage, then flows by gravity to the middle of the next stage. Reactor design is characterized by 60^{o} conical

bottom and with a cylindrical part aspect ratio of 1.2 (height/diameter). Heat from each reactor is removed by an external plate heat exchanger. Kinetic energy of the liquid exiting the heat exchanger is used for agitating the contents of the tank. Gases and foam are removed from the top of each reactor and washed in a perforated plate column and the gas is collected. Advances to the continuous process include the use of decanters and flocculent strain of *S.cerevisiae* to substitute the use of capital intensive centrifuges.

The residue leftover after distilling ethanol from the sugarcane fermentation medium is called stillage, which is rich in water, potassium, calcium, iron, phosphorous and other organic compounds. With its nutritional content and its ability to improve the soil porosity, stillage is being used as fertilizer. On an average production of 1 L of ethanol generates 12 to 15 L of stillage. Stillage can also be converted to biogas and biofertilizer following anaerobic digestion. One ton of sugarcane also produces 140 kg of bagasse (~ 50% $w_{water}/w_{bagasse}$), which finds use in thermal and electric energy generation.

2.4.2 Ethanol from Corn

Corn is presently the most important and economical feedstock for ethanol production in the United States. Nine states account for about 80% of the corn grown and 91% of the ethanol produced in the country (Shapouri et. al, 2002). As of 2004, there were 76 plants with a total production capacity of 11.4 billion L and an additional 12 plants under construction, each with a capacity of 1.9 billion L (Bothast and Schlicher, 2005). Over the past three decades, converting corn into ethanol has made significant improvement in terms of economic and technical feasibility mainly due to higher corn yields, lower energy use per unit of output in the fertilizer industry and advances in conversion technology. The corn kernel is comprised of 70 to 72% (dry weight basis) starch. Ethanol

from corn is produced either by a dry grind (67%) or wet milling process (33%). The process selected depends on the focus of resource utilization. The focus of a dry grind plant is to maximize capital return per gallon of ethanol while in a wet milling plant, the extent of capital investment allows for production of other valuable components. Compared to the dry grind process, the wet milling process is a capital and energy intensive process. Available technologies produce 10.6 L of ethanol per bushel of corn by dry grind process while the ethanol yield is 9.5 L in a wet milling process. Co-products of value produced from a dry grind process are distillers dried grain with or without solubles (DDGS) and from a wet milling process corn oil, corn gluten meal and corn gluten feed are produced.

In the dry grind process (Bothast and Schlicher, 2005), corn is cleaned, ground and slurried with water to form a mash. Alpha amylase is added at pH 6.0 to break down starch polymer into soluble dextrins. The mash is cooked at 100^{o}C for several minutes and cooled to 80-90^{o}C and additional alpha amylase added to further liquefy the starch. The mash is cooled and glucoamylase enzyme added at pH 4.5 to convert the dextrinized mash to glucose. The fermentable sugars are then converted to ethanol by the microorganisms. The mash is then cooled to 32^{o}C and transferred to fermentors where the added yeast converts the sugar to ethanol. A final ethanol concentration of 10 to 12% is obtained in 48 to 72 h. The distillation step separates ethanol from water and other impurities.

In a wet milling process (Bothast and Schlicher, 2005), corn is first separated into its four basic components: starch, fiber, gluten and germ by steeping in dilute sulfur dioxide solution at 52^{o}C for 24 to 48 h to break the starch and protein bridges. It is then coarsely

ground to break the germ loose from other kernel components. Subsequent saccharification, fermentation and distillation of ethanol are similar to the dry grind process.

Shapouri et al. (2002) indicate a Net Energy Value (NEV) of 5.86 MJ/L with a net energy ratio of 1.34 for corn based ethanol. However, significant differences in determining the NEV of corn by various authors exist due to different assumptions about corn yields, ethanol conversion technologies, fertilizer manufacturing efficiency, fertilizer application rates, co-product evaluation and the number of energy inputs included in the calculations.

Raw material cost, processing efficiency and exchange rates are the major sources of competitive advantage when comparing the feedstocks. When corn is compared with sugar as the feedstock, distinct differences arise due to the difference in the valuation of by-products. Corn processors return the protein and oil fraction to feed and food markets after starch extraction. Compared to corn, sugarcane does not yield byproducts of much value and the residue is being used in power plants for power generation.

2.4.3 Ethanol from Sweet Sorghum

The estimated cost for growing sweet sorghum is 296 USD per hectare, compared to the estimated cost of growing corn at 370 USD per hectare with a yield of 308 bu/hectare (Duffy and Smith, 2004; University of Georgia, 1999). Use of sweet sorghum for ethanol production has been extensively studied for the Piedmont, a geographic region in the eastern U.S. covering seven states, which is known for having drought-prone, low productivity soil (Worley and Cundiff, 1991). In the Piedmont, sweet sorghum has been shown to produce more carbohydrates per hectare than corn (Parrish et al. 1985). Unlike

corn in which carbohydrates are concentrated in the grain, sweet sorghum carbohydrates are stored in the stalk. This provides both advantages and disadvantages in using sweet sorghum for ethanol production. The primary advantage is that sugars are directly available for fermentation after simply pressing the biomass; no enzymatic treatment is necessary. Major disadvantages include the seasonal availability, sugar extraction and storability of the crop. Sweet sorghum is harvested seasonally at a high moisture content of 70 to 80%, which provides an ideal environment for the deterioration of available carbohydrates by the native microflora of the juice. The available sugars are known to deteriorate even when the stalks are bundled and stored at dry ambient temperatures (Coble et al. 1984). In contrast, Parrish and Cundiff, (1985) indicated that minimal carbohydrate losses occur in whole sorghum stalks that have been stockpiled for less than 30 days.

Various harvesting and processing models have been investigated for producing ethanol from sweet sorghum. One model system involved the development of a whole-stalk sorghum harvester which cuts and windrows stalks (Rains et al. 1990). In this system, the stalks could be stored for up to 30 days in the windrows, and were then transported to a processing site near the edge of the field. At the processing site, stalks were run through a screw press where the juice was collected and the pith presscake was ensiled in a nearby trench silo for later conversion (Worley and Cundiff, 1991). The juice was transported either to an evaporation plant for concentration or to a fermentation/distillation plant for immediate conversion to ethanol. A slight variation to this system was one in which a hypothesized pith combine was used instead of the whole-stalk harvester, where the forage was chopped and loaded onto a forage wagon (Worley and Cundiff, 1991). The

wagon was then taken to a nearby location for juice expression, similar to the previous scenario. The difference between these two models is that in the latter there was no storage of stalks, as the juice must be pressed within hours to avoid unacceptable losses. In both scenarios, it was envisioned that the ensiled presscake would then be transported to the fermentation plant for conversion of the cellulose to ethanol. Other studies have focused on enzymatic conversion of the cellulosic sorghum stalks to ethanol, and have demonstrated that a large portion of the insoluble carbohydrate from sorghum can be converted to ethanol by simultaneous saccharification and fermentation (Lezinou et al. 1995).

The above-mentioned studies share one common theme: each involves a scenario where juice and/or ensiled stalks are transported to a central processing plant where ethanol is produced, and the resulting process is not found to be economically feasible. The cost of ensiling and transporting, combined with the capital costs of the central processing plant, make these processes very costly.

Sugar concentrations of 15 to 22 ^{o}Brix have been reported for sweet sorghum, depending on growing location (Rains et. al, 1993). Typical sorghum juice expression ratios for roller mills have ranged from 0.47 to 0.58 g juice/g input biomass, depending on the specific crop (Lamb et al. 1982; Monroe et al. 1984). Nominal sorghum yields have been reported as about 75 tons/hectare (University of Georgia, 1999).

Using knowledge of current and projected yields and conversions, the expected ethanol yield from the proposed in-field process to produce ethanol from sweet sorghum can be estimated as follows:

Assumptions: Sweet Sorghum yield of 75 tons/hectare

Sugar content of stalks: 17%

Juice yield (with roller mill) = 0.55 g juice/ g biomass

Theoretical ethanol conversion from hexose sugars is 0.511 g ethanol/g sugar (Hettenhaus, 1998). Ethanol yields fall short of theoretical yields because approximately 5% of the sugar is used by the yeast for growth and for producing other minor products such as glycerol, acetic acid, lactic acid and fusel oils (Bothast and Detroy, 1981). Using a conversion rate of 95%, these estimates give an ethanol yield of approximately 3400 L of ethanol per hectare of sweet sorghum. As a point of comparison, ethanol yields from corn are estimated to be about 9.5 L/bu, and at a corn yield of 309 bu/hectare, that equates to 2930 L of ethanol per hectare of corn (Shapouri et. al, 2002). In terms of carbohydrate quantity and production capability, sweet sorghum has the potential to compete very favorably with corn.

The sugar content from fresh sorghum stalks have been found to deteriorate upon storage and hence needs to be extracted immediately. However under temperate conditions, Li (1997) reported storing stalks in the field for 4-5 months as silage.

Sorganol- Proposed Economics

Economics of in-field ethanol production from sweet sorghum juice is currently being studied at Oklahoma State University (Huhnke, 2006). Due to the lack of sweet sorghum

production data, many assumptions must be made to develop a set of scenarios to better determine economic feasibility. Base assumptions include: sweet sorghum is rotated with other row crops, fertility requirements are approximately one third that of corn, and pesticide requirements are comparable to grain sorghum production. In addition, the sweet sorghum crop is planted in May and harvested late September through mid-November using a single pass harvester-juicer system. A conservative sweet sorghum juice yield of 9400 L per hectare is pressed and fermented in 75,000-100,000 L collapsible bladders. Using a conversion efficiency of 95%, approximately 800 L of ethanol is produced per hectare. Estimated production cost is less than $0.50 per L of ethanol. A cost of $10.50 per 1000 L is assumed for transporting 38,000 L of ethanol over a distance of up to 100 kilometers. Net return on investment is over $400 per hectare with a payback period of less than seven years.

2.4.4 Ethanol from Lignocellulose

Lignocellulose represents the largest fraction of biomass available on earth. Common lignocellulosic biomass include agricultural and forestry residues, paper and municipal solid wastes, paper and pulp industry wastes, herbaceous and woody plants, softwood such as spruce and pine, grasses such as switchgrass and bermudagrass, crop residues, and solid animal waste. Lignocellulose is primarily composed of 40-60% cellulose, 20 to 40% hemicellulose and 10 to 25% lignin (Gnansounou et al. 2005). Conversion of lignocellulose to ethanol is a two step process. In the first step cellulose is hydrolyzed to fermentable sugars by the action of cellulase enzyme. In the second step, sugars are converted to ethanol by fermenting microorganisms. The crystalline structure of cellulose along with the protective hemicellulose and lignin sheath around the cellulose makes it

inaccessible for hydrolysis. Pretreatment of lignocellulose substrate by physical, physicochemical and biological methods results in delignification of the substrate thereby exposing the fermentable sugars. Pretreatment also reduces cellulose crystallinity and increases the porosity thereby improving the cellulose digestibility and the conversion efficiency.

2.4.5 Non-Sterilized Fermentation

Ethanol production under non-sterilized conditions has gained significant attention by researchers and industries because of its economic advantage. Hashiyada and Flor (1981) worked with raw digestion of starch and found that ethanol fermentation using a non-sterilized medium can save about 30 to 40% of the energy consumed compared to the sterilization commonly carried out in an industrial fermentation process. Sterilization of the fermenting substrate prior to fermentation also results in loss of sugar and nitrogen and undesirable pigmentation due to Maillard reaction. This can be avoided by sterilizing the reducing sugars and other nutrients separately and then mixing them aseptically. This is possible for a tailor made synthetic media, but is not possible when utilizing a biomass substrate and hence loss of nutrients is inevitable. Loss of 8 g/L of glucose has been reported by Kechang (1995). The above procedure also leads to process complexity and adds to the risk of cross contamination. Tao et al. (2005) also found that the glucose to ethanol conversion yield was higher in non-sterilized media compared to autoclaved media. The yield obtained was 0.488 g/g in non-sterilized media compared to 0.468 g/g in sterilized media. Gibbons and Westby (1989) found yeast inhibition in unpasteurized sweet sorghum juice was due to a combination of factors. One of the factors was the presence of high levels of bacterial contaminants. The source of the contaminating

bacteria in sweet sorghum is primarily from the soil and environment. The inhibitory effects of bacteria variety of feedstock fermentation are well documented. Another possible factor was the presence of inhibitory substances present in sorghum juice.

2.4.6 Comparison of Ethanol Production Technologies

Current processes for producing ethanol include either direct fermentation of sugars, enzymatic conversion of starch-based crops such as corn, or acid/enzymatic hydrolysis of lignocellulosic feedstocks. The majority of current ethanol production is from the fermentation of corn, accounting for 90% of total commercial production (Chum and Overend, 2001). The corn ethanol fermentation process involves enzymatic conversion of the starch to simple sugars, followed by yeast fermentation, and distillation to concentrate the ethanol. Ethanol production from lignocellulosic feedstocks such as wood, stover, and grasses requires more stringent enzymatic methods in order to break the cellulose into fermentable simple sugars, and this adds to ethanol production costs. Major disadvantages of current processes include high cost of enzymes, the formation of waste streams, long fermentation times, and federal subsidies required for economic returns, although major efforts are being extended to eliminate these disadvantages (Eriksson et al. 2002; Mielenz, 2001). As is evident, the level of difficulty in producing ethanol increases from sugar crops to starch-based crops to lignocellulosic plants as a result of the increasing complexity of the sugar components of the raw materials.

2.5 Environmental Influence on Fermentation

Physiology and molecular biology of stress responses in yeast cells has received widespread attention in recent years because of its practical implication in biotechnology. In alcoholic fermentation processes, yeast cells encounter several environmental stresses

that adversely affect their ability to perform efficiently and consistently in the conversion of sugars to ethanol (Figure 2.2). For instance, yeast cell death induced by extreme temperature or by toxic ethanol concentration levels has a direct impact on yeast fermentation performance. Environmental stress is also known to decrease cell viability. Atala et al. (2001) observed 60-70% reduction in cell viability with an increase in fermentation time. This was probably due to highly stressful conditions experienced as the fermentation progresses owing to high ethanol and cell concentrations.

Yeast management before, during, and after fermentation should endeavor to minimize physiological stresses imparted on the fermenting yeast cells. Stress may be imposed on fermenting yeast at pre-fermentation (e.g. acid washing, cold-shock, oxidative stress and nutrient starvation), primary and secondary fermentation (e.g. osmostress, ethanol toxicity, pH/temperature fluctuations and CO_2/hydrostatic pressure); and post-fermentation (e.g. mechanical shear, cold-shock, and nutrient starvation (Quain, 1990). An improperly managed ethanol fermentation process may also lead to stuck or sluggish fermentation. During a stuck fermentation the rate of sugar utilization is greatly reduced or protracted especially during the end of the fermentation process primarily due to the deficiency of vital nutrients in the fermentation media or due to inefficient control of the fermentation temperature.

2.5.1 Temperature

Yeasts employed in the ethanol industry are subjected to wide variations in temperature. Fermenting yeasts experiences temperatures lower than 5^oC during cold storage and higher than 40^oC in non-cooled fermentors. Temperature becomes critical when designing a fermentation process operating under environmental conditions. Optimal

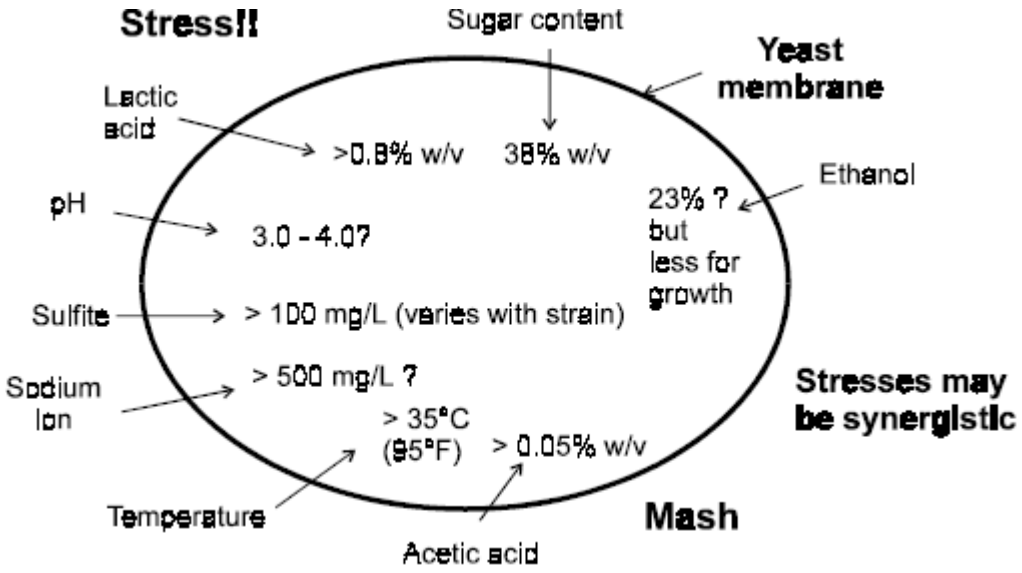


Figure 2.2 Typical yeast factors known to influence yeast fermentation capacity (Ingledew, 1999)

temperatures for ethanol production by brewing yeast are higher than those required for growth (Jones et al. 1981). The optimal temperatures for the growth of *S.cerevisiae* are in the range of 30-35^{o}C, and increasing the fermentation temperature from 30 to 39^{o}C resulted in higher ethanol productivity, but also caused greater cell death (Krouwel and Braber, 1979). The influence of temperature on fermentation kinetic parameters must be considered since it is usually difficult to control temperature during large scale alcoholic fermentation. Since the ethanol fermentation process is an exothermic process, small fluctuations in the temperature (2 to 4^{o}C) will deviate the optimal process temperature. Understanding the temperature influence on fermentation kinetics is a useful strategy for process optimization. Change in surrounding temperature can have a direct influence on metabolism characteristics and control, the structure of cells, enzyme reaction and cell permeability. Oh et al. (2000) found in the same study that in the range of 30 to 42.5^{o}C,

the maximum specific production rate decreases by 2.5% with every 1^{o}C rise in temperature above 30^{o}C. A typical ethanol fermentation process is exothermic, and in a process carried out at ambient temperatures (40^{o}C) with no cooling system, an increase of 11^{o}C can be experienced leading to limited ethanol productivity (Burrows, 1970). However, fluctuations in temperature cannot be avoided in large industrial fermentors due to difficulties related to agitation and cooling when the external temperature reaches high values. Currently, thermotolerant yeast strains capable of growth and ethanol production above 40^{o}C are being actively sought through screening, temperature adaptation, protoplast fusion, mutagenesis technique, molecular biology techniques and isolation techniques to improve upon the ethanol production efficiency (Banat et al. 1996).

Heat and high ethanol concentrations cause membrane disordering, protein denaturation, glycolysis inhibition and enhanced mutation. Exposure to lower temperatures may also prove to be lethal, especially in the presence of ethanol due to loss in essential cellular components through the lesions in the yeast cell membrane. Cold shock may also inhibit bud development, vacuolar rearrangement, and induction of 'cold-shock' proteins (Fargher and Smith, 1995).

2.5.2 Ethanol

The primary limiting factor during ethanol production is the influence of high ethanol concentration on yeast growth and fermentation. In general, as the initial amount of ethanol in the medium is increased, the specific growth rate and specific ethanol production rate decreases. Bailey and Ollis (1986) found that tolerance to high ethanol concentration is strain specific with a maximum allowable concentration of 10% w/v for

growth and 20% w/v for ethanol production exhibited by the most tolerant strains. However, Oh et al. (2000) observed that the maximum allowable ethanol concentration above which the cell stops growing was 8% w/v and the maximum allowable ethanol concentration, above which cells stop producing ethanol, was determined to be 10% w/v. The inhibitory effect of ethanol on ethanol production was more severe than that for the cell growth. Contradictory results by Bajpai and Margaritis (1982) indicate that high initial ethanol concentration is known to inhibit the maximum specific growth (μ_{max}) rate but had no effect on final ethanol concentration and cell yields or on sugar utilization.

Ethanol is known to alter the degree of polarity of the cell membrane and the cytoplasm, causing disruption of growth due to membrane fluidity (Lynd et al. 1991). Plasma membrane phospholipids have been shown to play a significant role in the ethanol tolerance of yeasts (Ingram and Buttke, 1984). At higher temperatures, higher saturated fatty acids such as palmitic and palmitoleic acids are formed in the cell membrane at the expense of unsaturated acyl chains such as oleic, linoleic and linolenic acid. This results in the loss of membrane fluidity required for various cellular activities.

Higher concentrations of membrane unsaturated fatty acids, vitamins and proteins (D'Amore and Stewart, 1987; Ingram, 1984) along with other physiological factors such as medium composition and mode of substrate feeding (Dombek and Ingram, 1986a, 1986b; Yamamura et al. 1988), intercellular ethanol accumulation (D'Amore et al. 1988), temperature and osmotic pressure can all lead to increased tolerance to ethanol (D'Amore and Stewart, 1987; Jones et al. 1981; Ohta and Hashiyada, 1983; Vienne and Stockar, 1985). In addition trehalose was found to act as both a membrane stabilizer and a

protectant for yeast cells under stressful conditions, especially under higher ethanol concentration (Kida et al. 1993; Majara et al. 1996).

2.5.3 Agitation

The purpose of liquid circulation (mixing) in a fermentation bioreactor is to homogeneously distribute the substrates and the products, and the physicochemical factors such as temperature and pH with respect to the microbial cells. Mixing is achieved by different means such as mechanical agitation, gas injection or by recirculating the contents of the fermentor. However, installing an agitator in a large scale process significantly adds to the cost, affecting the economic feasibility of the in-field process. Intensive mixing leads to mechanical destruction of the cells while insufficient mixing intensity inhibits substrate and oxygen mass transfer leading to cell damage. Galindo et al. (1989) observed a longer fermentation time by *S.cerevisiae* on sugarcane molasses when the fermentors were not agitated. The effect was more pronounced when the fermentor volume was scaled up due to increased residence time of CO_2, biomass, ethanol and sugar. They observed an increase of 36% in ethanol concentration and a reduction of 4 h in the fermentation time when the fermentors were agitated. Toma et al. (1999) observed that under intensive mixing conditions cells of *Z.mobilis* are prolonged while the cells of *S.cerevisiae* form continuous chains similar to hyphae and exhibit unusually high rate of substrate utilization (Berzins et al. 2001).

In contrast, Farid et al. (2002) found in their ethanol fermentation study with co-culture of *Aspergillus awamori* and *Saccharomyces cerevisiae* that the ethanol production decreased as the agitation speed increased. Maximum ethanol concentration was produced at 50 rpm and the lowest level of ethanol was produced at 200 rpm. This is

attributed to the fact that at 200 rpm there is an accumulation of inhibitory substances such as tryptophol (Hango et al. 1967). Similar findings were published by Converti et al. (1996) who observed a decrease in biological intensity due to increasing mixing intensity in *S.cerevisiae* cells, a phenomenon termed as turbohypobiosis. Shear stress caused by mechanical agitation also results in increased calcium ion channeling across the membranes of bacterial and yeast cells leading to synthesis of stress proteins (Namdev and Dunlop, 1995).

2.5.4 Acetic Acid and Other Weak Acids

Various growth inhibitors such as organic acids, aldehydes, higher alcohols and fatty acids produced by yeasts are known to accumulate during fermentation (Maiorella et al. 1983). Organic acids are known for their fungistatic and fungicidal effects which are maximal at low pH. Octanoic and decanoic acid in the presence of ethanol affect growth and thermal death parameters of *S.cerevisiae* (Viegas et al. 1985). Weak acids are also known to increase the ethanol induced thermal death by increasing the entropy of activation without affecting the enthalpy of activation of thermal death (Leao and van Uden, 1985).

The inhibitory effect of acetic acid on the growth rate is dependent on the pH of the medium, its dissociation and molar constants (Narendranath et al. 2001). Acetic acid is usually formed during the fermentation of D-glucose by *S.cerevisiae* (Fraenkel, 1982) and has been shown to inhibit growth and induce cell death (Moon, 1983; Pinto et al. 1989).

The inhibitory effect of acid can be explained by the classic weak acid theory (Maiorella et al. 1983). Toxicity of acetic acid is pH dependent, since the undissociated form of the acid diffuses through the cell membrane. Pampulha and Loureiro (1989) found that acetic acid inhibits *S.cerevisiae* in an exponential way, the effect becoming more pronounced at lower pH. At extracellular pH values below the pKa of acetic acid (pH 4.74), the fraction of undissociated acid and toxicity increases. The extent of pH inhibition is also influenced by medium composition, medium pH and buffering capacity of the acids. Hence interference of acetic acid results in an increased ATP requirement for cell maintenance. In other words, ATP available for cell mass is channeled for maintaining the cell pH homeostasis rather than growth resulting in decreased cell biomass.

2.5.5 Nitrogenous Constituents

Yeasts require low molecular weight nitrogenous compounds such as inorganic ammonium ion, urea, amino acids and peptides (Patterson and Ingledew, 1999). Deficiency of nitrogen results in stuck or sluggish fermentations. The growth is found to increase linearly in the presence of 100 mg/L of free amino nitrogen (Pierce, 1987). Higher levels were found not to have any influence on cell growth, but significantly improved the rate of fermentation. Absence of nitrogen in the fermentation system leads to catabolism of amino acids and degradation of proteins (Cooper, 1982). Generally nitrogen in the form of ammonium ion is provided to yeasts by supplementing the growth medium with urea, sulfates and phosphate salt. Urea is broken down by yeasts to provide two molecules of ammonium and one molecule of carbon dioxide.

Devine and Slaughter (1980) observed that ethanol production was stimulated only in the presence of NH_4^+ in a media containing glucose and not in presence of other carbon

sources such as maltose, fructose and sucrose, however NH_4^+ was observed not to influence the cell growth in the fermentation medium. Saita and Slaughter (1984) observed that the rate of fermentation by *S.cerevisiae* was dependent on the availability of NH_4^+ and an increase of 50 to 150 mg N/L improved the rate of ethanol production and ethanol yield. Yeasts cannot utilize all available amino acids. Schultz and Pomper (1948) observed that alanine, arginine asparagine, aspartic acid, glutamic acid, leucine and valine when used as a sole nitrogen source promoted yeast growth. The utilization of amino acids was strain dependent and hence a balanced mixture of amino acids is more efficient in providing nitrogen than a single source.

2.5.6 Magnesium

Magnesium is involved in many essential physiological and biochemical functions in yeast cells, including growth, cell division and enzyme activation. Yeasts have a very high growth demand for magnesium ions, and magnesium accumulation by yeast correlates closely with the progress of fermentation. Walker et al. (1996) found that supplementing fermentation media with magnesium resulted in stimulation of fermentation. Walker (1998) summarized the influence of magnesium in ameliorating the stressful effects of ethanol, high osmotic pressure, heat shock and heavy metals known to damage the yeast cell by affecting the structure and function of the plasma membrane. The results indicated that the availability of exogenous magnesium results in reducing the deleterious effects of ethanol concentration on the cellular magnesium homeostasis. Structurally, magnesium stabilizes the biological membrane by cross-linking the carboxylated and phosphorylated groups of membrane lipids (Cowan, 1995).

Functionally, magnesium stimulates the activity of membrane bound H^+-ATPase, which is necessary for the physiological function of the yeast cell (Rao and Slayman, 1996).

2.5.7 Other Growth Requirements

Yeasts require micro-aerobic conditions to supply approximately 8-20 ppm of oxygen required for necessary function and integrity of cell membranes (Ingledew, 1999). Oxygen in small amounts is required for the synthesis of sterols and unsaturated fatty acids. When yeast cells are grown in absence of oxygen, the available fatty acids and sterols become limited by sharing with new daughter cells and the cell stops growing.

Phosphorous and sulfur are the two important minerals required for the growth of yeasts. Phosphorus is required for sugar metabolism, lipid synthesis and production of nucleic acids in yeast cells. Sulfur is required at a concentration of 0.3 to 0.5% for the synthesis of sulfur containing amino acids. Yeasts also require other macro elements such as potassium, magnesium, calcium, iron, zinc and manganese at a concentration of 0.1 to 1.0 mM. Micro elements such as cobalt, boron, cadmium, chromium, copper, iodine, molybdenum, iodine, nickel and vanadium were required at a concentration of 0.1 to 100 µM (Matthews and Webb, 1991).

Berry and Brown (1987) indicated the importance of various vitamins such as biotin, pantothenic acid, inositiol, thiamin, nicotinic acid and pyridoxine as growth factors for yeasts. These vitamins play a major role in aerobic propagation of yeasts and may not prove to be critical during ethanol fermentation. Dry distillers yeast commonly employed during alcohol fermentation are known to multiply for only a few generations and hence the deficiencies of these vitamins may not prove to be critical.

CHAPTER III

EXPERIMENTAL MATERIALS AND METHODS

The overall goal of this study was to design and optimize an in-field process of ethanol production from sweet sorghum. The specific objective of the study was to determine the feasibility of performing the in-field fermentation of sweet sorghum juice with little or no process control. Laboratory scale experiments and two in-field experiments were conducted to answer the various research questions to establish the feasibility of in-field processing of sweet sorghum.

3.1 Laboratory Experiments

Laboratory experiments were conducted in 500 ml shake flasks using fermentation media prepared in reference to the composition of sweet sorghum juice. These experiments were conducted to test and refine the various process variables. Shake flasks were incubated under controlled conditions to test the adaptability and ethanol production capacity under similar in-field conditions. Objectives of the first experiment were:

- To test and compare the ethanol production capacity of different microorganisms belonging to *Saccharomyces cerevisiae* (Fermax and Superstart Distillers yeast), *Zymomonas mobilis, Kluyveromyces marxianus* species and their different combinations.
- To test different levels of incubation pH. The pH levels tested were 3.75, 4.3, 5.4 and 7.0.

- To test the adaptability and ethanol production of the microorganisms at different incubation temperatures of 15, 30 and 35^{o}C, and alternating 41 and 15^{o}C every 12 h.
- To develop analytical procedures for the estimation of total sugars, volatiles, cell biomass and nutritional composition of sweet sorghum juice and fermentation media.

Testing of different types of fermenting microorganisms, maintained at different levels of pH and incubation temperatures enabled us to understand the impact of these variables on the fermentation performance and the sugar-to-ethanol conversion efficiency during a fermentation process. The composition of sweet sorghum juice was used as a reference when preparing the fermentation media. Sugars in the fermentation media included sucrose, glucose, and fructose, at concentrations of 85 g/L sucrose, 45 g/L glucose and 40 g/L fructose. Yeast extract and peptone were added at 5g/L level to enable the growth and ethanol production of fermenting microorganisms.

To design an in-field process, it was necessary to carry out the fermentation without sterilization of the media. Ethanol production under non-sterilized conditions has gained significant attention by researchers and industries. All shake flask studies were conducted in 500-ml Erlenmeyer flasks with a working volume of 250 ml. The flasks were fitted with two-holed rubber stoppers. A disposable syringe with a 14-gauge blunt end needle was inserted through one of the holes. Flexible plastic tubing was connected to the other hole by using a plastic elbow fitting. The other end of the plastic tubing was inserted into a bottle containing water. The flexible tubing was used to obtain anaerobic conditions required for the fermentation process by venting the CO_2 produced during fermentation while preventing the influx of air into the fermentation vessel.

3.1.1 Yeast

One objective of this study was to test microorganisms from different sources for ethanol production and compare the process efficiency of sugar to ethanol conversion. The microorganisms tested were *S.cerevisiae* (Fermax yeast, Superstart Distillers yeast), *K.marxianus* and *Z.mobilis*. The microorganisms were also tested in different combinations to determine if conversion efficiency improved. The pH of the medium was adjusted to pH 4.3 by gradually adding 2N H_2SO_4 and 2N NaOH (if required). The flasks were incubated at 30^{o}C in an orbital shake incubator without agitation.

3.1.2 Temperature

Most yeast strains of interest operate within a finite optimum temperature range, and if left to environmental conditions, the temperature range for the in-field fermentation process could be very large. Temperature data was obtained from the Oklahoma Mesonet for the state of Oklahoma in the months of August, September, and October (likely harvest months for sweet sorghum) over the last ten years. The Oklahoma Mesonet is a joint effort by Oklahoma State University and the University of Oklahoma in recording real-time weather information from over 100 stations across the state (Brock et al. 1995). The average low temperature during this three-month period was 7^{o}C, and the average high temperature was 37^{o}C. In addition, the lowest temperature recorded was -7^{o}C and the highest temperature recorded was 43^{o}C. This obviously indicates a very large potential temperature range for fermentation. Incubation temperature conditions tested were: Fermax yeast at constant temperature of 15, 30, 35^{o}C, and alternating temperatures between 41 and 15^{o}C every 12 h by incubating the flask in incubators. The incubation temperatures were selected to determine the ability of the yeast to produce ethanol at

unfavorable fermentation temperatures. This was necessary from the research stand point because the in-field fermentation experiments were to be conducted under ambient conditions. By alternating the incubation temperature every 12 h between 41 and 15^{o}C the ambient environmental temperature was imitated.

3.1.3 pH

Laboratory scale tests were conducted to determine whether acidification of the media was necessary if the fermentation was carried out in the absence of sterilization. The theory behind these tests was that sufficient acidification may help in inactivating the native microflora present in the sorghum juice. However, acidification of the fermentation is an added expense resulting in increased processing cost. The levels of pH were selected based on initial screening experiments where the amount of ethanol produced at different pH levels was compared. Experiments to evaluate the effects of pH included fermentation using Fermax yeast at pH 3.75, 4.3 and 5.5. and *Z.mobilis* at pH 4.3 and 7.0. pH 5.5 is the native pH of the sweet sorghum juice. The initial screening experiments indicated that the rate of ethanol production at pH 3.75 was superior to pH 4.3, but produced similar concentrations of ethanol. The pH of the medium was adjusted to pH 4.3 by gradually adding 2N H_2SO_4 and 2N NaOH (if required). The flasks were incubated at 30^{o}C in orbital shake incubator without agitation.

3.1.4 Agitation

Agitation helps to provide uniform heat and mass transfer inside a fermentation vessel; however, installing an agitator in a large scale process significantly adds to the cost. The objective of this study was to test the influence of agitation on fermentation. Experiments were conducted using *Z.mobilis* and Fermax yeast in combination. Conditions tested

were: *Z.mobilis* and Fermax Yeast with agitation, *Z.mobilis* with agitation, and *Z.mobilis* and Fermax Yeast without agitation. All flasks were incubated at alternating 15 and 41^{o}C every 12 h. The agitated flasks were incubated in an orbital shake incubator placed in a cold room at 4^{o}C. The lower temperature of the room helped to better stabilize the fluctuations in incubation temperature.

3.2 In-Field Experiments

3.2.1 Experiment I

The objective of the study was to determine the effect of several fermentation variables on ethanol yield. The variables tested included two different yeasts (Fermax yeast from Martrex Inc., www.martrexinc.com, and Superstart Distillers yeast from Crosby and Baker), two different pH levels (4.3 and 5.4- the natural pH of the sweet sorghum juice) and with and without added urea. The levels of pH were chosen based on the preliminary results from the laboratory experiments which indicated the ethanol yield at pH 4.3 and 3.75 was similar. Hence, pH 4.3 was selected as one of the pH levels and compared with the native pH of the sweet sorghum juice at pH 5.4.

Experimental Design

A factorial design was used which included three factors, each at two levels to give a 2^3 factorial design, plus replicates. Treatments were tested in triplicate, to produce 24 different fermentation samples. Factor levels were chosen following an extensive literature search and preliminary laboratory experiments. The experimental setup for the twenty four vessels is shown in Table 3.1.

Table 3.1. Experiment I Design of Experiment

Drum #	Size (L)	Temperature Logger	Yeast	pH	Nutrient	Temperature
1	3.8	1-Submerged	Fermax	4.3	Urea	Ambient
2	3.8	None	Fermax	4.3	Urea	Ambient
3	3.8	None	Fermax	4.3	Urea	Ambient
4	3.8	1-Submerged	Fermax	4.3	Without Urea	Ambient
5	3.8	None	Fermax	4.3	Without Urea	Ambient
6	3.8	None	Fermax	4.3	Without Urea	Ambient
7	3.8	1-Submerged	Fermax	5.4	Urea	Ambient
8	3.8	None	Fermax	5.4	Urea	Ambient
9	3.8	None	Fermax	5.4	Urea	Ambient
10	3.8	1-Submerged	Fermax	5.4	Without Urea	Ambient
11	3.8	None	Fermax	5.4	Without Urea	Ambient
12	3.8	None	Fermax	5.4	Without Urea	Ambient
13	3.8	1-Submerged	SD	4.3	Urea	Ambient
14	3.8	None	SD	4.3	Urea	Ambient
15	3.8	None	SD	4.3	Urea	Ambient
16	3.8	1-Submerged	SD	4.3	Without Urea	Ambient
17	3.8	None	SD	4.3	Without Urea	Ambient
18	3.8	None	SD	4.3	Without Urea	Ambient
19	3.8	1-Submerged	SD	5.4	Urea	Ambient
20	3.8	None	SD	5.4	Urea	Ambient
21	3.8	None	SD	5.4	Urea	Ambient
22	3.8	1-Submerged	SD	5.4	Without Urea	Ambient
23	3.8	None	SD	5.4	Without Urea	Ambient
24	3.8	None	SD	5.4	Without Urea	Ambient

SD- Superstart Distillers yeast

Source of Sweet Sorghum Juice

Sweet sorghum juice was obtained from the Kerr Center, Poteau, OK. Sorghum stalks (Dale variety) were harvested manually and then pressed in a mechanical roller press by Kerr Center staff (Figure 3.1a and b). Extracted juice was filtered and collected in 19-L buckets and prepared for fermentation within 4 h of juice pressing.

Fermentation

Fermentation experiments were conducted under unsterilized conditions in twenty-four, 3.8-L PETE vessels (Rubbermaid, Item # 66273, 100-400 Cap Size). Fermentation vessels were equipped with a cylindrical air trap filled with water (Cellar Homebrew). A 14-gauge blunt end syringe (Fisher Scientific) was inserted into each lid using a rubber stopper for sample collection. A submerged temperature logger (Type: HOBO Pendant Temp/Alarm 8K, Onset Computer corporation) was submerged in one fermentation vessel in each treatment set to monitor the temperature throughout the fermentation process. A temperature logger was also used to monitor the ambient temperature. Figure 3.2a shows the schematic setup of the fermentation vessel.

Dry yeast was weighed @ 0.05% w/v and hydrated using water at 35^{o}C for 1 h. Juice (3.5 L) was measured using a graduated cylinder and filled into each of the fermentation vessels. Hydrated yeast was added to each fermentation vessel. Urea (MN, www.martrexinc.com) was added at 200 ppm if required based on the experimental plan. The pH of fermentation media was reduced to pH 4.3 by the gradual addition of 36 N Sulfuric Acid as required based on the experimental plan. The contents were mixed thoroughly and allowed to ferment under ambient conditions (Figure 3.2b). Samples were

taken at 24 h intervals and analyzed. Fermentation was allowed to continue until the sugar in the media was completely consumed by the fermenting yeast.

Figure 3.1. Pretreatment of sweet sorghum stalks at Kerr Center a) Manual harvesting, and b) Mechanical pressing using roller press

Figure 3.2. Fermentation vessel (3.8-L) setup: a) Schematic representation, and b) Ambient temperature fermentation

3.2.2 Experiment II

The objective of the second experiment was to examine the influence of process variables on the fermentation performance at a larger scale and determine the ethanol yield under field conditions. The original experimental plan was laid out in a completely randomized design with triplicate treatments for each of the test variables. The experimental plan was to use three 760-L, twenty-one 209-L, nine 19-L, and twenty-four 3.8-L fermentation vessels, requiring a total sweet sorghum juice volume of 7000 L. The objective of the extensive experimental plan were to understand the influence of agitation, influence of vessel scale up, mass transfer properties, the influence of two different varieties of *S.cerevisiae*, two levels of pH, and urea addition on the fermentation performance. It was also planned for the experiment to be conducted in the field. The plan relied heavily on the successful operation of the prototype harvester/presser juice system. Since the design and development of the harvester/presser juice system was still in its infancy, the availability of the juice became the major constraint. Due to the delay in availability of the fully functional harvester/presser juice system, harvesting of the sweet sorghum was delayed until the month of November when the environmental temperatures became unfavorable for in-field fermentation. Henceforth, the experimental plan was revised to accommodate the limited juice volume availability and to conduct the experiment under controlled temperature conditions.

The revised experimental plan tested the process variables fermentation vessel size (scale-up effect) and agitation. Temperature data was recorded at the top and bottom of the fermentation vessel to understand heat transfer during fermentation. Analysis samples were taken from the top and bottom of the fermentation vessel to understand mass

transfer during fermentation. Temperature and analysis samples were taken at the same level of the fermentation tank to provide similar points for comparison.

Experimental Design

A 2 by 2 experiment was designed to test process variables. Two sizes of fermentation vessels were used. Analysis samples were taken from the top and bottom of the fermentation vessels. The statistical design of the experiment is shown in Table 3.2. Detailed experimental plan is shown in Table 3.3.

Table 3.2. In-field Experiment-II Layout

Class	Levels	Description
Vessel Size	2	19-, 209-L
Sampling Location	2	Top, Bottom

Sweet Sorghum Source

Sweet sorghum juice for the experiment was obtained from a 50-acre field on S&S farms, Hinton, Oklahoma (Agricultural producer: Dean Smith). Sweet sorghum was harvested and pressed in the field using a prototype harvester/presser system (Source: Lee McClune, OH). A total of 600 L of juice was pressed from 1 acre of sorghum harvest. Figure 3.3a shows the sorghum plot at Hinton. Figure 3.3b shows the rear view image of the harvester/presser juice system. Figure 3.4a shows the harvest of the sweet sorghum crop. Figure 3.4b shows the incubation of the fermentation vessels under controlled condition.

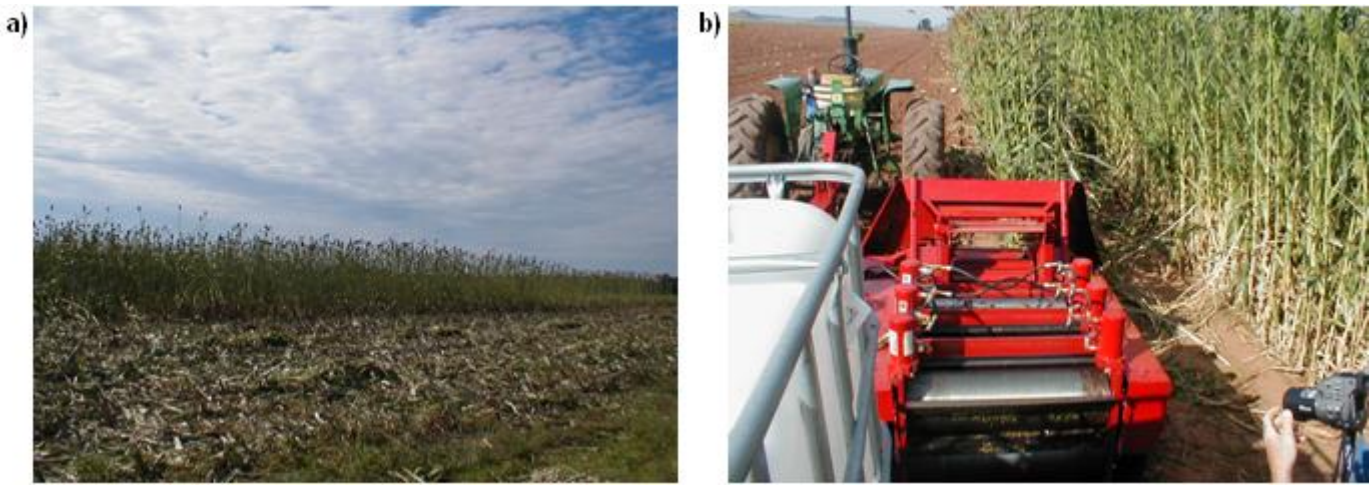


Figure 3.3. In-field experiment II at S&S Farms, a) Sweet sorghum plot, and b) Prototype harvester.

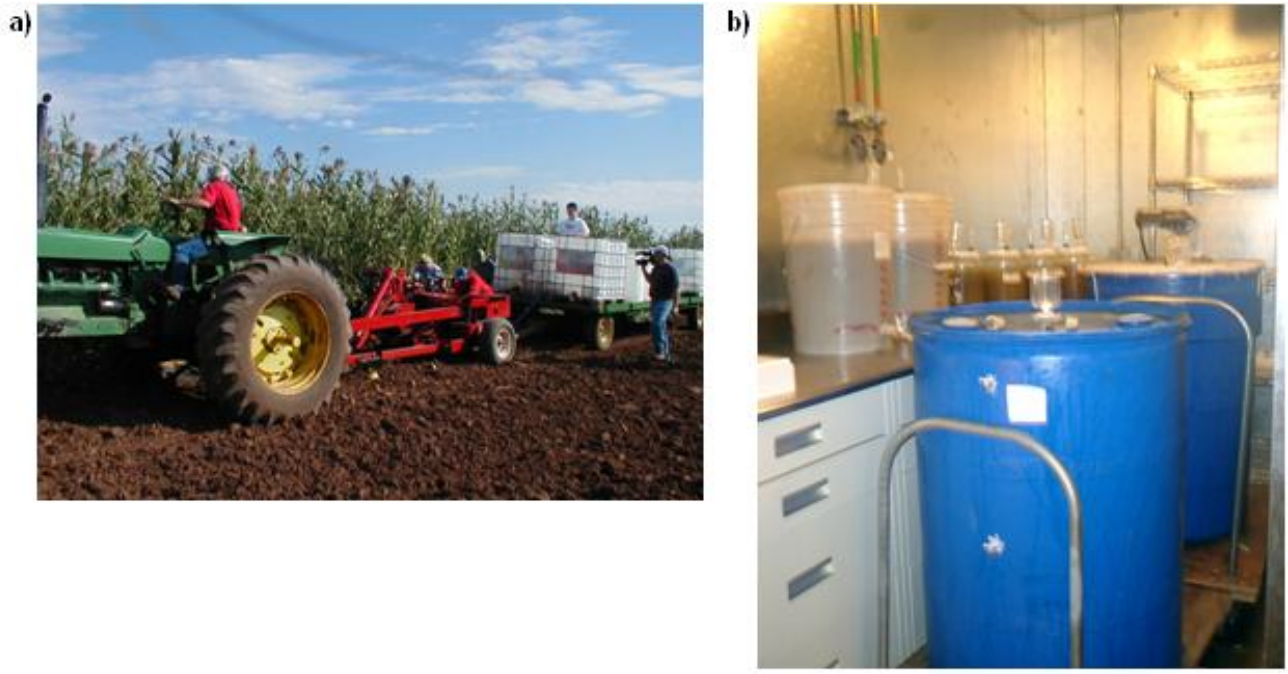


Figure 3.4. In-field Experiment II, a) Mechanical harvest of sweet sorghum plot, and b) Controlled condition fermentation of the fermentation vessel.

Table 3.3. Design of Experiment II

Drum #	Size (L)	Agitation	Sampling Port	Temperature Logger	Yeast	Yeast Quantity (g)	pH	Temperature	Urea Quantity (g)
1	209	Yes	Top & Bottom	Top & Bottom	Fermax	105	5.5	7 & 37◦C	41.8
2	209	None	Top & Bottom	Top & Bottom	Fermax	105	5.5	7 & 37◦C	41.8
3	19	None	Top & Bottom	1-Submerged	Fermax	10	5.5	7 & 37◦C	3.8
4	19	None	Top & Bottom	1-Submerged	Superstart Distillers	10	5.5	7 & 37◦C	3.8

Fermentation Vessel Setup and Design

Experiments were conducted in two 209-L and two 19-L high density polyethylene (HDPE, Airgas Mid-South, OK, www.airgas.com) vessels. The vessels were setup at the Food and Agricultural Products Research and Technology Center (FAPRTC, OSU, OK). Figure 3.5a shows the 19-L fermentation vessel assembly. The assembly consisted of two sampling ports, one temperature logger and an air lock. Samples from the top of the vessel were collected using a disposable syringe fitted with a 14-gauge blunt end needle (Cat # 14-825-16N, www.fishersci.com) inserted in the lid using a rubber stopper. Samples from the bottom were collected through an existing spigot located at 0.05 m from the base of the vessel. Anaerobic conditions in the fermentation vessel were maintained using a cylindrical air lock filled with water (Cellar Homebrew, www.cellarhomebrew.com). The air lock expels the CO_2 produced during fermentation and prevents air from entering the vessel, thereby creating an anaerobic environment. One temperature data logger (Type: HOBO Pendant Temp/Alarm 8K, Onset Computer corporation, www.onset.com.) was placed at the bottom of the vessel to monitor the temperature throughout fermentation. One data logger was also used to monitor ambient temperature.

The 209-L fermentation vessel assembly included two sampling spouts, two temperature loggers and an air lock (Figure 3.5b). One of the 209-L vessels was fitted with an agitator drive assembly. The vessels were 1m in height with an internal diameter of 0.5 meter. The vessel lids were removable and were secured by screw fit and a metal clamp. The vessels were fitted with two sampling ports to collect samples from the top and bottom of the drum. One 0.076 meter air lock was fitted on the top of the lid by using a Number 7

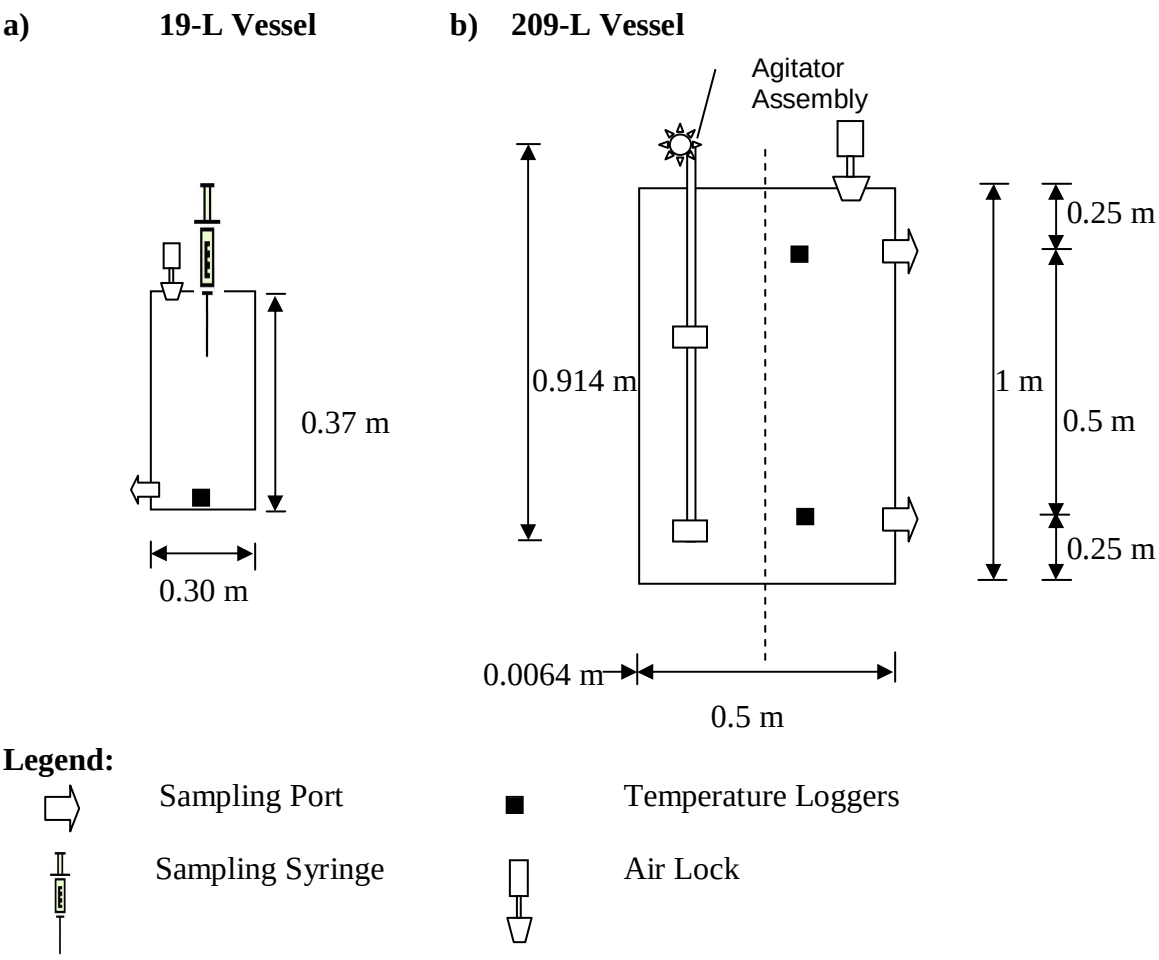


Figure 3.5 Experiment II assembly of fermentation vessels, a) 209-L, and b) 19-L vessel

rubber adapter. Two temperature loggers (Type: HOBO Pendant Temp/Alarm 8K, Onset Computer corporation, www.onset.com) were suspended from the lid by means of a stainless steel link chain to measure the temperature at the top and bottom level of the drum. The sampling ports and the temperature loggers were fixed at a distance of 0.25 m from the top and bottom of the drum.

The agitator assembly consisted of a drive (Magmotor Corporation, P/998, Grainger Corporation 3 hp, 300-3000 rpm, 100 psi,) and impeller (Figure 3.6). The impeller shaft was 0.865 m in length with a diameter of 0.0095 m and made from 316 SS (Figure 3.6a). The agitator drives were mounted on the lid and supported by guide rollers (Figure 3.6b).

The impeller blades were paddle type and made from polytetrafluroethylene (PTFE) with a dimension of 0.102 x 0.127 m (Figure 3.6c). Three holes of 1 cm diameter were punched on the impeller blades for greater mixing properties. The blades were screwed to the impeller shaft.

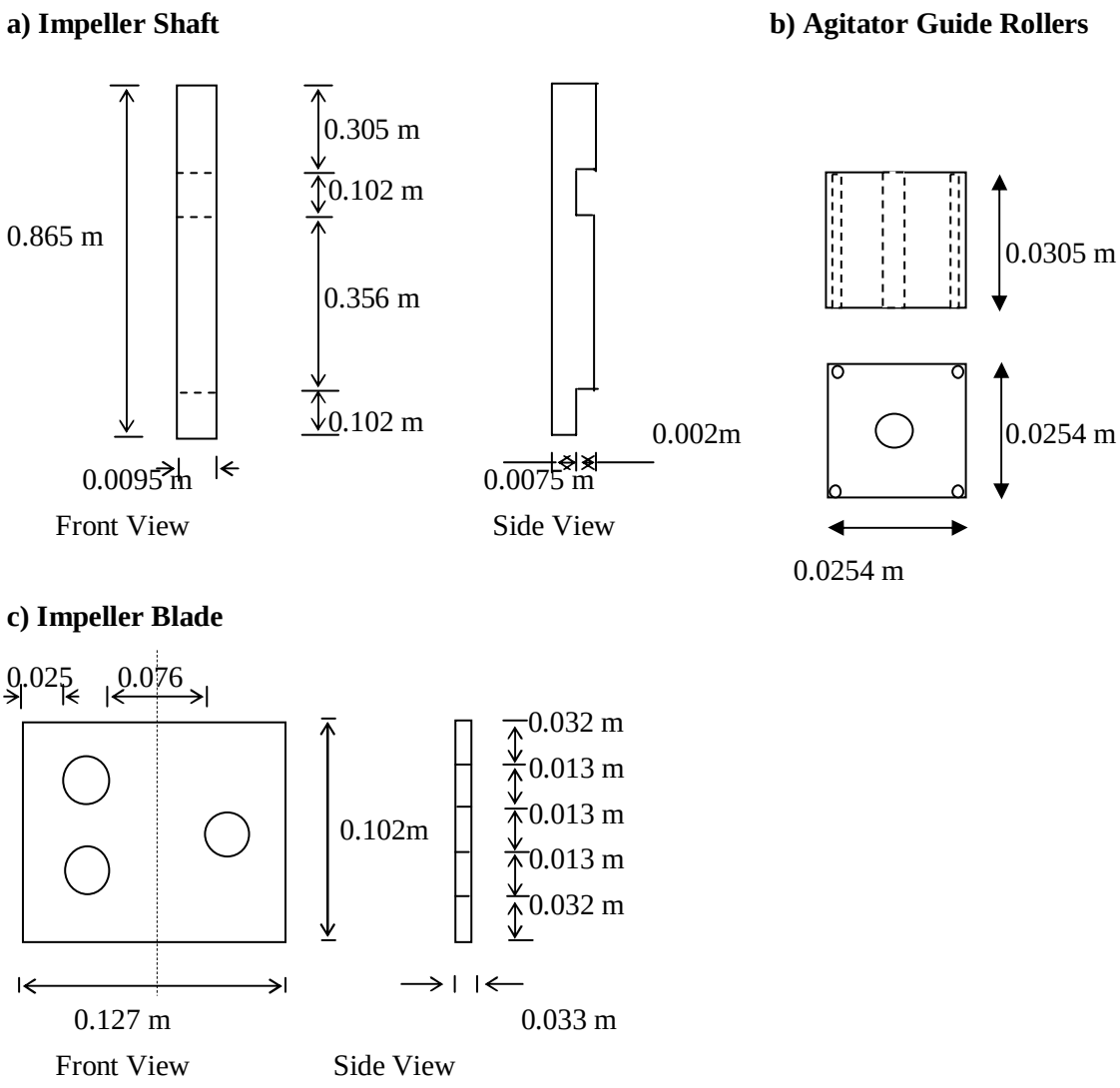


Figure 3.6. Fermentation vessel agitator design a) Impeller shaft; b) Agitator guide rollers; c) Impeller blade

Fermentation

Fermentation experiments were carried out under unsterilized conditions. Dry distillers yeast was weighed @ 0.05% w/v and hydrated using water at 35^{o}C for 1 h. The amount of water used for hydration was approximately 10 times the culture volume. Urea (MN, www.martrexinc.com) was added at 200 ppm. 600 L of sweet sorghum juice was pressed on the field using the harvester/presser system. The pressed juice was filtered using an inline sieve and filled into a collection unit mounted at the back of the harvester. Juice from the collection tank was pumped simultaneously into the fermentation vessels on an attached flatbed trailer. Sweet sorghum juice was filled into each fermentation vessel to give a working volume of 200 L and 18 L in the larger and smaller vessels, respectively. Hydrated yeast and urea were added while filling the sweet sorghum juice to ensure uniform mixing. Fermentation vessels were then allowed to ferment under controlled conditions at the Advanced Technology Research Center (ATRC, OSU, OK). The vessels were alternated between incubation temperatures of 37 and 7^{o}C every 12 h to imitate the environmental temperature conditions. The incubation of the non-agitated vessel was started at 37^{o}C while the agitated vessel started incubating at 7^{o}C due to the space constraint in the incubation room. Samples were taken every 24 h and analyzed. The fermentation was continued until the sugar was completely utilized by the yeasts.

3.3 Microbiology

Saccharomyces cerevisiae

Dry industrial distillers yeast was procured from two suppliers. Fermax dry distillers yeast was procured from Fermax Inc. (MN, www.Fermaxinc.com) and Superstart distillers yeast was procured from Superstart (Catalogue # 9804A, Crosby and Baker,

Atlanta, GA www.crosby-baker.com). Both yeasts were stored under refrigerated conditions (4^{o}C) until used.

Zymomonas mobilis

Freeze dried *Zymomonas mobilis* spp. *mobilis* culture (ATCC 10988) was obtained from American Type Culture Collection (ATCC, VA, USA, www.atcc.org) and stored at 4^{o}C until used.

The freeze dried culture was revived using ATCC *Zymomonas* medium consisting of glucose- 20 g/L; yeast extract- 5 g/L; deionized water- 1000 ml; pH- 4.8$\pm$0.2; and agar- 20g/L (for plating). The media was autoclaved at 121^{o}C for 15 minutes and cooled to room temperature. Under anaerobic conditions, 0.5 ml of broth is transferred from a single test tube containing 5-6 ml of broth into the culture vial. The aliquot is aseptically transferred back into the broth tube. The tubes are incubated under anaerobic conditions at 30^{o}C for 48 h, which corresponds to the ideal condition for achieving exponential growth of the cells. Following incubation the broth tubes are refrigerated at 4^{o}C until used.

The cultures were subcultured every 30 days using a media consisting of glucose- 20 g/L; peptone- 5g/L; yeast extract- 5 g/L; deionized water- 1000 mL and pH- 7.0. The sterile media was inoculated with 10% of previously grown culture and incubated anaerobically at 30^{o}C for 48 h. The flasks were stored at 4^{o}C until used.

Kluyveromyces marxianus

Freeze dried *Kluyveromyces marxianus* culture (ATCC 12708) was obtained from American Type Culture Collection (ATCC, VA, USA, www.atcc.org) and stored at 4°C until used.

The freeze dried culture was revived using ATCC YM medium No. 200 consisting of yeast extract-3 g/L; malt extract-3 g/L; peptone-5 g/L; dextrose- 10 g/L; deionized water-1000 ml; and agar-20 g/L (for plating). The pH of the media was adjusted to pH 4.3±0.2 using 2 N sulfuric acid. The media was autoclaved at 121°C for 15 minutes and cooled to room temperature. Using a sterile pipette, 0.5 ml of sterile distilled water was applied directly to the pellet. The suspension was then aseptically transferred to a test tube containing 5 ml of sterile distilled water. The freeze dried culture was allowed to hydrate for 12 h at 25°C. One ml of this suspension was then inoculated into YM liquid medium and incubated at 28°C for 48 h.

The cultures were subcultured monthly using a sterile media consisting of glucose-20g/L; malt extract-5 g/L; yeast extract- 5 g/L; peptone-5 g/L; and deionized water- 1000 ml at pH-4.3. The sterile media was inoculated with 10% of previously grown culture and incubated anaerobically at 30°C for 48 h. The flasks were stored at 4°C until used.

3.4 Sweet Sorghum Juice Composition and Stability

Two liters of pressed sweet sorghum juice was collected and immediately refrigerated at 4°C for analyzing the nutritional composition and determining the total sugar availability over extended periods of refrigerated storage. The objective of this study was to ascertain the storage stability of the sweet sorghum juice. The nutritional composition of the juice

was analyzed in terms of total sugars, anions, cations, proteins and pH. To assess the stability of nutrients when stored at 4^{o}C, the total sugar concentration of the juice was analyzed every 24 h. The measure of change in sugar concentration was indicative of the change in the availability of the nutrients.

3.4.1 Anion Analysis

Availability of anions such as fluoride, chloride, acetate, nitrate, phosphate, citrate and sulfate in the sweet sorghum juice was analyzed using liquid chromatography Dionex DX-600 (Dionex Corporation, Sunnyvale, CA, www.dionex.com) under the following conditions:

Equipment:	LC20 chromatography module GP50 Gradient Pump ED50 Electrochemical Detector
Column:	IonPac AS11 Analytical IonPac AG11 Guard IonPac ATC-1 Anion Trap
Eluent:	E1: Deionized water E2: 5.0 mM Sodium Hydroxide E3: 100 mM Sodium Hydroxide
Flowrate:	2.0 ml/min
Detection:	Suppressed Conductivity, ASRS AutoSuppression recycle mode
Injection Volume:	10 µL
Background Conductivity:	0.5 mM NaOH: < 1 µS 35 mM NaOH: < 3.5 µS

Operating Backpressure: 11 MPa (1600 psi)

Wave Form: Fluoride, Chloride, Acetate, Nitrate, Phosphate, Citrate, Sulfate

Gradient:

Time	E1	E2	E3
Initial	90	10	-
2.00	90	10	-
6.00	-	100	-
18.00	-	62	38
18.10	90	10	-
25.00	90	10	-

Stock analyte standard solutions were prepared by dissolving the amount of each salt listed in Table 3.4 in deionized water to obtain 1000 mg/L (1000 ppm) solutions.

Table 3.4. Anion standard preparation table

Analyte	Salt	Amount (g)
Fluoride	Sodium fluoride	2.210
Chloride	Sodium chloride	1.648
Acetate	Sodium acetate	1.389
Nitrate	Sodium nitrate	1.371
Phosphate	Potassium dihydrogen phosphate	1.433
Citrate	Citric acid	1.000
Sulfate	Potassium sulfate	1.814

3.4.2 Cation Analysis

Availability of cations such as sodium, ammonium, magnesium, calcium, and potassium in the sweet sorghum juice was analyzed using liquid chromatography Dionex DX-600 (Dionex Corporation, Sunnyvale, CA, www.dionex.com) under the following conditions:

Equipment:	LC20 chromatography module GP50 Gradient Pump ED50 Electrochemical Detector
Column:	IonPac CS12A Analytical (4 mm) IonPac CG12 Guard (4 mm) IonPac CTC-1 Cation Trap
Eluent:	E1: DI Water E2: 100 mM Methanesulfonic acid
Flowrate:	1.0 ml/min
Injection Volume:	25 µL
Detection:	Suppressed Conductivity, CSRS AutoSuppression recycle mode
Operating Backpressure:	1300 psi
Wave Form:	Sodium, Ammonium, Magnesium, Calcium, Potassium

Gradient:

Time	E1	E2
Initial	84	16
5.00	84	16
5.01	60	40
10.00	60	40
10.01	84	16

Stock analyte standard solutions were prepared by dissolving the amount of each salt listed in Table 3.5 in deionized water to obtain 1000 mg/L (1000 ppm) solutions.

Table 3.5. Cation standard preparation table

Analyte	Salt	Amount (g)
Sodium	Sodium chloride	2.542
Ammonium	Ammonium chloride	2.964
Potassium	Potassium chloride	1.906
Calcium	Calcium chloride hydrate	3.668
Magnesium	Magnesium chloride hexahydrate	8.365

3.4.3 Protein Estimation

Protein content in the sorghum juice was estimated based on the bicinchoninic acid (BCA) colorimetric detection and quantification of total protein method (Application number 23227, Pierce, Rockford, IL, www.piercenet.net). The method is based on the biuret reaction principle where Cu^{+2} is reduced to Cu^{+1} followed by selective colorimetric detection of the cuprous cation (Cu^{+1}) by using BCA. The reaction results in a purple colored end product which exhibits a strong absorbance at 562 nm that is nearly linear at a broad protein concentration range (20-2000 µg/ml).

The procedure involved preparing a dilute bovine serum albumin standard over the required concentration range. The working reagent was prepared by mixing reagent A with reagent B in a 50:1 ratio. 0.1 ml of sample was mixed with 2.0 ml of working reagent. The vials were then incubated in a water bath at 37^{o}C for 30 minutes and cooled immediately to room temperature by placing the vials in a ice water bath. An ultraviolet spectrophotometer was zeroed using distilled water blank and the sample absorbance was

measured at 562 nm within 10 minutes. Standard samples were analyzed in triplicate and corrected averages were used to determine the standard curve. Determination of the standard curve for protein estimation is shown in Table 3.6 and Figure 3.7.

Table 3.6. Calculation of BSA Standard Assay

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Conc.	Corr. Avg. Abs (562 nm)
A	0	300 ml of stock	2000 µg/ml	1.8225
B	125 ml	375 ml of stock	1500 µg/ml	1.5022
C	325 ml	325 ml of stock	1000 µg/ml	1.0992
D	175 ml	175 ml of vial B dilution	750 µg/ml	0.8749
E	325 ml	325 ml of vial C dilution	500 µg/ml	0.6088
F	325 ml	325 ml of vial E dilution	250 µg/ml	0.3123
G	325 ml	325 ml of vial F dilution	125 µg/ml	0.1542
H	400 ml	100 ml of vial G dilution	25 µg/ml	0.0251
I	400 ml	0	2000 µg/ml	0.0000

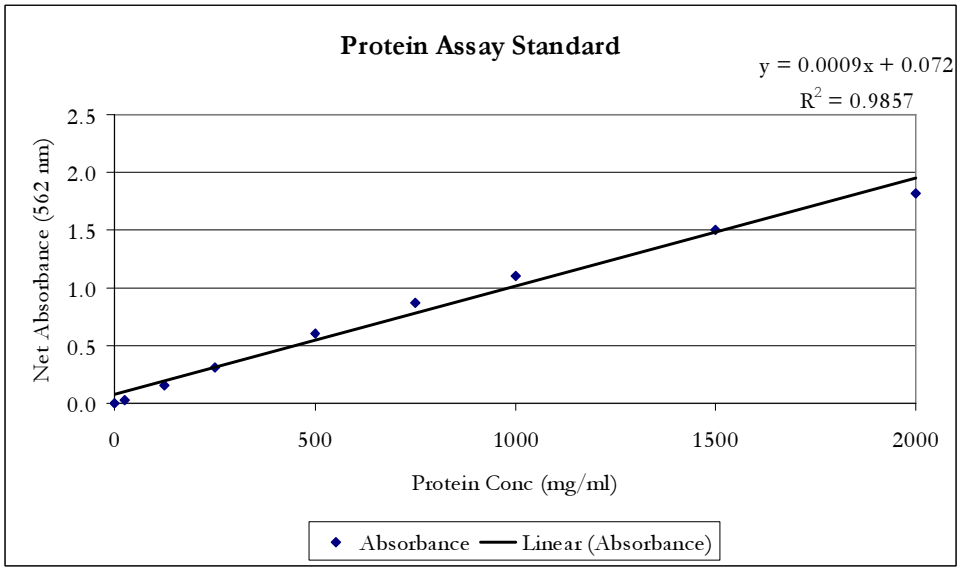


Figure 3.7. Standard curve for protein estimation using the BCA assay.

3.4.4 pH

pH of the sweet sorghum juice was measured using the digital pH meter (Model 310, Orion Research Inc., MA). The reported pH value was the average of the three pH readings.

3.5 Analysis of Fermentation Samples

Fermentation media samples were taken every 24 h using the inserted 10 ml disposable syringe (Cat # 14-823-2A, Fisher Scientific., www.fishersci.com). A sample volume of 3 ml was taken at each sampling time after thoroughly mixing the fermentation media. Samples for cell biomass estimation were taken and tested immediately.

3.5.1 Cell Biomass Determination

Cell growth was determined by measuring the optical density (OD) of the fermentation sample (Bulawayo et al. 1996). Cell concentration was determined by measuring the OD at 570 nm on a UV Vis spectrophotometer (Cary 50 Bio UV visible spectrophotometer, varianinc.com). The measured OD values were read using a computer interface using the Cary WinUV Simple Reads software supplied by the vendor. The instrument was zeroed with the media blank using the same dilution factor as that of the analysis sample. Media for the blank preparation was stored at 4^{o}C until used. Analysis samples were diluted using Ultrapure Milli-Q water to a final sample volume of 1.5 ml. The sample volume required for estimating the cell biomass differed as the fermentation progressed in order to ensure that the measured OD fell within an OD range of 0.2 and 1.0 which corresponds to the linear limits on the standard curve. A dilution factor of 6 was used for the initial sample prepared by diluting 0.25 ml of the sample in 1.25 ml of deionized water (DI). In a similar fashion, the dilution factor for the other sample intervals was prepared. In

general, a dilution factor of 15 was used for 24 and 48 h sample and for the rest of the sampling intervals a dilution factor of 30 was used. Standard curve for determining the cell biomass was obtained by correlating the absorbance of the cell suspension versus with the dry weight of the yeast (Cramer et al. 2002). Table 3.7 shows the dilutions made for obtaining the standard curve. The standard curve is shown in Figure 3.8. Standard curves were prepared for each of the fermenting microorganisms.

Table 3.7. Table for the biomass standard determination

Sample #	Dilution	Water	SS	Initial Weight (g)	Final Weight (g)	Biomass Weight (g/L)	Λ (570 nm)
1	25%	37.5	12.5	1.0686	1.1136	0.90	1.6603
2	20%	40.0	10	1.065	1.1073	0.85	1.5102
3	15%	42.5	7.5	1.0667	1.0916	0.50	1.3193
4	10%	45.0	5	1.0639	1.0843	0.41	1.0333
5	5%	47.5	2.5	1.0667	1.0771	0.21	0.5963
6	0%	50.0	0	1.0664	0	0.00	0

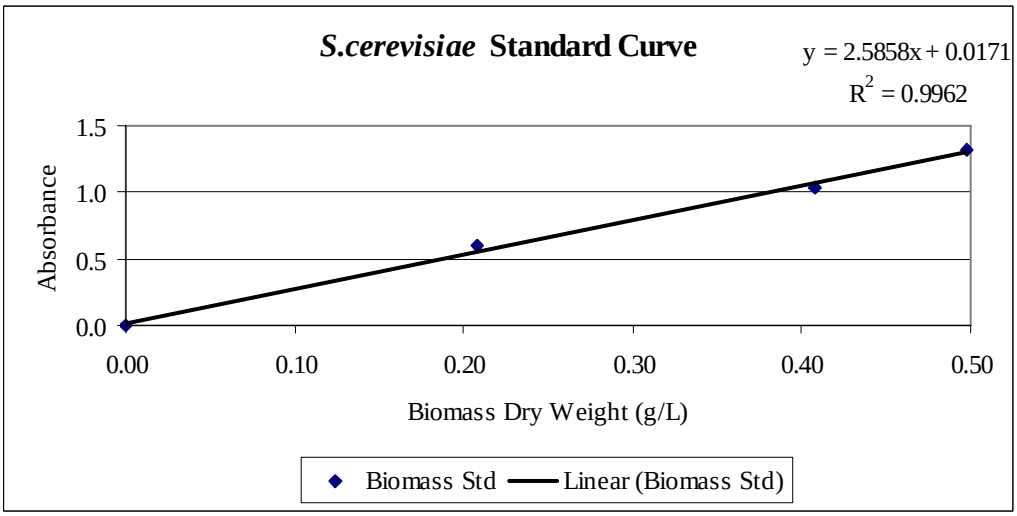


Figure 3.8. Biomass Standard Curve

3.5.2 Sample Preparation for Volatile and Total Sugar Estimation

A fermentation media sample volume of 1.5 ml was pipetted into a 1.5 ml micro-centrifuge tube (Cat # 05-408-129, www.fishersci.com). The tubes were centrifuged at 14000 rpm for 15 min in a microcentrifuge (Model Force 14, Labnet International Inc.). Immediately after centrifuging, 125 µL of the supernatant pipetted into ion chromatography vials (Cat # 038008, www.dionex.com) and diluted 40 times using deionized water (18 MΩ-cm). The vials were capped (Cat # 038009, www.dionex.com) and the samples tested on a Dionex ion chromatography system for sugar estimation. For the volatile estimation, 1.0 ml of the supernatant was transferred into gas chromatography vials (Cat # 21141, www.restek.com). The vials were capped (Cat # 24486, www.restek.com) immediately to prevent the loss of any volatiles. Samples were tested on Agilent gas chromatography system for volatile estimation.

3.5.3 Volatiles Estimation

Volatile (Methanol, Ethanol, Acetic Acid, Propionic Acid, Butyric Acid and Butanol) were analyzed using a 6890 Gas Chromatograph (Agilent Technologies, Wilmington, DE). The operating conditions are listed below.

Oven:	Initial Temperature:	200°C
	Initial Time:	13 minute
	Maximum Temperature:	225°C
	Equilibration Time:	0.50 minute
Front Inlet:	Initial Temperature:	200°C
	Flow:	25.8 ml/min
	Carrier Gas:	Nitrogen

Column:	Packed Column	
	Model:	Porapak QS 80/100, 8 feet
	Maximum temperature:	250^{o}C
	Mode:	Constant flow
	Nominal Initial Flow:	25.0 mL/min
Front Detector:	Flame Ionization Detector (FID)	
	Temperature:	250^{o}C (On)
	Hydrogen Flow:	30.0 mL/min (On)
	Air Flow:	400.0 mL/min (On)
	Mode:	Constant makeup flow
	Makeup Flow:	25.0 mL/min (Off)
	Makeup Gas Type:	Nitrogen
Signal 1:	Data Rate:	20 Hz
Injection Volume:		1.0 µL

3.5.4 Sugar Analysis

Soluble sugars were analyzed by Ion Chromatography (IC) using a Dionex DX-600 equipped with a CarboPac MA1 anion-exchange column and a pulsed amperometric detector (Dionex, Sunnyvale, CA). The mobile phase was 700 mM NaOH with a flow rate of 0.40 ml/min at room temperature. Glucose, fructose, sucrose and cellobiose standards were prepared by dissolving 12.5 g in 1000 ml deionized water to give 12500 mg/L standard solution. Subsequent dilutions for each sugar were prepared by dissolving the standard solution in deionized water to give a final volume of 5 ml (Table 3.8). Standard curves for the individual sugars and the equation are shown in Figure 3.9.

Table 3.8. Sugar standard table

Sugar Conc. (mg/L)	Vol. of Stock Sol (ml)	Vol. of DI H_2O (ml)	Glucose Area	Fructose Area	Sucrose Area	Cellobiose Area
0	0	5.00	0.0000	0.0000	0.0000	0.0000
25	10	4.99	0.0101	0.0034	0.0080	0.0098
125	50	4.95	0.0507	0.0206	0.0258	0.0340
625	250	4.75	0.2510	0.1140	0.1473	0.1547
1250	500	4.50	0.4948	0.2592	0.2976	0.3264
2500	1000	4.00	0.9558	0.5180	0.5765	0.6493
5000	2000	3.00	1.9243	0.9913	1.0964	1.3473

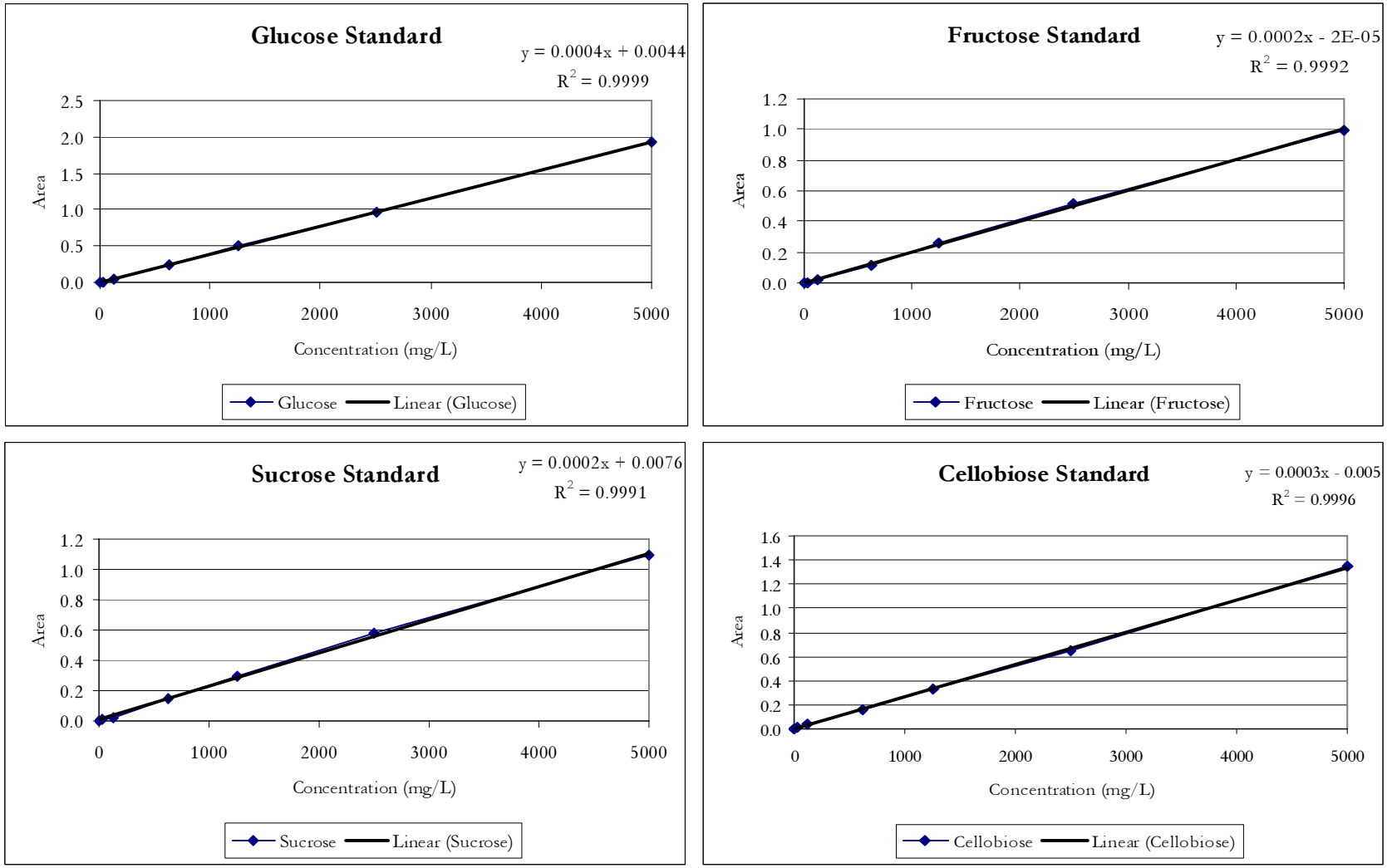


Figure 3.9. Sugar standard curves

3.5.5 Sugar Conversion Efficiency

The ability of yeasts to produce ethanol from the available sugar can be expressed as the sugar conversion efficiency given by the following equation (de Manchilla and Pearson, 1984):

$$SCE = \frac{\text{alcohol content (\% w/v) x 100}}{\text{media sugar content (\%) x 0.504}}$$

3.6 Statistical Analysis

Design of experiments using factorial design helps in estimating both the main effects and interactions in contrast to the classical design in which each response is investigated for each factor in turn while keeping the other factors constant (Miller, 1988; Montgomery, 1997).

Dependent variables used to interpret the data were: cell biomass content, ethanol concentration, and total residual sugar. Statistical differences were tested using analysis of variance and multiple comparisons of means. Analysis of variance of data was conducted using the General Linear Model procedure of SAS (SAS Institute Inc., 1989). Multiple comparison of means were tested by LSD (Least Significant Differences) at $\alpha = 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter contains a description of results obtained from the laboratory and in-field experiments. Parameters tested were: type of fermenting microorganism, effect of temperature conditions, effect of pH conditions, and effect of agitation.

4.1 Laboratory Experiments

A series of laboratory experiments was conducted to screen the optimal conditions for sweet sorghum fermentation. The tests included screening different microorganisms for highest sugar to ethanol conversion efficiency; comparison of fermentation efficiency at different pH and temperature levels; and determination of effects of agitation during fermentation. All the fermentations were conducted in prepared media containing sucrose, glucose, fructose, proteins and minerals at levels similar in composition to the sweet sorghum juice composition.

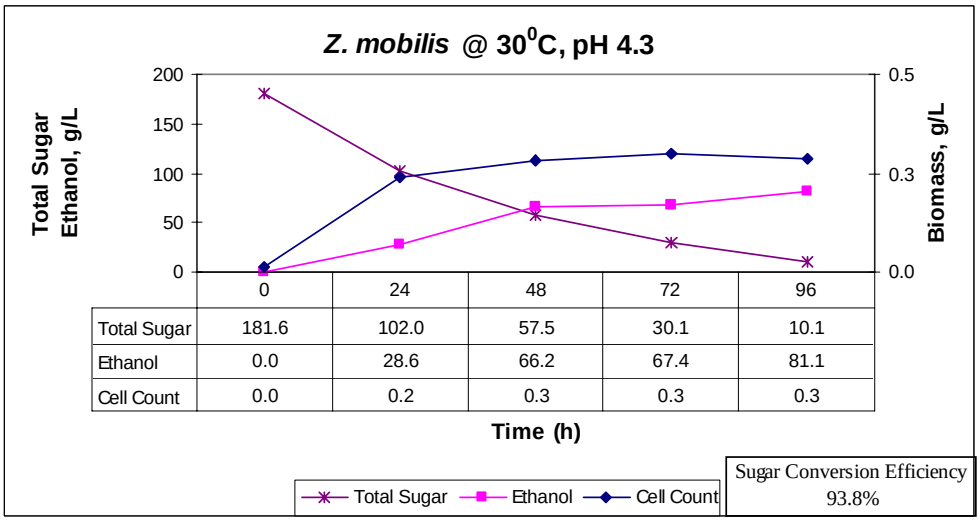
In the first set of experiments, the fermenting ability of different microorganisms, both individually and in combination, at 30^{o}C and pH 4.3 was compared (Figure 4.1 and 4.2). Microorganisms tested were *Z.mobilis*; *K.marxianus*; Superstart Distillers yeast; Fermax yeast; *Z.mobilis* and Fermax Yeast; and *Z.mobilis*, Fermax yeast and *K.marxianus*.

Comparing the curves in Figure 4.1, it can be seen that *Z.mobilis* resulted in highest sugar conversion efficiency (SCE) of 93.8% with an ethanol production of 81.1 g/L. Superstart

Distillers yeast produced 57.0 g/L of ethanol with a SCE of 73.0%. The results are similar to earlier findings of high sugar utilization and ethanol yield with a low biomass production by *Z. mobilis*. Comparing the curves in Figure 4.2 it was observed that Fermax yeast resulted in the highest SCE of 94.1% with an ethanol production of 80.2 g/L. Comparing both Figures 4.1 and 4.2, Fermax yeast resulted in the highest SCE compared to all the other fermenting microorganisms. It is also interesting to note that the available sugars were utilized by Fermax in 72 h compared to 96 h required by *Z.mobilis*, *K.marxianus* and Superstart Distillers yeast which indicates a higher rate of ethanol production under similar fermentation condition.

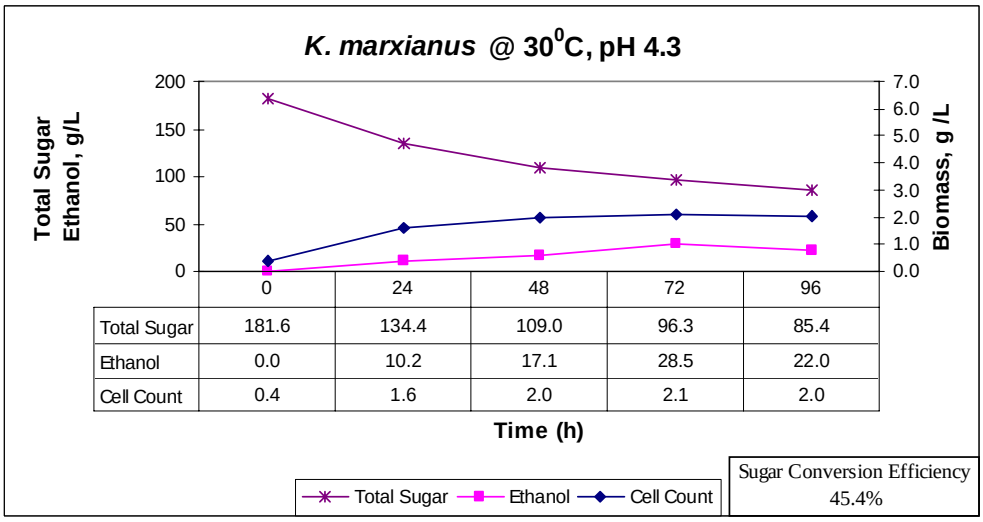
The mixed culture of *Z.mobilis* and Fermax yeast resulted in 79.2 g/L of ethanol with a SCE of 86.5%. The mixed culture of Fermax yeast, *Z.mobilis* and *K.marxianus* resulted in a lower ethanol yield of 63.8 g/L of ethanol with a SCE of 69.9%. The results indicate that the conversion efficiency was lower when the microorganisms were used in combination when compared to individual fermentation performance. However, the sugars were completely utilized by the mixed culture in 72 h compared to the 96 h required by the individual culture of *K.marxianus, Z.mobilis* and Superstart Distillers yeast. The result suggests that some of the sugars are utilized for the maintenance of mixed culture rather than for ethanol production, hence a higher rate of ethanol production but with lower SCE.

a)



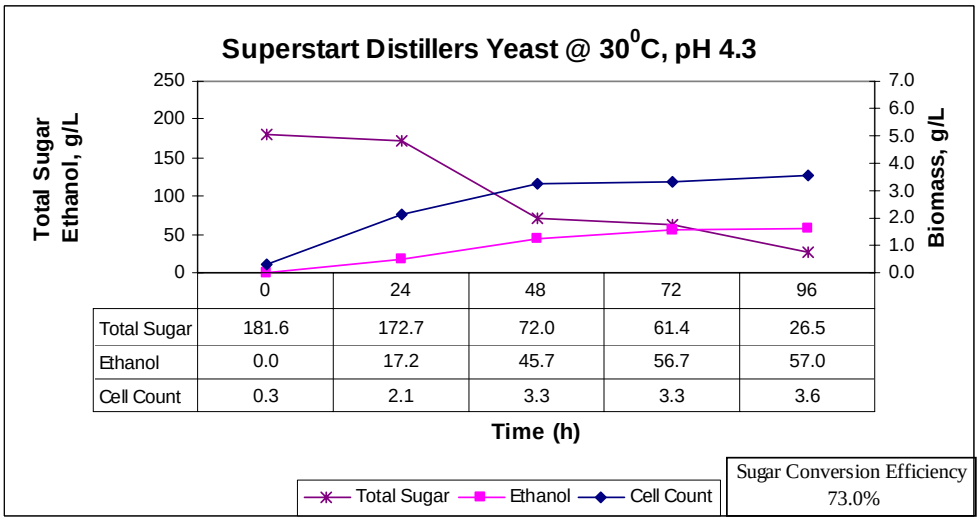
	0	24	48	72	96
Total Sugar	181.6	102.0	57.5	30.1	10.1
Ethanol	0.0	28.6	66.2	67.4	81.1
Cell Count	0.0	0.2	0.3	0.3	0.3

b)



	0	24	48	72	96
Total Sugar	181.6	134.4	109.0	96.3	85.4
Ethanol	0.0	10.2	17.1	28.5	22.0
Cell Count	0.4	1.6	2.0	2.1	2.0

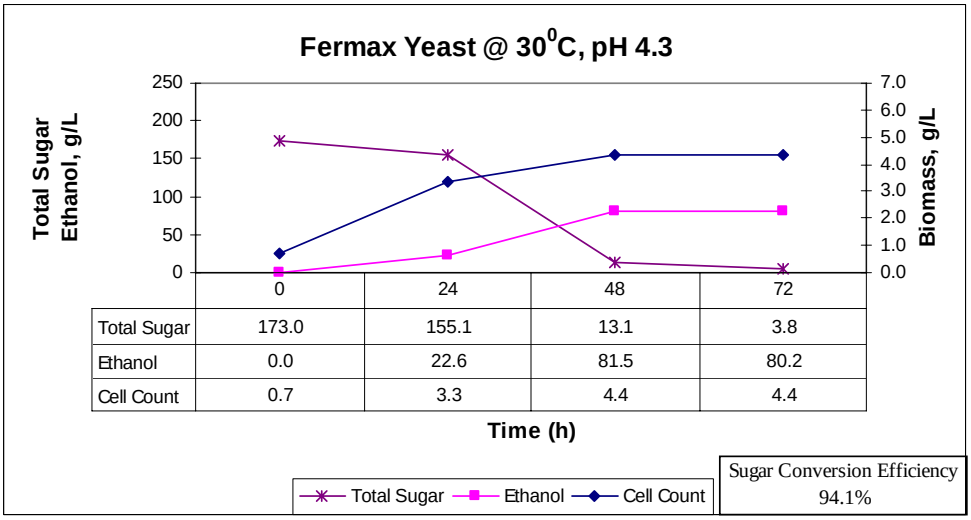
c)



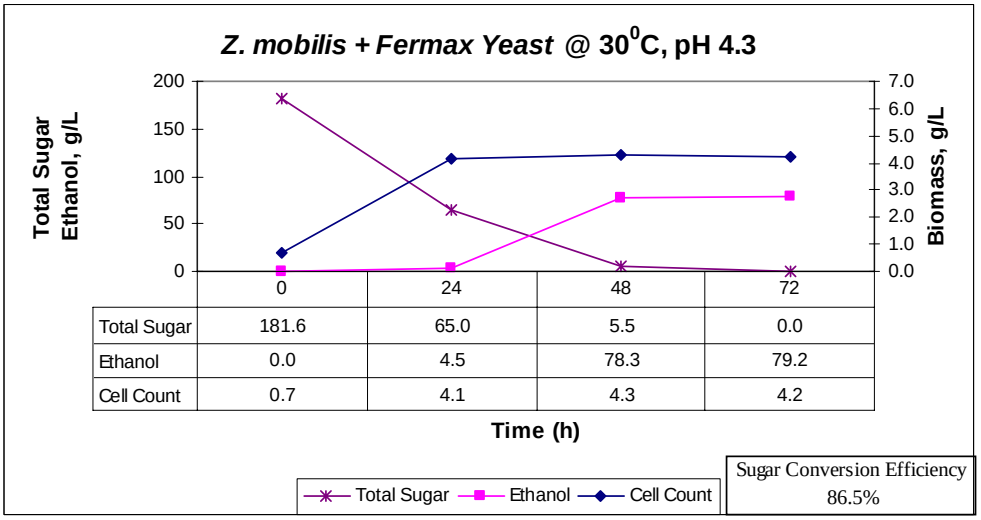
	0	24	48	72	96
Total Sugar	181.6	172.7	72.0	61.4	26.5
Ethanol	0.0	17.2	45.7	56.7	57.0
Cell Count	0.3	2.1	3.3	3.3	3.6

Figure 4.1. Comparison of fermentation performance of a*) Z. mobilis*, b) *K. marxianus* and c) Superstart Distillers yeast at 30^{o}C and at pH 4.3.

a)



b)



c)

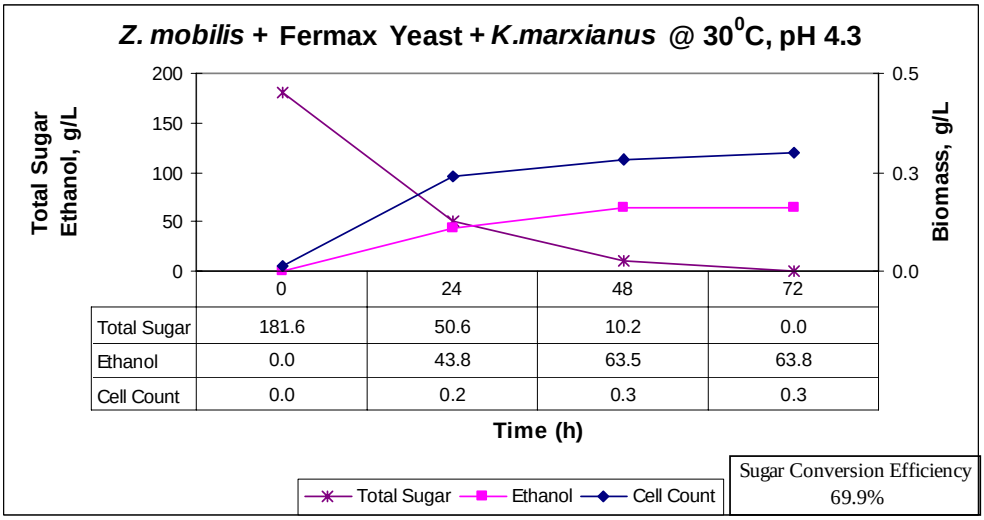
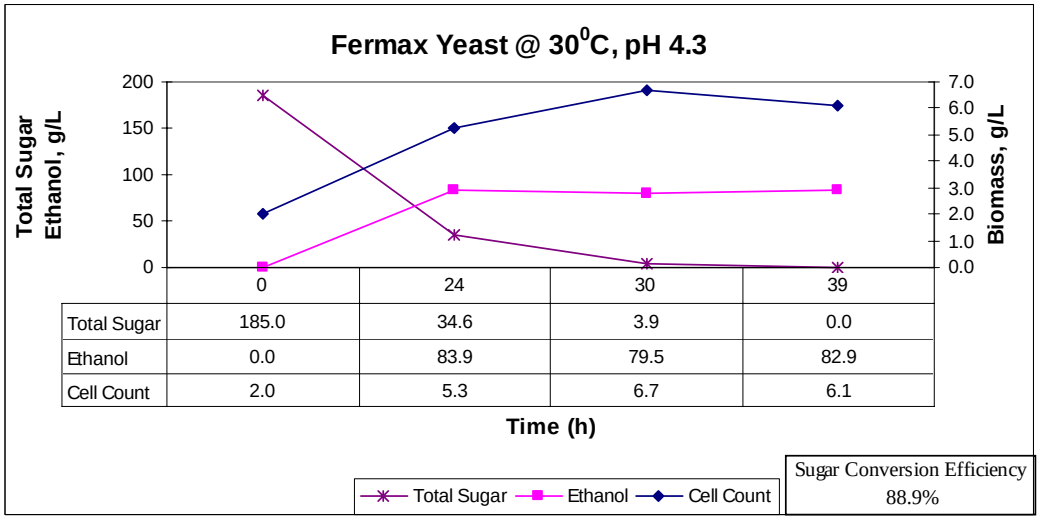


Figure 4.2. Comparison of fermentation performance of a) Fermax yeast, b) *Z. mobilis* + Fermax yeast and c) *Z.mobilis* + Fermax yeast + *K. marxianus* at 30°C and at pH 4.3.

The goal of the second laboratory scale experiment was to determine the fermentation performance of Fermax yeast at pH 4.3 and at different temperature conditions of 15, 30, and 35^{o}C, and alternating between 41 and 15^{o}C. Fermentation results are shown in Figures 4.3 and 4.4. Fermax yeast performed best at 35^{o}C, producing 84.8 g/L of ethanol from a starting total sugar concentration of 185 g/L, giving a SCE of 95.7%. The result indicates that Fermax yeast was able to produce higher ethanol levels at elevated temperatures. At 30^{o}C (the published optimal temperature for growth of Fermax yeast), it produced 82.9 g/L of ethanol from a starting total sugar of 185 g/L, giving a SCE of 88.9%. However, the rate of ethanol production was higher at 30^{o}C with the available sugars being utilized in 39 h compared to the rate of ethanol production at 35^{o}C. This ensures a more efficient use of available facilities for potentially greater process efficiency through quicker turnaround of the fermentation equipment.

Fermax yeast also produced 50 g/L of ethanol when the fermentation was carried out at 15^{o}C indicating the large temperature range over which it can perform. When the fermentation vessels were incubated at temperatures alternating between 41 and 15^{o}C, Fermax yeast produced 67.8 g/L of ethanol. This indicates that the yeast is able to ferment at extreme incubating temperatures and perform equally well at fluctuating temperature conditions compared to the fermentation at optimum temperature. This fermentation ability of the Fermax yeast makes it a good candidate for ethanol production under field conditions.

a)



b)

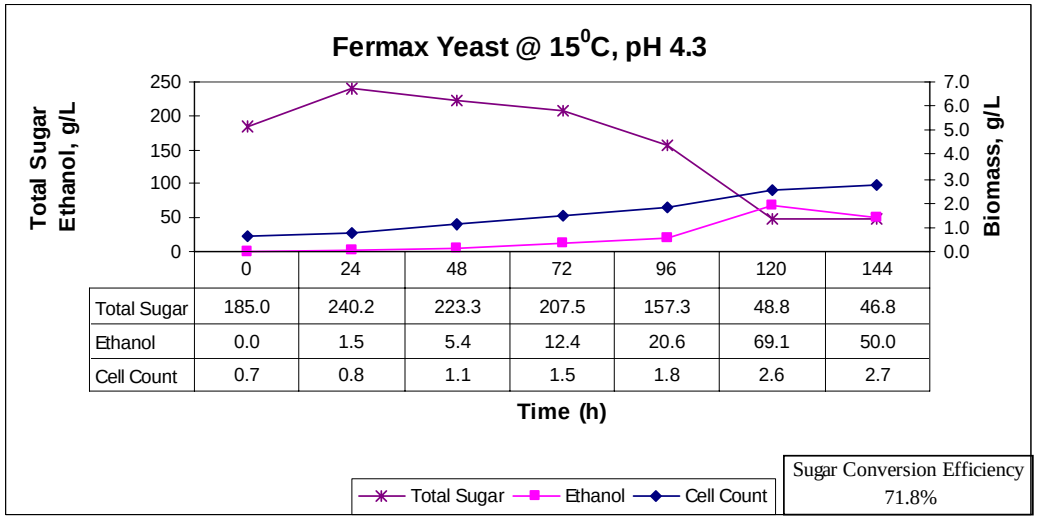
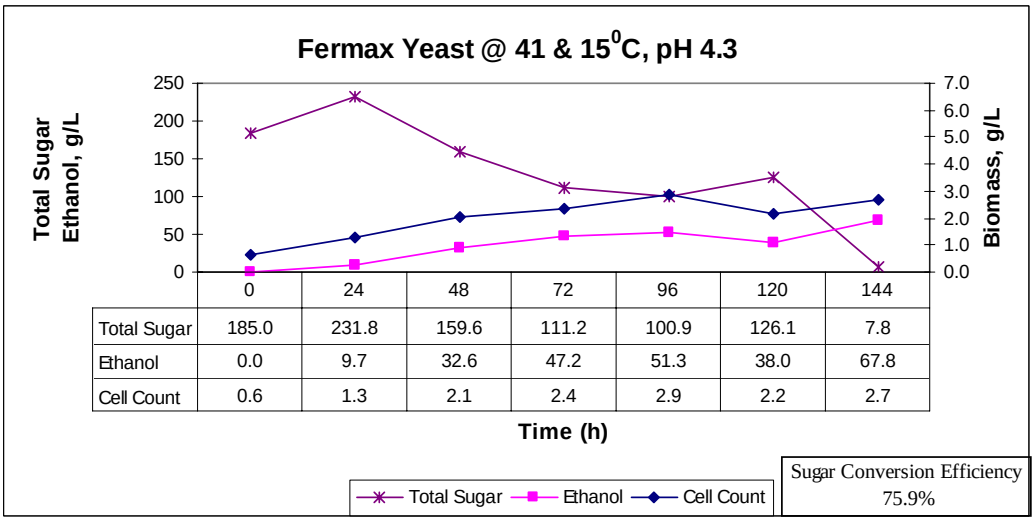


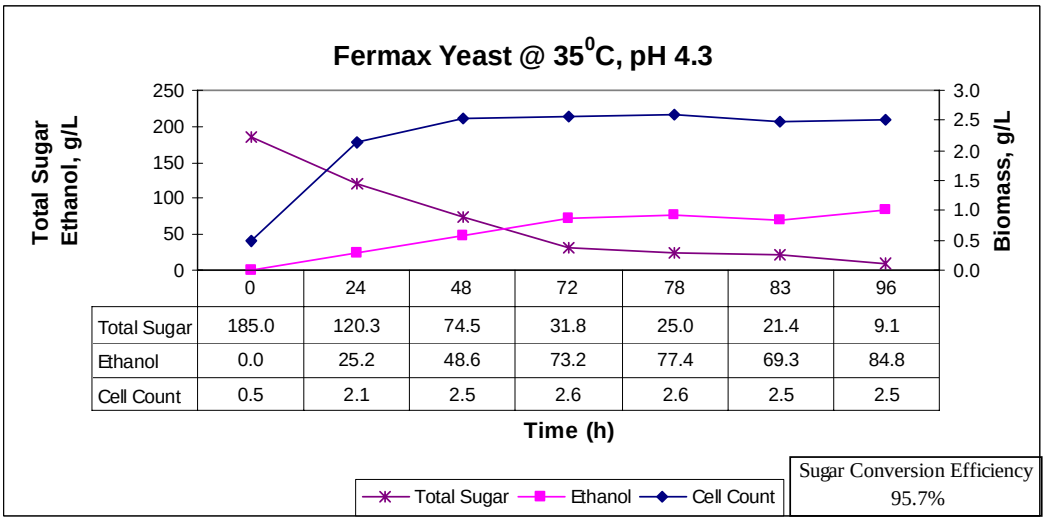
Figure 4.3 Comparison of fermentation performance of Fermax yeast at pH 4.3 and incubating at a) 30°C and b) 15°C.

a)



	0	24	48	72	96	120	144
Total Sugar	185.0	231.8	159.6	111.2	100.9	126.1	7.8
Ethanol	0.0	9.7	32.6	47.2	51.3	38.0	67.8
Cell Count	0.6	1.3	2.1	2.4	2.9	2.2	2.7

b)

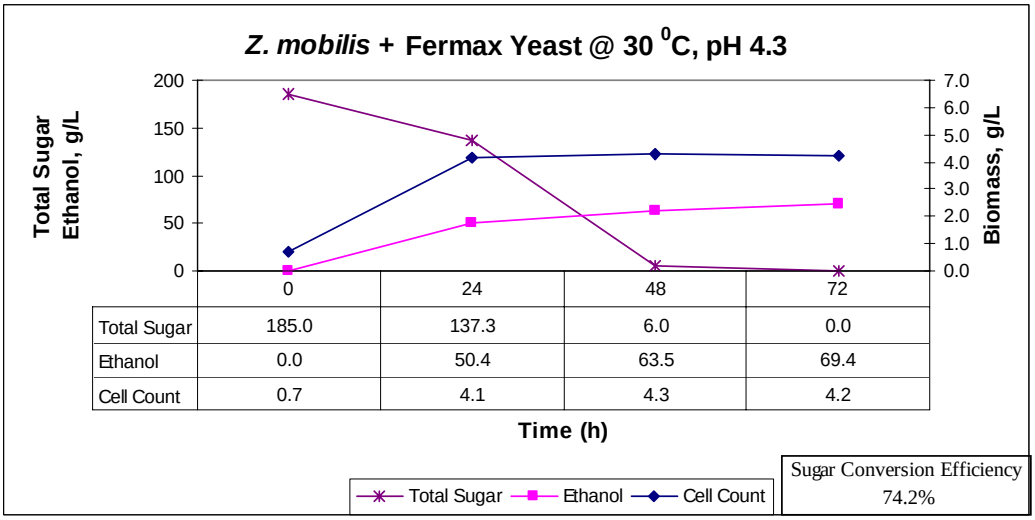


	0	24	48	72	78	83	96
Total Sugar	185.0	120.3	74.5	31.8	25.0	21.4	9.1
Ethanol	0.0	25.2	48.6	73.2	77.4	69.3	84.8
Cell Count	0.5	2.1	2.5	2.6	2.6	2.5	2.5

Figure 4.4. Comparison of fermentation performance of Fermax yeast at pH 4.3 and incubating at a) alternating between 41 & 15^{o}C and b) 35^{o}C.

The third laboratory-scale experiment was performed to understand the effect of using different fermenting microorganisms in combination and the effect of incubating at alternating temperatures of 41 and 15^{o}C on the ethanol yield. The pH of the fermentation media was maintained at pH 4.3. Results shown in Figures 4.5 and 4.6 compare ethanol production at alternating temperature to optimum incubation temperature of 30^{o}C. From Figure 4.5, maximum ethanol (69.4 g/L) was produced by the mixed culture of *Z.mobilis* and Fermax yeast at 30^{o}C. When the incubating temperature was alternated between 15 and 41^{o}C, only 36.1 g/L of ethanol was produced, giving a SCE of 67.2%. The mixed culture of *Z.mobilis*, Fermax yeast and *K.marxianus* produced 63.8 g/L of ethanol at 30^{o}C. However when the fermentation vessels were alternated between 15 and 41^{o}C, only 40.8 g/L of ethanol was produced giving a SCE of 59.4%. It can be observed that the ethanol yield was much higher when the fermentation was carried out with a single microorganism than with mixed culture. Even under unfavorable fermentation temperatures, the SCE of individual microorganisms was higher than for the combination.

a)



b)

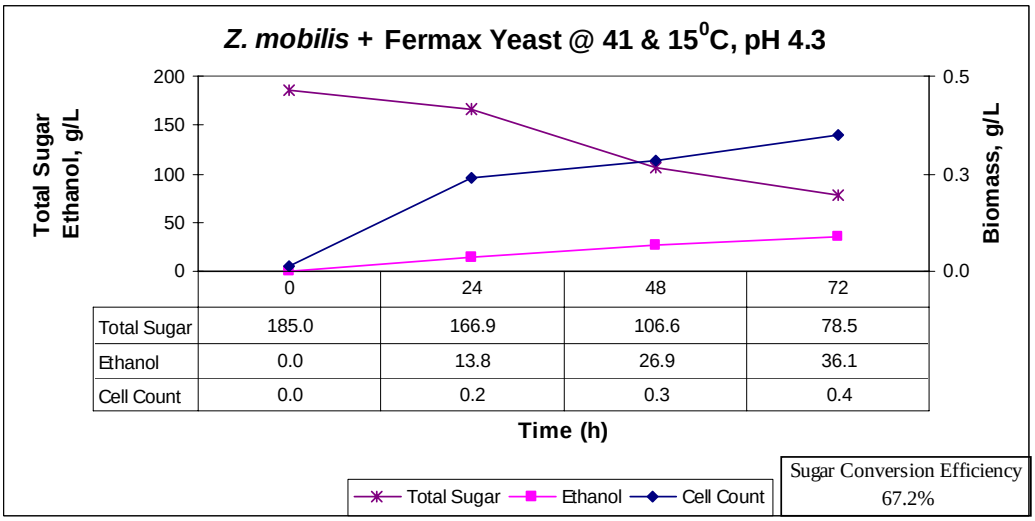
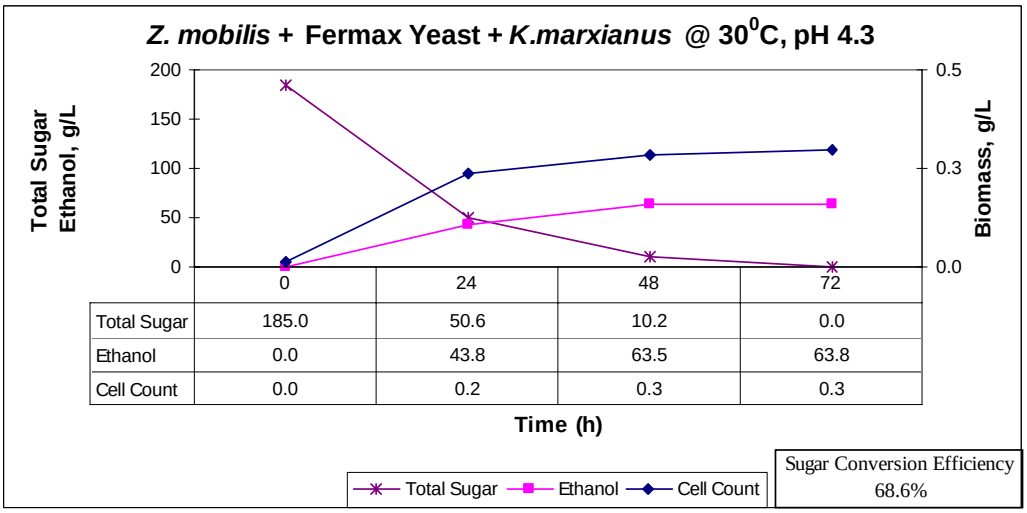


Figure 4.5. Comparison of fermentation performance of combination of *Z.mobilis* + Fermax yeast at pH 4.3 and incubating at a) 30°C and b) alternating between 41 & 15°C.

a)



b)

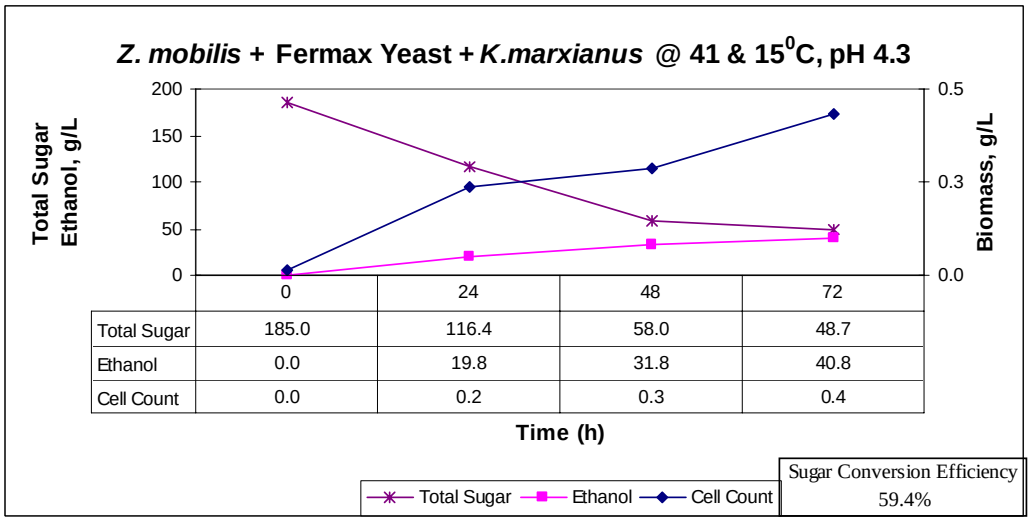
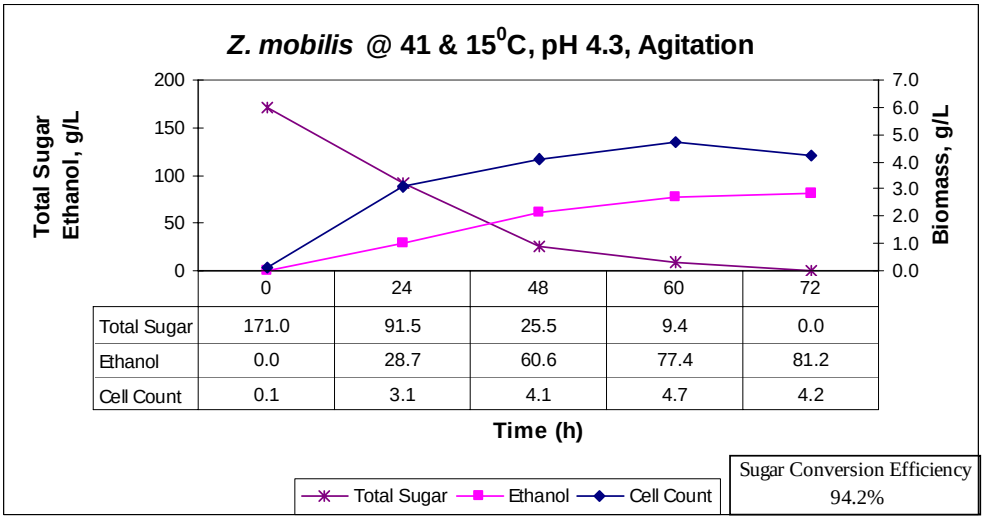


Figure 4.6. Comparison of fermentation performance of combination of *Z.mobilis* + Fermax yeast + *K.marxianus* at pH 4.3 and incubating at a) 30°C and b) alternating between 41 & 15°C.

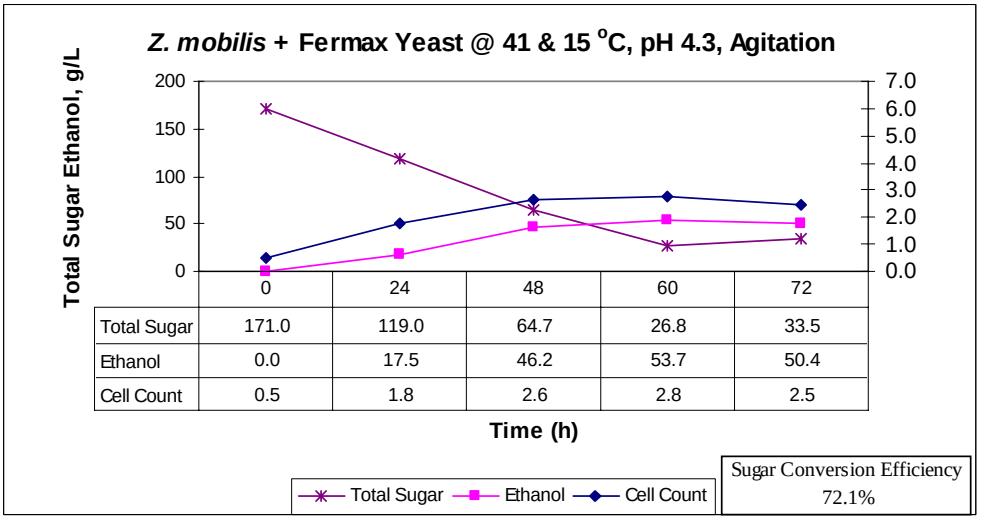
Figure 4.7 shows results from a laboratory experiment to understand the impact of agitation on ethanol production. Agitation was achieved using an orbital shaker and the flasks were agitated at 75 rpm. Results (Figure 4.7a) indicated that under agitated conditions, *Z.mobilis* produced 81.2 g/L of ethanol with a SCE of 94.2% even when the fermentation temperature was alternated between unfavorable temperatures of 41 and 15^{o}C. This ethanol yield was higher than the non-agitated fermentation at an optimal temperature of 30^{o}C which had a SCE 93.5% (Figure 4.1a). The results indicate that the agitation appears to have a marked positive influence on the ethanol production by *Z.mobilis*. Figures 4.7b and 4.7c compare the ethanol fermentation by a mixed culture of *Z.mobilis* and Fermax yeast under agitated and non agitated conditions. The SCE is observed to be 80.6% with an ethanol yield of 69.5 g/L under non agitated conditions compared to SCE of 72.1% with an ethanol yield of 50.4 g/L under agitated conditions. This indicated that while agitation was favorable for the growth and ethanol production of *Z.mobilis*, it had a negative effect on Fermax yeast.

a)



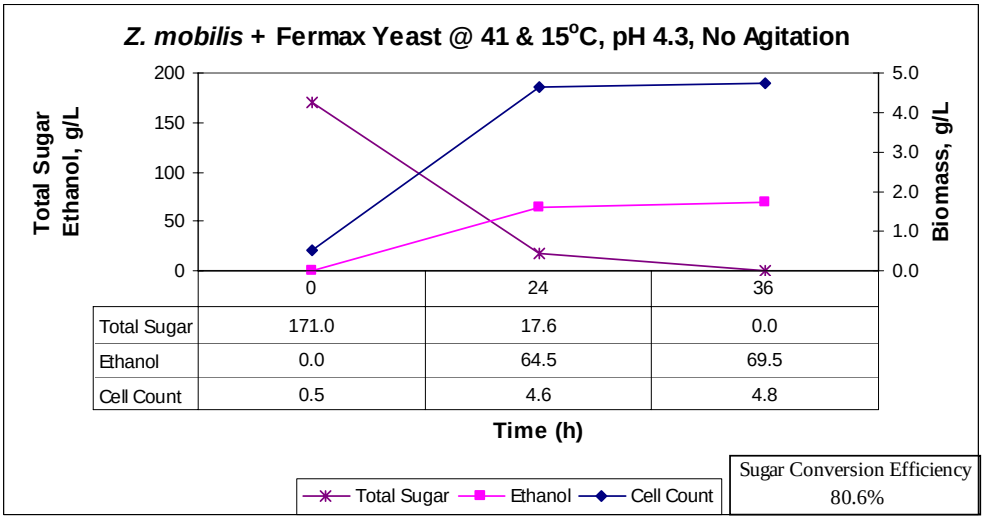
	0	24	48	60	72
Total Sugar	171.0	91.5	25.5	9.4	0.0
Ethanol	0.0	28.7	60.6	77.4	81.2
Cell Count	0.1	3.1	4.1	4.7	4.2

b)



	0	24	48	60	72
Total Sugar	171.0	119.0	64.7	26.8	33.5
Ethanol	0.0	17.5	46.2	53.7	50.4
Cell Count	0.5	1.8	2.6	2.8	2.5

c)

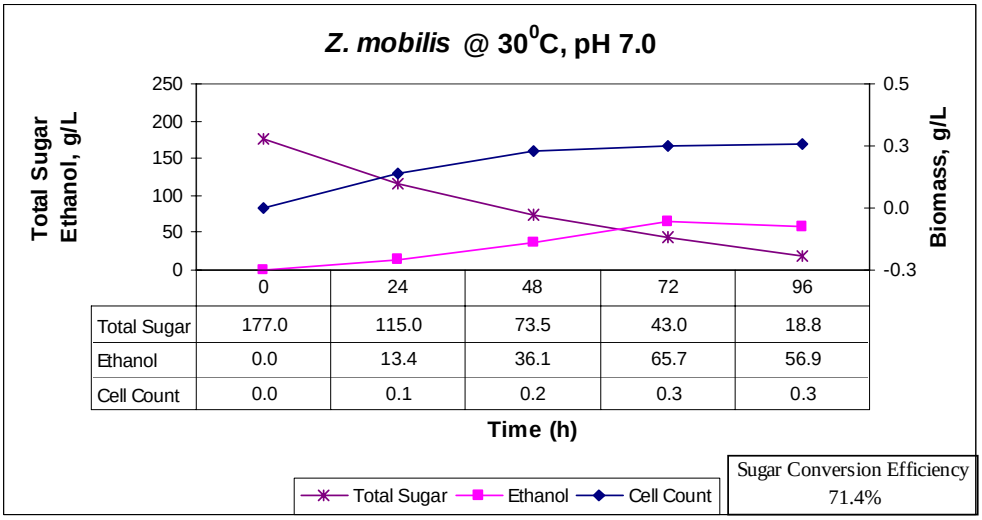


	0	24	36
Total Sugar	171.0	17.6	0.0
Ethanol	0.0	64.5	69.5
Cell Count	0.5	4.6	4.8

Figure 4.7. Impact of agitation on the microbial fermentation performance of a) *Z. mobilis*, b) *Z.mobilis* + Fermax yeast, and c) *Z.mobilis* + Fermax yeast at pH 4.3 and at an incubating temperature of 15 and 41°C.

The next laboratory experiment was conducted to understand the impact of pH on the fermentation performance of Fermax yeast and *Z.mobilis.* Figure 4.8 compares the fermentation of *Z.mobilis* at pH 7.0 and pH 4.3. *Z.mobilis* is observed to produce higher amounts of ethanol (56.9 g/L) at pH 7.0 with a SCE of 71.4% compared to 65.2% SCE at pH 4.3 with an ethanol yield of 54.4 g/L. The optimal pH for growth and maintenance of *Z.mobilis* is pH 7.0 and lowering the pH to 4.3 appears to have an inhibitory effect on its fermentation performance.

a)



b)

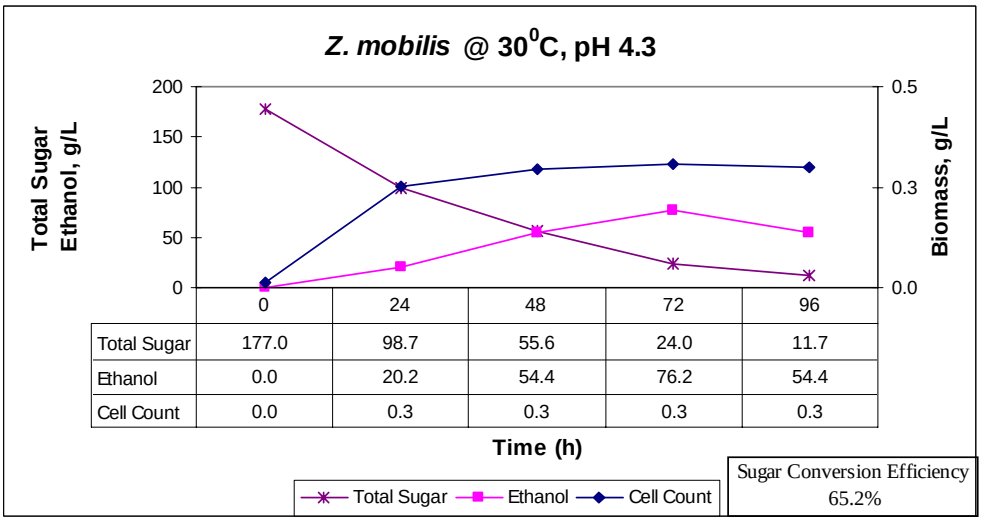
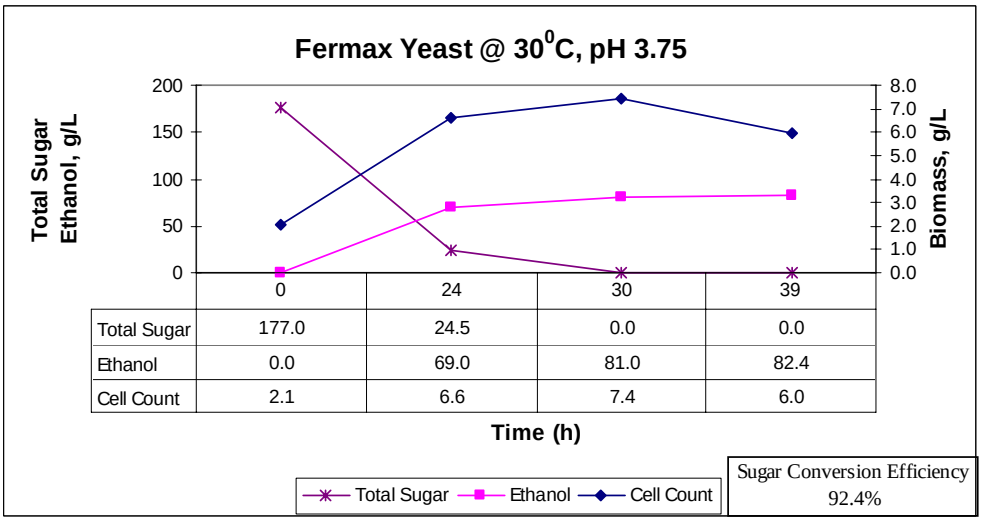


Figure 4.8. Comparison of fermentation performance of *Z.mobilis* incubating at 30°C and at a) pH 7.0 and b) pH 4.3.

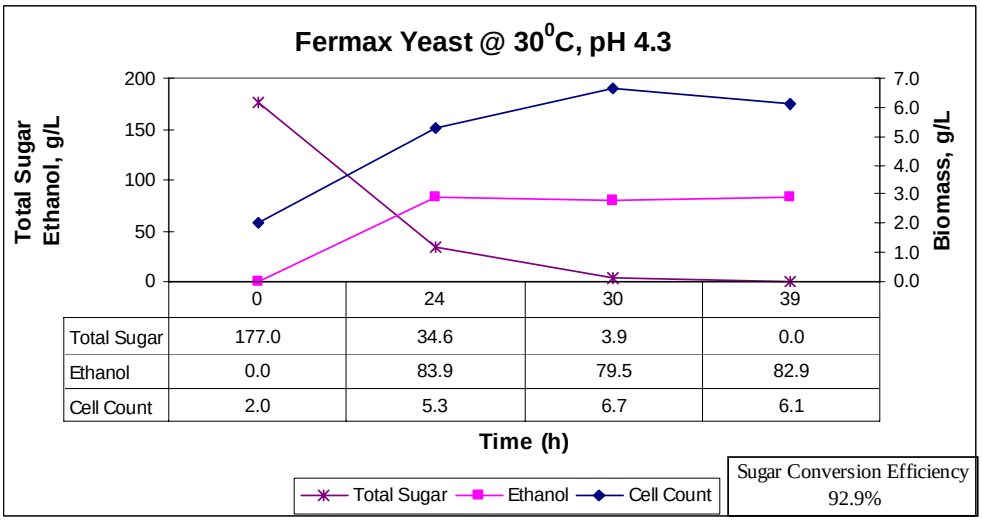
Figure 4.9 compares the fermentation performance of Fermax yeast at pH 3.75, 4.3 and 5.4. It was observed that Fermax yeast produced higher amounts of ethanol at pH 4.3 compared to pH 3.75 and 5.4, with a SCE of 92.9% at pH 4.3 compared to SCE of 92.4% and 90.9% at pH 3.75 and 5.4, respectively. Comparing the ethanol production capacity at pH 4.3, it was observed that Fermax yeast produced 82.9 g/L with a SCE of 92.9 % compared to 54.4 g/L of ethanol with a SCE of 65.2% by *Z.mobilis* (Figures 4.8b and 4.9b). The rate of ethanol production was also found to be higher for Fermax yeast with the available sugars utilized in 39 h compared to 96 h by *Z.mobilis*.

The results indicate that lowering pH to 4.3 appears to have a positive effect in improving the SCE of Fermax yeast. However, when the pH was lowered to 3.75, the pH seems to have created an unfavorable environment for the growth of the yeast as no marked increase in ethanol yield is observed. Appendix A.1 compares the SCE for each of the laboratory experiments.

a)



b)



c)

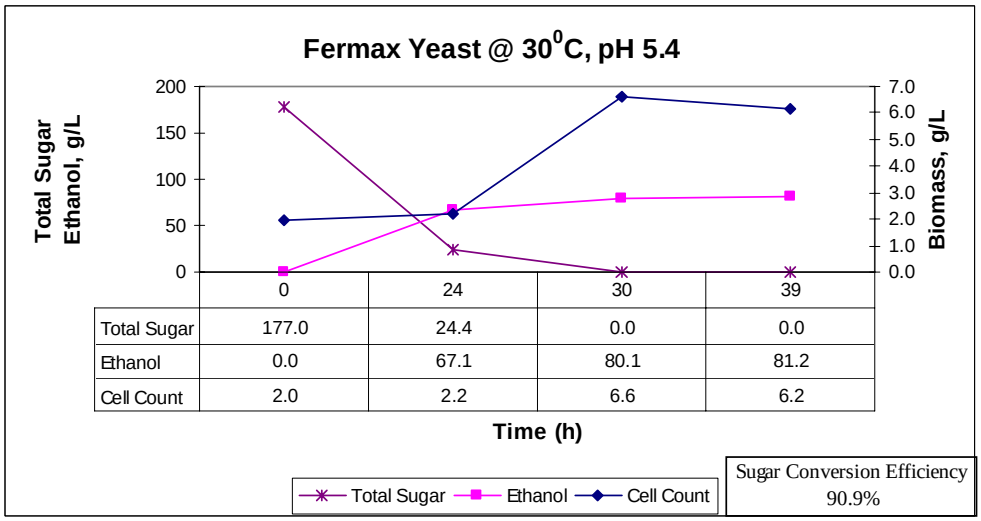


Figure 4.9. Comparison of fermentation performance of Fermax yeast incubating at 30°C and at a) pH 3.75, b) pH 4.3 and c) pH 5.4.

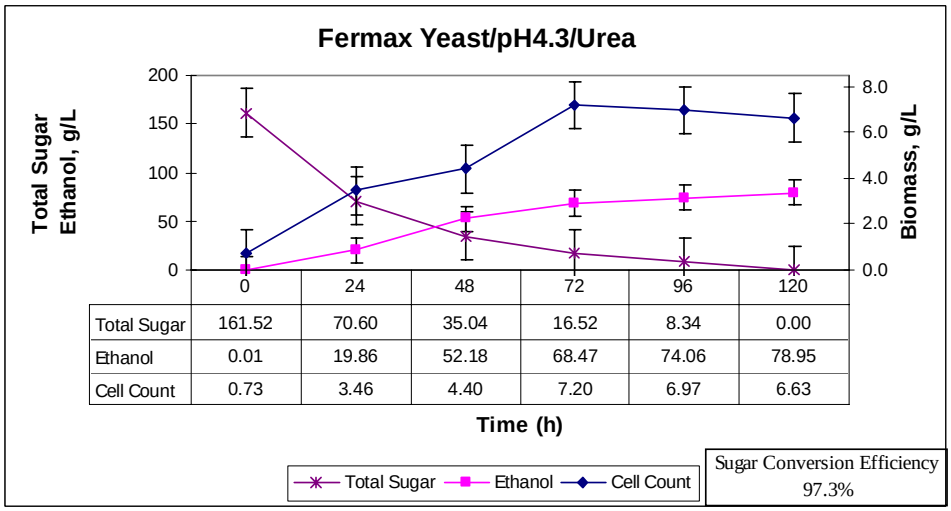
4.2 In-Field Experiment 1

The objective of the first in-field experiment was to compare the fermentation performance of Fermax and Superstart Distillers yeast at different levels of pH (4.3 and 5.4), with and without added urea. All the experiments were conducted under ambient conditions in 3.8-L fermentation vessels using sweet sorghum juice. All curves are the average of three replicate treatments. The SCE at each of the treatment conditions was comparable with one another with all values above about 95%. As can be seen from the Figures 4.10 and 4.11, sugars are completely utilized in 120 h, and final ethanol concentrations are about 79 g/L. One of the possible explanation for the better performance of the in-field experiments compared to the laboratory experiments is the availability of necessary nutrients for the yeast growth and fermentation in the sweet sorghum juice.

Figures 4.10 and 4.11 compare the sugar consumption, ethanol production and cell count for fermentation treatments with Fermax yeast at pH 4.3 and 5.4, with and without added urea. Parts a and b of each figure show the treatments with and without added urea, respectively. As can be seen, there is no apparent difference in fermentation performance with the addition of urea. Maximum ethanol (79.6 g/L) with a SCE of 97.8% is produced at pH 5.4 with no added urea. Fermax yeast produced 79.6 g/L of ethanol with a SCE of 97.8% at pH 4.3 with no urea added compared to 78.9 g/L of ethanol with added urea. At pH 5.4 Fermax yeast produced 78.9 g/L of ethanol with no urea added compared to 77.1 g/L when urea was added to the medium. At either pH treatment, a higher ethanol production level and a higher SCE was observed when no urea was added compared to added urea treatment. This indicates that pH has a minimal effect in improving the

ethanol yield with Fermax yeast. Also, the addition of urea does not appear to improve the ethanol production by Fermax yeast.

a)



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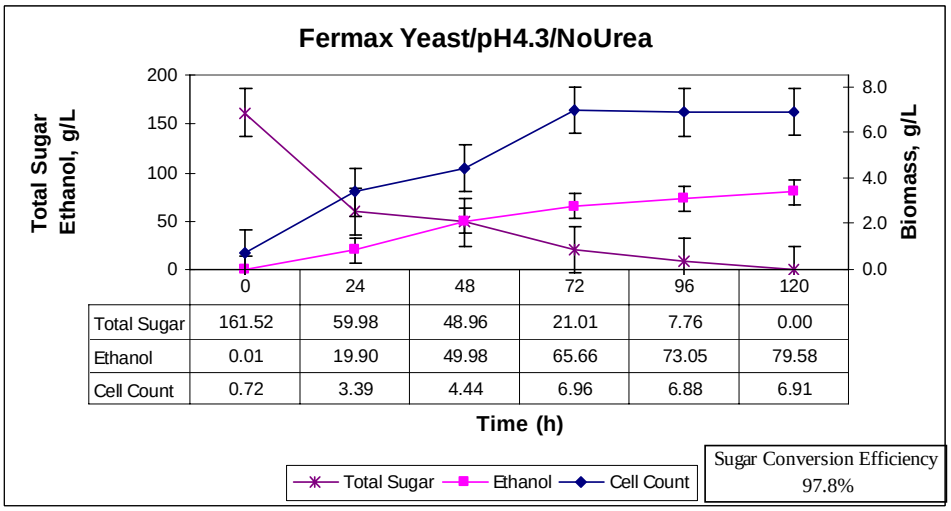
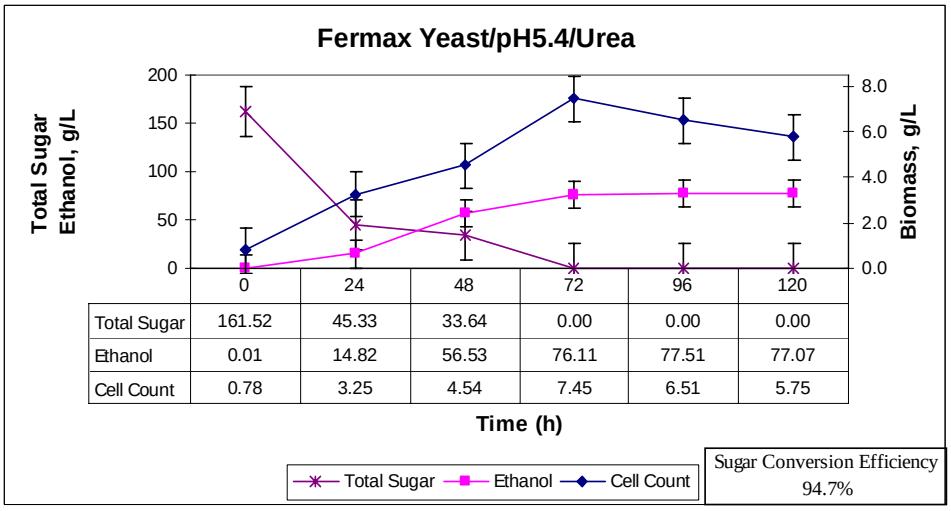


Figure 4.10. Comparison of fermentation performance of Fermax yeast at pH 4.3 and with a) Urea and b) No urea added.

a)



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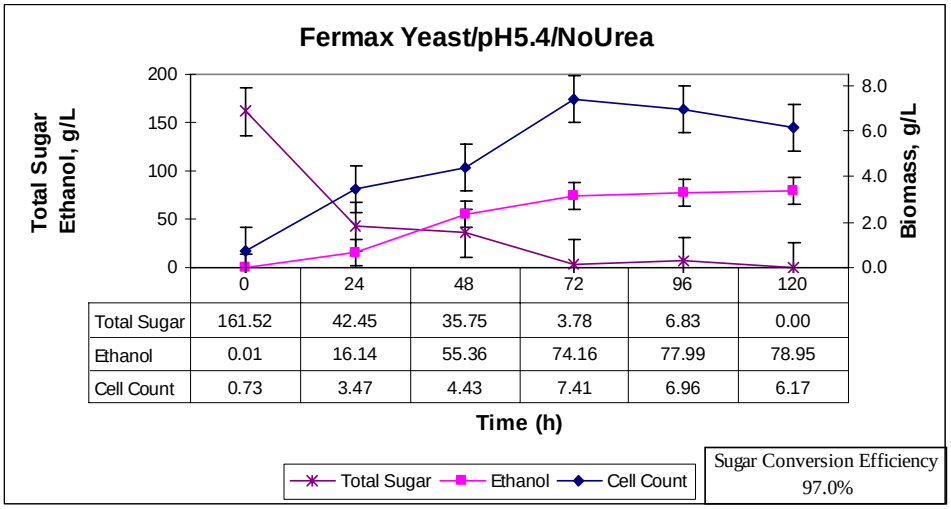


Figure 4.11. Comparison of fermentation performance of Fermax yeast at pH 5.4 and with a) Urea and b) No urea added.

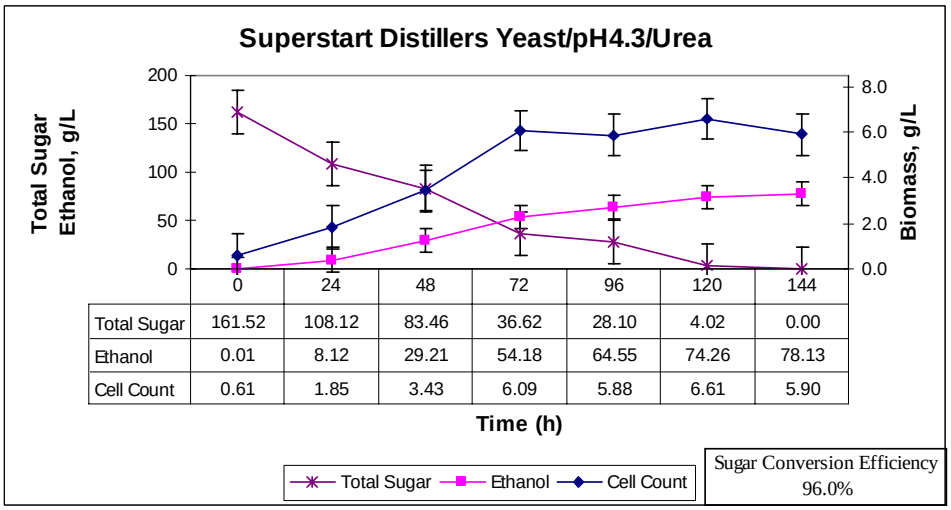
Figures 4.12 and 4.13 compare the sugar consumption, ethanol production and cell count for fermentation treatments with Superstart Distillers yeast at pH 4.3 and 5.4, with and without added urea. Maximum ethanol (78.1 g/L) was produced by Superstart Distillers yeast at pH 4.3 with added urea. When no urea was added the yeast produced 76.8 g/L with a SCE of 94.4%. At pH 5.4, Superstart Distillers yeast produced a higher concentration of ethanol (77.0 g/L) when no urea was added, compared to ethanol concentration of 74.7 g/L (SCE of 91.8%) when urea was added. The results indicate that the urea appears to enhance the ethanol yield for Superstart Distillers yeast when the pH environment was lower than optimum for the yeast. The yeast is able to produce comparable concentrations of ethanol at pH 5.4, at either nutrient condition, indicating that it is able to ferment in the presence of native microflora in the sweet sorghum juice, and further acidification is not necessary.

Addition of urea appears to have a greater influence on the ethanol production by Superstart Distillers yeast at pH 4.3 than at pH 5.4. Addition of urea did not enhance the ethanol production by Fermax yeast. Results obtained for Superstart Distillers yeast at pH 4.3 are in accordance with the results obtained by Nain and Rana (1988) in their nutrient optimization study during the production of ethanol by *S.cerevisiae*. They observed that supplementation with either nitrogen in the form of ammonium sulfate or urea (0.025%) or phosphate (0.003%) or in combination resulted in greater ethanol yield with greater fermentation efficiency and reduced fermentation time.

However comparing the fermentation performance of Fermax and Superstart Distillers yeast it can be observed that the Fermax yeast is able to utilize all the available sugars in 120 h, whereas the Superstart yeast took 144 h to completely utilize the available sugar

under identical fermentation conditions. Appendix A.2 compares the sugar conversion efficiency (SCE) for each of the fermentation treatments.

a)



b)

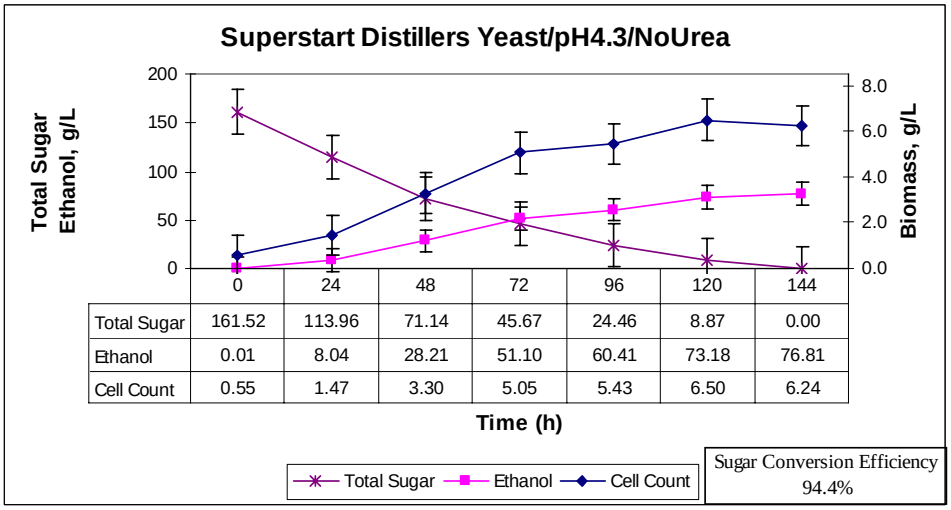
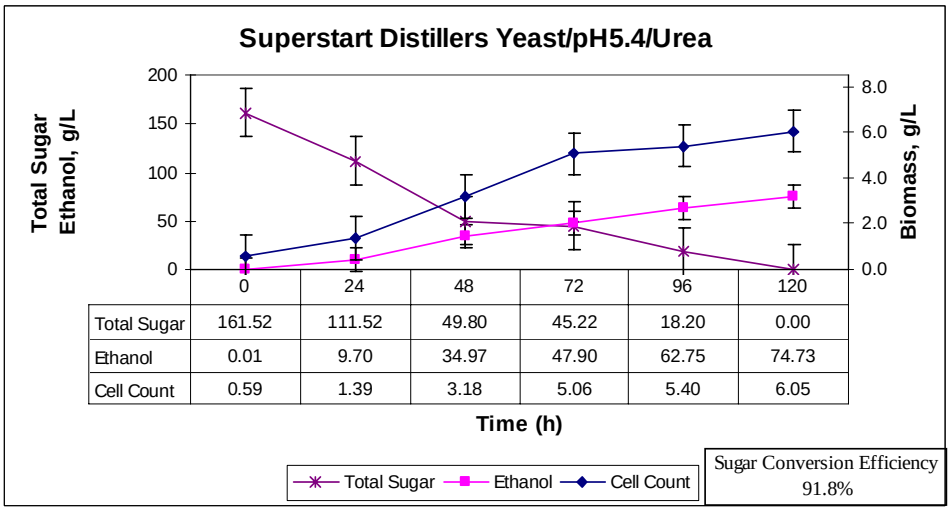


Figure 4.12. Comparison of fermentation performance of Superstart Distillers yeast at pH 4.3 and with a) Urea and b) No urea added.

a)



b)

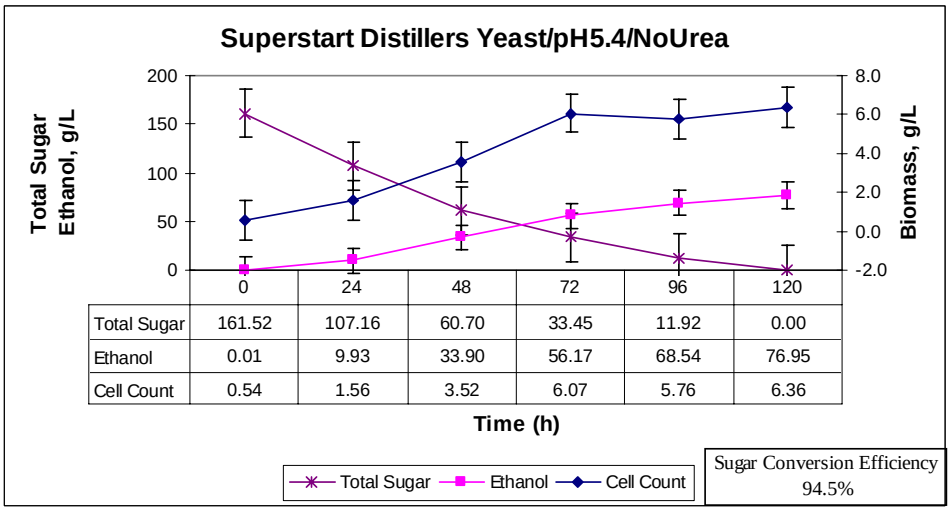


Figure 4.13. Comparison of fermentation performance of Superstart Distillers yeast at pH 5.4 and with a) Urea and b) No urea added.

4.2.1 In-Field Experiment I: Rates of Reaction

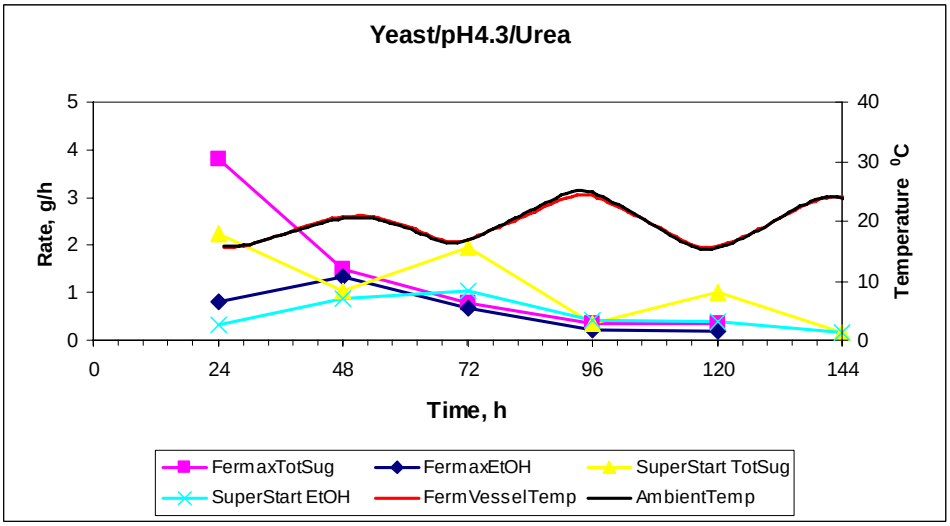
Figures 4.14 and 4.15 show a comparison of ethanol production rates for Fermax and Superstart Distillers yeast at pH 4.3 and 5.4 with and without urea addition. The curves shown are the average of three replicate treatments. Rate of ethanol production by Fermax yeast was generally observed to be higher than Superstart Distillers yeast. In

general, rate of ethanol production by Fermax yeast increased for the first 48 h of fermentation and then decreased. However, for Superstart Distillers yeast, the rate of ethanol production increased to a maximum at 72 h and then decreased with the progress of fermentation. When urea was added to the fermentation media, both yeasts exhibited a slightly higher initial rate of ethanol production.

For all the treatments except at pH 5.4 with added urea, Fermax yeast exhibited higher rate of ethanol production for the first 48 h compared to Superstart Distillers yeast. The trend reversed after 48 h, with Superstart Distillers yeast exhibiting a higher rate of ethanol production. At pH 4.3, addition of urea enhanced the rate of ethanol production for the Superstart Distillers yeast. Comparing pH 4.3 and 5.4 for Superstart Distillers yeast, it was observed that the yeast took 144 h to convert the available sugars into ethanol at pH 4.3 while it took 120 h at pH 5.4, which indicates that pH 5.4 is favored for the Superstart Distillers yeast activity. Fermax yeast, on the other hand, exhibited a higher rate of ethanol production with added urea for the first 72 h, but afterwards the rate was higher without added urea. This trend was seen at both pH 4.3 and 5.4. Addition of urea did not enhance the rate of ethanol production by Fermax yeast significantly.

Fermentation vessel temperature and ambient temperature monitored throughout the fermentation process are also represented on the curves. The fermentation vessel temperature fluctuated between 31 and 10^{o}C, nearly identically overlapping the ambient temperature curve. This indicates that there seems to be a uniform heat distribution in the 3.8-L fermentation vessels. Although the fermentation process is exothermic, the expected increase in the fermentation vessel temperature was not observed, probably due to the small size of the vessel and the influence of the ambient temperature.

a)



b)

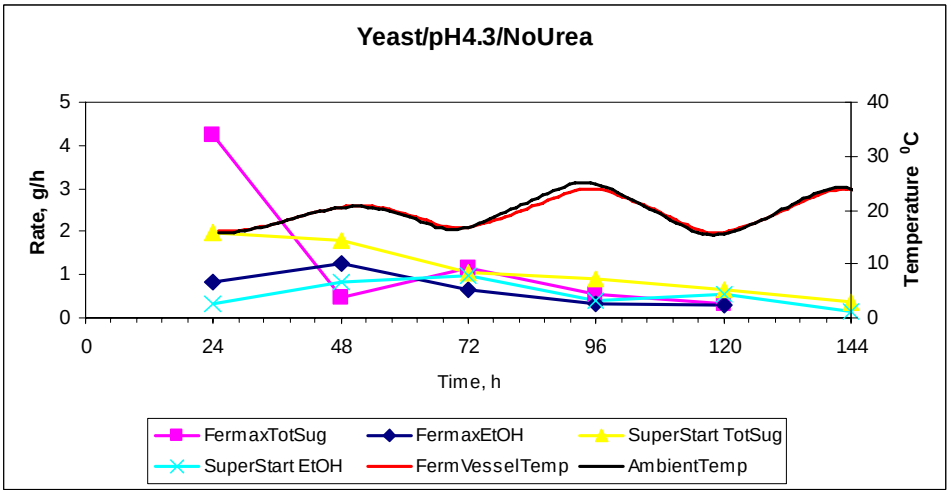
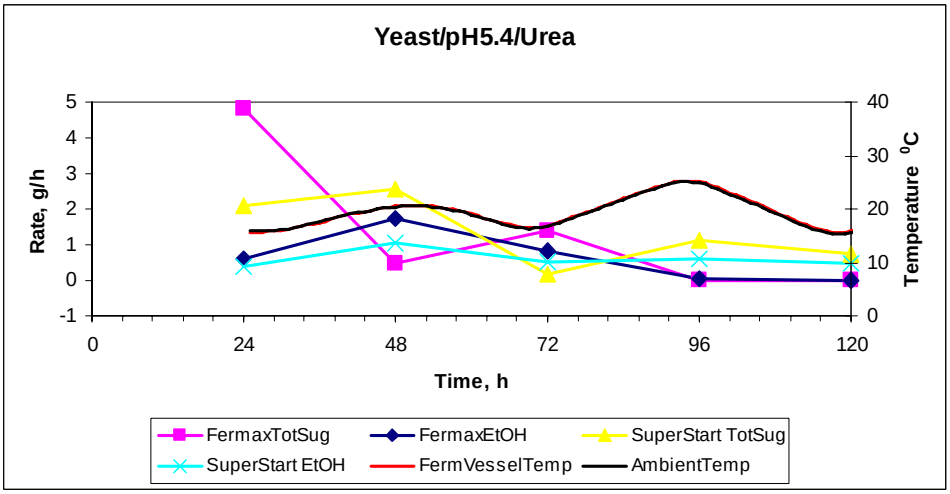


Figure 4.14. Comparison of rate of ethanol production of Fermax and Superstart Distillers yeast at pH 4.3 and with a) Urea and b) No urea added. Average temperature in the fermentation vessel is also plotted.

a)



b)

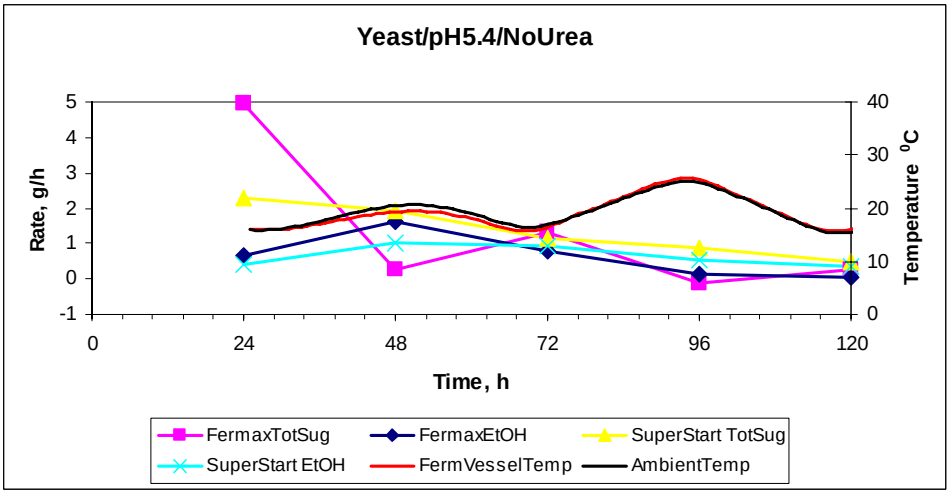


Figure 4.15. Comparison of rate of ethanol production of Fermax and Superstart Distillers yeast at pH 5.4 and with a) Urea and b) No urea added. Average temperature in the fermentation vessel is also plotted.

4.2.2 In-field Experiment I: Statistical Analysis

The three process variables Yeast, pH and Nutrient were laid out in a 2 by 2 by 2 Factorial Arrangement within a Completely Randomized Design (CRD) experiment. Response variables were analyzed based on the Mixed Procedure by testing the fixed effects for each of the factorial effects. Standard error and the estimated value of each treatment combination were obtained using the Least Square Means (LSM). Each pair of treatment combinations was compared and the estimated difference determined. Significant differences were obtained by determining the level of significance at $\alpha = 0.05$.

An analysis of variance was conducted to determine significant factorial effects for the factors of pH, yeast, and nutrient addition. The analysis was conducted for all three response variables (ethanol, sugar, and cell mass) at every 24-h interval of sample collection. The results of most significance are those related to ethanol concentration, which are shown in Table 4.1. Part (a) lists the significant and non-significant factorial effects at each time interval, and part (b) shows the estimated values for ethanol concentration for the significant interactions at the end of the fermentation (T = 120 h). From the table, it can be seen that significant interactions occur throughout the fermentation process.

A significant two-level interaction between yeast and pH on the ethanol production level was observed at the end of fermentation (T=120 h). Fermax yeast was found to produce significantly higher levels of ethanol than Superstart Distillers yeast. Superstart Distillers yeast produced a higher level of ethanol at pH 5.4 (estimated value 75.84) than at pH 4.3 estimated value 73.72), however, Fermax yeast shows a slightly higher ethanol level at pH 4.3 (estimated value 79.27) than at pH 5.4 (estimated value 78.01).

Table 4.1. Statistical summary for the variable ethanol

a) Significant and non-significant effects for the dependent variable ethanol at various sampling time intervals

Source Effect	24 h	48 h	72 h	96 h	120 h
Yeast	S	S	S	S	S
pH	S	S	S	S	NS
B v b Yeast x pH	NS	NS	S	NS	S
Nutrient	NS	S	NS	NS	S
Yeast x Nutrient	NS	NS	S	NS	NS
pH x Nutrient	NS	NS	S	S	S
Yeast x pH x Nutrient	NS	NS	S	S	NS

S- Significant
NS- Not Significant

b) Estimated ethanol values for significant interactions at a sampling time = 120 hrs

Time	Standard Error	Yeast	pH	Nutrient	Estimated Value
120 h	0.35	Fermax	4.3	-	79.27
		Fermax	5.4	-	78.01
		SuperStart	4.3	-	73.72
		SuperStart	5.4	-	75.84
	0.35	-	4.3	Urea	76.38
		-	4.3	No-Urea	76.61
		-	5.4	Urea	77.95
		-	5.4	No-Urea	75.90

A significant interaction between pH and nutrient on the ethanol production levels was also observed at the end of the fermentation. With the pH of the fermentation media initially adjusted to 4.3, addition of urea did not have a significant effect on the ethanol production levels. However, when the fermentation was carried out at the native pH of the sweet sorghum juice, adding urea resulted in slightly higher ethanol production levels (estimated value 77.95) compared to when no added urea (estimated value 75.90).

Fixed effects showing significant and non significant interactions for the three process variables, Yeast, pH and Nutrient are shown in Appendix A.3. The estimated values for each of the significant source of interaction between the independent variables on the dependent variables, cell count, ethanol and total sugar, are tabulated in Appendix A.4 through Appendix A.6.

An analysis of variance indicated that there was a significant three-level interaction amongst yeast, pH and nutrient on the cell biomass and ethanol production levels observed during the growth stage of the yeast, i.e. between 48 and 96 h of fermentation. A significant three level interaction was also observed with respect to the sugar utilization at the end of fermentation, however it is interesting to note that this effect is mainly due to the null estimates of available sugars in the media.

Difference in least square means (LSM) of the process variables on the cell biomass and ethanol is tabulated in Appendix A.5 and A.6 respectively, at a statistical level of significance, $\alpha = 0.05$. Significant differences were observed for the ethanol concentration at the end of 72 and 96 h of incubation. Fermax yeast shows higher difference between Urea and No Urea condition on ethanol levels at pH 4.3 [p=0.03] than

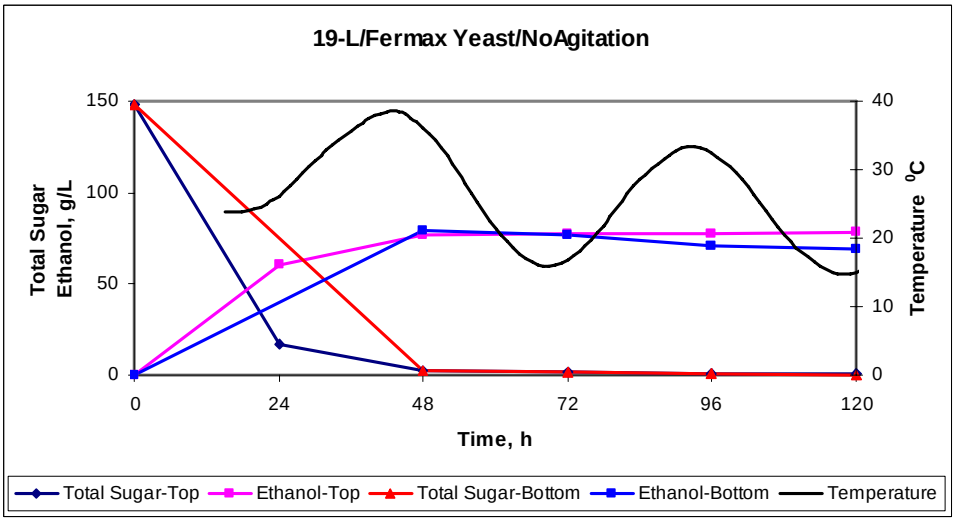
at pH 5.4 [p=0.12]. Superstart Distillers shows significantly larger difference in ethanol production at both pH 4.3 [p=0.02] and pH 5.4 [p<0.0001]. However at the end of 96 h, no significant difference is observed for Fermax yeast [p>0.05] while significantly larger difference is expressed by Superstart Distillers yeast [p<0.0001]. While for a given pH and nutrient condition Fermax yeast shows a significantly higher biomass and ethanol production levels than Superstart Distillers yeast [p<0.0001].

Comparing the two yeasts at the pH, nutrient and temperature conditions encountered in the in-field Experiment I, it was observed that Fermax yeast produced the greatest amount of ethanol in the shortest time.

4.3 In-Field Experiment II

The second in-field experiments were carried out in 19- and 209-L fermentation vessels under controlled incubation temperature. The objectives of the experiment were to understand the influence of scale-up and agitation on fermentation performance and ethanol production. As was mentioned previously, the extensive experiments initially planned were not possible due to a lack of available juice volume. All resulting fermentations were carried out using sweet sorghum juice at the native pH 5.4 of the juice. Temperature data were also collected to understand the heat distribution within a fermentation vessel. Analysis samples were taken from the top and bottom of the fermentation tank and analyzed to understand the distribution of mass during the fermentation process.

a)



b)

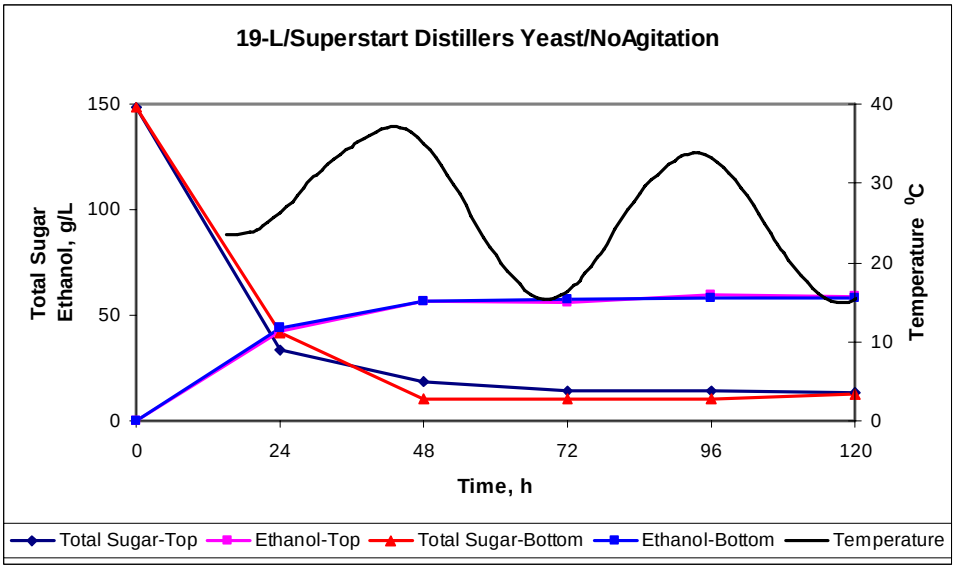


Figure 4.16. Comparison of fermentation performance of a) Fermax yeast and b) Superstart Distillers yeast in 19-L fermentation vessel. Average temperature in the fermentation vessel is plotted.

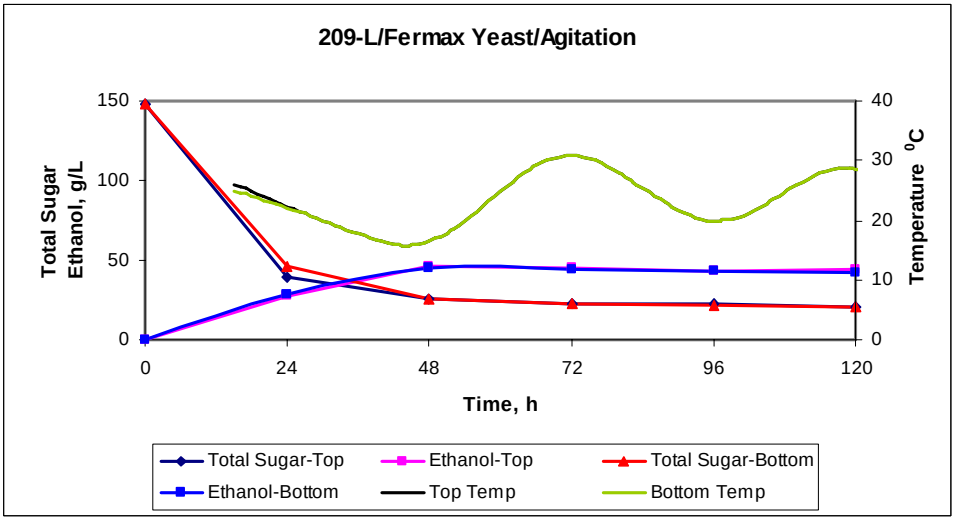
Figure 4.16 compares the fermentation performance of Fermax and Superstart Distillers yeast in 19-L fermentation vessels without agitation. Temperature in the vessels varied between 39 and 8^{o}C. Both yeasts produce maximum ethanol concentration within 48 h of fermentation. However Fermax yeast produces greater amounts of ethanol (77.8 g/L) in 120 h compared to 63.8 g/L produced by Superstart Distillers yeast. Alternating the

incubation temperature between 7 and 37^{o}C does not seem to inhibit the ethanol fermentation. However the rate of ethanol production is generally reduced or remains constant once the yeast cells are exposed to extreme incubation temperatures possibly due to the cold-shock experienced by the yeast cells. This is evident from the flattening of the curve after 48 h of incubation. The impact is much more dramatic on Superstart Distillers yeast as the cells are unable to breakdown the available sugars to ethanol and residual sugar is still available at the end of fermentation.

Figure 4.17 compares the fermentation results for Fermax yeast in 209-L fermentation vessels with and without agitation. Samples taken from the top and bottom of the fermentation vessel were analyzed for total sugar and ethanol concentration every 24 h. The time required for the completion of fermentation was 120 h. The ethanol yield was comparatively lower compared to the treatment conditions in the first in-field experiment possibly due to larger vessel volume, greater cell settlement and non-uniform distribution of the sugars and other nutrients. A lead time of approximately 5 h was also involved from the time that the juice was pressed and the time that the yeast was pitched into the fermentation vessel leading to competitive inhibition of the fermentation process by the native microflora.

As is evident from the plots, the ethanol and sugar curves for the top and bottom samples overlap each other, indicating that there is uniform mixing within each vessel. The temperature profiles at the top and bottom of the vessel are identical for the agitated vessel, while an average difference of 3^{o}C is observed for the non agitated vessel. A steep increase in ethanol concentration with a subsequent decrease in total sugar level is observed within 48 h of fermentation in the non-agitated vessel after which the levels

a)



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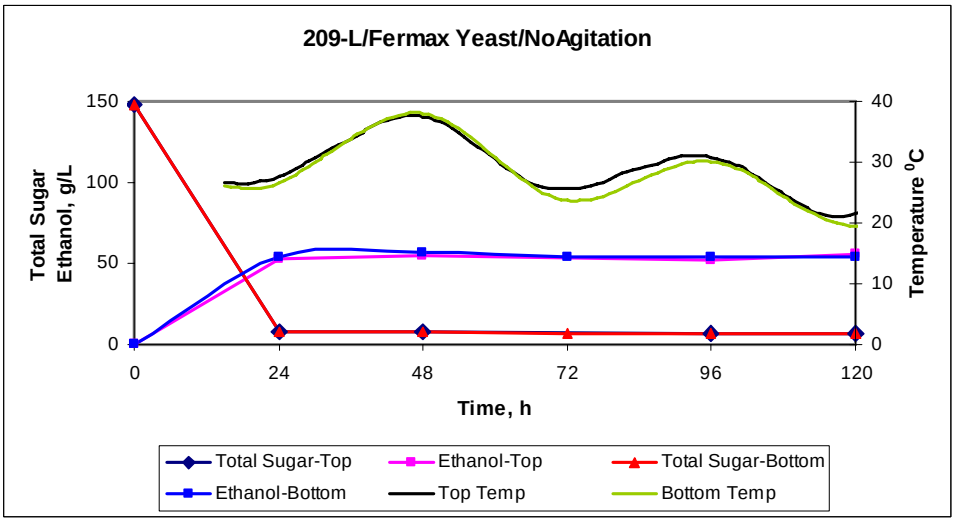

Figure 4.17. Impact of a) Agitation and b) No agitation on the fermentation performance of Fermax yeast in 209-L fermentation vessel. Average temperature at the top and bottom of the fermentation vessel is plotted.

plateau. Ethanol concentration in the non-agitated vessel was higher (61.1 g/L) than the agitated vessel (50.7 g/L). This is primarily due to the different incubation regimes employed for the two fermentation vessels. The agitated vessel incubation was started at 7^{o}C while the incubation of the non-agitated vessel was started at 37^{o}C. It is known that the optimum temperature for the growth of *S.cerevisiae* is 28^{o}C and the optimum

temperature of fermentation is 35^{o}C. Yeasts cells exposed to low temperature, experience 'cold shock' causing impairment of protein synthesis and reduced membrane fluidity (Fargher and Smith, 1995). In addition to this, agitation may have increased oxygen levels above the micro-aerobic levels, thereby switching the yeast cells from ethanol production to biomass generation. Similar observations were reported by Banat et al. (1996). Levels of oxygen greater than required, will also lead to stuck or sluggish fermentation (Sablayrolles et al. 1996).

Compared to the first in-field experiment, the ethanol levels produced in the experiment II were lower due to the lower concentration of fermentable sugars available in the sweet sorghum juice, however, the sugar conversion efficiency for both experiments were comparable. Sweet sorghum stalks for the experiment I were hand harvested in the month of October, while the stalks for experiment II were harvested in the month of November using a prototype harvester. Studies have indicated that higher fermentable sugars were obtained when the stalks were hand harvested compared to mechanical harvesting (Eiland et al. 1983). From a commercial perspective, it is a trade off between the rate of harvesting and the fermentable sugars and it is not a feasible option to harvest the stalks by hand. Time of harvest also has a significant bearing on the fermentation sugar availability in the juice as observed by Collier (1884).

4.3.1 In-Field Experiment II: Rates of Reaction

Figures 4.18-4.20 compare the rate of reaction under different fermentation conditions. All the treatments were incubated under controlled temperature conditions alternating between 7 and 37^{o}C to imitate the ambient conditions. Fermentations were carried out using sweet sorghum as the media and at the native pH of the sweet sorghum juice.

Figure 4.18 compares the rate of ethanol production of Fermax and Superstart Distillers yeast in the 19-L fermentation vessels. After 24 h of fermentation, Fermax yeast shows a significantly higher rate ethanol production (2.59 g/h) compared to Superstart Distillers yeast (1.81 g/h). The rate of reaction is observed to decrease from start to 72 h of fermentation and then remain constant until the end of the fermentation. Both yeasts show similar rates of ethanol production as the fermentation progresses after 72 h. Temperature curves for both yeasts overlap each other, indicating no significant differences in the fermentation temperature as the process progresses.

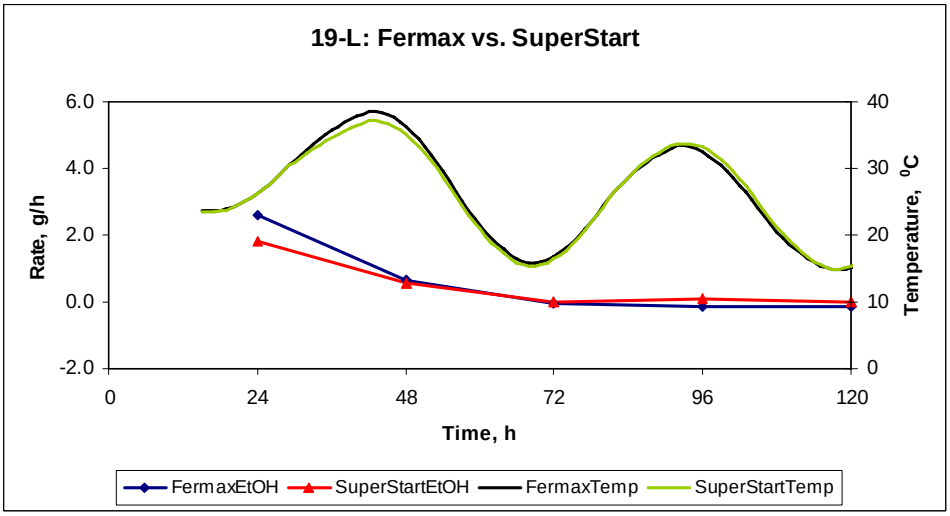

Figure 4.18. Comparison of rate of ethanol production for Fermax and Superstart Distillers yeast on the rate of ethanol production in 19-L fermentation vessels. Temperature in the fermentation vessels is also plotted.

Figure 4.18 compares the rate of ethanol production in the agitated and non-agitated 209-L fermentation vessels. The non agitated fermentation vessel shows a significantly higher rate ethanol production (2.21 g/h) compared to the agitated fermentation vessel (1.15 g/h). However the rate curve tends to flatten out at 48 h for the non-agitated drum and at 72 h for the agitated drum. The rate curves overlap each other after 72 h of

incubation. The initial lower rate observed in the agitated fermentation vessel was primarily due to the start of incubation at 7^{o}C while the non-agitated drum started incubating at 37^{o}C. Start of fermentation at low incubation temperatures might have caused 'cold shock'. Sensitivity of the yeast cells to cold shock causes lethal and sublethal injuries. Cold shock is a cascade of physiological, biochemical and genetically controlled events leading to loss of protein and ATP synthesis and vacuolar rearrangement. Other contributing factors affecting rate of ethanol production could have been mechanical shear of the yeast cells and the incorporation of oxygen, similar to the results obtained by Berzins et al. (1989).

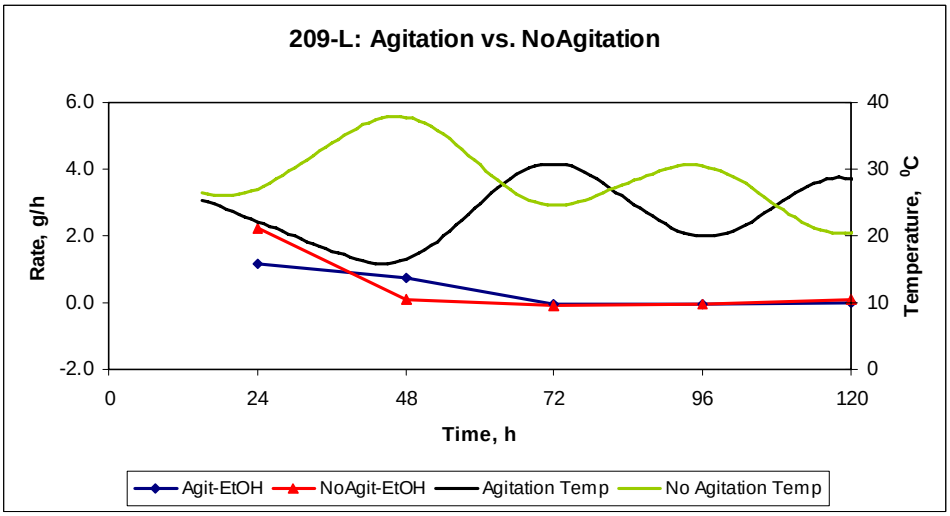

Figure 4.19. Impact of agitation on the rate of ethanol production by Fermax yeast in 209-L fermentation vessels. Temperature in the fermentation vessels is also plotted.

Figure 4.20 compares the fermentation performance of Fermax yeast in 209- and 19-L fermentation vessels. Rates of ethanol production by Fermax yeast show similar patterns irrespective of the size of the fermentation vessel. The rates were found to be maximum after 24 h of fermentation, then decrease as the fermentation progresses. Rates are very

similar after 72 h of incubation for both treatment conditions. The rate of ethanol production was higher in the 19-L fermentation vessel (2.59 g/h) compared to the 209-L vessel (2.21 g/h). In the 209-L fermentation vessel, the rates of ethanol production and sugar utilization decreased from start to 48 h of fermentation and then remain constant until the end of the fermentation. However, for the 19-L fermentation vessel, the rate decreased until 72 h of incubation and then remained constant until the end of fermentation.

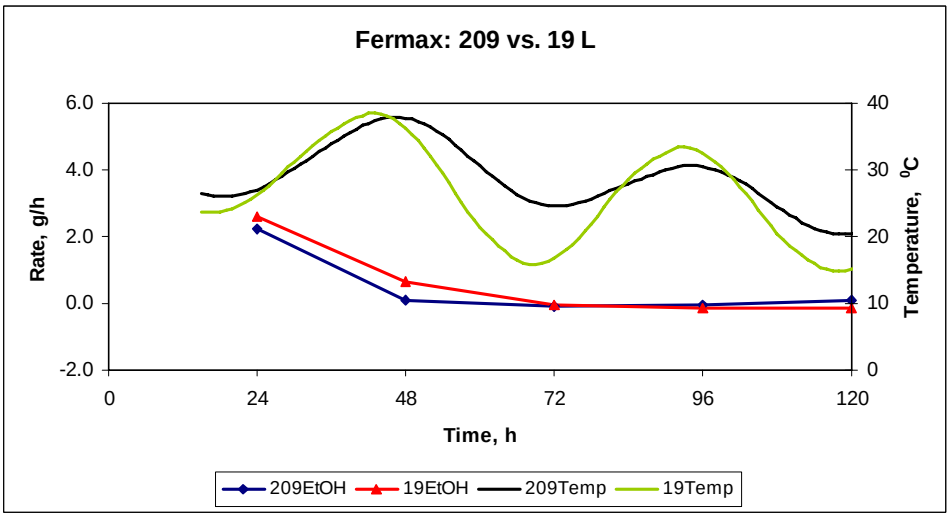

Figure 4.20. Comparison of fermentation vessel size (209- and 19-L) on the rate of ethanol production for Fermax yeast.

It is also interesting to compare the temperature profiles within the two different vessel sizes. It can be seen in Figure 4.20 that the temperature fluctuation in the smaller 19-L vessel is significantly larger than in the 209-L vessel. This confirms the fact that larger vessels with larger thermal mass and a larger surface area to volume ratio will tend to be more resistant to large ambient temperature fluctuations.

4.3.2 In-Field Experiment II: Statistical Analysis

For experiment II, the effect of sampling location, fermentation vessel size and their interaction on the ethanol production and total sugar utilization during the fermentation process were tested using the SAS GLM procedure. Variables were laid out as a split plot in a Completely Randomized Design (CRD). Results are tabulated in Appendix B.1 and B.2. Since the experiments did not involve replications, the observations from the data analysis are suggestive and indicative. Following a larger experimental design, it can be expected that some of the interactions between the variables will be significant, thereby providing conclusive observations. All p values > 0.05 were interpreted as insignificant. Cell biomass, one of the dependent measured variables, was not statistically interpreted due to the difficulty in obtaining a representative sample. This situation arose due to the sedimentation of the yeast cells in the fermentation vessel.

Significant differences in ethanol production ($p=0.02$) and total sugar utilization ($p=0.01$) was observed at the end of 120 h of incubation. Sugar concentration was significantly different between the top and bottom sample locations in the 19-L fermentation vessel ($p=0.00$). However no significant difference was observed in the 209-L fermentation vessel (Appendix B.2). Ethanol concentration, on the other hand differed significantly between the top and bottom samples taken from the 19-L ($p=0.00$) and 209-L ($p=0.01$) fermentation vessels.

4.4 Sweet Sorghum Juice Stability and Nutrient Analysis

Table 4.2 shows the analysis of nutrients available in sweet sorghum juice. The juice was analyzed immediately after pressing. Results indicate that sweet sorghum juice has

various cations and anions available. The juice contains on average 16% fermentable sugar, 0.23% protein, and the pH of the juice is around 5.4.

Pressed sweet sorghum juice was stored under refrigerated conditions (4^{o}C) and analyzed for total sugar at a sampling frequency of 24 h for a period of eight days. Results from the study are shown graphically in Figure 4.21. An error of 2 g/L in estimation of the sugar content is also marked on the curve. The initial sugar in the sweet sorghum juice was analyzed to be 146.7 g/L. The sugar level in the juice remained stable over a period of 8 days, as is evident from the curve. The native microflora in the sweet sorghum juice did not breakdown the sugar. Refrigerated storage conditions appear to inhibit the activity of the native microflora, thus aiding in stabilizing the sugar level. The nutritional stability of sweet sorghum juice in the present study is similar to the results obtained by Mamma et al. (1996), where they periodically analyzed the stalk composition of sweet sorghum and observed no significant difference when stored at -20^{o}C for six months.

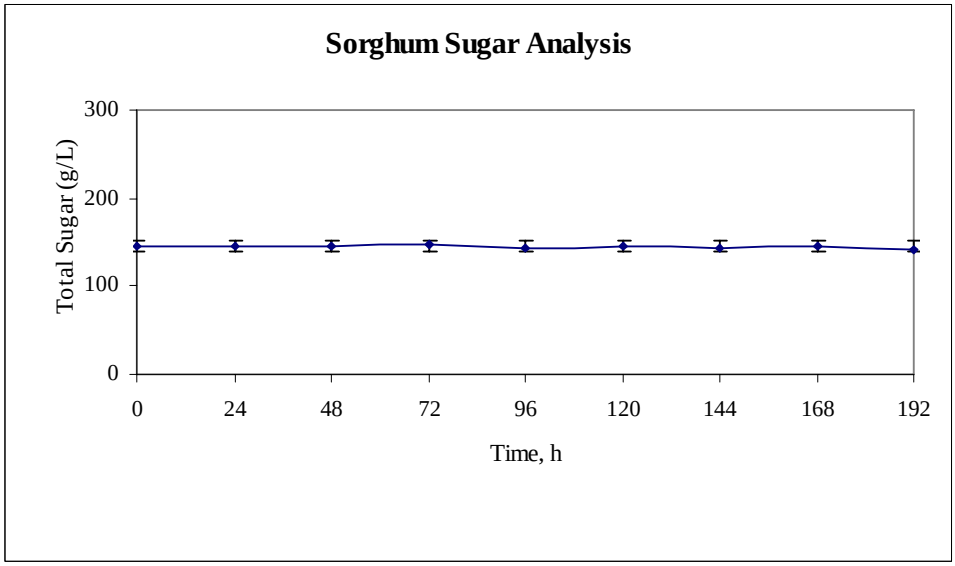

Figure 4.21. Stability of total sugar available in the sweet sorghum juice.

Table 4.2. Composition analysis of sweet sorghum juice

Parameter	Method	Component	Amount
Cation	Ion Chromatography	Sodium	0.13 g/L
		Ammonium	0.05 g/L
		Magnesium	0.79 g/L
		Calcium	2.42 g/L
Anion	Ion Chromatography	Fluoride	-
		Chloride	-
		Acetate	-
		Nitrate	2.06 g/L
		Phosphate	0.13 g/L
		Citrate	-
		Sulfate	0.014 g/L
Sugars	Ion Chromatography	Glucose	41.26 g/L
		Fructose	26.72 g/L
		Sucrose	93.54 g/L
		Total Sugar	**161.52 g/L**
Protein	BCA Protein Assay	Total Protein	2.33 g/L
pH	pH Meter	Initial	5.40

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Specific conclusions from the in-field fermentation experiments are summarized below.

1) Comparison of the fermentation performance of the yeasts.

It was observed from the series of laboratory scale and in-field fermentation experiments using sweet sorghum juice as the fermentation media, that Fermax yeast was the better candidate for carrying out the in-field process for ethanol production. Fermax yeast produced 79.6 g/L of ethanol under field conditions in 120 h compared to Superstart Distillers yeast (78.1 g/L). Fermax yeast performed equally well under different fermentation conditions of temperature, pH and nutrients. Fermax yeast also showed significantly superior rates of sugar utilization and ethanol production compared to Superstart Distillers yeast.

2) Heat and Mass distribution in fermentation vessel.

Results indicate that uniform heat and mixing takes place in the fermentation vessels and no significant difference exists between the concentration of ethanol and total sugar in the fermentation vessel.

3) Addition of urea

Results indicated that ethanol production was not significantly different when urea was added to the medium. Native sweet sorghum juice contains the necessary nutrients and fermentable sugars to produce viable amounts of ethanol.

4) Comparison of fermentation pH on ethanol production.

Results obtained from the in-field experiments indicate that reducing the pH of sweet sorghum juice from pH 5.4 to 4.3 did not enhance fermentation rate or increase ethanol production. It was hypothesized that reducing pH would inhibit the native microflora present in the sweet sorghum juice, making the nutrients in the media completely available for the fermenting yeasts. However, Fermax yeast showed no significant difference in the ethanol production levels at pH 4.3 (79.58 g/L) and pH 5.4 (79.0 g/L). Superstart Distillers yeast also showed no significant differences in ethanol production levels at pH 4.3 (76.8 g/L) and pH 5.4 (77.0 g/L). These findings are economically significant by reducing the cost required for the adjustment of the media pH.

5) Ethanol yield due to scale-up

No significant difference in ethanol production was observed among fermentation vessel volumes of 3.8-L, 19-L and 209-L. A uniform heat and mass (ethanol and fermentable sugars) transfer was observed in the vessel irrespective of the vessel volume.

6) Results from agitation were inconclusive

Agitation appeared to have a detrimental effect on the fermentation performance and ethanol production. Ethanol production in the agitated fermentation vessel was found to be 45.8 g/L compared to 58.5 g/L in the non-agitated fermentation vessel. However,

because the agitated and non-agitated vessels were fermented under two different temperature regimes, the effects of agitation were inconclusive.

7) Sorghum juice composition stability under refrigerated storage conditions

Analysis of the sweet sorghum juice over a period of eight days indicated that the available nutrients and fermentable sugars were stable under refrigerated conditions. This result is encouraging in terms of storing the sweet sorghum juice, thereby making the in-field process flexible.

From the present study it appears that in-field fermentation of sweet sorghum juice can be carried out under ambient conditions with no pre-sterilization of the fermentation media and equipment, no temperature control, no added nutrients, no pH adjustment and is a feasible process option for ethanol production.

5.2 Recommendations for Future Research

1. Perform fermentation experiments in 1000, 10000 and 250000 L fermentation vessels with a view to commercialize and further understand the influence of scale-up on fermentation.
2. Investigate simultaneous saccharification and co-fermentation of sweet sorghum biomass and make an economical comparison to the submerged fermentation process.
3. Develop an in-field process for distillation of ethanol. Consider the economics of using sweet sorghum bagasse as a source of energy for the distillation unit.
4. Perform experimental trials to further understand the impact of agitation on the fermentation performance.
5. Perform heat and mass balance study of the in-field fermentation process.

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APPENDIX A.1

Sugar Conversion Efficiency (SCE) for different process variables as observed in laboratory shake flask under different treatment conditions.

Microorganism	pH	Temperature (^{o}C)	Sugar Conversion Efficiency (%)
Z.mobilis	4.3	30	93.8
K.marxianus	4.3	30	45.4
Superstart Distillers Yeast	4.3	30	73.0
Fermax Yeast	4.3	30	94.1
Z.mobilis + Fermax Yeast	4.3	30	86.5
Z.mobilis + Fermax Yeast + *K.marxianus*	4.3	30	69.9
Fermax Yeast	4.3	30	88.9
Fermax Yeast	4.3	15	71.8
Fermax Yeast	4.3	41 & 15	75.9
Fermax Yeast	4.3	35	95.7
Z.mobilis + Fermax Yeast	4.3	30	74.2
Z.mobilis + Fermax Yeast	4.3	41 & 15	67.2
Z.mobilis + Fermax Yeast + *K.marxianus*	4.3	30	68.6
Z.mobilis + Fermax Yeast + *K.marxianus*	4.3	41 & 15	59.4
Fermax Yeast	3.75	30	92.4
Fermax Yeast	4.3	30	92.9
Fermax Yeast	5.4	30	90.9
Z.mobilis	7.0	30	71.4
Z.mobilis	4.3	30	65.2

APPENDIX A.2

Sugar Conversion Efficiency for Fermax and Superstart Dry Distillers yeast under Urea and No Urea added conditions and at different treatment pH conditions

Microorganism	pH	Nutrient	Sugar Conversion Efficiency (%)
Fermax Yeast	4.3	Urea	97.0
Fermax Yeast	4.3	No-Urea	97.8
Fermax Yeast	5.4	Urea	94.7
Fermax Yeast	5.4	No-Urea	97.0
Superstart Distillers Yeast	4.3	Urea	96.0
Superstart Distillers Yeast	4.3	No-Urea	94.4
Superstart Distillers Yeast	5.4	Urea	91.8
Superstart Distillers Yeast	5.4	No-Urea	94.5

APPENDIX A.3

Table of fixed effects for Experiment I showing significant and non significant effects for the independent process variables (Yeast x pH x Nutrient). Results are tabulated for each of the dependent variables (cell count, ethanol, total sugar). [$\alpha = 0.05$]

A) Cell Count

Source Effect	0 h	24 h	48 h	72 h	96 h	120 h
Yeast	S	S	S	S	S	NS
pH	NS	S	NS	NS	NS	S
Yeast x pH	S	NS	NS	NS	NS	NS
Nutrient	S	NS	NS	NS	NS	NS
Yeast x Nutrient	S	NS	NS	NS	NS	NS
pH x Nutrient	NS	S	NS	S	S	NS
Yeast x pH x Nutrient	NS	NS	S	S	NS	NS

B) Ethanol

Source Effect	0 h	24 h	48 h	72 h	96 h	120 h
Yeast		S	S	S	S	S
pH		S	S	S	S	NS
Yeast x pH		S	NS	S	NS	S
Nutrient		NS	S	NS	NS	S
Yeast x Nutrient		NS	NS	S	NS	NS
pH x Nutrient		NS	NS	S	S	S
Yeast x pH x Nutrient		NS	NS	S	S	NS

C) Total Sugar

Source Effect	0 h	24 h	48 h	72 h	96 h	120 h
Yeast		S	S	S	S	S
pH		S	S	S	S	S
Yeast x pH		S	NS	NS	NS	S
Nutrient		NS	NS	NS	NS	S
Yeast x Nutrient		NS	NS	NS	NS	S
pH x Nutrient		NS	NS	NS	NS	S
Yeast x pH x Nutrient		NS	NS	NS	NS	S

S- Significant
NS- Not Significant

APPENDIX A.4

Table showing the significant source of interaction between the process variables (Yeast, pH and Nutrient) for the dependent variable Cell Count as observed in Experiment I. Results are tabulated for each of the sampling times.

Time	Significant Source	Standard Error	Yeast	pH	Nutrient	Estimated Value
24 h	pH x Nutrient	0.06	-	4.3	Urea	2.65
			-	4.3	No-Urea	2.43
			-	5.4	Urea	2.32
			-	5.4	No-Urea	2.51
	Yeast	0.04	Fermax	-	-	3.39
			SuperStart	-	-	1.57
48 h	Yeast x pH x Nutrient	0.08	Fermax	4.3	No-Urea	4.44
			Fermax	4.3	Urea	4.40
			Fermax	5.4	No-Urea	4.43
			Fermax	5.4	Urea	4.54
			SuperStart	4.3	No-Urea	3.30
			SuperStart	4.3	Urea	3.43
			SuperStart	5.4	No-Urea	3.52
			SuperStart	5.4	Urea	3.18
72 h	Yeast x pH x Nutrient	0.30	Fermax	4.3	No-Urea	6.96
			Fermax	4.3	Urea	7.20
			Fermax	5.4	No-Urea	7.41
			Fermax	5.4	Urea	7.45
			SuperStart	4.3	No-Urea	5.05
			SuperStart	4.3	Urea	6.09
			SuperStart	5.4	No-Urea	6.07
			SuperStart	5.4	Urea	5.06
96 h	pH x Nutrient	0.10	-	4.3	Urea	6.43
			-	4.3	No-Urea	6.16
			-	5.4	Urea	5.96
			-	5.4	No-Urea	6.36
	Yeast	0.07	Fermax	-	-	
			SuperStart	-	-	
120 h	pH	0.09	-	4.3	-	6.08
			-	5.4	-	6.66

APPENDIX A.5

Table showing the significant source of interaction between the process variables (Yeast, pH and Nutrient) for the dependent variable Ethanol as observed in Experiment I. Results are tabulated for each of the sampling times.

Time	Significant Source	Standard Error	Yeast	pH	Nutrient	Estimated Value
24 h	Yeast x pH	0.50	Fermax	4.3	-	19.88
			Fermax	5.4	-	15.48
			SuperStart	4.3	-	8.08
			SuperStart	5.4	-	9.81
48 h	Yeast	0.35	Fermax	-	-	53.51
			SuperStart	-	-	31.57
	pH	0.35	-	4.3	-	39.90
			-	5.4	-	45.19
	Nutrient	0.35	-	-	No-Urea	41.86
			-	-	Urea	43.22
72 h	Yeast x pH x Nutrient	0.85	Fermax	4.3	No-Urea	65.66
			Fermax	4.3	Urea	68.47
			Fermax	5.4	No-Urea	74.16
			Fermax	5.4	Urea	76.11
			SuperStart	4.3	No-Urea	51.10
			SuperStart	4.3	Urea	54.18
			SuperStart	5.4	No-Urea	56.17
			SuperStart	5.4	Urea	47.88
96 h	Yeast x pH x Nutrient	0.57	Fermax	4.3	No-Urea	73.05
			Fermax	4.3	Urea	74.06
			Fermax	5.4	No-Urea	77.98
			Fermax	5.4	Urea	77.51
			SuperStart	4.3	No-Urea	60.41
			SuperStart	4.3	Urea	64.55
			SuperStart	5.4	No-Urea	68.55
			SuperStart	5.4	Urea	62.75
120 h	Yeast x pH	0.35	Fermax	4.3	-	79.27
			Fermax	5.4	-	78.01
			SuperStart	4.3	-	73.72
			SuperStart	5.4	-	75.84
	pH x Nutrient	0.35	-	4.3	Urea	76.38
			-	4.3	No-Urea	76.61
			-	5.4	Urea	77.95
			-	5.4	No-Urea	75.90

APPENDIX A.6

Table showing the significant source of interaction between the process variables (Yeast, pH and Nutrient) for the dependent variable Total Sugar as observed in Experiment I. Results are tabulated for each of the sampling times.

Time	Significant Source	Standard Error	Yeast	pH	Nutrient	Estimated Value
24 h	Yeast x pH	3.91	Fermax	4.3	-	65.29
			Fermax	5.4	-	43.89
			SuperStart	4.3	-	111.04
			SuperStart	5.4	-	109.34
48 h	Yeast	3.23	Fermax	-	-	38.35
			SuperStart	-	-	66.27
	pH	3.23	-	4.3	-	59.65
			-	5.4	-	44.97
72 h	Yeast	2.83	Fermax	-	-	10.33
			SuperStart	-	-	40.24
	pH	2.83	-	4.3	-	29.96
			-	5.4	-	20.61
96 h	Yeast	2.16	Fermax	-	-	5.73
			SuperStart	-	-	20.67
	pH	2.16	-	4.3	-	17.16
120 h: No Significant Interaction considered						

APPENDIX A.7

Table showing the differences in least square means for the three level interaction between the process variables (Yeast x pH x Nutrient) for the dependent variable Cell Count as observed in Experiment I. Results are tabulated for each of the sampling times.

Time	Yeast	pH	Nutrient	Estimate	p-Value	Significance
48 h	Fermax	4.3	Urea	0.04	0.72	Not Significant
	Fermax	5.4	Urea	-0.11	0.36	Not Significant
	SuperStart	4.3	Urea	-0.22	0.30	Not Significant
	SuperStart	5.4	Urea	0.34	0.01	Significant
	Fermax	4.3	No-Urea	1.14	<0.0001	Highly Significant
	Fermax	4.3	Urea	0.97	<0.0001	Highly Significant
	Fermax	5.4	No-Urea	0.90	<0.0001	Highly Significant
	Fermax	5.4	Urea	1.36	<0.0001	Highly Significant
72 h	Fermax	4.3	Urea	-0.24	0.58	Not Significant
	Fermax	5.4	Urea	-0.03	0.94	Not Significant
	SuperStart	4.3	Urea	-1.04	0.03	Significant
	SuperStart	5.4	Urea	1.01	0.03	Significant
	Fermax	4.3	No-Urea	1.91	0.00	Highly Significant
	Fermax	4.3	Urea	1.11	0.02	Significant
	Fermax	5.4	No-Urea	1.34	0.01	Significant
	Fermax	5.4	Urea	2.38	<0.0001	Highly Significant

APPENDIX A.8

Table showing the differences in least square means for the three level interaction between the process variables (Yeast x pH x Nutrient) for the dependent variable Ethanol as observed in Experiment I. Results are tabulated for each of the sampling times.

Time	Yeast	pH	Nutrient	Estimate	p-Value	Significance
72 h	Fermax	4.3	Urea	-2.18	0.03	Significant
	Fermax	5.4	Urea	-1.95	0.13	Not Significant
	SuperStart	4.3	Urea	-3.08	0.02	Significant
	SuperStart	5.4	Urea	8.27	0.00	Highly Significant
	SuperStart	4.3	No-Urea	14.58	0.00	Highly Significant
	SuperStart	4.3	Urea	14.29	0.00	Highly Significant
	SuperStart	5.4	No-Urea	17.99	0.00	Highly Significant
	SuperStart	5.4	Urea	21.93	0.00	Highly Significant
96 h	Fermax	4.3	Urea	-1.01	0.23	Not Significant
	Fermax	5.4	Urea	0.47	0.57	Not Significant
	SuperStart	4.3	Urea	-4.15	0.00	Highly Significant
	SuperStart	5.4	Urea	5.80	0.00	Highly Significant
	SuperStart	4.3	No-Urea	12.64	0.00	Highly Significant
	SuperStart	4.3	Urea	9.51	0.00	Highly Significant
	SuperStart	5.4	No-Urea	9.44	0.00	Highly Significant
	SuperStart	5.4	Urea	14.76	0.00	Highly Significant

APPENDIX B.1

Table showing the significant source of interaction (p-value) between the process variables (Sampling Location x Fermentation Vessel Size) for the dependent variable a) Ethanol and b) Total Sugar as observed in Experiment II. Results are tabulated for each of the sampling times. [$\alpha = 0.05$]

a) Ethanol

Time	Sample Location	Vessel Size	Size x Location
24 h	0.06	0.51	0.27
48 h	0.61	0.29	0.62
72 h	0.93	0.26	0.37
96 h	0.35	0.17	0.19
120 h	**0.00**	0.20	**0.02**
144 h	0.18	0.23	0.22
168 h	0.99	0.24	0.74
192 h	0.26	0.19	0.34

b) Total Sugar

Time	Location	Size	Size x Location
24 h	0.37	0.94	0.98
48 h	0.39	0.53	0.45
72 h	0.40	0.50	0.43
96 h	0.37	0.50	0.43
120 h	**0.00**	0.54	**0.01**
144 h	0.07	0.52	0.23
168 h	0.83	0.53	0.06
192 h	0.16	0.53	0.83

APPENDIX B.2

Table showing the least square means for the significant two level interaction observed at T=120 h in Experiment II. Results are tabulated for the dependent variables (Total Sugar and Ethanol). [$\alpha = 0.05$]

Dependent Variable	Source	p-Value	Significance
Total Sugar	19-L Top vs. 19-L Bottom	0.00	Significant
	209-L Top vs. 209-L Bottom	0.15	Not Significant
Ethanol	19-L Top vs. 19-L Bottom	0.01	Significant
	209-L Top vs. 209-L Bottom	0.00	Significant

VITA

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Title of Study: "SORGANOL®": IN-FIELD PRODUCTION OF ETHANOL FROM SWEET SORGHUM

Pages in Study: 132 Candidate for the Degree of Master of Science

Major Field: Biosystems and Agricultural Engineering (Food Processing)

Scope and Method of Study: Specific objective of the study was to determine if it was really possible to carry out in-field fermentation of sweet sorghum juice to ethanol with little or no process control. The main objectives of present research are to determine the effect of numerous process variables (yeast type, pH, nutrient and agitation) on in-field ethanol production from sweet sorghum and to determine the nutrient stability of sorghum juice. Testing of parameters enabled us to understand the impact of each variable on the fermentation performance and the sugar to ethanol conversion efficiency. Initial laboratory experiments were conducted to screen different types of fermenting microorganism (*Zymomonas mobilis*, *Kluyveromyces marxianus* and industrial dry distillers yeast, *Saccharomyces cerevisiae*), optimum temperature conditions (7, 37, and 7 and 41^{o}C), optimum pH conditions (pH 4.3, 4.3 and 5.4) and the agitation effect. Two in-field factorial design experiments were conducted using various vessel sizes: 3.8 L, 19 L and 209 L with variables being yeast from two different suppliers (Fermax and Superstart Distillers *S.cerevisiae*), nutrients (with and without urea) and pH (5.4 and 4.3).

Findings and Conclusions: Results indicated that both varieties of yeast tested were able to carry out fermentation under extreme temperature (7 and 37^{o}C). Maximum ethanol produced was 7.9% w/v in 120 h from an initial sugar concentration of 16%. Fermax yeast showed significantly greater amount of ethanol production compared to other fermenting microorganisms. Other process variables such as scaling-up of fermentation vessel, adding urea or lowering pH did not significantly affect sugar to ethanol conversion efficiency of yeasts. Uniform heat and mass distribution (ethanol and sugars) was observed during fermentation in absence of agitation and scaling-up. Sorghum juice composition was stable under refrigerated conditions. Based upon the experimental findings we are able to conclude that in-field fermentation of sorghum juice is possible with no temperature control, no added nutrients, and no pH adjustment and is potentially a feasible process for ethanol production.

ADVISER'S APPROVAL: Dr. Danielle Bellmer