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thesis entitled

MODELING THE EFFECTS OF INITIAL NITROGEN AND TEMPERATURE ON THE FERMENTATION KINETICS OF HARD CIDER

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in BIOSYSTEMS ENGINEERING

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10/27/06
Date

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MODELING THE EFFECTS OF INITIAL NITROGEN AND TEMPERATURE ON FERMENTATION KINETICS OF HARD CIDER

By

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A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTERS OF SCIENCE

Department of Biosystems and Agricultural Engineering

2006

ABSTRACT

MODELING THE EFFECTS OF INITIAL NITROGEN AND TEMPERATURE ON FERMENTATION KINETICS OF HARD CIDER

By

Shantanu Kelkar

The combined effect of nitrogen and temperature on fermentation of apple juice to produce hard cider was studied. Flasks containing apple juice (400 ml) were inoculated with yeast and supplemented with three different levels (100, 300, 600 ppm) of nitrogen in the form of Diammonium phosphate (DAP). The apple juice was then allowed to ferment under isothermal conditions at three temperatures between 11 and 22 °C.

Yeast cell, nitrogen, ethanol and sugar concentrations at various times were evaluted. A simple mechanistic primary model based on Monod kinetics was proposed to describe the process. Kinetic parameters of the primary model were estimated non-linearly via Runge-Kutta method for best fit to one set of experimental data. These kinetic parameters were fit to a secondary model that proposed an Arrhenius relationship with the two independent variables, temperature and initial nitrogen content. The model predictions were validated using a second set of experimental data.

Raw data and secondary model fitting showed that nitrogen did not have a significant Arrhenius effect ($p=0.12$) on growth rate. Temperature had a significant Arrhenius effect ($p<0.05$) on four of the model parameters. The model gave satisfactory predictions for three of the dependent variables, nitrogen, ethanol and sugar. The study showed that dessert apples could be used for hard cider manufacture with ethanol concentrations of over 6.5% and that more complete fermentations could be achieved at higher temperatures and by supplementing nitrogen at the onset of fermentation.

DEDICATION

To my parents, Mohan and Meenal Kelkar and my sister Shivangi for their unconditional love, support and faith in me. To all my friends for their encouragement and best wishes.

ACKNOWLEDGEMENT

A big thank you to my major professor, Dr. Kirk Dolan, for his guidance, patience, encouragement and positive attitude. And for helping me out weekend after weekend! Many thanks to Dr. Pat Oriel for being kind enough to allow the use of his laboratory facilities and for his very valuable inputs on my research. A very special thank you to Dr Janice Harte for her unstinting support and encouragement. Thanks also to my committee members, Dr Gale Strasburg and especially, Dr Bradley Marks, for their support and advice.

Mavis Tan and Darclee Popa, my partners in crime on the hard cider project, thank you for everything! Norm Matella, Ritu Saini and Maria Suparno: thank you for being my peer mentors, for you advice and encouragement. I would also like to express my deepest gratitude to Mitzi Ma, Dharmendra Mishra and Pankaj Kumar for tolerating me, encouraging me and for being there for me, always. Thanks also to Patnarin Benyathiar, Harlem Suniaga, George Nyombaire and Kathy Lai.

I would like to thank Michigan Apple Committee, USDA Rural Development and Uncle John's Cider Mill for supporting this project.

And of course, a big thank you to my family and all my friends who have made my life exciting and worth living; for all their love, support, prayers and best wishes.

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INTRODUCTION

Hard Cider is an alcoholic apple beverage made from fermentation of apple juice. Hard cider is popular in Europe and was consumed widely in the United States in the 18th and 19th centuries. Prohibition laws and easy availability of beer after the American Civil War dealt a blow to consumption of hard cider. After its reintroduction in the 1990s in North America, hard cider has been growing exponentially in popularity. At the same time, a combination of factors has led to excess production of apples, Michigan's most valuable crop (Michigan Apple Committee, 2006). Hence, Michigan apple growers are looking to manufacture value-added products from their excess apple produce.

Hard cider is made from cider apples, which are bitter, sharp and sour. However, North America grows only dessert apples and currently states like Michigan are experiencing an excess apple production. Dessert apples lack the sugar, acidity and tannin levels found in cider apples and are not suitable for making hard cider. However, economics dictate the manufacture of hard cider from dessert apples in the US. Every hard cider brewed has its own history because many of the fermentation variables like type of yeast, composition of apple juice, temperature, nitrogen and other nutrients, fermentation vessel and presence of other microorganisms can change and affect fermentation and the eventual sensory characteristics of hard cider. As hard cider continues to grow in popularity, the scale of production will rise, and there will a need to study and predict fermentation performance. As the industry continues to grow, cider production will move from microbreweries to larger-scale productions. A mechanistic model that could predict the fermentation kinetics of hard cider production would be a useful tool for understanding and designing processes.

Few researchers have studied hard cider fermentation; fewer still have attempted to model the fermentation kinetics. Hard cider fermentation is a process very similar to wine fermentation. Slow and incomplete fermentations are a chronic problem for the wine and beer industries and the factors leading to sluggish and stuck fermentations have been extensively studied (Bisson 1999; Cramer et al. 2002; Del Nobile et al. 2003). Nitrogen compounds are often present in a small amount in grape juice which can be the limiting factor for yeast growth and activity (Bisson and Butzke 2000; Cramer et al. 2002; Del Nobile et al. 2003; Malherbe et al. 2004).

Temperatures affect the rate of fermentation by giving higher rates and shorter fermentations at higher temperatures. It has been shown that temperature can affect the assimilation and uptake of nitrogen and sugar and consequently alter the fermentation rate (Malherbe et al. 2004; Sablayrolles et al. 1996). The Arrhenius relationship between temperature and rate of alcoholic fermentation has also been established (Phisalaphong; et al. 2006).

Hence, the objective of this study was to apply a simple mechanistic model to predict the fermentation kinetics of hard cider made from Michigan apples. The novelty of this work lies in modeling hard cider fermentation, which has not been attempted before. This study is helpful in investigating the fermentation characteristics of dessert apple juice. This study is also amongst the very few to attempt modeling the effect of initial nitrogen levels and temperature on fruit fermentations concurrently. The basic mechanistic model proposed in this study can help explain the process of fermentation as well predict the rate of ethanol production and nitrogen consumption.

Chapter 1. Literature Review

1.1 American Apple and Cider Industry

1.1.1 Hard Cider: Introduction and History

Hard cider, also known as cider, refers to an alcoholic apple beverage. It is manufactured by fermenting apple juice, a process similar to wine making. In some cases, the product is called apple wine. The distinction between hard cider and apple wine is usually made based on alcohol content, but there is much overlap between the two products. Apple wine is usually above 7% alcohol content, and hard cider is usually below 7% (Rowles 2003).

Although hard cider is popular in Europe, it was reintroduced to the American market only in 1990. The product was consumed widely in the 18th and 19th centuries in the U.S., particularly along the East Coast. Hard cider came to the U.S. with the first English settlers, who brought apple seeds with them to plant in their new home. Most of the apple crop was used for the production of hard cider. In fact, in 1767, the per capita consumption of hard cider in Massachusetts is estimated to have been about 40 gallons per person annually (Fabricant 1997)!

Hard cider was a family drink in colonial America (Miller 2004). Many people, even children, drank hard cider with meals. President John Adams was known to drink a pint of hard cider each morning to settle his stomach. Fermented cider sometimes offered a safe alternative to water because the alcohol prevented bacterial contamination. Cider mills were common throughout New York and New England. Families even kept barrels of cider in their basements.

Cider remained a popular beverage until the Civil War when beer began to take its place in the American market. The influx of German immigrants to the U.S. boosted the popularity of beer. Beer was cheaper and easier to produce than hard cider and therefore, it was more attractive to produce commercially. Early in the 20th century, Prohibition dealt the final blow to hard cider's popularity in the U.S. until its recent resurgence.

1.1.2 Michigan Apple Industry

Apples are Michigan's largest and most valuable fruit crop, worth $150 million to the growers and generating a total of nearly $500 million of economic activity in Michigan annually(Committee 2005). Michigan is the nation's largest producer of apple slices for pie filling and frozen pies, and also produces applesauce, dried apples and fresh-cut apple slices. Today, the demand in the U.S. for apple juice and cider exceeds, by far, that for fresh apples. Over the past decade, U.S. apple exports have increased because of liberalization of export markets, substantial industry export promotion efforts and increased disposable income in developing countries. However, in the last few years, U.S. market share of total world apple exports has dropped. China, the European Union and New Zealand have gained market share, while the United States market share of exports has declined (Miller 2004). China has become a major world apple juice producer and a significant supplier to the U.S. market. China has affected the demand for Michigan apples, leading to excess apple production in Michigan. Therefore, Michigan apple growers are looking at ways to utilize their excess apples through manufacture of value-added products, such as hard cider.

Since 1990, the U.S. market for cider has grown rapidly every year with over 4.6 million cases of hard cider sold nationally today, and is expected to exceed 75 million cases in the next ten years (Rowles 2003). Historically, there has been little alcohol production in Michigan because of regulation in Michigan and Canada. Legislation changed in 1996 and the production, as well as the demand in microbreweries and wineries continues to grow ever since.

Michigan being one of the nation's leading producers of apples, most of the infrastructure needed to create a hard cider industry already exists. Trends in the wine and microbrewery industries suggest that locally produced high-quality products are being accepted and sought after by consumers (Proulx 1997). With this in mind, numerous cider mills and microbreweries across the state are entering the hard cider business. The local hard cider market is a small but growing one. Hard cider thus provides an important potential value-added product for Michigan apple producers, brewers and vintners.

1.1.3 Beneficial Effects of Cider Consumption

Many people who do not regularly drink alcohol enjoy hard cider, due to its fruity flavor and low alcohol content. Within this population, studies have shown that females prefer the pleasant flavor of hard cider to beer (Anonymous 1998). Research also suggests that drinking cider may be good for health, as cider is rich in antioxidants known as polyphenols. Antioxidants may help stop cell damage, prevent cancer and degenerative diseases like dementia. These factors will go a long way in making cider the alcoholic beverage of choice in America in future.

Research also suggests that drinking cider may be good for health, as cider is rich in antioxidants known as polyphenols (Guyot 2003). Antioxidants may help stop cell damage, prevent cancer, and decrease the risk of heart disease as well as degenerative diseases like dementia.

Polyphenols in the diet are becoming increasingly recognized as important in long-term health and reduction in the risk of chronic disease. Various experimental studies have investigated the effects of the consumption of food products rich in polymeric polyphenol content such as tea, onions, apples and wine on diseases such as cardiovascular diseases and cancer. These polymeric compounds are also thought to have anti-oxidant properties and are still under investigation.

Additionally, polymeric flavan-3-ols also known as procyanidins are major phenolic constituents in juices and fermented beverages as they are involved in many quality criteria such as bitterness, astringency and shelf life (Alonso-Salces 2001).

1.1.4 Dessert Apples

Apples are the primary raw material for cider making. Traditionally, European hard cider is made from bittersweet or bittersharp 'cider' apples. Polyphenols present in cider apples are responsible for mouthfeel characteristics such as the astringency, and bitter flavor generally associated with fermented beverages (Lea 1990).

Cider apples like Taylor's or Brown's Apple have higher sugar levels, a more fibrous structure and high tannin content as compared to dessert apples like Jonathan or Macintosh. Although traditionally cider was made from true cider cultivars, not all ciders are made from true cider apples; many may contain dessert and culinary varieties. It is

however, rare for cider to be made from single cultivar only because the balance of sugar, acid and tannin required for a successful product is difficult to achieve from any single cultivar. A mix of fresh juice and apple juice concentrates along with other fermentable sugars from cane, beet or high fructose corn syrup are now widely used in English cidermaking and are permissible to a limited extent in France.

Michigan does not grow cider apples. Michigan apples are sweet and classified as 'dessert' apples. Michigan grows more than 20 varieties of apples on a regular basis. Some of the popular and common varieties are Jonathan, Gala, McIntosh, Northern Spy, Red Delicious etc. Michigan grown apples may not have the balance of sugar, acids and tannins required for manufacturing a successful alcoholic product. Michigan apples also have lower tannin content, and consequently, lower procyanidin content. Thus, cider made from Michigan apples may not have the same sensory attributes as that made from cider apples.

1.2 Mainstream Cidermaking (Lea 2004; Proulx 1997)

1.2.1 Juice Preparation

The fruit used in cidermaking is generally ripe and stored for a few weeks after harvest so that all the starch can be converted to sugar. Ripe apples are pulped & pressed; the form of pressing is a specific to region. Most major cider-makers use a high-speed grater mill that feeds a horizontal piston press in a semi-continuous system.

The juice is clarified and collected in tanks. Before fermentation, the juice is blended with fermentable sugar sources such as fresh juice, apple juice concentrate and glucose syrups to the required levels. This mixture may have a specific gravity as high as 1.080-

1.100 to give a final alcohol of 10-12 %, which is then diluted before retail. Nutrients are also added to ensure a complete and speedy fermentation to dryness. Diammonium phosphate maybe added to bring up the levels of free amino nitrogen in the must, which has lower levels than those in grape musts or beer worts. Vitamins like thiamin (0.2 ppm), panthothenate (2.5 ppm) and biotin (7.5 ppb) may also be added.

If clarified concentrates and adjuncts are to be fermented, a source of insoluble solids is often helpful. This source allows the yeast cells a solid surface to rest on and from which ethanol and carbon dioxide can be liberated. Otherwise the yeast tends to compact at the bottom of the vat and a thin layer of these toxic end products builds up around each cell, so that the metabolic activity ceases. Many cider makers also add pectolytic enzymes like pectinase prior to fermentation of fresh.

The most significant adjunct is sulfur dioxide in the form of potassium metabisulfite that controls the growth of acetic and lactic acid bacteria and suppresses the activity of yeasts. The activity of sulfur dioxide decreases after 24 hours and yeast can then be added.

1.3.2 Yeast Fermentation

In traditional cidermaking, no external source of yeast is added. However, since the apples themselves contain mixed yeast microflora, spontaneous fermentation commences within a few hours if the temperature of the juice is above 10 °C.

When no yeast is added and no sulfite is used, the first few days are dominated by non-*Saccharomyces* species, which multiply quickly to produce a rapid evolution of gas and alcohol. They hence generate a distinctive range of flavors. If sulfite is added to the

initial juice, non-*Saccharomyces* yeast and most bacteria are suppressed or killed. This situation gives the *Saccharomyces* time to multiply after a lag phase of several days and the fermentation proceeds to dryness with a more homogenous and benign microflora than in the case of unsulfited juice.

The use of active dry wine yeasts has become almost universal since the 1980s in the mainstream cider industry. Typically used are *S. bayanus* strains and *S. uvarum* or their mixed inoculum on grounds that the *uvarum* will provide a speedy start but *bayanus* copes better with higher alcohol levels and the fermentation to dryness conditions. The strain of yeast used significantly affects the flavors in cider.

The juice is fermented in wood or stainless steel vats or barrels at 15–25°C without mixing. Hard cider is a product of apple juice that has undergone two different kinds of fermentation. The first fermentation is carried out by yeasts in anaerobic conditions, which converts fermentable sugars to alcohol (Figure 1.1).

Figure 1.1 Conversion of fermentable sugars to ethanol

$C_6H_{12}O_6$	$\rightarrow$	$2C_2H_5OH$	+	$2CO_2$
Sugar (Glucose, Fructose)		Alcohol (Ethyl Alcohol)		Carbon Dioxide (Fermentation Gas)

Most UK cidermakers take the view that a complete 'dry' fermentation of cider apple juice yields 10-12% alcohol in as little as two weeks is a desirable objective. Incomplete fermentation can be obtained by removing the yeast halfway throughout the process, thus retaining less alcohol and more fermentable sugars than 'dry' hard ciders. In the U.S, commercial hard ciders usually contain about 5.5% alcohol and most are carbonated (Lea 2004; Proulx 1997).

Cider made from traditional methods is frequently subjected to malolactic fermentation following the yeast fermentation. Malolactic fermentation is the decarboxylation of malic to lactic acid and the consequent evolution of gas. Malolactic fermentation is favored by lack of sulfiting, storage and nutrients released from yeast autolysis. The main organisms effecting this change are *Leuconostoc oenos* and *Lactobacilli* species. In modern factory cidermaking, malolactic fermentation is considered a nuisance and is not encouraged; the possibility is minimized by the use of sulfite.

1.3.3 Racking and Clarification

Once fermentation is complete, the yeast is separated from the cider by a process known as racking. Racking consists of drawing off the cider into clean casks, which causes the suspended yeast to become dormant and sink to the bottom of the liquid. Racking is best done by means of a pump and it may be necessary to repeat the operation one or two more times for best results.

In commercial processes, racking is immediately followed by a clarification process for blending and packing of the final product. In smaller units, the cider may be racked into inert or oak tanks for a maturation period of several weeks. If this process is carried out in traditional wooden vats, it is known as maturation. Maturation is an active microbial process where bacterial inocula present in the pores of wooden tanks are responsible for the flavor character of the cider. If the same process is carried out in vessels made of oak, there is flavor transfer from the oak barrels to cider and the process in known as aging.

Initial clarification may be performed by natural settling of well flocculating yeast, by centrifugation, by fining or a combination of all three. Typical fining agents are gelation or bentonite.

1.3.4 Blending, Final Filtration and Storage

Nearly all ciders are blended before sale. In a large factory, several fermentations may be running concurrently from different must sources and intended for different products. These products form the base ciders from which blending is performed according to the cidermaker's requirement.

Blending involves more than the cider itself. Water may be added to high-alcohol bases for correcting the alcoholic strength for retail sale. Sugar and other sweeteners, malic and other acids, permitted food colors and preservatives may all be added to obtain the final product. The cider is also carbonated. Nearly all cidermakers add 50 ppm of SO_2 at filling to give an equilibrium of 25 ppm free SO_2 in the beverage to inhibit any residual yeast.

Final filtration may take place just before and after blending. Generally, powder filters or coarse disposable sheets are used to produce a bright product, followed by membrane filtration to remove all yeasts and most bacteria. Cross-flow ultra filtration systems are now becoming widespread in the cider industry despite occasional problems of membrane blockage and poor throughput. Most ciders are then pasteurized and carbonated into the final pack. Most large factories have a HTST treatment in a flow-through pasteurizer followed by a chiller and aseptic filling conditions.

Small filters suitable for cider making on the farm are also available. Small filters may use a method of filtration in which the cider under pressure through a vessel containing a quantity of wood or paper pulp. For the production of a clean sparkling natural cider with even a slight degree of sweetness, a filter is almost indispensable. The only substances removed from the liquid by filtering consist of yeast, particles of pomace and dirt. The body, flavor and aroma of the cider remain relatively unchanged.

The storage of cider after the fermentation is over naturally or has been artificially arrested requires extreme care to avoid transformation of the alcohol into vinegar. With the end of the fermentation, protection afforded by the gas released during fermentation is no longer available. Hence, precautions must be taken to exclude the air as much as possible and maintain a low, even temperature in the storehouse. No air lock is now required but the casks should be completely filled and all wastage caused by evaporation through the wood made good from time to time.

1.3 Modeling of Alcoholic Beverage Fermentation

1. 3.1 Justification for research

Production of alcoholic beverages always undergoes changes due to modernization. Improvements to the fermentation step, which, despite being a major part of the fermentation process, is still carried out empirically, are particularly important. Mathematical modeling of fermentation kinetics will enable better process control and thus improve the efficiency of the fermentation process. Mathematical modeling techniques can be used to scale-up from lab scale to commercial beverage production. For example, such models would help cidermakers to predict the effect of sugar and

nitrogen addition on the progress of fermentation and quality of final product. Mathematical models also reduce the number of trial-and-error experiments needed and allow prediction of trends.

Slow and incomplete alcoholic fermentation is a chronic problem for the alcoholic beverage and especially the wine industry. Under certain circumstances, fermentations may take significantly longer than usual to finish or leave residual sugar greater than 0.4%, which is classified as sluggish and stuck fermentation (Cramer et al. 2002; Salmon and Barre 1998). These abnormal fermentation kinetics are considered a serious problem in an industrial setting and can lead to loss of tank capacity due to longer processing time and the potential for further fermentation of the final product due to the residual sugar (Sainz et al. 2003). Hence, early diagnosis of the cause of such fermentation arrest is critical. Currently, an incomplete fermentation is not recognizable until the rate of sugar consumption has been observed to decrease. Thus the ability of a model to predict the fermentation kinetics prior to yeast inoculation and based solely on the apple juice characteristics will be a useful tool. Such a model can then be used to prevent and combat fermentations that may tend be sluggish or stuck.

1. 3.2 Nitrogen Limitation

The most studied cause of sluggish and stuck fermentations is low nitrogen levels. Nitrogen in the apple juice is made up of an ammonia component and a more complex amino-acid based nitrogen component. Nitrogen compounds are often present in a small amount in the must and it can be the limiting factor for yeast growth and activity (Bisson and Butzke 2000). Addition of ammonium ions during the stationary phase can partially

reactivate the hexose transport system and hence increase the fermentation rate (Salmon 1989). Several groups have reported a transition point which may correspond to the point at which biomass no longer increases with increasing initial nitrogen in some juices (Bely 1990, Ingeldew 1985).

Other have shown that nitrogen addition throughout the course of fermentation is also effective to varying degrees in assuring rapid completion of sugar utilization, especially when the nitrogen level is low (Bely et al. 2003). Researchers (Jiranek et al. 1995) found specific amino acids that might limit fermentation in cases and also that amino acids could be grouped into three categories based on the utilization pattern with one group (including arginine) preferentially depleted from the medium. While a detailed mechanism of regulation based on nitrogen components of juice has not been established, it is clear that nitrogen can play a key role in determining both the rate and extent of normal and problem fermentations (Cramer et al. 2002).

It has been observed that exhaustion or near exhaustion of nitrogen corresponds with the time of cessation of the exponential phase of cell growth. In the later stages of fermentation that follow exhaustion of nitrogen, the rate-limiting macronutrient is only sugar.

Slow and incomplete fermentations are a chronic problem for the wine and beer industries. The factors leading to sluggish and stuck fermentations have been extensively studied (Bisson 1999; Cramer et al. 2002; Del Nobile et al. 2003). Nitrogen has also been linked with low cellular activity and resultant biomass concentration in yeast (Monteiro and Bisson 1991; Spayd et al. 1994). Supplementation of nitrogen in the form of

diammonium phosphate or sulfate can alleviate problems that arise from low initial nitrogen level.

Cider apples need 100 ppm of supplemented nitrogen in the form of free alpha-amino nitrogen for complete fermentation. Nitrogen is generally supplemented in the form of Diammonium phosphate (DAP); 100 mg/L of DAP provides 21.1 mg/L of atomic nitrogen that is entirely assimilable (Malherbe et al. 2004). The permissible limit for use of nitrogen for alcoholic beverage production in US is 203 mg/L.

1. 3.3 Temperature Effect

Temperatures affect the rate of fermentation by giving higher rates and shorter fermentations at higher temperatures. The yeast cell membrane permeases have been shown to be highly temperature-dependent due to conformational changes in these molecules (Entian and Barnett 1992). Leao and van Uden (1985, 1982a, 1982b) and Sa-Correia and Van Uden (1983) have shown that for temperatures from 15 to 25°C, glucose transport and glycolytic flux increase steadily with temperature.

Lower temperatures also result in lower rate of amino acid assimilation, which is consistent with the observation of lower rates of fermentation and yeast growth (Lopez et al., 1996). For anisothermal fermentations, when temperatures were raised to the value of an isothermal curve, reactivation was more marked for must with the highest level of assimilable nitrogen curves (Malherbe et al. 2004; Sablayrolles et al. 1996). The rate of fermentation has also been shown to increase almost linearly with temperature and give curves with very similar and normalized superimposed curves (Malherbe et al. 2004).

Although several kinetic models have described single-temperature isothermal conditions(Marin 1999), most fermentations are carried out under anisothermal conditions. A study published recently investigated the effect of temperature on the kinetic parameters of ethanol fermentation (Phisalaphong; et al. 2006). They concluded that the kinetic parameters of the proposed model have an Arrhenius relationship with temperature. The study also observed that temperatures higher than 35 °C led to a decrease in ethanol and cell yields.

Thus, temperature can affect the assimilation and uptake of nitrogen and sugar and consequently alter the fermentation rate. Hence, we have extended our study to include the effect of different fermentation temperatures on fermentation kinetics.

1.3.4 Modeling of Fermentation Kinetics

Currently, there is no available literature on modeling of cider fermentation. However, significant research has been conducted in enology and viticulture. An analogy can be drawn between cider and wine making, as they are both yeast fermentations of fruit or fruit juice.

Fermentation kinetics of products like cider are complex and challenging to model since it may involve numerous yeast strains that adapt to highly variable environmental conditions. The yeast utilizes chemical signals to determine the concentrations of some nutrients, such as fermentable sugars, assimilable nitrogen, oxygen, vitamins, ergosterol and the presence of inhibitory substances such as ethanol, agrochemical residues, killer toxins, and so on to adapt to changes in the extra cellular environment during wine fermentation. While the causes of problem fermentations have

been well documented, we have not yet understood the basic mechanism that results in the cessation of the conversion of sugar to ethanol. Such complexity accounts for the difficulty in predicting the kinetic behavior of the fermentation process. Hence, the construction of an 'exact' model for fermentation is unrealistic. A model may be considered satisfactory if it can be used to predict the state of the bioreactor or fermentation at any point in time in terms of measurable quantities that can be changed or modified for fermentation management (Malherbe et al. 2004).

Several models for microbial growth have been developed over the last 40 years (Marin 1999). Many of these models may be classified as empirical (McKellar and Knight 2000, Peleg 1996, Schaffner 1995) and they describe sigmoidal functions that approximate bacterial growth curves (cell concentration compared with time). Whereas empirical models are useful for correlating a wide range of batch growth data and have predictive value, they fail to provide any real insight into the underlying mechanisms controlling cell growth. On the contrary, mechanistic models, which are more complex from a mathematical point of view, give a description of phenomena involved in cell growth providing valuable quantitative information that can be advantageously used to control microbial growth.

Mechanistic models developed have typically used growth expressions previously established by Monod (1956) or Baranyi (1994). Thus, these models have indirectly linked cell growth and ethanol production using classic kinetic structures. Kinetic parameters in these models are simultaneously dependent on yeast strain, must composition and the development of fermentation, which vary significantly with different fermentation practices.

One of the first comprehensive kinetic models for wine fermentation was reported by Boulton (1980). This mechanistic model included the influence of glucose and fructose levels, ethanol levels, and temperature on sugar utilization and captured the general trend found in practice. Caro et al (1991) used a similar mechanistic model to describe sugar utilization but also included sugar utilization pathways other than ethanol production through respiration in order to address the mismatch in ethanol concentration found in previous models.

Two of the most recent mechanistic models in enology have been proposed by Cramer et al (2002) and Del Nobile et al (2003). Both are unstructured mechanistic models for wine fermentation kinetics and assume nitrogen as the only growth-limiting nutrient. However, Cramer et al. uses Monod relationship to describe the specific growth rate while Del Nobile et al uses the 'inhibition function' derived by Baranyi and Roberts (1994) to describe the specific growth rate. This specific growth rate accounts for the dependence of yeast cell growth rate on nitrogen concentration in the must.

During our experiments, it was observed that once assimilable nitrogen had been exhausted, the growth rate stopped its exponential rise. This phenomenon has been confirmed by other researchers (Cramer et al. 2002).

1.3.5 A Generic Mechanistic Model

When yeast cells are inoculated into a new extracellular environment like apple juice, the cells try to adapt to the new media. Their growth as well as death rate is negligible during this time and this phase is thus known as the lag phase (Baranyi et al 1993a, 1993b). The environmental conditions are favorable for cell growth with high

nutrient concentration in form of fermentable sugars and low levels of toxic metabolites like alcohol. Hence, once the lag phase is over and the cells adapt to the media, the cell proliferation rate rises exponentially while the death rate remains negligible (exponential phase). As the cells grow, they reduce the nutrient concentration and release metabolites that are toxic for them and their growth rate starts dropping until it drops to zero (stationary phase). As this continues, the growth finally becomes less than the death rate leading to a decrease in the cell concentration (death phase). To predict this growth curve, it is necessary to describe the proliferation and death rate of cells as well as the rate of change in the extracellular environment.

Most mechanistic models proposed to describe the fermentation kinetics of alcoholic beverage have the following common features:

1. Nitrogen is the primary growth-limiting nutrient for yeast cell growth (Bailey and Ollis 1986b; Cramer et al. 2002)
2. Sugar consumption and ethanol production is proportional to the viable yeast cell concentration;
3. The death rate of cells is proportional only to the alcohol content (Ansaney-Galeote et al. 2001; D'Amore et al. 1990)

Simple mechanistic models can be described in terms of four differential equations that describe the kinetics of yeast cell, total nitrogen, sugar and ethanol concentrations.

The growth rate of microorganisms is given by the following equation:

$$\frac{dX_V}{dt} = \mu \,.\, X_V - k_d \,.\, X_V \qquad (1.1)$$

where X_V = viable yeast cell concentration, μ = specific growth rate h^{-1}, k_d = ethanol dependent death rate h^{-1}.

The rate of nitrogen consumption or depletion is given by:

$$\frac{dN}{dt} = \frac{\mu . X_V}{Y_{X/N}} \tag{1.2}$$

where $Y_{X/N}$ = Yield co-efficient of biomass on nitrogen.

The rate of sugar depletion in cider is given by:

$$\frac{dS}{dt} = \frac{\beta . X_V}{Y_{E/S}} \tag{1.3}$$

where $Y_{E/S}$ = Yield co-efficient of ethanol on sugar,

β = specific ethanol production rate.

The rate of ethanol production is given by:

$$\frac{dE}{dt} = \beta . X_V \tag{1.4}$$

The above set of differential equations needs to be solved simultaneously and the model predictions need to be compared with the experimental data for validation.

To date, very few researchers have studied hard cider production from dessert apples (Wilson et al. 2003). None of these studies has attempted to study the fermentation kinetics of the process. Although several kinetic models have been proposed in enology literature, very few (Malherbe et al. 2004) have studied the effect of temperature on fermentation kinetics, even though a majority of fermentations are performed under

anisothermal conditions. Further, none of these models have been applied to hard cider manufacturing.

1.3.6 Objectives

Based on information presented in the preceding sections, it is hypothesized that initial nitrogen and external temperature during fermentation will have a significant Arrhenius type effect on fermentation kinetics (Cramer et al. 2002; Del Nobile et al. 2003; Malherbe et al. 2004). Hence, the objectives of the present study were:

1) To show that a mechanistic model based on Monod kinetics can adequately describe hard cider fermentation from dessert apples,
2) To validate the hypothesis that nitrogen is the rate-limiting nutrient for cell growth and that the concentration of initial nitrogen has a significant ($p<0.05$) effect of the Arrhenius type on hard cider fermentation (Cramer et al. 2002; Del Nobile et al. 2003; Malherbe et al. 2004),
3) To validate the hypothesis that external temperature has a significant ($p<0.05$) effect of the Arrhenius type on fermentation kinetics of hard cider (Phisalaphong; et al. 2006).

Hard cider's popularity continues to grow in the US and large-scale production will increase the demand for methods that will help understand and forecast fermentation results. A simple, mechanistic model that could predict the fermentation kinetics of hard cider production, especially from dessert apples, will prove useful in understanding and designing processes.

CHAPTER 2: Materials and Methods

2.1 Fermentation Setup

Apple Juice

Hard cider is generally made from fermentation of a blend of as many as dozen different varieties of mostly cider and some dessert apples. However, using such a blend for fermentation experiments would increase the variability in the composition of apple juice and add additional factors to the model. Cider apples are not cultivated on a large scale in North America. Efforts to grow them in parts of United States have not been very successful. Since dessert apples are facing the problem of excess production, it makes economic sense to produce hard cider from dessert apples. Consequently, only one variety of American dessert apples, i.e., Jonathan apples, was selected for experimentation.

Jonathan apples are generally small to medium in size and dark to bright red in color. They are used for cooking and baking as well as fresh eating. The peak time of the year for the availability of Jonathans is early fall to late winter, and they are the third highest-volume apple produced in Michigan. However, Red Delicious, Golden Delicious, Gala and Jonagold have a higher consumer demand, resulting in an excess production of Jonathan apples (Michigan Apple Committee 2005). Jonathan apples are juicy, aromatic and moderately tart. All these factors make Jonathan apples ideal candidates for manufacturing value-added products such as cider.

Michigan Jonathan apple juice was obtained at Uncle John's Cider Mill (St. Johns, MI, USA) and stored at –18°C in plastic one-gallon containers for a period of 3-8 months.

The approximate sugar concentration of the apple juice was 120 g/L and the nitrogen concentration was 45 mg/L. The pH was measured to be 3.26 ± 0.03.

Yeast

Lalvin's DV10 *Saccharomyces cerevisiae (bayanus)* yeast (Lallemand Inc, Rexdale, ON) was selected. The juice was inoculated with 0.3 g dry weight/L of DV10. As per the manufacturers' recommendations, the yeast was not rehydrated before inoculation. DV10 is one of the most widely used strains for champagne production and is the most recommended strain for cider and mead (an alcoholic beverage made from fermented honey and water) production. DV10 has strong fermentation kinetics over a wide temperature range (10-35° C) with relatively lower oxygen and nitrogen demands. DV10 is known for clean fermentations that avoid bitter sensory attributes associated with many other strains. It has an 18% alcohol tolerance and can ferment under stressful conditions of low pH or high SO_2. It is also low foaming and low volatile acid production, a factor that may affect the sensory attributes of the final cider (Lallemand Inc, Rexdale, ON).

Nitrogen Supplementation

Apple juice contains considerably less free amino nitrogen than grape must or beer worts and this lack of nitrogen can place a severe limit on yeast growth,. Therefore, common practice is to bring the level up to ca 100 mg nitrogen per liter (Lea 2004), which can be achieved by adding 250 ppm ammonium sulfate or phosphate. 100 ppm of diammonium phosphate (Sigma Chemical Co., St. Louis, MO), henceforth referred to as

DAP, provides 21.1 mg/L of atomic nitrogen, entirely assimilable (Malherbe et al. 2004). The permissible limit for nitrogen supplementation in the form of DAP in the US is 960 mg/L of DAP, corresponding to 203 mg of nitrogen/L (203 ppm).

Experimentation

Frozen Jonathan apple juice stored in 1-gallon containers was thawed. The juice was sterilized by addition of 50 ppm of potassium metabisulfite (Sigma Chemical Co., St. Louis, MO) and left to stand for 24 hours. Potassium metabisulfite inhibits the growth of spoilage yeasts and bacteria thus permitting the desirable fermenting yeasts (such as *Saccharomyces cerevisiae* or *uvarum*) used for inoculation to multiply and dominate the conversion of sugar to alcohol. For apple juice with pH of 3.0 to 3.3, addition of 50-75 ppm potassium metabisulfite is recommended (Lea 2004). After 24 hours, the sulfited juice was transferred into 500-ml flasks filled up to the 400-ml level and inoculated with the DV10 yeast. For each condition of temperature and initial nitrogen level, triplicates were fermented (Table 2.1). All flasks were fitted with fermentation locks (Michigan Brewing Company, Webberville, MI). The control was flasks containing sulfited apple juice that did not contain any added yeast inoculum or DAP. This control sample allowed us to track any unforeseen changes in experimental conditions or any contamination of samples after the start of experiments. The fermentation flasks were stored in incubators with temperature control during experimentation. The flasks were also minimally agitated at 60 rpm using flask shaker tables for uniformity of the samples and their temperature.

Four levels of initial nitrogen and three levels of temperature were selected. The four levels of nitrogen were selected to represent the range of total initial nitrogen

between the residual concentration (in apple juice) of 45 ppm to the legally permissible limit of 200 ppm (Lea 2004; Malherbe et al. 2004). Hard cider fermentations are usually performed at cooler temperatures. Traditional fermentations are carried out at temperatures of >10 °C and a range of 15-25 °C is considered preferable (Lea 2004). It has been found from sensory analysis (unpublished data) that pure, unblended hard cider produced at higher temperature (>20 °C) has sensory attributes that many consumers do not like. Hence the three temperatures, 11 °C, 17 °C and room temperature of 22 °C were selected. Temperature measurements of air inside the incubator at periodic intervals showed that the variation in these temperatures was ±2 °C. The cider samples were not measured for temperature. It was assumed that for the small volumes of fermenting cider, use of shakers and the natural agitation resulting from the formation of carbon dioxide inside the cider would help maintain uniformity of temperature of the sample. However, since fermentation is an exothermic process, it is possible that the internal sample temperature may be higher than the external environment. However, this is akin to an actual fermentation in microbreweries where only the external environment is controlled and the fermenting samples may be at a much higher temperature.

The experimental plan, tabulated below, was repeated three more times giving a total of four sets of data. One of these sets of data was rejected due many errors in analyzing the samples. Of the three remaining sets of data, one data set was used for estimating the parameters of the proposed mathematical model. This data set is henceforth referred to as the 'parameter estimation data set'. The two remaining data sets were combined and used to validate the model. This combined data is henceforth referred to as the 'model validation data set'.

Table 2.1: Experimental Plan

Flask	Temperature (°C)	Yeast (0.3g/L) Y = Yes, N = No	DAP (mg/L)
1	11	N	0
2	11	N	0
3	11	N	0
4	11	Y	0
5	11	Y	0
6	11	Y	0
7	11	Y	100
8	11	Y	100
9	11	Y	100
10	11	Y	300
11	11	Y	300
12	11	Y	300
13	11	Y	600
14	11	Y	600
15	11	Y	600
16	17	N	0
17	17	N	0
18	17	N	0
19	17	Y	0
20	17	Y	0
21	17	Y	0
22	17	Y	100
23	17	Y	100
24	17	Y	100
25	17	Y	300
26	17	Y	300
27	17	Y	300
28	17	Y	600
29	17	Y	600
30	17	Y	600
31	22	N	0
32	22	N	0
33	22	N	0
34	22	Y	0
35	22	Y	0
36	22	Y	0
37	22	Y	100
38	22	Y	100
39	22	Y	100
40	22	Y	300
41	22	Y	300
42	22	Y	300
43	22	Y	600
44	22	Y	600
45	22	Y	600

Figure 2.1 Fermentation Setup

2.2 Methods of Analysis

Sample Preparation

Prior to sampling, each flask was mixed by swirling in order to suspend all solids and achieve uniformity. Samples were drawn at various time intervals using 10-mL disposable pipettes to prevent cross contamination, and stored in disposable plastic test-tubes (Corning Inc., Corning, NY). Each test-tube was agitated to mix the sample. 50 μL of the sample was withdrawn in triplicate into wells of a 96-well plate for enumeration of yeast cell counts. Another 3ml was withdrawn and centrifuged (Beckman Coulter Inc., Fullerton, CA) at 3000 rpm for 5 minutes to settle suspended solids. The centrifuged samples were then further clarified by filtration through 25 mm Nylon 0.2 μm disposable syringe filters (Waters Corp., Milford, MA). The purified samples were stored in 1.5 ml microcentrifuge vials (BioDot Inc., Irvine, CA) and frozen for analysis later. The original unfiltered samples were also stored frozen in test tubes.

Due to lack of sufficient resources and the large number of samples that needed sampling and analysis, it was not possible to take samples at periodic intervals. Additionally, some of samples did not generate any data points due to errors during analysis. Hence, the number of observed data points differs for different conditions and variables.

Viable Yeast Cell Concentration

Viable cell numbers were estimated microscopically using a Neubauer-type Bright Line Counting Chamber (Hausser Scientific, Horsham, PA). Triplicates of each sample (50 µL) were withdrawn into wells of a 96-well plate. To each well, 50 µl of 0.4% (w/v) Trypan blue was added to dye the non-viable cells and ensure that only viable cells were enumerated (Nielsen et al. 1991). The well was mixed thoroughly using a pipette. This mixture of sample and trypan blue formed a 1:2 dilution of the cells. The haemocytometer and the cover slip were cleaned and dried. The cover slip was then placed over the counting surface prior to loading the cell suspension. From each well, 10 µl of cell mixture was transferred to each of the two V-shaped wells with a Pasteur pipette. The chamber fills by capillary action. The charged counting chamber was then placed on the microscope (Reichert Microscope Services, Depew, NY) stage and brought into focus at 40X power.

Because the counting chamber has an exact volume under the cover slip, one can determine the concentration (cells/mm^3) of live and dead cells in the chamber. The cell concentration of the original cell suspension will be the same as that of the chamber. Dead cells take up the trypan blue dye and appear blue under the microscope. Living cells

exclude trypan blue, and appear white. Thus, the percentage of viable cells can be calculated.

The viable cell density of the original mixture was determined according to the following formula:

The number of cells per cubic millimeter = number of non-dyed cells counted per square millimeter of counting chamber X Dilution X 10

Assimilable Nitrogen

Nitrogen that can be utilized by yeast cells for their growth or maintenance is known as assimilable nitrogen. Assimilable nitrogen in cider is in the form of ammonium ions and free α-amino nitrogen compounds. It is necessary to estimate the concentration of both these forms as they represent all the total nitrogenous compounds that are directly utilized by the yeast for growth. Detailed below are the two procedures for determining ammonium ion concentration and the α –amino nitrogen concentration.

Ammonium ion concentration was determined using an Ammonium Ion Selective Electrode (Cole-Parmer Instrument Co., Vernon Hills, IL). The electrode was connected to a pH meter (Corning pH meter 440, Cole-Parmer Instrument Co., Vernon Hills, IL) and the reading scale was changed to millivolt. The Ion Selective Electrode method was selected for its ease of use and has been shown to be accurate and consistent with readings obtained by an enzymatic assay (Turbow, S.B., Wehmeier, G.H., et al., 2002).

The electrode was first calibrated as recommended by the manufacturer. A 1000-ppm standard ammonium solution was prepared by adding 2.97 grams of reagent grade ammonium chloride (Sigma Chemical Co., St. Louis, MO) to 1 liter of distilled water in a

volumetric flask. Standard solutions of 100 and 10-ppm ammonium solutions were prepared by dilutions from this 1000-ppm solution. A standard curve of ammonium ions versus voltage reading was obtained by plotting the readings in triplicates for the above solutions. The concentration of ammonium ions was determined from the standard curve and the voltage reading of the cider samples. All readings were taken in triplicate at room temperature with a 3 ml sample in a 25 ml test tube. Sodium chloride (5M) was added at a concentration of 2ml for 100ml sample as a standard base ion concentration.

The α -amino nitrogen was determined using the enzymatic method of Dukes and Butzke (1998). The method uses a spectrophotometric procedure to measure the primary amino nitrogen fraction and thus quantify the levels of yeast assimilable nitrogenous compounds in cider. The assay is based on the derivatization of primary amino groups with an o-phthaldialdehyde/N-acetyl-N-cysteine (OPA/NAC) reagent. The resulting isoindole derivatives form rapidly and are stable (absorbance) at 335 nm.

The reagent solution consisted of 0.671 g of ortho-phthaldialdehyde or OPA dissolved and made to 100 ml with 95 % ethyl alcohol. This solution was added to a 1000-ml volumetric flask that contained an aqueous solution of 3.84 g sodium hydroxide, 8.47 g ortho-boric acid and 0.816 g N-acetyl-L-cystiene. The flask was then made up to volume with deionized water. The same buffer was made without OPA. Both the solutions were stored at 4 °C and are stable for three weeks. All of the above chemicals were sourced from Sigma-Aldrich Chemical Co., St. Louis, MO.

For analyzing α-amino nitrogen, 50 µl of the centrifuged cider sample was placed in a 10 ml test tube to which 3 ml of the above reagent containing OPA was added. The tube was vortexed and decanted into a UV-grade methyl acrylate cuvette. A juice blank

was also analyzed using the reagent buffer that did not contain OPA. The absorbance of the samples was measured at 335 nm using a DU 520 General Purpose UV/Vis spectrophotometer (Beckman, Fullerton, CA, USA). All measures were carried out at room temperature and the net absorbance was calculated by subtracting the absorbance of the blank from than of the sample.

Total assimilable nitrogen was determined from the sum of nitrogen in the form of ammonium ions and α -amino nitrogen.

Sugar

Individual sugars were measured using a Waters 6100 HPLC system with Hamilton PRP-X300 Ion Exclusion column. The HPLC apparatus consisted of 717 Autosampler, a 996 Refractive Index Detector and Breeze 32 Manager System. The method used a mobile phase containing 1 mM Sulfuric acid and 0.001 N H_2SO_4. The sample injection volume was 20 µl while the elution conditions included an isocratic gradient and a flow rate of 2ml/min. The column and detector were both at room temperature (~25 °C).

Standard peaks of glucose, fructose and sucrose (Sigma-Aldrich Chemical Co., St. Louis, MO) were monitored at ambient column temperature. The samples were loaded into the HPLC in a Waters 1-ml glass vial with polyethylene snap cap for a run time of 5 minutes.

Apple juice and hard cider samples were spiked with glucose to validate the above process. The chromatograms are shown in Appendix E.

Ethanol

Ethanol concentration was measured using a Waters 6100 HPLC system with Hamilton PRP-X300 Ion Exclusion column. The HPLC apparatus consisted of 717 Autosampler, a 996 Refractive Index Detector and Breeze 32 Manager System. The method used a mobile phase containing 1 mM sulfuric acid. The sample injection volume was 10 µl while the elution conditions were as follows: isocratic gradient, flow rate of 1ml/min. The column and detector were both at room temperature (~25 °C).

Standard peaks of 99.9 % pure ethanol (Sigma-Aldrich Chemical Co., St. Louis, MO) were measured at ambient column temperature to develop a standard curve. The samples were loaded into the HPLC in a Waters 1-ml glass vial with polyethylene snap cap for a run time of 15 minutes.

The above process of ethanol detection was validated by spiking hard cider samples with ethanol. The chromatograms are shown in Appendix E

2.3 Model Development & Validation

2.3.1 Primary Model

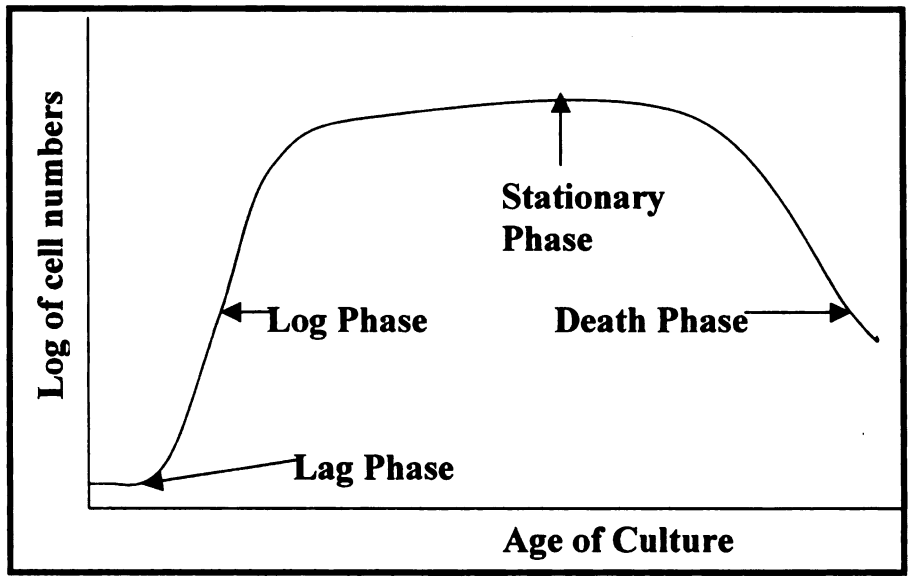


Figure 2.2. Yeast Life Cycle

When yeast cells are inoculated into a new extracellular environment like apple juice, the cells try to adapt to the new media. Their growth, as well as death rate, is negligible during this time. This phase is thus known as the lag phase (Baranyi and Roberts 1993a; Baranyi and Roberts 1993b). The environmental conditions are favorable for cell growth with high nutrient concentration in form of fermentable sugars and low levels of toxic metabolites like alcohol. Hence, once the lag phase is over and the cells adapt to the media, the cell proliferation rate rises exponentially while the death rate remains negligible (exponential phase). As the cells grow, they reduce the nutrient concentration and release metabolites that are toxic for them and their growth rate starts dropping until it drops to zero (stationary phase). As this continues, the growth rate finally becomes less than the death rate leading to a decrease in the cell concentration

(death phase). To predict this growth curve, it is necessary to describe the proliferation and death rate of cells as well as the rate of change in the extracellular environment.

Most mechanistic models proposed for alcoholic beverage fermentation kinetics have the following common features:

1. Nitrogen is the primary growth-limiting nutrient for yeast cell growth (Cramer et al. 2002);
2. Sugar consumption and ethanol production are proportional to the viable yeast cell concentration;
3. The death rate of cells is proportional only to the alcohol content (Ansaney-Galeote et al. 2001)

A simple mechanistic model that describes the kinetics of yeast cell, total nitrogen, sugar and ethanol concentrations in terms of four ordinary differential equations was used to describe the fermentation process.

The growth rate of microorganisms is given by equation (1.1):

$$\frac{dX_V}{dt} = \mu \,.\, X_V - k_d \,.\, X_V$$

Where X_v = viable yeast cell concentration, cells/L, μ = specific growth rate (h^{-1}),

k_d = ethanol dependent death rate (g L ethanol^{-1} h^{-1}).

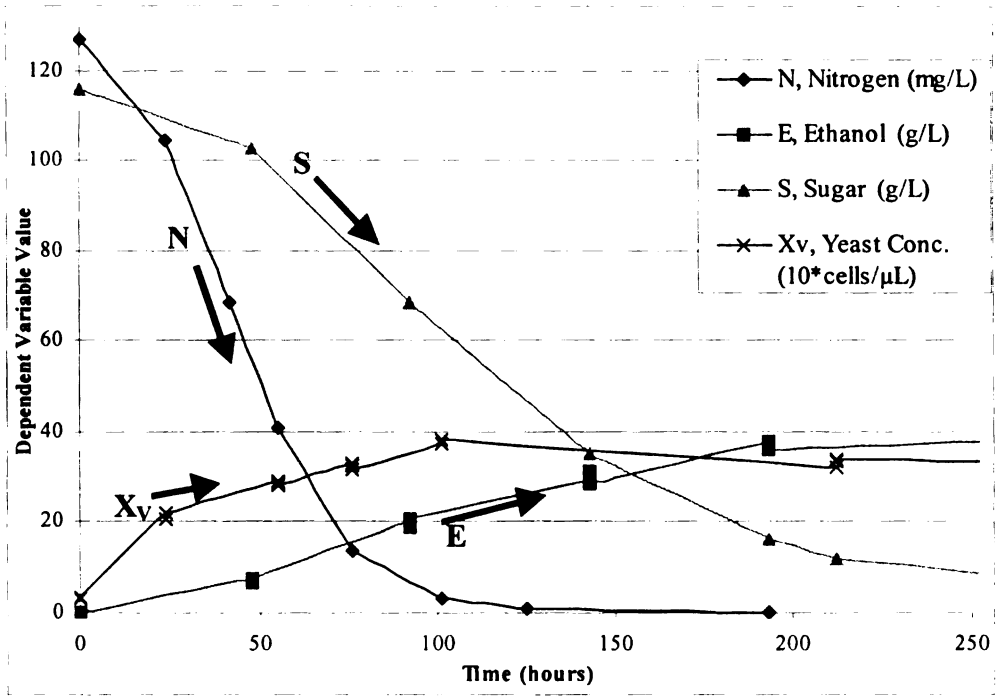


Figure 2.2. Generic Plot of Dependent Variables vs. time

The proposed model is based on Monod kinetics. The figure above shows a generic plot for the four dependent variables as a function of time and helps explain the principles behind Monod kinetics. *Y* represents any dependent variable. It has been proven that the growth rate of microorganisms is hyperbolic in nature when only one nutrient is growth limiting and all other nutrients remain the same (Bailey and Ollis 1986a). Monod (1942) proposed a functional relationship between the specific growth rate μ and the limiting nutrient's concentration (Bailey and Ollis 1986a). It has been observed in this and other studies that the sugar concentration does not change appreciably until the end of the exponential phase of growth and the beginning of the stationary phase (Cramer et al. 2002; Del Nobile et al. 2003; Malherbe et al. 2004). Most of the sugar is utilized during the stationary phase; thus, sugar is not a growth rate-

limiting nutrient. It has also been observed that near exhaustion of nitrogen in the fermenting juice coincides with the end of the exponential phase of cell growth and the start of the process of significant ethanol production (Cramer et al. 2002; Del Nobile et al. 2003; Malherbe et al. 2004). Hence, nitrogen was considered as the rate-limiting nutrient and cell growth was proportional to the total nitrogen concentration. Of the same form as the Langmuir adsorption isotherm (1918) and the standard rate equation for enzyme-catalyzed reactions with single substrate (Henri, 1902 and Michaelis and Menten, 1913), the Monod equation states that

$$\mu = \frac{\mu_m N}{K_N + N} \tag{2.1}$$

Where μ_m = maximum specific growth rate (hr^{-1})

N=nitrogen concentration (ppm)

K_N= Monod constant for Nitrogen (ppm)

The rate of nitrogen consumption or depletion is given by (1.2):

$$\frac{dN}{dt} = \frac{\mu . X_V}{Y_{X/N}}$$

where $Y_{X/N}$ = Yield co-efficient of biomass on nitrogen. This is different from Y, which represents any dependent variable.

The rate of sugar depletion in cider is given by (1.3):

$$\frac{dS}{dt} = \frac{\beta . X_V}{Y_{E/S}}$$

where $Y_{E/S}$ = Yield co-efficient of ethanol on sugar. This is different from Y, which represents any dependent variable.

β = specific ethanol production rate, (g ethanol/cell-hour).

It has been shown that most of the conversion from sugar to ethanol occurs during the stationary phase and is non-growth associated (Cramer et al. 2002; Del Nobile et al. 2003; Ludeking and Piret 1959). Although this does not rule out ethanol formation during the growth phase, it assumes that ethanol produced during the growth phase is an insignificant part of the total ethanol production, a trend also shown by the experimental data. Hence the model does not include any term to describe the relationship between growth rate and ethanol production rate.

The model does not distinguish between the types of sugars in fermenting juice. Although most of the sugar is in the form of glucose and fructose and can be directly used by the yeast, a small quantity is sucrose. Yeast breaks down sucrose into assimilable components, namely, glucose and fructose, using the enzyme invertase synthesized by the yeast itself.

Thus

$$\beta = \frac{\beta_m S}{K_S + S} \tag{2.2}$$

Where β_m= maximum specific ethanol production rate, (g ethanol/cell-hour)

S=sugar concentration (g/L)

K_S= Monod constant for sugar (g/L)

This form of specific ethanol production rate is consistent with previous observations that sugar transport is facilitated by diffusion, which in turn, is governed by the concentration of sugar and ethanol.

The rate of ethanol production is given by equation (1.4):

$$\frac{dE}{dt} = \beta \,.\, X_V$$

The primary model is defined as the set of equations from (1.1) to (1.4) and equations (2.1) and (2.2).

2.3.2 Procedure for Parameter Evaluation

The following plan of action was adopted to estimate the model parameters:

Step 1: Initial Estimates for Parameters

The primary model contains seven parameters of which six can be estimated initially from previous literature and our experimental data. Observations from studies by (Cramer et al. 2002) and (Del Nobile et al. 2003) were used to obtain initial estimates.

The estimate for μ_m, maximum specific growth rate (hr^{-1}), was obtained from the slope of the semi log plot of viable cell concentration and versus time. These values were plugged into the model and the model was solved using a program in MATLAB© to compare the predictions with raw data. The parameter was then manually adjusted along with other parameters until a 'visual fit' to the experimental data was obtained.

βm, specific ethanol production rate, (g ethanol/cell-hour), was similarly obtained from equation (1.3) from the slope of a plot of specific ethanol production rate versus time.

The yield co-efficient of biomass on nitrogen, $Y_{X/N}$ (no. of cells/g N), is the stoichiometric yield coefficient of biomass on nitrogen. The initial estimate for $Y_{X/N}$ was obtained from literature (Shuler and Kargi 2001).

k_d (g L ethanol^{-1} h^{-1}) is the ethanol-independent death rate constant and was obtained from literature (Cramer et al. 2002). Its value was fixed at 0.005. This value cannot be

easily calculated from experimental data and little previous literature is available on the subject. The ethanol independent death rate constant shows a noticeable effect on the stationary and death phases of cell growth; however it was observed to have a minor effect on the prediction of other dependent variables.

The yield co-efficient of ethanol on sugar, $Y_{E/S}$ (g ethanol/ g sugar), is the stoichiometric yield coefficient of ethanol on nitrogen and can be obtained from simple stoichiometric calculations from the equation in figure 1.1 on the experimental data.

$C_6H_{12}O_6$	$\rightarrow$	$2C_2H_5OH$	+ 2CO2
Sugar		Alcohol	Carbon Dioxide
(Glucose, Fructose)		(Ethyl Alcohol)	(Fermentation Gas)
~116 g		~ 45 g	

Based on experimental data it was found that the conversion of sugar into ethanol was approximately 38%. Hence the value of this parameter, $Y_{E/S}$ (g ethanol/ g sugar), was fixed at 0.38.

From preliminary data fitting, it was observed that due to complexity of non-linear parameter estimation, the model would not converge and terminated without results when more than five parameters were included in the parameter estimation process. Hence, the five more important parameters that showed an effect of change in independent variables were selected for the non-linear estimation process. The remaining two, $Y_{E/S}$ and k_d were given fixed values and were excluded from the non-linear parameter estimation process.

Step 2: Primary Model & Non-Linear Estimation of Parameters

To obtain a solution to the primary model, the above set of non-linear, coupled, ordinary differential equations need to be solved simultaneously using numerical integration techniques. A program was written in MATLAB© (Mathworks Inc, Natick, MA) to solve the set of four differential equations using 4th-order Runge Kutta formula. The model consists of ordinary differential equations (ODE) for which we have only one value for each of the dependent variables, that is, the value at time t=0. A numerical solution to this problem can be obtained as given below.

The model is a coupled set of 1st order differential equations of the form

$$\frac{dy}{dt} = f(y,t) \tag{2.3}$$

Where f, y = n-dimensional vectors; y (or Y) is any dependent variable, t is time.

Formulae of the Runge-Kutta type are among the most widely used for numerical solutions to ODE (Hornbeck 1975). The Runge-Kutta formulae require the calculation of several intermediate values of the function between t_j and $t_{(j+1)}$. Before each of these values can be calculated, a corresponding 'y' value must be found.

When we have a coupled set of equations as in the proposed model, complete vectors, that is, all the vectors in this coupled set must be calculated at each intermediate point before moving to the next intermediate point.

The 4th order Runge-Kutta formula is as follows:

$$y_{j+1} = y_j + \Delta t[\frac{1}{6} f(y_j, t_j) + \frac{1}{3} f(y^*_{j+1/2}, t_{j+1/2}) + \frac{1}{3} f(y^{**}_{j+1/2}, t_{j+1/2}) + \frac{1}{6} f(y_{j+1}, t_{j+1})] \tag{2.4}$$

Where

$$y^*_{j+1/2} = y_j + \frac{\Delta t}{2}.f(y_j, t_j)$$

$$y^{**}_{j+1/2} = y_j + \frac{\Delta t}{2}.f(y^*_{j+1/2}, t_{j+1/2})$$

$$y^*_{j+1} = y_j + \Delta t.f(y^{**}_{j+1/2}, t_{j+1/2})$$

Since there is a cross coupling between the equations for these vectors, it becomes necessary to update all the components of each vector before moving on to the next vector. The intermediate values must be computed in the order given above because they are interdependent. This formula requires four evaluations of *f*, which is quite time consuming. It is essential to verify that the step size is sufficiently small to give accurate answers.

The advantages of using Runge-Kutta formulae are (Hornbeck 1975):

1) Ease of programming,
2) Good stability characteristics,
3) Step-size can be changed without complications,
4) Self starting.

The disadvantages are:

1) Require significantly more computing time than other methods,
2) Local error estimates are difficult to obtain

The initial parameter estimates were substituted into the model, which was solved using 4th order Runge-Kutta formula and the software MATLAB©. Graphs comparing the experimental data with the model predictions were plotted in MATLAB©. The initial parameter estimates were manually adjusted until a good visual fit with the experimental

data was observed. The 'parameter estimation data set' (~ 110 data points per each condition), that is, data from one set of experiments was selected as the experimental data for this procedure.

Non-linear fitting estimates the coefficients (parameters) of a non-linear regression function using least squares estimation. The values of the parameters were estimated using non-linear regression for best fit to experimental data in MATLAB©.

Since the numerical value of X_V, yeast cell concentration was many magnitudes higher than the rest of variables; the error was highly magnified, skewing the non-linear regression fitting process. This skewing led to termination of the data-fitting process without any results or incorrect estimations of model parameters and poor fits to experimental data. Weights were therefore assigned to each of the dependent variables for the least squares estimation. Even for very small weights for the dependent variable Xv, there were errors in the execution of the data-fitting process. Hence it was decided to assign a zero weight to this variable.

$W_{Xv} = 0$

$W_N = 1$

$W_E = 1$

$W_S = 1$

Where W= weight assigned for non-linear least squares estimation

Although X_v, yeast cell concentration, was assigned a zero weight for least squares estimation, all the dependent variables were inter-connected. Due to cross coupling of all the model equations, the parameters affecting X_v were estimated with reasonable accuracy. Consequently, all the dependent variables including X_v were

predicted with reasonable accuracy in most cases. However, as a result of excluding X_v from the data fitting process, prediction and asymptotic confidence bands for this variable were not obtained.

The confidence and predictions bands for the predicted dependent variables and the confidence intervals for the non-linear estimated parameters were calculated in MATLAB© using the following commands:

ci=nlparci (P_{FINAL},*r*,*J*)

where *ci*=confidence interval for the parameters

P_{FINAL} = vector of all the non-linear estimations of parameters

[*ypred, delta*] = nlpredci ('function', *t*, P_{FINAL}, r, J, 0.05, 'on', 'curve')

Where *ypred*= predicted dependent variables;

t= time;

J=Jacobian matrix of predicted data

r=residuals from model predictions

The function 'curve' and the value '0.05' tells MATLAB© to calculate 95% confidence bands for the mean, the predicted curve for the dependent variable.

Similarly, the use of function 'observation' results in calculation of 95% prediction bands for the observed data.

2.3.3 Secondary Model & Linear Estimation of Parameters

It is hypothesized that the kinetic parameters of the model have an Arrhenius relationship with the independent variables. This relationship can be described in terms of a secondary model as follows:

$$k = f(T, N_i)$$

or

$$\ln k = \ln k_r + \frac{E_T}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right) + \frac{E_N}{R}\left(\frac{1}{N_i} - \frac{1}{N_r}\right) \qquad (2.5)$$

Where k= non-linearly estimated parameter from the previous section

T= temperature at which parameter was evaluated (°K)

N_i=initial nitrogen level at which parameter was evaluated (ppm)

T_r=reference temperature (°K)

E_T=activation energy for temperature effect (J/gmol)

E_N=activation energy for nitrogen effect (J/gmol)

k_r= value of the same parameter at reference temperature and reference initial nitrogen

R= Universal gas constant, 8.31 J K^{-1} mol^{-1}.

The non-linear parameter estimates from Step 2 were fitted to the above equation using multiple linear regression in Excel©. The linear estimation yielded parameters k_r, E_T/R and E_N/R for each non-linearly estimated parameter. The linearly estimated parameters were substituted into the above equation to determine all k for each condition of temperature and initial nitrogen.

Step 2.3.4 Procedure for Model Validation

The non-linear parameter estimates were fitted by multiple linear regression to equation (2.5). This secondary model was then used to calculate the predicted parameter

values for 12 different conditions of temperature, T and initial nitrogen content, N_i using the 'model validation data set'. Note that this data consists of more data points (~250 per condition of nitrogen and temperature) and is different from the 'parameter estimation data set' used for estimating the parameters. These predicted parameters were substituted into the primary model to get the model predictions for the four dependant variables (X_v, N, S, E) over time. These predicted values of X_v, N, S, and E were compared to the validation data set.

A plot of each dependent variable versus time at every temperature-initial nitrogen condition was obtained. This plot included the experimental and predicted data as well as the asymptotic confidence bands and prediction bands (using function 'nlpredci' in MATLAB©.) for the three dependent variables, N, E and S. A plot of the predicted versus observed values was obtained for each of the 4 variables X_v, N, E and S to evaluate the accuracy of predictions along with the root mean square error. The root mean square error (RMSE) of the prediction was calculated as

$$RMSE = \sqrt{\frac{SS}{n-p}} \tag{2.6}$$

Where SS=sum of squared errors between predicted and experimental value of each dependent variable

n=number of data points

p=number of estimated parameters.

Thus the effect of initial nitrogen content and temperature on fermentation kinetics was studied.

Chapter 3. RESULTS & DISCUSSIONS

3.1 Initial Estimates for Parameters

The initial estimates for the seven parameters were obtained from literature and experimental data as described in section 2.3.1.

Table 3.1: Initial Estimates for Model Parameters

Temp	DAP added	Nitrogen	μ_m	β_m	K_N	K_S	$Y_{X/N}$
(°C)	(ppm)	(ppm)	(hr^{-1})	(g ethanol $cell^{-1}$ hr^{-1})	(g N/L)	(g sugar/L)	(no. of cells/ g N)
11	**0**	**45**	0.14	7.37E-10	172.32	14.589	5.00E+06
11	**100**	**65**	0.1	8.00E-10	2000	10	1.50E+06
11	**300**	**110**	0.2	8.00E-10	2000	10	1.50E+06
11	**600**	**165**	0.45	1.50E-09	4000	90	2.00E+06
17	**0**	**45**	1.25	5.00E-10	500	10	2.00E+06
17	**100**	**65**	1.9	9.00E-10	2000		1.00E+06
17	**300**	**110**	2	1.00E-09	5000	50	1.00E+06
17	**600**	**165**	2.3	9.10E-10	7700	10	1.20E+06
22	**0**	**45**	2.17	9.71E-10	243.11	100	1.00E+06
22	**100**	**65**	2.45	1.20E-09	200	40	2.00E+06
22	**300**	**110**	2.9	1.50E-09	2500	80	1.80E+06
22	**600**	**165**	3	9.00E-10	14200	38	2.50E+06

3.2 Non-Linear Parameter Estimation from Primary Model

Using these initial estimates, the model was solved for each condition by the ode15s routine in MATLAB© that uses 4th-order Runge-Kutta formula. After solving the model once using the initial estimates, the program compared the predictions of the model with the observed values. It then used non-linear regression to iterate and estimate the parameters for the best fit (minimization of least squares) to experimental data. The program code and the experimental data are elaborated in Appendix A.

Due to the complexity of representing all the information obtained from fitting the model to data, the Results section will demonstrate the working of the model for one condition of independent variables, temperature (22 °C) and initial nitrogen content (65 ppm, corresponding to 100 ppm of added DAP). This condition was selected as good fits of the model predictions to experimental data were observed for this time-temperature condition. Data and results for all other combinations of temperature and initial nitrogen concentration are included in the Appendix B. Other conditions may or may not show better fits to experimental data.

Figure 3.1 shows the four dependent variables with the plots of observed data and the best-fit predictions obtained from the model by using the above described procedure for T=22 °C and N_i =65 ppm (DAP=100 ppm). The asymptotic confidence bands (95% C.B.) and the 95% prediction bands (95% P.B.) are also depicted for three dependent variables. As Xv was given a weight $W_{Xv} = 0$ for the parameter estimation process, MATLAB© was unable to calculate the C.B. and P.B. for yeast cell concentrations. In figure 3.2, 3.3 and 3.4, the band formed by two thin lines on either side of the model prediction line represents the 95% confidence band for the model prediction line. The thick lines form a band outside of this confidence band. This band represents the 95% prediction band, that is, it represents the region in which the data points predicted by the model would lie 95% of the time.

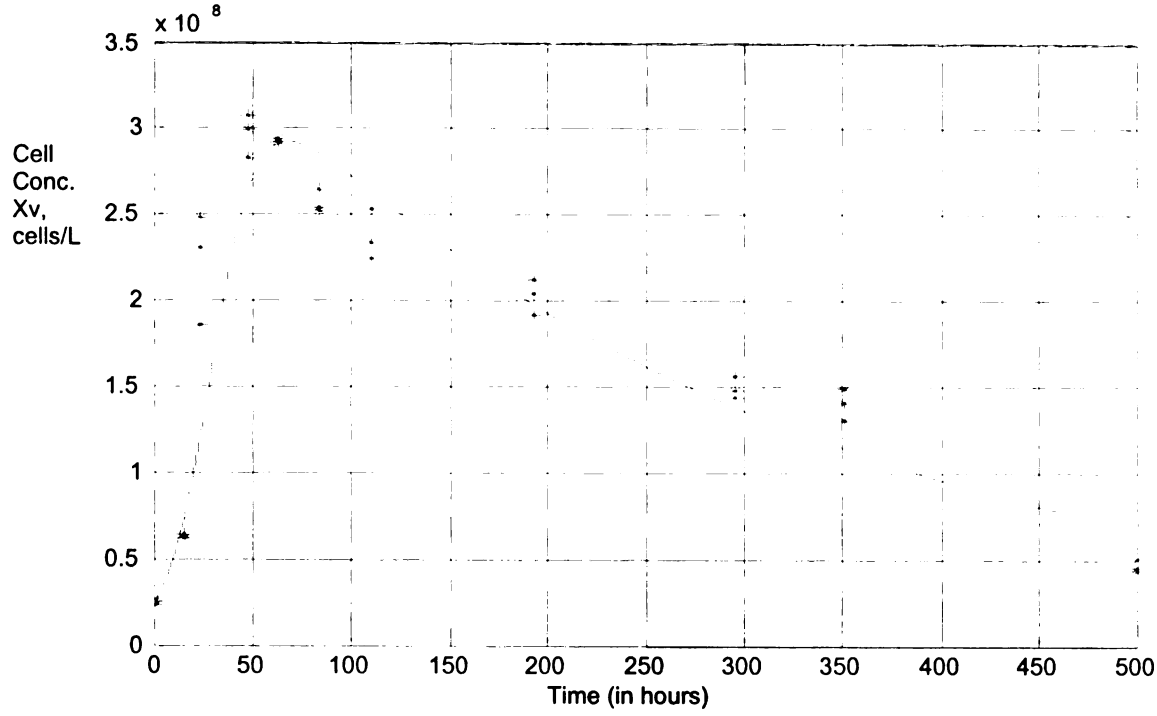


Figure 3.1. Non-Linear Data Fitting: Predicted and Observed Yeast Cell Concentration for T=22 °C and N=65 ppm (DAP=110ppm)

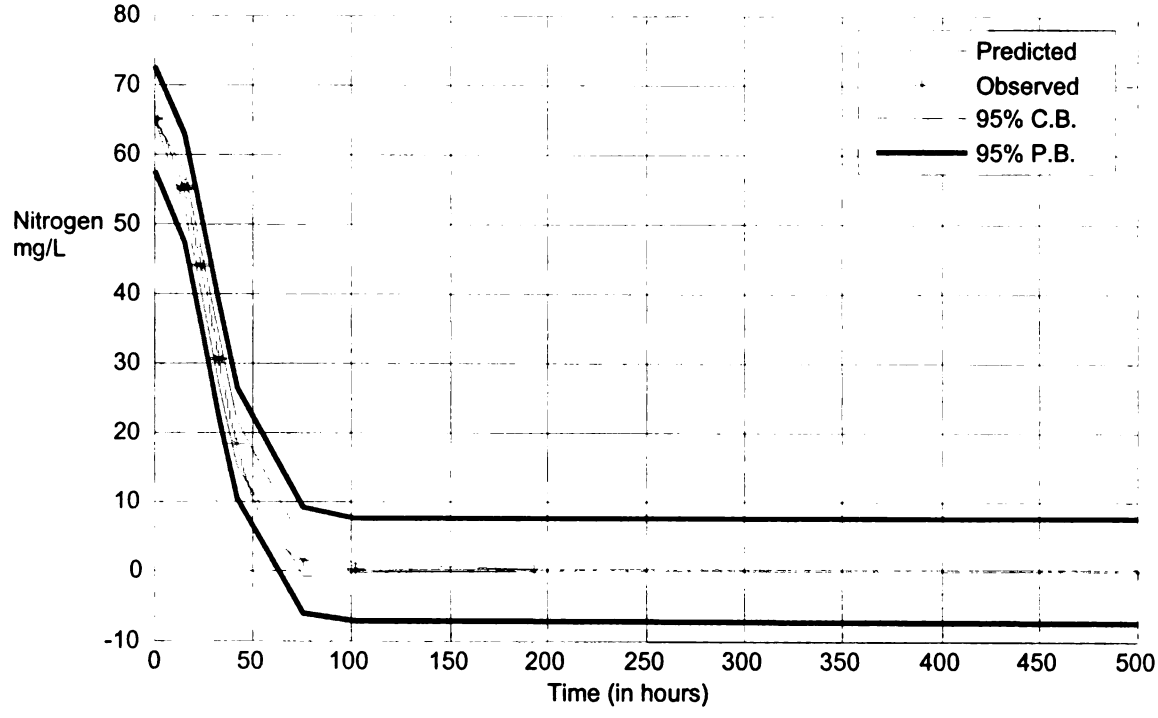


Figure 3.2. Non-Linear Data Fitting: Predicted and Observed Nitrogen for T=22 °C and N=65 ppm (DAP=100ppm)

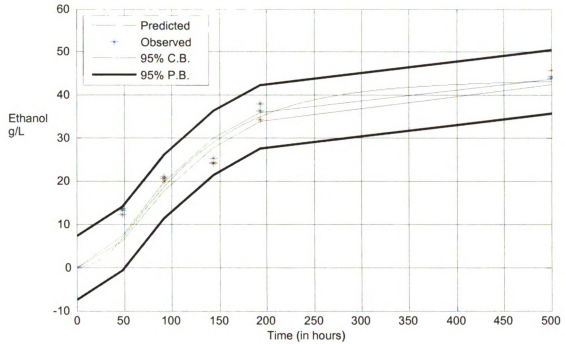


Figure 3.3. Non-Linear Data Fitting: Predicted and Observed Ethanol for T=22 °C and N=65 ppm (DAP=100ppm)

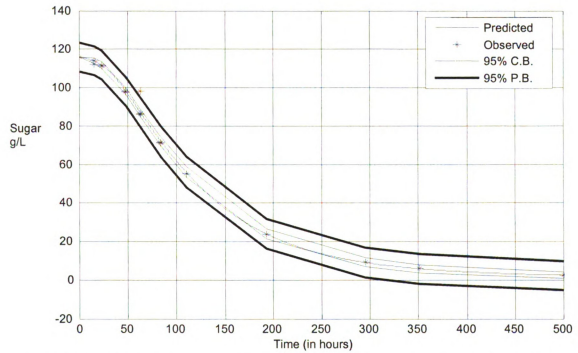


Figure 3.4. Non-Linear Data Fitting: Predicted and Observed Sugar for T=22 °C and N=65 ppm (DAP=100ppm)

Similarly, the non-linear regression routine was applied to all the conditions and the non-linear parameters estimates were obtained (Table 3.2).

Table 3.2: Non-linear Estimated Parameters

Temp (°C)	Nitrogen (ppm)	μ_m (hr^{-1})	β_m (g ethanol $cell^{-1}$ hr^{-1})	K_N (g N/L)	K_S (g sugar/L)	$Y_{X/N}$ (no. of cells/ g N)
11	45	0.1442	7.37E-10	172.32	14.589	5.00E+06
11	65	0.16443	4.17E-10	206.96	3.9181	5.00E+06
11	110	0.39818	1.43E-09	1991	9.8417	8.50E+05
11	165	0.50891	1.56E-09	2546.5	35.945	2.00E+06
17	45	1.9244	3.39E-10	769.26	8.5546	1.10E+07
17	65	2.5465	5.09E-10	2492.2	5.0503	5.00E+06
17	110	2.6444	8.52E-10	5104	128.06	8.00E+06
17	165	2.8017	1.89E-09	7705.5	279.97	1.00E+07
22	45	2.9714	1.25E-09	1199.4	35.631	5.00E+06
22	65	3.0157	1.82E-09	2252.6	72.028	5.00E+06
22	110	3.2545	3.48E-09	4984.7	208.03	5.00E+06
22	165	4.0419	5.99E-09	7073	297.44	4.50E+06

The non-linearly estimated parameters above (Table 3.2) may be used directly in the primary model to predict fermentation kinetics. However this would mean that the model is applicable only for the three temperatures and four levels of nitrogen used for in current experiments. The use of a secondary model (2.5) not only increases the applicability of the model, it also gives the level of significance of the effect of nitrogen or temperature on the model parameters and dependent variables. In this particular case, the fitting of the above (Table 3.2) to secondary model (2.5) will show if an Arrhenius relationship exists between the model parameters and the two independent variables, fermentation temperature and initial nitrogen content.

3.3 Linear Parameter Estimation from Secondary Model

The figure 3.18 below shows a plot of ln μ_m vs $(1/N - 1/N_{ref})$. It can be seen that the log of specific growth rate increases with increasing nitrogen and also with increasing temperature. Although figure 3.18 shows that nitrogen and temperature have an Arrhenius type effect on the growth rate, whether this effect is significant or not cannot be determined at this time.

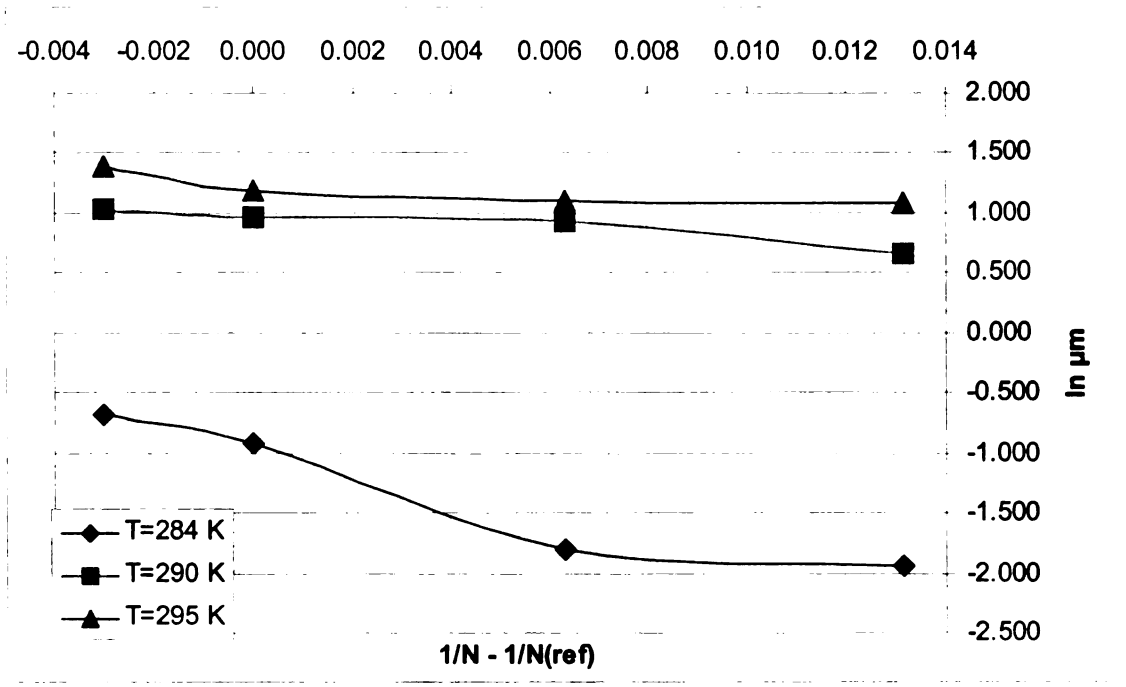


Figure 3.18 Secondary Model Fitting of ln μ_m vs $(1/N - 1/N_{ref})$

The non-linear parameter estimates were different for each condition of temperature and initial nitrogen levels (Table 3.2). The multiple linear regression estimated parameters fitted to the Arrhenius equation (2.5) are shown in Table 3.3.

Table 3.3: Linearly Estimated Coefficients from Arrhenius Fits

μ_m					
	Coefficients	Std Error	Lower 95%	Upper 95%	P-value
$ln\ k_r$	0.51303	0.17927	0.10749	0.91856	0.018
E_T/R	-19707	2777.4	-25990	-13424	$5.7\ X10^{-05}$
E_N/R	-40.609	24.0389	-94.988	13.7712	0.12
β_m					
	Coefficients	Std Error	Lower 95%	Upper 95%	P-value
$ln\ k_r$	-20.157	0.19019	-20.588	-19.727	$3\ X10^{-15}$
E_T/R	-7609.7	2946.61	-14275	-944.01	0.03
E_N/R	-84.373	25.5035	-142.07	-26.681	0.009
K_N					
	Coefficients	Std Error	Lower 95%	Upper 95%	P-value
$ln\ k_r$	8.16322	0.17806	7.76042	8.56601	$5.6\ X10^{-12}$
E_T/R	-12287	2758.64	-18528	-6046.7	0.0016
E_N/R	-146.15	23.8765	-200.17	-92.141	0.00018
K_S					
	Coefficients	Std Error	Lower 95%	Upper 95%	P-value
$ln\ k_r$	4.25128	0.32425	3.51778	4.98479	$3.6\ X10^{-07}$
E_T/R	-16989	5023.56	-28353	-5624.6	0.008
E_N/R	-142.03	43.4798	-240.39	-43.674	0.01
$Y_{X/N}$					
	Coefficients	Std Error	Lower 95%	Upper 95%	P-value
$ln\ k_r$	15.2493	0.22498	14.7403	15.7582	$1.7\ X10^{-13}$
E_T/R	-5362.9	3485.52	-13248	2521.9	0.16
E_N/R	32.0288	30.1678	-36.215	100.273	0.32

From Table 3.3, the following observations can be made:

1) The P-value for μ_m with respect to initial nitrogen is 0.12. This shows that nitrogen does not have a significant effect of the Arrhenius-type on the specific

growth rate at the 95% confidence level (p=0.12). Temperature however does have a significant effect on the growth rate (p<0.05).

2) Fermentation temperature and initial nitrogen content have a significant Arrhenius-type effect on β_m and K_S. This means that sugar consumption and ethanol production are significantly affected. In many cases, the confidence level is much higher than 95%.

3) $Y_{X/N}$, that is, the parameter that represents the yield of biomass per gram of nitrogen, does not show an Arrhenius relationship with fermentation temperature (p=0.16) or initial nitrogen level (p=0.32) in the fermenting juice. Thus, it may be possible to fix the value of this parameter and exclude it from the data fitting process.

Thus, hypothesis (4), which proposes that the kinetic parameters of the primary model have an Arrhenius-type relationship with the two independent variables, temperature and initial nitrogen, proves to be true only for three parameters β_m , K_S and K_N at a 95% confidence level. Temperature is shown to have a very significant (<0.05) effect on μ_m, βm, K_S and K_N proving hypothesis (3). Nitrogen does not have a very significant (<0.05) Arrhenius-type effect on the specific growth rate; however it should be noted that the effect is still significant at an 88% confidence level. Thus, even though hypothesis (2) may have be true at a 95% confidence level, its still holds true at an 88% confidence level.

Plugging the secondary model coefficients from Table 3.3 in equation (2.9), the parameters for various temperature-initial nitrogen conditions were predicted (Table 3.4).

Table 3.4: Predicted Parameters from Secondary Model Eq. 2.9

Temp (°C)	Nitrogen (ppm)	μ_m (hr^{-1})	β_m (g ethanol $cell^{-1}$ hr^{-1})	K_N (g N/L)	K_S (g sugar/L)	$Y_{X/N}$ (no. of cells/ g N hr)
11	45	0.233	$3.33X10^{-10}$	209.710	3.150	$4.32X10^{+06}$
11	65	0.980	$5.80X10^{-10}$	513.299	10.859	$6.39X10^{+06}$
11	110	3.100	$9.05X10^{-10}$	1052.567	29.308	$8.75X10^{+06}$
11	165	0.308	$5.93X10^{-10}$	569.662	8.318	$3.47X10^{+06}$
17	45	1.294	$1.03X10^{-09}$	1394.338	28.677	$5.13X10^{+06}$
17	65	4.093	$1.61X10^{-09}$	2859.218	77.402	$7.03X10^{+06}$
17	110	0.397	$1.01X10^{-09}$	1429.197	20.335	$2.84X10^{+06}$
17	165	1.670	$1.76X10^{-09}$	3498.187	70.105	$4.20X10^{+06}$
22	45	5.284	$2.74X10^{-09}$	7173.354	189.220	$5.74X10^{+06}$
22	65	0.450	$1.30X10^{-09}$	2225.522	31.273	$2.58X10^{+06}$
22	110	1.889	$2.27X10^{-09}$	5447.319	107.813	$3.81X10^{+06}$
22	165	5.977	$3.54X10^{-09}$	11170.229	290.995	$5.21X10^{+06}$

The predicted parameters in Table 3.4 show that the specific growth rate increases with temperature for a particular level of initial nitrogen. The specific growth rate also increases with increasing initial nitrogen at constant temperature. K_N and K_S also show this trend. No such continuous trend is observed for specific sugar consumption rate, β_m, or the parameter for the yield of biomass on nitrogen, $Y_{X/N}$. The parameters predicted above (Table 3.4) from the secondary model were plugged into the primary model. The results from solving the primary model with the predicted parameters for the 12 conditions of temperature and initial nitrogen represent the model predictions.

3.4 Model Validation

The model predictions as compared with the experimental values are shown below. The figure below shows predicted and observed values of yeast cell concentration, nitrogen concentration, ethanol concentration, and sugar concentration versus time for cider

fermentation at T=22 °C and N_i =65 ppm (DAP=100 ppm). The predictions were based on parameters estimated from the 'parameter estimation data set' while the experimental values that these predictions were compared with are from the 'model validation data set'. As mentioned before, the 'model validation data set' is much bigger than the 'parameter estimation data set'.

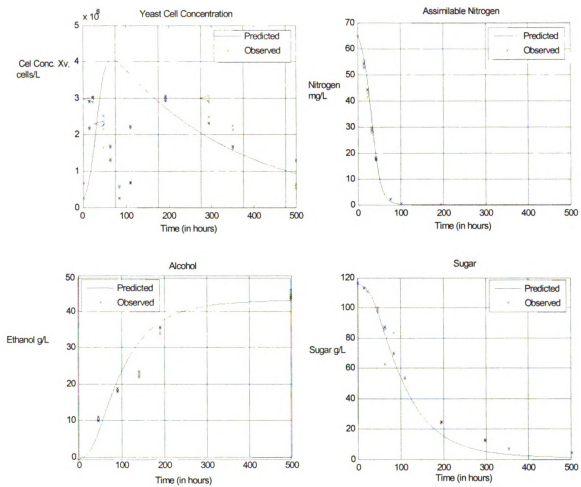


Figure 3.5. Model Validation for T=22 °C and N_i=65 ppm (DAP=100 ppm)

As can been seen from the above figure, the model is able to predict the production of ethanol, *E*, consumption of nitrogen, *N*, and sugar, *S*, with reasonable accuracy for T=22 °C and N_i =65 ppm (DAP=100 ppm). Comparisons between the model predictions and experimental data for other conditions are shown in Appendix C.

Nitrogen depletion curves were predicted with reasonable accuracy by the model for all most conditions of temperature (T=22 and 17°C) and initial nitrogen. For some conditions, particularly, figure C.3. (T=17°C and DAP=600 ppm) and at T=11 °C (figures C.8 to C.11), nitrogen curve predictions were not very good. However, for all conditions, the model predicted the nitrogen exhaustion point (N~0 ppm) quite accurately.

The model was able to predict the trend for ethanol production curves very well for all cases except T=11° C, DAP=0 ppm (figure C.8.). The model under predicted the final ethanol levels in cider slightly for all conditions except T=22° C, DAP=0 ppm (figure C.3.) T=17° C, DAP=300 ppm (figure C.5.) and T=22 °C and DAP=100 ppm (figure 3.5.).

Although the model captured the trend for sugar consumption very well for all conditions, the predictions were not very accurate for most cases except T=22 °C and DAP=100 ppm (figure 3.5.), T=17 °C and DAP=600 ppm (figure C.4.), T=22 °C and DAP=300 ppm (figure C.6.), T=11 °C and DAP=100 ppm (figure C.9.) and T=11 °C and DAP=600 ppm (figure C.11.).

Further study of figure 3.5 not only helps understanding of the operation of the model but also explains the errors on model predictions for except T=22 °C and DAP=100 ppm. The exponential phase of growth coincides with the exhaustion of

nitrogen. At this point in time (t~90 hours), there is considerable sugar (> 70 g/L) still left in the sample, and only 15 g/L approx. of a total of 45 g/L approx. of ethanol has been produced at this time. However, this does not mean that no ethanol production occurred during the growth phase, but that this value is not significant. From the observed and predicted value of viable yeast cell concentration, X_V, it is seen that the cell concentration reaches its peak around 50 hours. At this time, there is insignificant production of ethanol and less than 15% of the total sugar available has been consumed. This concurs with the model's assumption that cell growth is not dependent on sugar or ethanol concentration. This does not mean that no ethanol is produced during the growth phase, only that the growth rate is ethanol independent. At the same time, however, ethanol production is determined by the cell concentration, especially during the stationary phase.

The model predicts the depletion of nitrogen, N, and its exhaustion point accurately, as seen in figure 3.5. The ethanol production, E, is over predicted but the final value of E produced is slightly less than the observed value. This coincides with the model predictions for viable cell concentration, X_V. The model overestimates the X_V during the exponential phase and the peak value of X_V. The model predictions also show an absence of a stationary phase. This may have resulted in the final predicted E at the end of fermentation being lower than the observed E, even though E was being over predicted during fermentation.

According to the model assumptions, the consumption of sugar, S, is strongly related to both E and X_V. The model over predicts the consumption of S but is able to predict the final residual S value accurately. This concurs with the discussion above on the model predictions for E and X_V. Higher sugar consumption during the period after the

exponential phase results in higher production of ethanol. Ethanol being toxic to cell viability, the model prediction for viable cells is less than the observed value. The model reacts to the over-prediction of sugar consumzption by under predicting the viable cell concentration. Thus, although the model does not predict sugar consumption accurately, it shows the effect of change in sugar consumption on ethanol production and cell viability.

The model is not able to predict the yeast cell concentration X_V very well for T=22 °C and N_i =65 ppm (DAP=100 ppm), as seen in figure 3.5. The observed X_V is very scattered and shows the presence of a stationary phase up to 300 hours. However, the model overestimates the growth rate and the maximum X_V. The model predictions also show an absence of a stationary phase.

The model predicts the change in cell concentration very well for the following conditions: T=17 ° (figures C.2, C.3. and C.4.), T=11 °C and DAP=300 ppm (figure C.10.) and T=11 °C and DAP=600 ppm (figure C.11.). In some of the cases like T=22 °C and DAP=300 ppm (figure C.6.) and =11 °C and DAP=0 ppm (figure C.8.), the experimental data itself is extremely scattered making the model predictions inaccurate by default. For most conditions, the model is able to predict the transition out of the exponential phase into the stationary phase, which is crucial to ethanol and sugar predictions.

For the current set of experiments, certain common traits were observed as below. The model predictions for T=11 °C seemed to be less accurate than for the other two temperatures. The predictions of X_V were inaccurate for a majority of temperature-initial

nitrogen conditions. These observations may change when the model is applied to a new case; the reasons for which are identified in the following paragraphs.

The current study has shown that it is possible to obtain ethanol levels of as high as 6.5% (>50 g/L for a $Y_{E/S}$=0.38 g ethanol/g sugar) in hard cider with more complete fermentations. Nitrogen was the key limiting nutrient and supplementation of apple juice with appropriate amount of nitrogen in the form of diammonium phosphate helps achieve better fermentation yields. It has been recommended that hard cider fermentations should start with a nitrogen level of 100 ppm for good results (Lea 2004). This was shown to be true in the current study. Initial nitrogen levels of less than 110 ppm gave incomplete or sluggish fermentations as can be seen in figure 3.5, model validation for T=22 C and N=65 ppm (DAP added=100 ppm), where even at the high temperature, there is some residual sugar left in the product. Thus the model can be used to predict sluggish or stuck fermentations.

The effect of temperature on hard cider fermentation too was distinct. At T=11 °C, sluggish fermentations were a norm. Fermentations at this temperature seemed to reach completion only at very high nitrogen levels. Although fermentations at this temperature may give us lower ethanol levels in the final hard cider, other by-products and residual sugar may prove help improve the sensory attributes (such as aroma, flavor profile and sprarking effect due to release of CO_2) of the final product. Similarly, although fermentations at a higher temperatures like T=22°C may yield faster and more complete fermentation at lower nitrogen levels, the effect on the sensory characteristics of such fermenting conditions may not be positive (unpublished observations).

At this point, it is necessary to note that the comparison of model predictions with experimental data above only applies to the experiments conducted during this research. Some of the comparisons and the accuracy of model predictions will change when applied to other cases or a different set of experiments. The model depends on good data for growth and growth rates. Lack of data during the exponential phase and inaccuracy in measurements will affect the model predictions.

Reasons for inaccuracies in model predictions, especially, the high errors in estimation of yeast cell concentration may have resulted from the following:

a) $W_Y = 0$ to X_V during the non-linear data fitting process: A weight of zero was assigned to dependent variable X_V (viable yeast cell concentration) to allow the program in MATLAB© to execute without errors. This meant that the effect of cell concentration, X_V, was ignored during the non-linear parameter estimation process and best fits to experimental data were obtained only for the other three dependent variables *E, S* and *N*. However, X_V affects all the other three dependent variables. This may have been an important reason for inaccuracy in model predictions. At the same time, it should be noted that despite the exclusion of X_V from the non-linear parameter estimation process, the predicted data, even for X_V, compared well with its experimental counterpart.

b) Model too simplistic: Biological processes are extremely complex. However to study them using mathematical models, it is necessary to keep the model simple by making assumptions or ignoring certain unimportant variables. This helps solve the model without excessive effort and helps us understand the

process without making it too complex. However, these assumptions may cause the model to predict with less accuracy.

c) Inaccurate assumptions made by the model: The model assumes that all of the viable cells will take part in the process of converting sugar into alcohol. However, this may not always be true. As alcohol concentrations increase, they have a increased toxicity effect on the transport of products to and from the cell (Ansaney-Galeote et al. 2001; Bisson and Butzke 2000). Thus, as the fermentation progresses, some cells may be alive but may not contribute to the alcohol formation process making the model predictions inaccurate.

d) Errors in Observed Cell Concentration Values: Total viable cell concentrations (X_v) were established using haemocytometry (Hausser Scientific, Horsham, PA). Haemocytometry is a labor intensive process and the possibility of humar error in cell enumeration cannot be eliminated (Cramer et al. 2002). The method was preferred nevertheless, due to its ease of use and comparable accuracy with other methods like plate counts.

e) Parameter Estimation Method: The non-linear parameter estimation process employs 4^{th} order Runge Kutta formulae. Runge Kutta methods have their disadvantages as explained in section 2.3.2. Combined with all of the other factors listed above, the use of this method may have led to errors in estimation of X_v.

The accuracy of model predictions can be determined by a plot of predicted versus experimental data directly as shown below. The line on the plot was a 45-degree line intersected at (0, 0).

Figure 3.6 below shows a plot of all predicted versus observed yeast cell concentrations at all conditions. These data points were obtained as a result of non-linear parameter estimation process. Since the 'parameter estimation data set' was used for non-linear parameter estimation, the observed data consists of all the yeast cell concentrations in the 'parameter estimation data set'. The total number of such data points for $X_V \sim 600$.

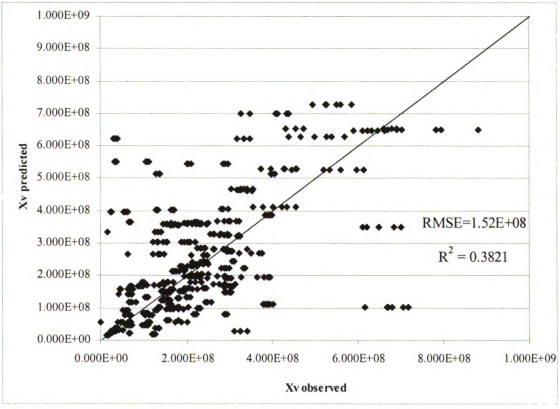


Figure 3.6. Model Validation for Viable Cell Concentration (X_V), cells/ml. Total number of data points = 600

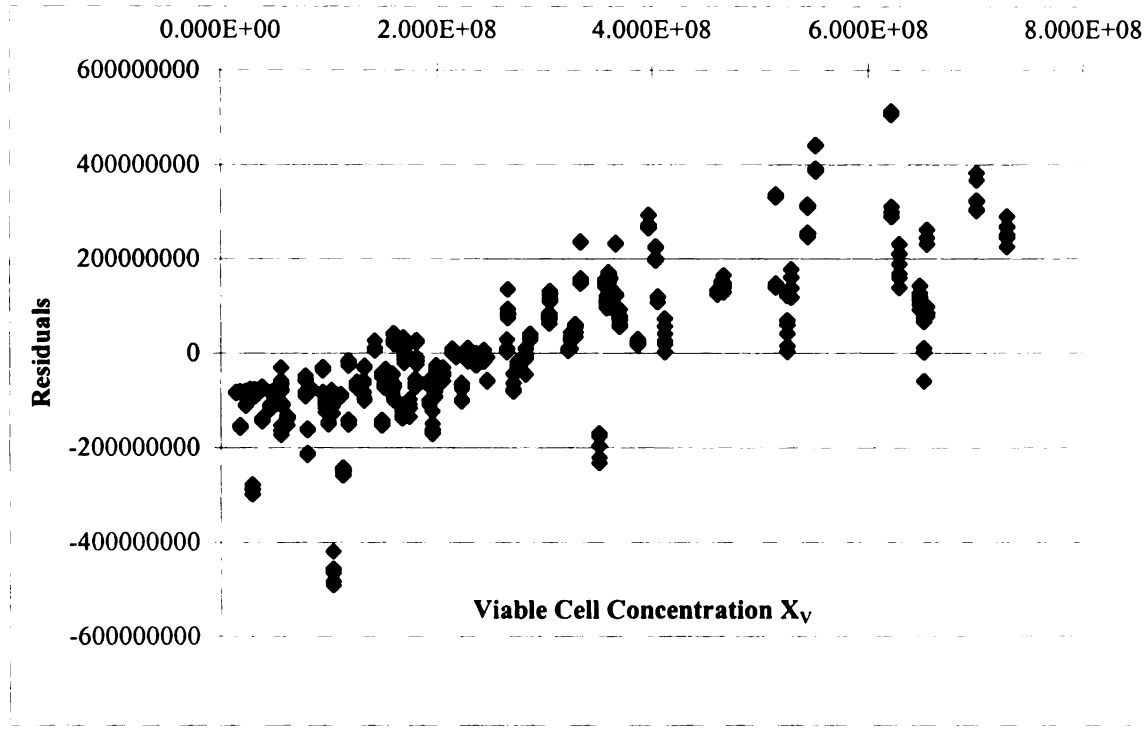


Figure 3.7. X_V predicted vs. Residuals: Total number of data points = 600

From the above data analysis, it is seen that plot 3.6 of X_v predicted versus observed values has a $R^2 = 0.3059$. Although this may seem like a poor fit, most of the data points lie close to the 45 degree line while those that do not have a great variation leading to a lower R^2 value. This can also be observed from figures 3.7 and 3.8. It is also observed from figure 3.7 that the residuals for lower X_V values are smaller than those for higher X_V values. The highest X_V values correspond with the end of the exponential phase and the peak cell concentration value. This means that the errors in predicting the end of the exponential phase and the stationary phase are higher which corresponds well with what is observed in figure 3.1. The possible reasons for incorrect predictions of X_V have already been enumerated in section 3.4 on model validation.

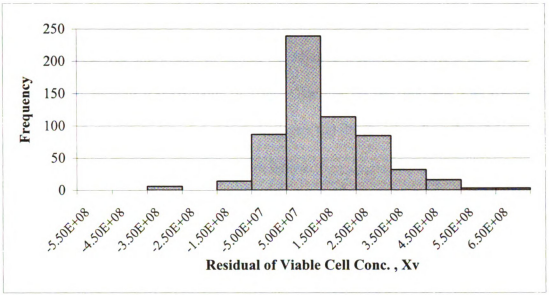


Figure 3.8. Frequency of X_V Residuals

The figure below shows a comparison of all predicted and observed nitrogen concentration. The predictions were based on parameters estimated from the parameter estimation data set. The total number of data points in this plot ~575. The total number of data points changes as the number of observations made for a particular dependent variable at various conditions is different.

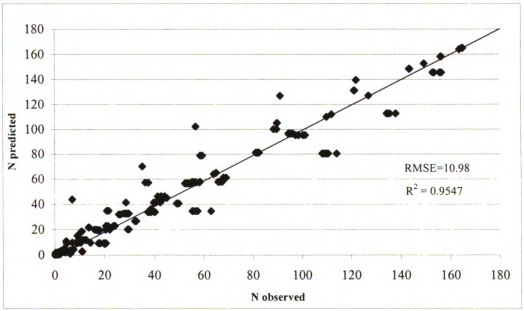


Figure 3.9. Model Validation for Nitrogen, N: Total number of data points = 575

From the above plot, it can be seen that there is good agreement (R^2=0.9547) between the predicted and observed values for ethanol production. This is also proven by a relatively low overall RMSE value of 10.98. The line on the plot was a 45-degree line intersected at (0, 0). The plot of residuals between the observed and predicted values and the model predictions for nitrogen is shown below.

The residual plot in figure 3.10 and the frequency plot in figure 3.11 show that there is a trend for the model to predict lower nitrogen consumption than actual. The residuals seem to show a tendency towards the negative side in the frequency plot for low values. However, the mean of the residuals was only 8.6 x 10^{-14}. From figure 3.11, it is seen that positive residuals large in value compensate for a large number of small negative residuals giving a mean very close to zero.

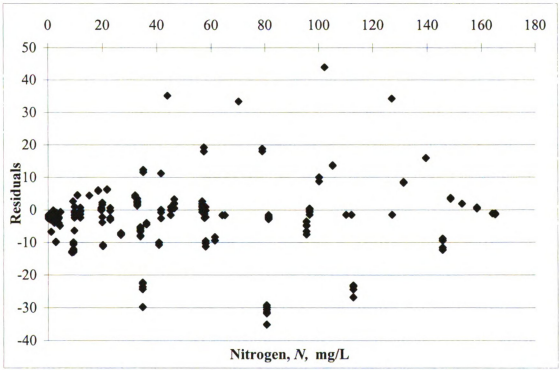


Figure 3.10. N predicted vs. Residuals

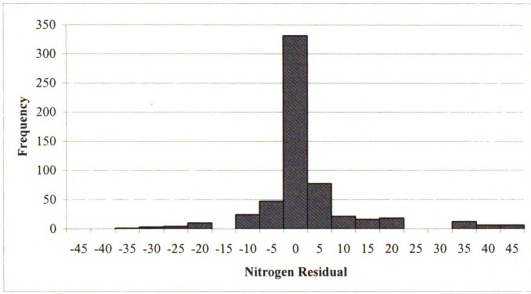


Figure 3.11. Frequency of Residual for Nitrogen

The figure below shows a Comparison of All Predicted and Observed Ethanol Production. The predictions were based on parameters estimated from the parameter estimation data set. Total number of observed points for this plot = 510.

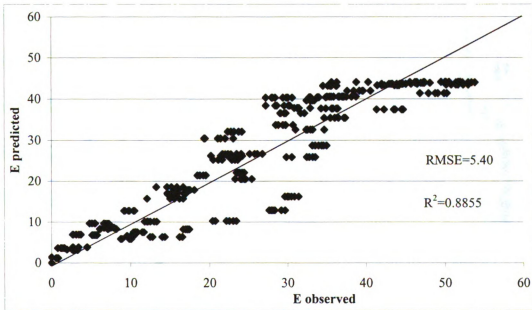


Figure 3.12. Model Validation: Predicted and Observed Ethanol Comparison, Total number of data points = 510

From the above plot, it can be seen that there is good agreement (R^2=0.8855) between the predicted and observed values for ethanol production. This is also proven by the RMSE value of 5.40, which is less than 10% of the total range of 0-55 g/L for ethanol. The line on the plot was a 45-degree line intersected at (0, 0).

The plot of residuals between the observed and predicted values and the model predictions for sugar is shown below. The residual plot above shows that there is more deviation in the model predictions from the observed ethanol values as fermentation progresses and ethanol concentration rises. The residuals seem to show a tendency towards the positive side in the frequency plot but have a very low mean of 2.76×10^{-14}. This is observed in the figure for frequency of residuals given below. Thus the model seems to be under-predicting ethanol concentrations very slightly.

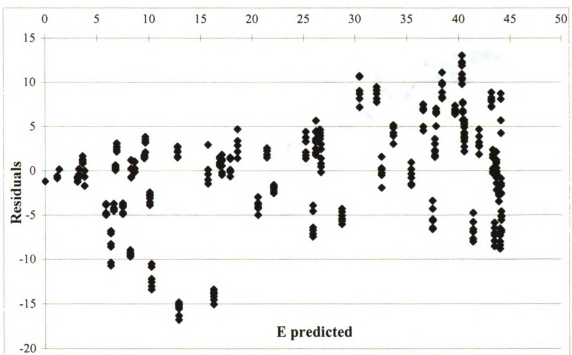


Figure 3.13. E predicted vs. Residuals

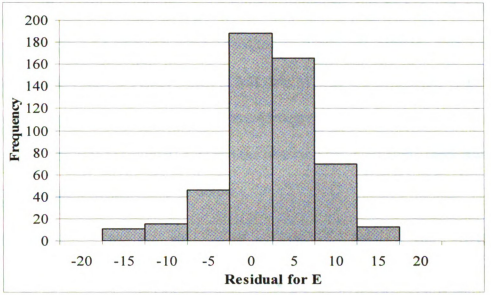


Figure 3.14. Frequency of Residual for Ethanol

The figure below shows a Comparison of All Predicted and Observed Sugar Consumption. The predictions were based on parameters estimated from parameter estimation data set. Total number of observed points = 672.

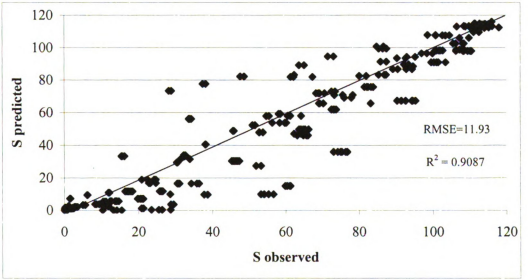


Figure 3.15. Model Validation: Predicted and Observed Sugar Comparison, Total number of observed points = 672

From the above plot, it can be seen that there is a high correlation (R^2=0.9077) between the predicted and observed values for sugar consumption. This is also proven by the RMSE value of 11.93, which is less than 10% of the total sugar concentration range of 0-120 g/L. The line on the plot was a 45-degree line intersected at (0, 0).

The plot of residuals between the observed and predicted values and the model predictions for sugar is shown below.

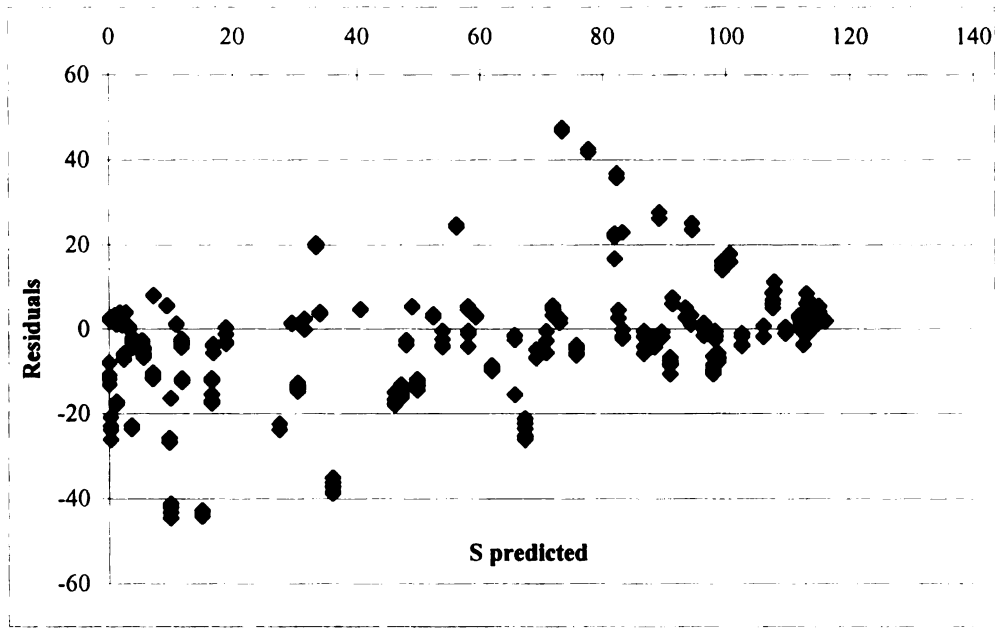


Figure 3.16. S predicted vs. Residuals

The residual plot above shows that the model is able to predict sugar concentrations at the start and end of fermentations more accurately than at the time points between them. The residuals seems have a tendency to be on the positive side (in quantity) showing that the model tends to over predict the consumption of sugar slightly. This is observed in the figure for frequency of residuals given below. However, the mean of the residuals was found to 2.69×10^{-13}.

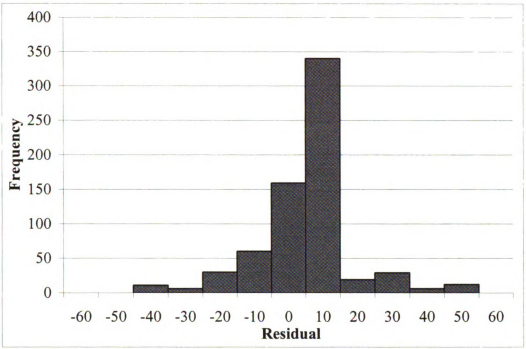


Figure 3.17. Frequency of Residuals for Sugar, S, from model predictions

The model is mechanistic in nature, thus it is able to explain the cider fermentation process in terms of well established and accepted principles in biochemistry (Bailey and Ollis 1986a). Many other models that been non-mechanistic give good predictions but do not help understanding of the process. The model has only seven parameters, two of which are fixed. This makes the model relatively simple to use and solve. However its simplicity does not prevent the model predicting with reasonable accuracy, especially when compared with many other complex and non-mechanistic models in wine (Marin 1999).

Although the model in its current state may not be an accurate predictor of yeast concentrations, it utility lies in its ability to accurately predict ethanol and nitrogen rates while giving us a scientific understanding of the process. Although the effects nitrogen and sugar addition midway through the process of fermentation was not studied, other

researchers have shown that similar models (mechanistic, based on Monod kinetics) are able to predict satisfactorily even in such cases (Cramer et al. 2002; Malherbe et al. 2004).

The model proves that a significant Arrhenius relationship exists between four of the model kinetic parameters and fermentation temperature ($p<0.05$). The level of initial nitrogen also affected the sugar consumption and ethanol production rates significantly ($p<0.05$). On the other hand, specific growth rate was significantly affected by nitrogen at $p=0.12$ only. Thus the model may be used 88% confidence to predict the specific growth rate and fermentation kinetics.

To further evaluate whether growth was nitrogen-limited, log of raw experimental data, log Xv was plotted versus time for all conditions. These plots are shown in Appendix A. These plots showed that the level of nitrogen did not seem to have a strong effect on the growth rate; however, it did affect the final concentration attained by the yeast cells at the end of the exponential phase.

This research showed that an increase in temperature within the range of 11-22 °C and initial nitrogen level within the range of 45 to 165 ppm would yield more complete fermentation with higher ethanol concentration. It was observed that higher temperature and initial nitrogen level resulted in higher cell concentration at the end of exponential phase. This in turn gave more complete fermentation and higher level of ethanol in the final product.

3.5 Conclusions

A simple, mechanistic model based on Monod kinetics for predicting fermentation kinetics was successfully applied to hard cider made from dessert apples. The non-linear data fitting process used 4th order Runge Kutta and functions available in MATLAB© to estimate model parameters.

The model gave satisfactory predictions for three of the dependent variables, Nitrogen consumption (RMSE=10.98, R^2=0.95), sugar consumption (RMSE=5.4, R^2=0.90), and Ethanol production (RMSE=11.93, R^2=0.88) for fermentation experiments conducted in the temperature range 11 to 22 °C and initial nitrogen levels of 45 to 165 ppm (corresponding to supplementation of DAP in the range 0 ppm to 600 ppm). While yeast cell concentrations were not predicted accurately (RMSE=1.52 x 10^{+08}, R^2=0.3821), this does not prevent the usability of the model since the predictions for other three dependent variables, which may be more crucial to hard cider manufacture, were reasonably accurate.

The model shows that a significant Arrhenius relationship exists with temperature for four of the model kinetic parameters, β_m , K_S and K_N , μ_m ($p<0.05$). An Arrhenius relationship between initial nitrogen level and parameters β_m and K_S was also established ($p<0.05$) proving that sugar consumption and ethanol production rates were significantly affected by nitrogen. The parameter for maximum specific growth rate, μ_m , showed an Arrhenius relationship with the level of initial nitrogen at p=0.12, however the effect was not significant at p=0.05.

A significant effect of initial nitrogen on growth rate was not seen in plots of raw growth data (log Xv versus time). From the above observation and information obtained

from secondary model data fitting, it may be concluded that the hypothesis that cell growth rate was significantly nitrogen-limited was false at a 95% confidence level and was only true at a weaker 88% confidence level.

The current study showed that dessert apples could be used to obtain hard cider with ethanol concentrations of over 6.5% and that more complete fermentations could be achieved at higher temperatures and by supplementing nitrogen at the onset of fermentation. Using data generated from simple experimentation, the proposed model can be used with reasonable success to predict the effect of change in initial nitrogen content and temperature on fermentation kinetics of hard cider production. The mechanistic nature of the model helps explain and study the fermentation process. The model provides a framework, using which; models with better predictive capabilities may be established.

3.6. Novelty of work

The current study is the first known application of a mathematical model to hard cider fermentation process. Additionally, unlike regular hard cider manufacturing which uses mostly cider apples, this study specifically involved the fermentation of hard cider from locally grown dessert Jonathan apples. This model is amongst the few mechanistic models applied to fermentation of alcoholic beverages. Most models proposed are non-mechanistic and do not help understanding of the process. Even with mechanistic models proposed in wine, none have attempted non-linear least squares fitting of the data.

Although many researchers have attempted to study the effect of nitrogen on wine fermentations, none using mechanistic models have studied the combined effect of temperature and nitrogen on the fermentation process. Further, no known study has established an Arrhenius relationship between its model parameters and the independent variables of temperature and initial nitrogen content.

3.7. Future Work

1) Larger Fermentation Volumes: In the current experimental setup, the volume of fermenting apple juice was small (400 ml) due to limited resources. Larger samples (> 2 liters) may be used in future experiments. This will be a better replication of cider manufacturing in microbreweries. Large fermentations may exhibit characteristics that are slightly different from the current setup and will be a better test of the model's predictive capabilities.
2) Sensory Analysis: The ultimate test for a hard cider manufacturer is the likeability of the sensory characteristics of hard cider by consumers of wine, beer or other alcoholic beverages. Although making hard cider at a particular temperature may give higher alcohol levels it may not be the best tasting hard cider. Hence, a sensory analysis of the hard ciders made at different temperatures with apple juice containing various amounts of initial nitrogen levels will help determine the best tasting cider.
3) GC Analysis of Volatile Products: For fruit-based alcoholic beverages, the 'taste' or likeability of the product depends not only on the flavor and texture of the beverage but also on the aroma from the volatile compounds being released. It has been shown by other researchers that the concentration and variety of aromatic compounds released by an alcoholic beverage changes with chemical composition and fermenting conditions of the raw material. A gas chromatograph of the volatile products from hard cider will determine the type and concentration of these compounds. Together with sensory analysis, it will be possible to determine which compounds are produced under specific conditions and their effect on the

sensory attributes. GC analysis is also the preferred method of evaluating ethanol levels in hard cider samples and may be used instead of HPLC.

4) Sluggish and stuck fermentations: Sluggish and stuck fermentations are major problems faced by the industry. The effect of nitrogen or sugar addition midway through the process of such fermentations and the ability of the model to predict the effect of this addition can be studied.

5) Initial sugar concentration: The current study dealt with temperature and initial nitrogen levels in apple juice. However, the amount of sugar in apple juice also affects the rate of fermentation and the amount of alcohol produced. Hence, a study of effect of sugar on fermentation will help better prediction of the process.

6) Algorithms in MATLAB© : The 4^{th} order Runge-Kutta formula is very labor intensive and may not be the most accurate. It is, however, widely used due to its simplicity and good results. For modeling hard cider fermentation based on Monod kinetics, this numerical technique is unable to converge in MATLAB© for more than 5 parameters and is very time consuming for smaller time steps. However our model contains a maximum of 7 parameters that may be non-linearly estimated from the primary model and 21 parameters that may be linearly estimated from the secondary model. This is not possible using the current numerical techniques. Hence, a more efficient method for solving the model and non-linear data fitting may improve results.

Appendix A

Semi-Log Plot of Raw Growth Data

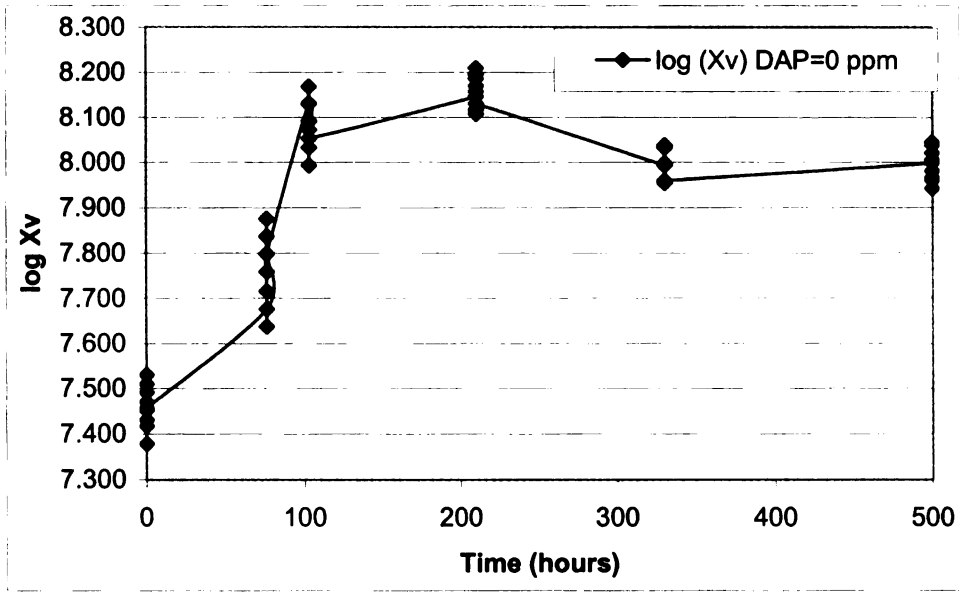


Figure A.1. Semi-log plot of raw X_V versus time for T=11 C, DAP=0 ppm: Slope=

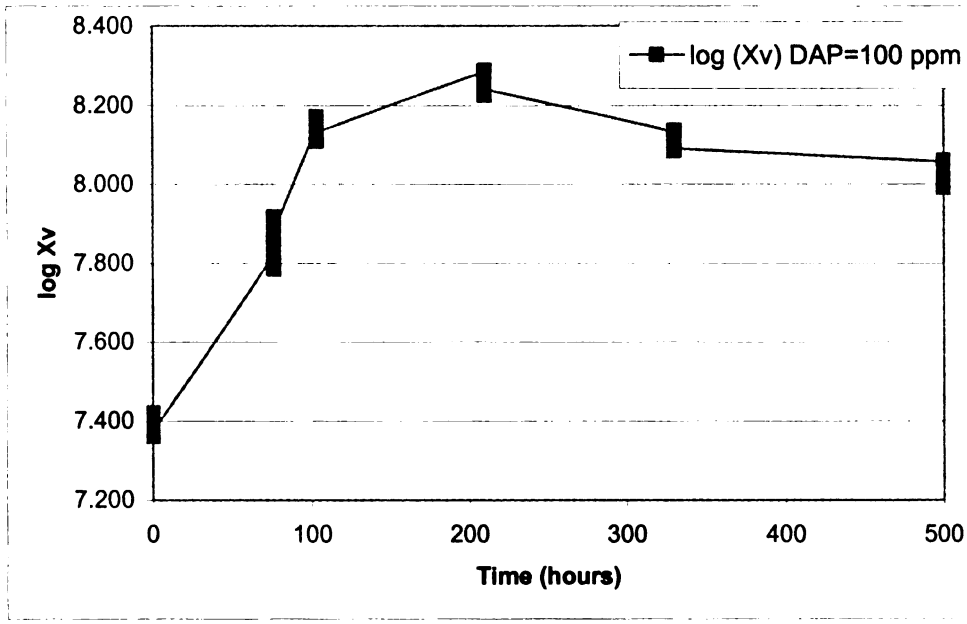


Figure A.2. Semi-log plot of raw X_V versus time for T=11° C, DAP=100 ppm

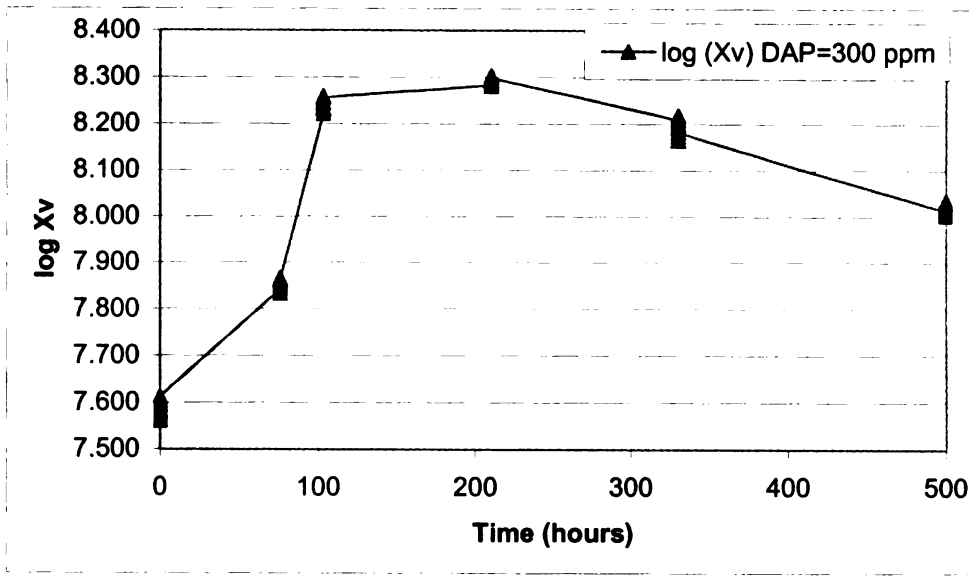


Figure A.3 Semi-log plot of raw X_V versus time for T=11°C, DAP=300 ppm

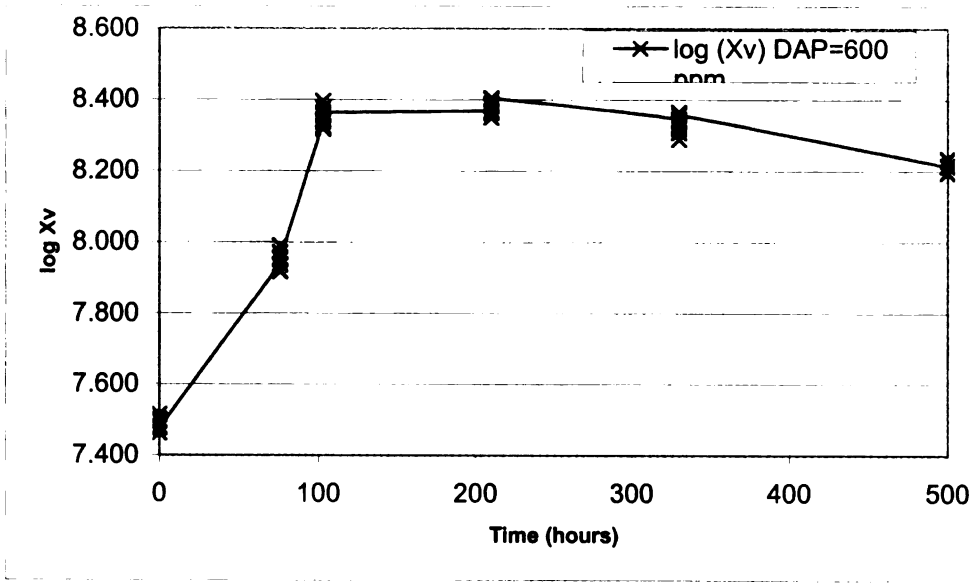


Figure A.4. Semi-log plot of raw X_V versus time for T=11° C, DAP=600 ppm

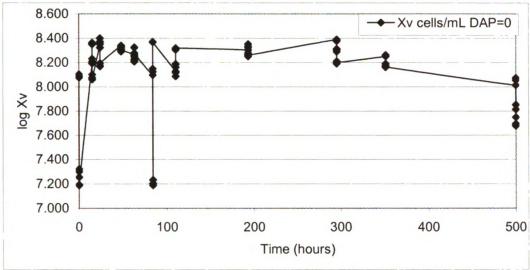


Figure A.5. Semi-log plot of raw X_V versus time for T=17° C, DAP=0 ppm

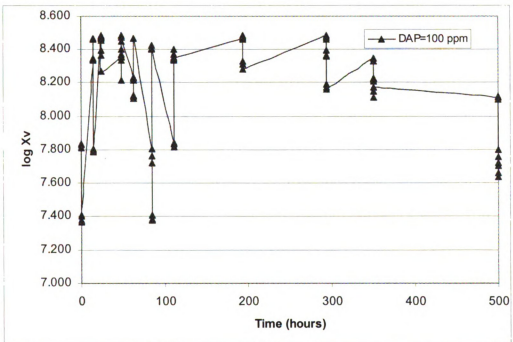


Figure A.6. Semi-log plot of raw X_V versus time for T=17° C, DAP=100 ppm

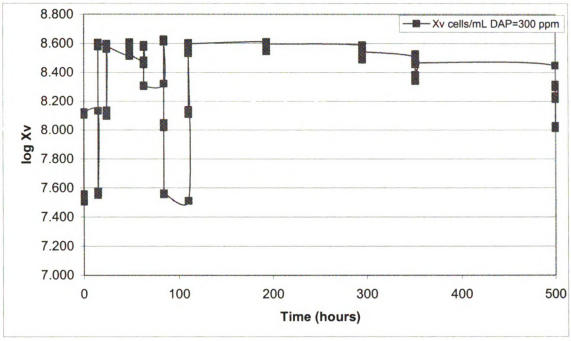


Figure A.7. Semi-log plot of raw *Xv* versus time for T=17° C, DAP=300 ppm

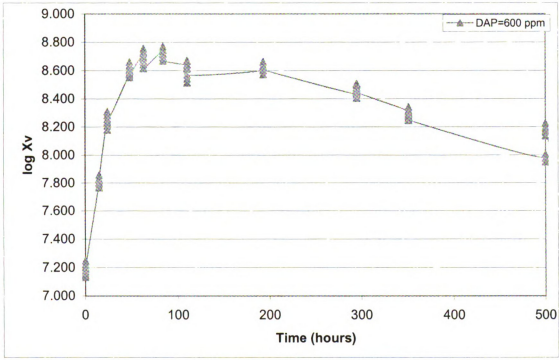


Figure A.8. Semi-log plot of raw *Xv* versus time for T=17° C, DAP=600 ppm

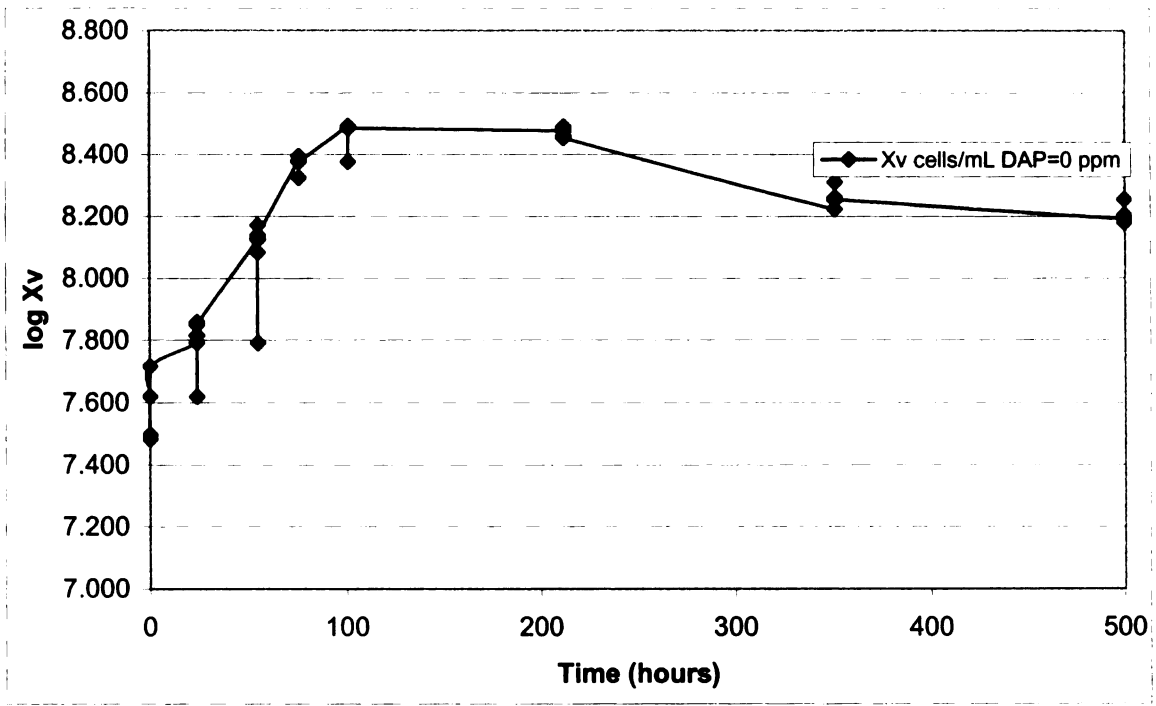


Figure A.9. Semi-log plot of raw *Xv* versus time for T=22° C, DAP=0 ppm

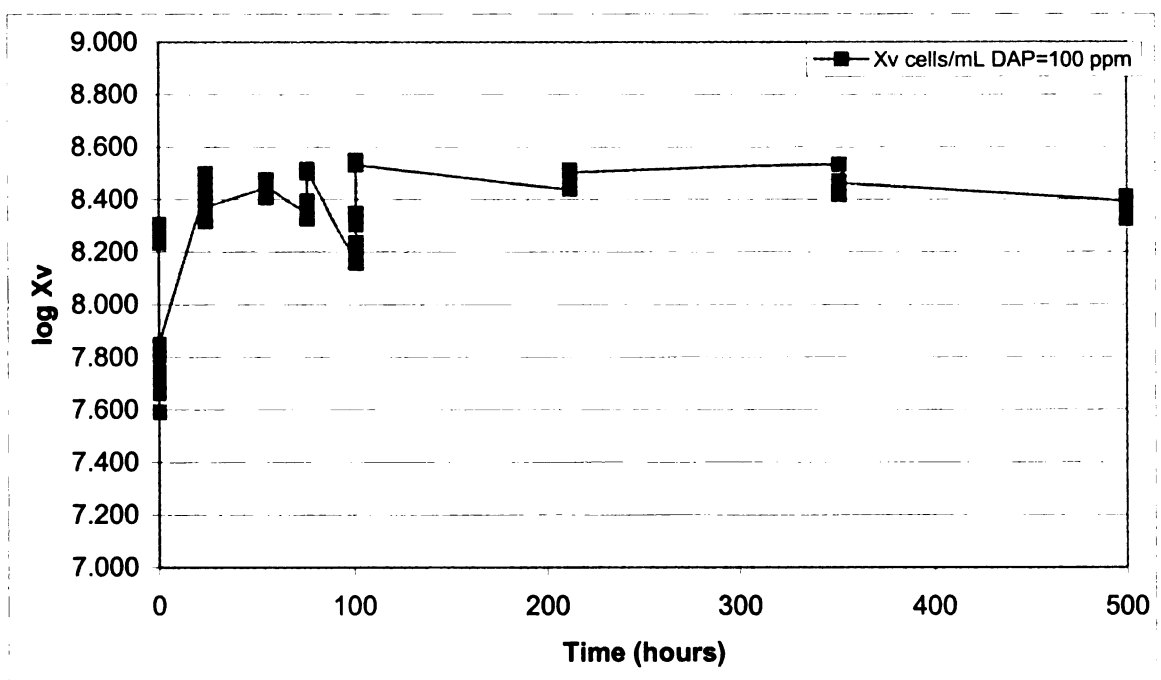


Figure A.10. Semi-log plot of raw *Xv* versus time for T=22° C, DAP=100 ppm

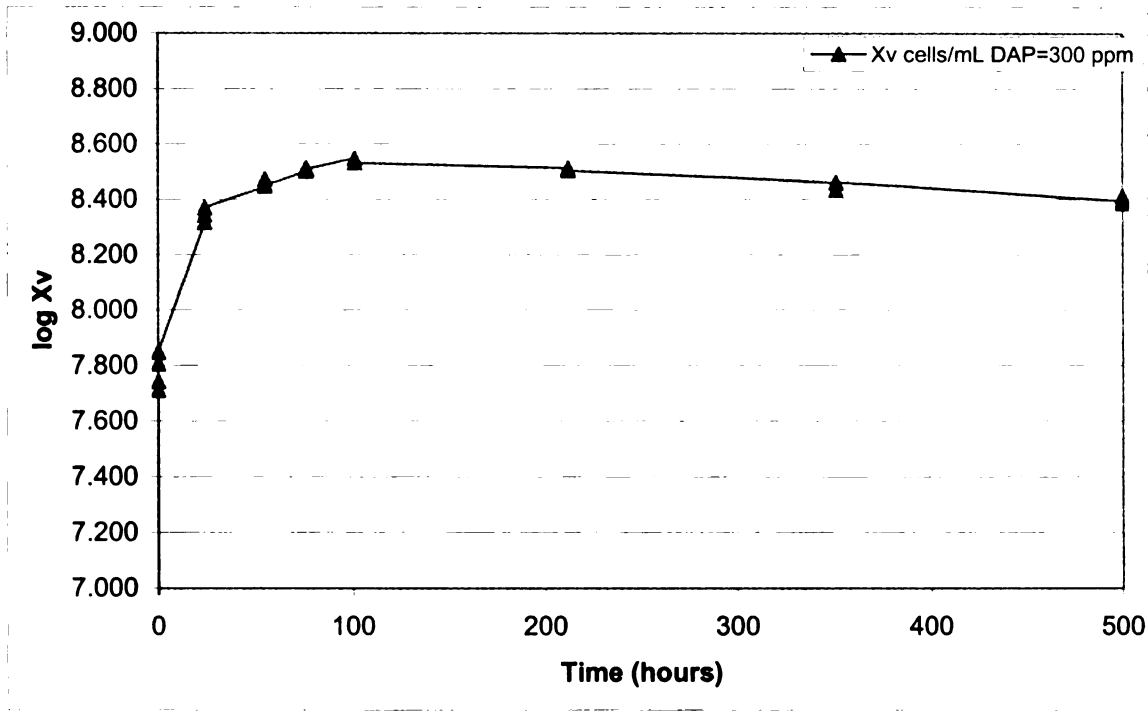


Figure A.11. Semi-log plot of raw *Xv* versus time for T=22° C, DAP=300 ppm

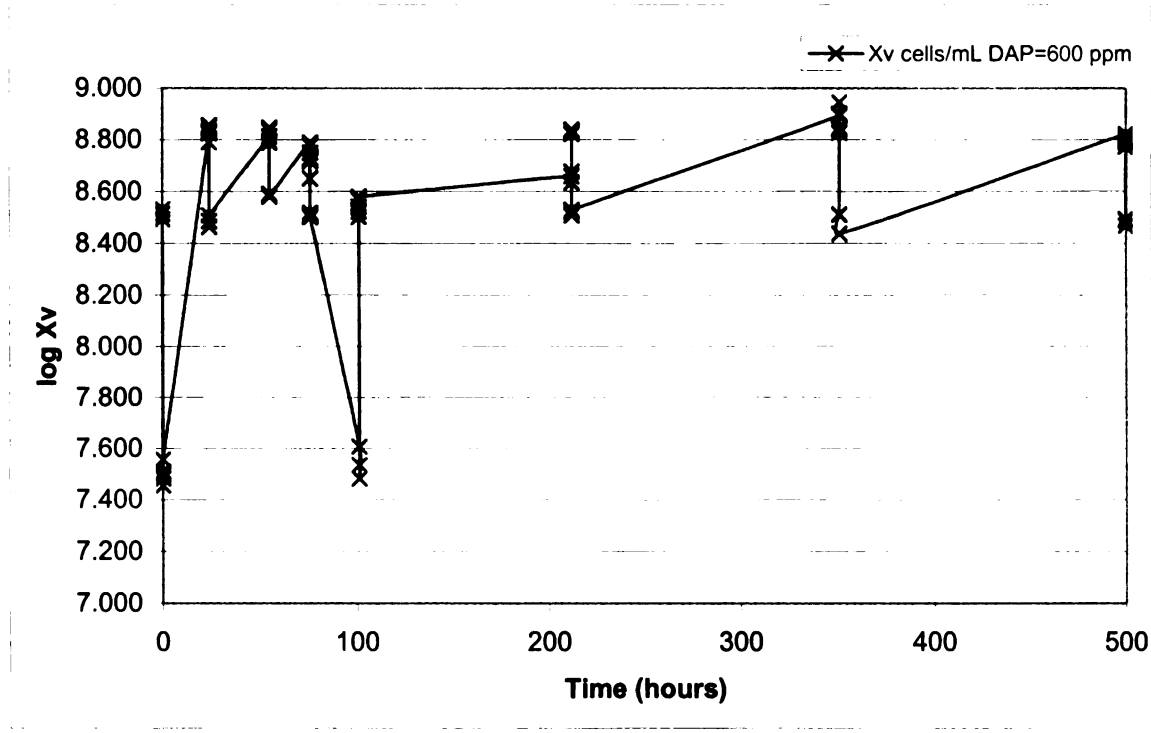
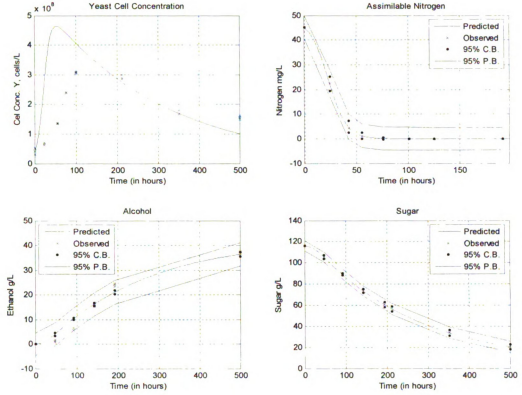


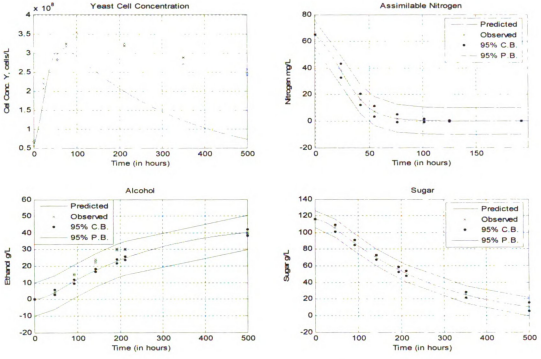
Figure A.12. Semi-log plot of raw *Xv* versus time for T=22° C, DAP=600 ppm

Appendix B

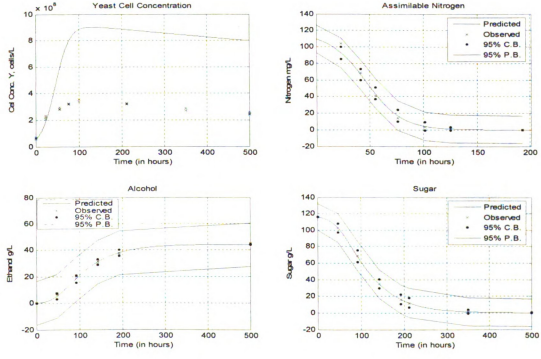
Plots of Predicted & Observed Dependent Variables from Parameter Evaluation



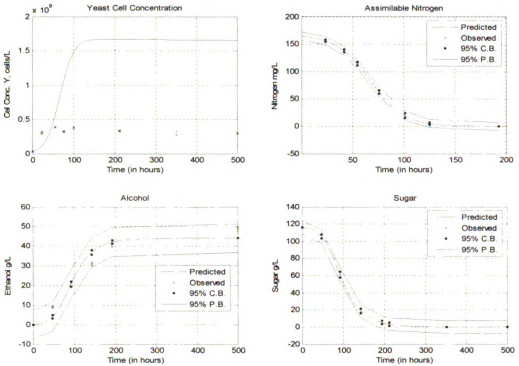
B.1. Non-Linear Parameter Estimation for T=17° C, DAP=0 ppm



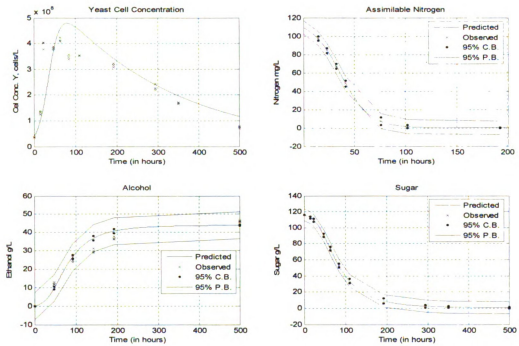
B.2. Non-Linear Parameter Estimation for T=17° C, DAP=100 ppm



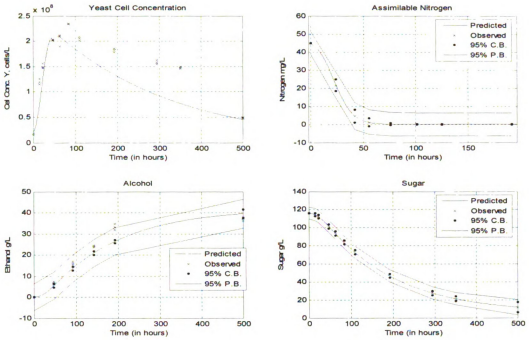
B.3. Non-Linear Parameter Estimation for T=17° C, DAP=300 ppm



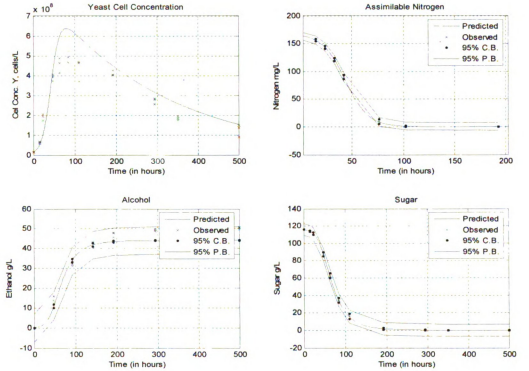
B.4. Non-Linear Parameter Estimation for T=17° C, DAP=600 ppm



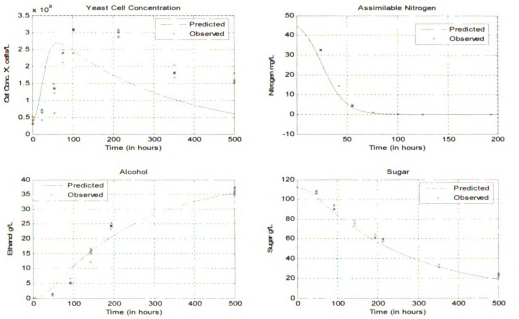
B.5. Non-Linear Parameter Estimation for T=22° C, DAP=0 ppm



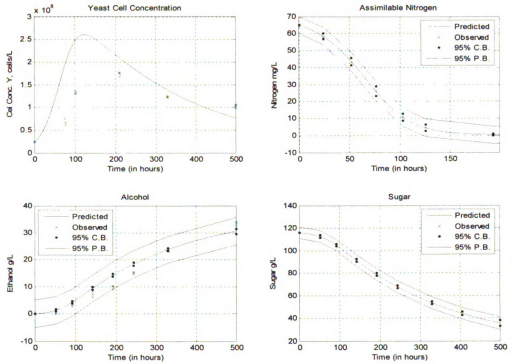
B.6. Non-Linear Parameter Estimation for T=22° C, DAP=300 ppm



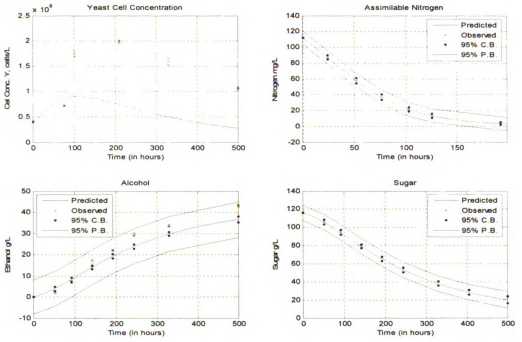
B.7. Non-Linear Parameter Estimation for T=22° C, DAP=600 ppm



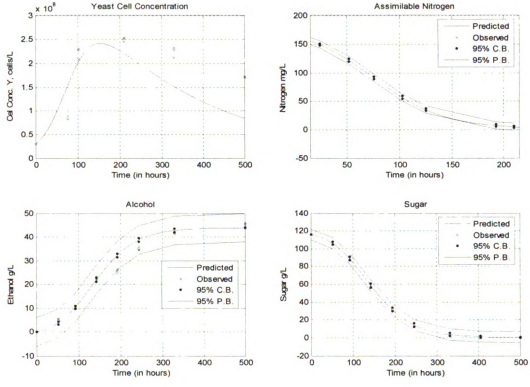
B.8. Non-Linear Parameter Estimation for T=11° C, DAP=0 ppm



B.9. Non-Linear Parameter Estimation for T=11° C, DAP=100 ppm



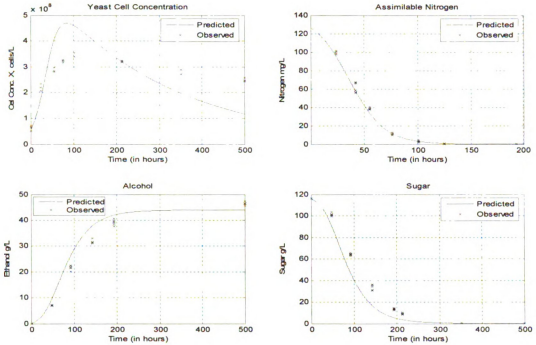
B.10. Non-Linear Parameter Estimation for T=11° C, DAP=300 ppm



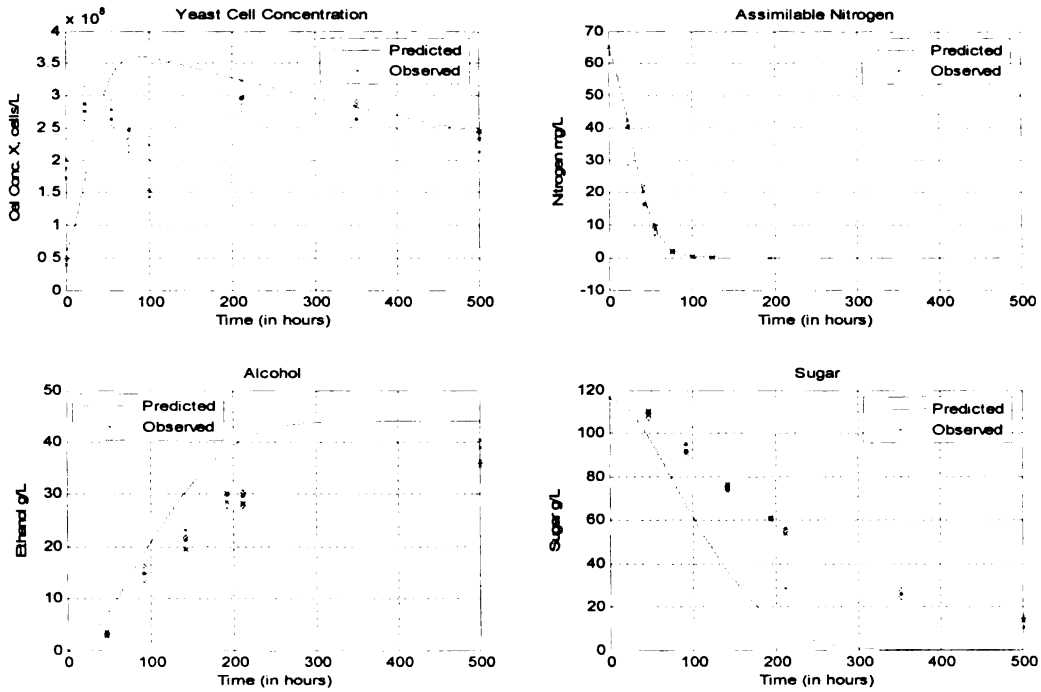
B.11. Non-Linear Parameter Estimation for T=11° C, DAP=600 ppm

Appendix C

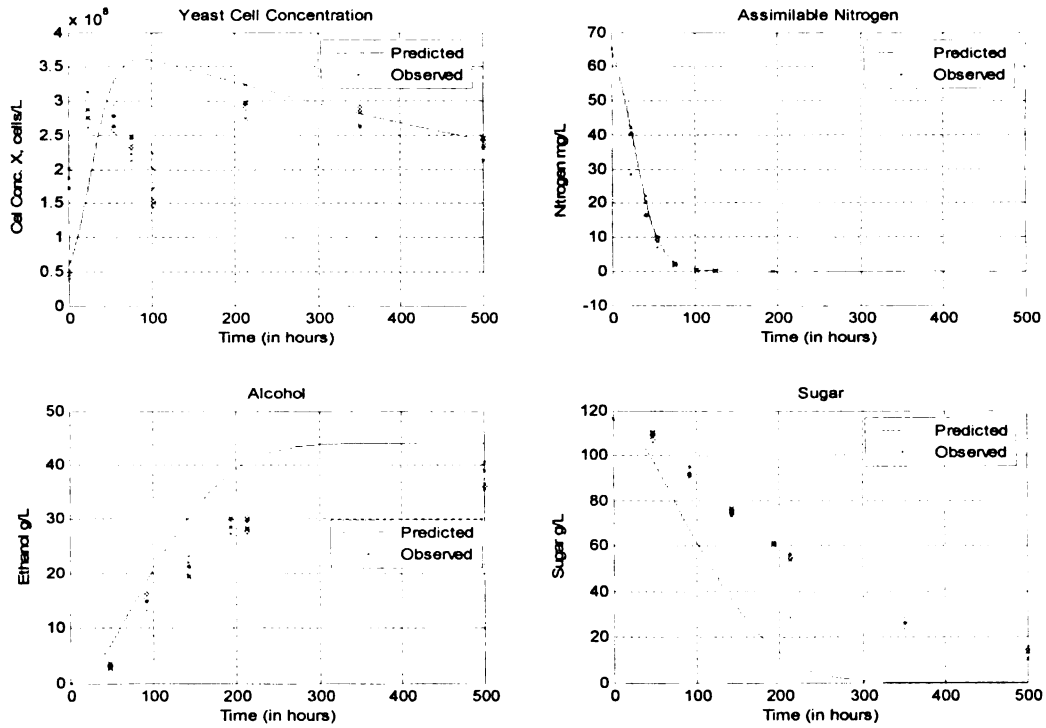
Plots of Predicted & Observed Dependent Variables from Model Validation



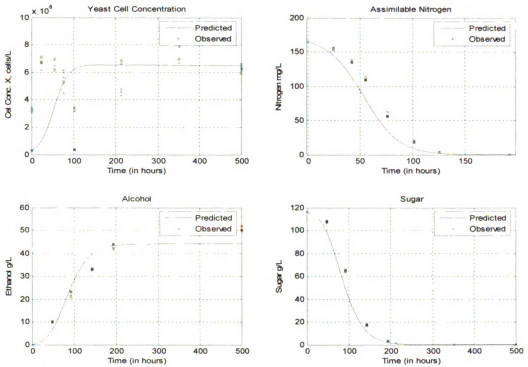
C.1. Model Validation for T=17° C, DAP=0 ppm



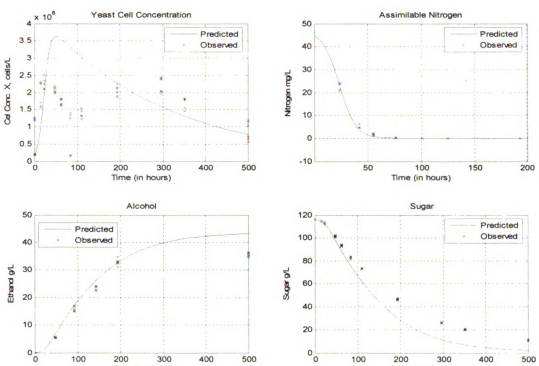
C.2. Model Validation for T=17° C, DAP=100 ppm



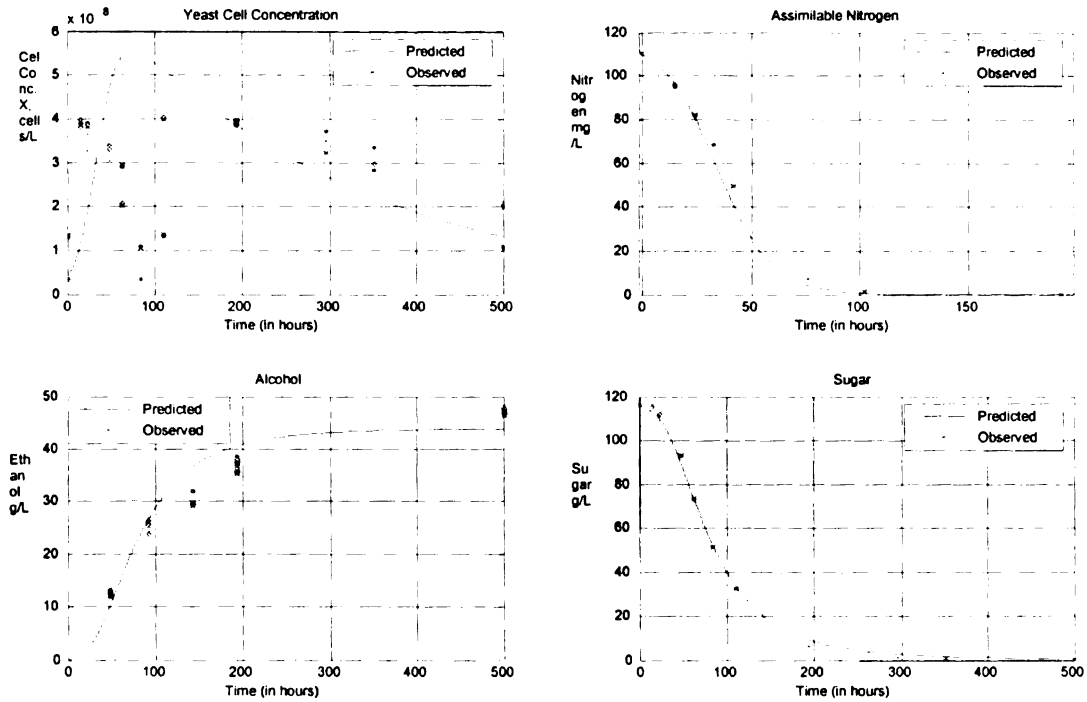
C.3. Model Validation for T=17° C, DAP=300 ppm



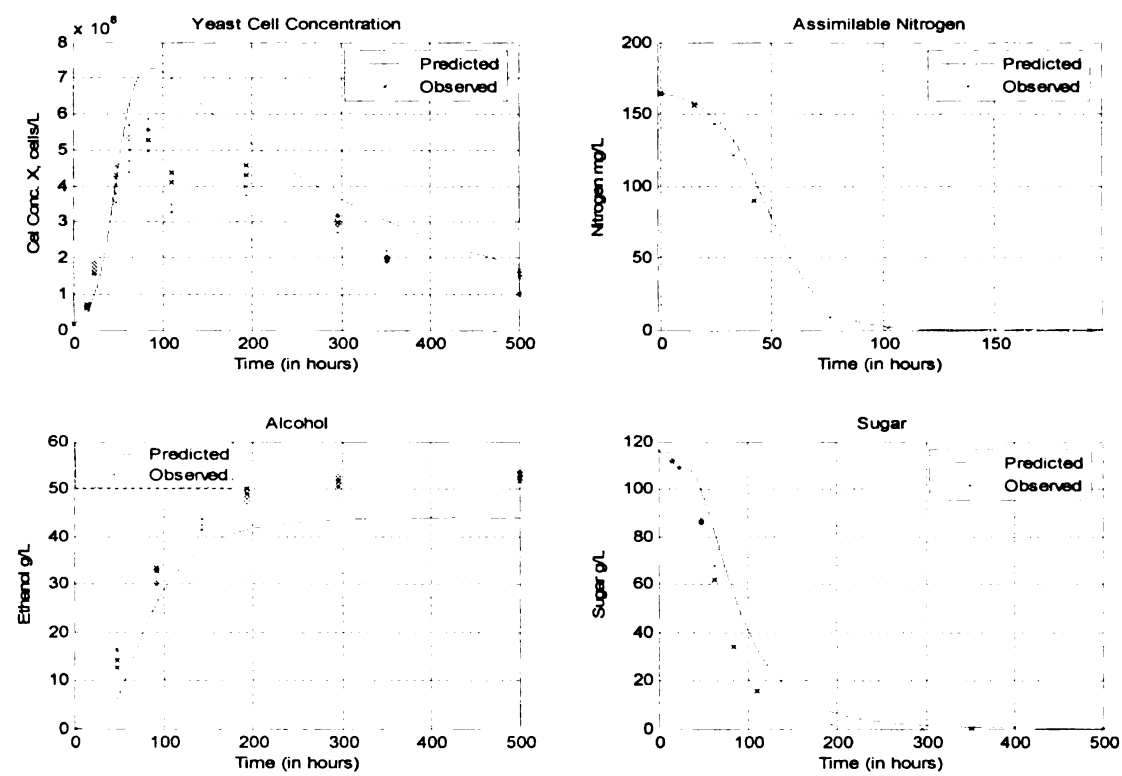
C.4. Model Validation for T=17° C, DAP=600 ppm



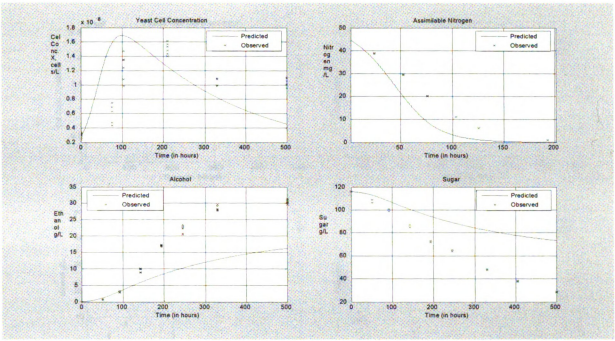
C.5. Model Validation for T=22° C, DAP=0 ppm



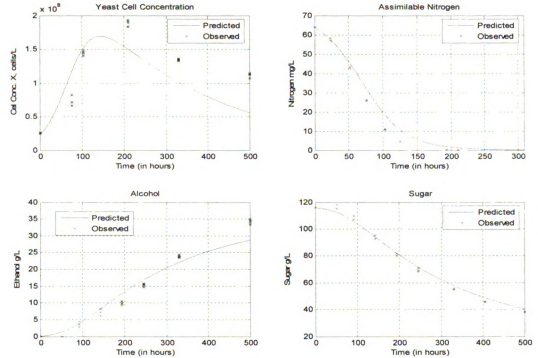
C.6. Model Validation for T=22° C, DAP=300 ppm



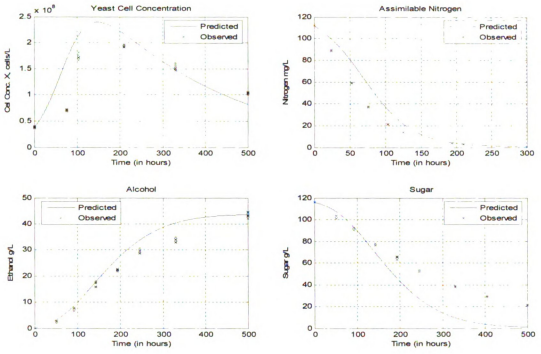
C.7. Model Validation for T=22° C, DAP=600 ppm



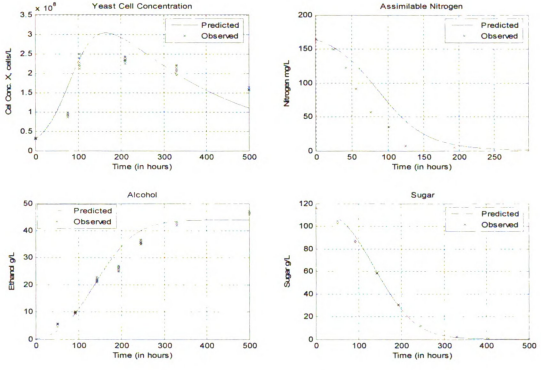
C.8. Model Validation for T=11° C, DAP=0 ppm



C.9. Model Validation for T=11° C, DAP=100 ppm



C.10. Model Validation for T=11° C, DAP=300 ppm



C.11. Model Validation for T=11° C, DAP=600 ppm

Appendix D

Program A: Non-Linear Parameter Estimation in MATLAB© at T=22 C and DAP=100 ppm

'nlf.m'

```
% 22 C DAP=100 added

%Read all observed data. Raw observed data is read from an Excel© file.

clear all
global t4 y4 pf4 p1 p2 p3 p4 p5 p6 p7 tn ts te tx n s e x;
global nhat shat ehat xhat that tmpt0 sumsq count2 counter;

t4=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A35:A109');
y4=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B35:B109');

tx=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A2:A34');
tn=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A35:A58');
te=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A59:A76');
ts=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A77:A109');

x=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B2:B34');
n=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B35:B58');
e=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B59:B76');
s=xlsread('C:\...\Thesis\expt-1-data.xls', 'SSQ7', 'B77:B109');

po4=textread('C:\MATLAB7\work\New Files\par4.txt')
% this is a text file containing the initial parameter estimates

count2=1;
counter=0;
%Call another function for non-linear data fitting
[pf4,r,J] = nlinfit(t4,y4,'nested4',po4);

pf4=pf4

subplot (2,2,1)
    plot(that,xhat, '--
rs','LineWidth',1,'MarkerEdgeColor','k','MarkerFaceColor','g','MarkerSize',2)
        hold all % hold plot and cycle line colors
        plot(tx,x)
        hold off
        grid on
```

```
        xlabel('Time (in hours)')
        ylabel(' Cel Conc. X, cells/uL')
        title('Yeast Cell Concentration')

    subplot (2,2,2)
    plot(that,nhat, '--rs', 'LineWidth',1,'MarkerEdgeColor','k','MarkerFaceColor','g','
MarkerSize',2)
        hold all % hold plot and cycle line colors
        plot(tn,n)
        hold off
        grid on
        xlabel('Time (in hours)')
        ylabel('Nitrogen g/L')
        title('Assimilable Nitrogen')

    subplot (2,2,3)
    plot(that,ehat, '--rs','LineWidth',1,'MarkerEdgeColor','k','MarkerFaceColor','g',
'MarkerSize',2)
        hold all % hold plot and cycle line colors
        plot(te,e)
        hold off
        grid on
          xlabel('Time (in hours)')
          ylabel('Ethanol g/L')
          title('Alcohol')

    subplot (2,2,4)
    plot(that,shat, '--rs','LineWidth',1,'MarkerEdgeColor','k','MarkerFaceColor','g',
'MarkerSize',2)
        hold all % hold plot and cycle line colors
        plot(ts,s)
        hold off
        grid on
        xlabel('Time (in hours)')
        ylabel('Sugar g/L')
        title('Sugar')
```

Program B: Solve model in MATLAB© using 4th-order Runge-Kutta formula for T=22 C and DAP=100 ppm

'nested4.m'

```
% This program solves the model using 4th-order Runge-Kutta formula
```

```
function out=nested4(po4,t4)
    global p1 p2 p3 p4 p5 p6 p7 counter nhat shat ehat xhat that;
    global tx tn te ts x n e s;
    opts=optimset('disp','iter','TolX',1e-0012);
    %options=['Vectorized' 'On']
  % Input initial parameter estimates%
    p1=po4(1);
    p2=po4(2);
    p3=3.5E-003;
    p4=po4(3);
    p5=po4(4);
    p6=po4(5);
    p7=0.38;

    %ICs =txtread('C:\MATLAB7\work\New Files\ic1.txt')
    ICs =[23E6 65 0 116];
    tmpt0=[0:0.01:500];
    count=0;

% Call function ode15s to solve coupled differential equations in ode1.m

    [t,Y]=ode15s(@ode1,tmpt0,ICs,opts);
    counter=counter+1


%       for i=1:[length(tx)]
%           temp=tx(i);
%           Ytemp=find(t==temp);
%           %pause
%           out(i)=(Y(Ytemp,1));
%       end
%       count=count+i;

% Select the output of [t,Y] those predicted values that correspond to the observed data

    for i=1:[length(tn)]
        temp=tn(i);
        Ytemp=find(t==temp);
        out(count+i)=Y(Ytemp,2);
    end
    count=count+i;

    for i=1:[length(te)]
        temp=te(i);
        Ytemp=find(t==temp);
```

```
%           out(count+i)=Y(Ytemp,3)/7.89;
          out(count+i)=Y(Ytemp,3);
    end
    count=count+i;

    for i=1:[length(ts)]
        temp=ts(i);
        Ytemp=find(t==temp);
        out(count+i)=Y(Ytemp,4);
    end


  out=out';
  nhat=Y(:,2); shat=Y(:,4); ehat=Y(:,3); xhat=Y(:,1); that=t;

  return
  end
```

Program C: Compare raw observed data with model predictions and check accuracy of initial estimates

'odesolver.m'

```
clear all

po4=textread('C:\MATLAB7\work\New Files\par4.txt')

    global p1 p2 p3 p4 p5 p6 p7 counter nhat shat ehat xhat that;
    global tx tn te ts x n e s;
    opts=optimset('disp','iter','TolX',1e-012);
    %options=['Vectorized' 'On']
    p1=po4(1);
    p2=po4(2);
    p3=3.5E-003;
    p4=po4(3);
    p5=po4(4);
    p6=po4(5);
    p7=0.38;
    %ICs =txtread('C:\MATLAB7\work\New Files\ic1.txt')
    ICs = [23E6 65 0 116];
    tmpt0=[0:0.01:500];
    count=0;
    [t,Y]=ode15s(@ode1,tmpt0,ICs,opts);
```

```
   nhat=Y(:,2); shat=Y(:,4); ehat=Y(:,3); xhat=(Y(:,1)); that=t;
%    ehat=(Y(:,3)/7.89);

tx=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A2:A34');
tn=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A35:A58');
te=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A59:A76');
ts=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'A77:A109');

x=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B2:B34');
n=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B35:B58');
e=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B59:B76');
s=xlsread('C:\...\expt-1-data.xls', 'SSQ7', 'B77:B109');

subplot (2,2,1)
     plot(that,xhat)
        hold all % hold plot and cycle line colors
         plot(tx,x, '--
rs','LineWidth',2,'MarkerEdgeColor','k','MarkerFaceColor','g','MarkerSize',2)
         hold off
         grid on
         xlabel('Time (in hours)')
         ylabel(' Cel Conc. X, cells/uL')
         title('Yeast Cell Concentration')

    subplot (2,2,2)
    plot(that,nhat)
         hold all % hold plot and cycle line colors
         plot(tn,n, '--
rs','LineWidth',2,'MarkerEdgeColor','k','MarkerFaceColor','g','MarkerSize',2)
         hold off
         grid on
         xlabel('Time (in hours)')
         ylabel('Nitrogen mg/L')
         title('Assimilable Nitrogen')

    subplot (2,2,3)
    plot(that,ehat)
         hold all % hold plot and cycle line colors
         plot(te,e, '--
rs','LineWidth',2,'MarkerEdgeColor','k','MarkerFaceColor','g','MarkerSize',2)
         hold off
         grid on
          xlabel('Time (in hours)')
          ylabel('Ethanol g/L')
          title('Alcohol')
```

```
    subplot (2,2,4)
    plot(that,shat)
        hold all % hold plot and cycle line colors
        plot(ts,s, '--
rs','LineWidth',2,'MarkerEdgeColor','k','MarkerFaceColor','g','MarkerSize',2)
        hold off
        grid on
        xlabel('Time (in hours)')
        ylabel('Sugar g/L')
        title('Sugar')
```

Program D: Program describing the ordinary differential equations that constitute the proposed model

```
function dy = ode1(t,y)

dy = zeros(4,1);
global p1 p2 p3 p4 p5 p6 p7;
%PARAMS=[MEUmax BETAmax kd Kn Ks yx/n yE/S]

% dy = [ Xv N E S ]

%Equations (1.1) to (1.4) and equations (2.1) and (2.2)

%dXv/dt
%dy(1)= (((params1(1) * y(1) /(params1(4) + y(1))) * y(1)) - (params1(3)*y(1)))

%dN/dt
dy(2)= ((-1)*y(1)*(p1*y(2)/((p4+y(2))))/p6);

%dE/dt
dy(3)= (p2*y(4)/(p5+y(4)))*y(1) ;

%dS/dt
dy(4)= ((-1)*y(1)*(p2*y(4)/(p5+y(4))))/p7;

return
end
```

Appendix E

HPLC Analysis of Ethanol, *E*

The figures below show a typical HPLC chromatogram for hard cider followed by a chromatogram for hard cider spiked with ethanol.

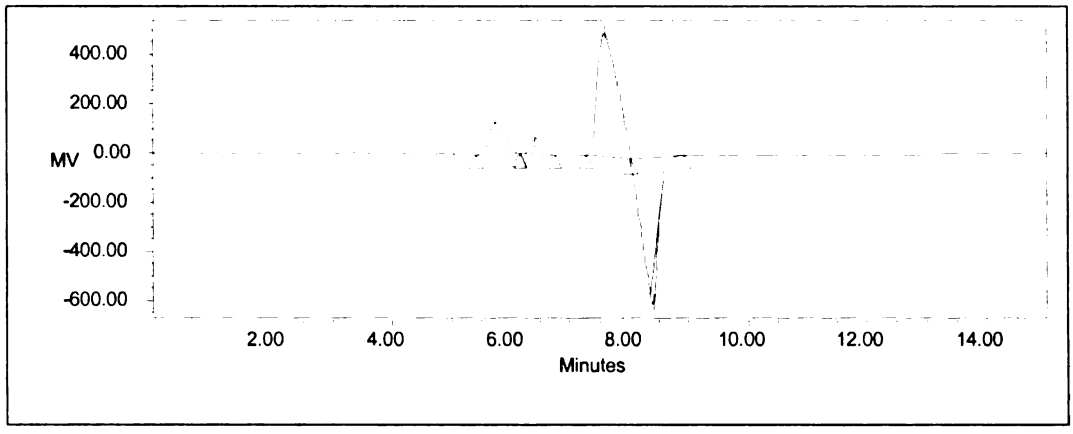


Figure E.1. HPLC Chromatogram for hard cider: Ethanol is detected by a positive peak at 7.5 minutes

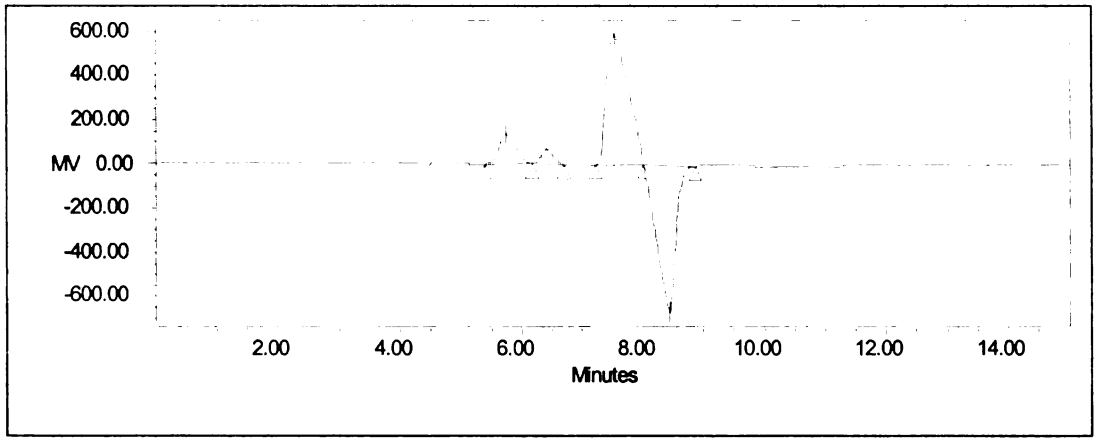


Figure E.2. HPLC Chromatogram for hard cider spiked with 99.9% pure ethanol: The positive peak at 7.5 minutes is greater in height and area

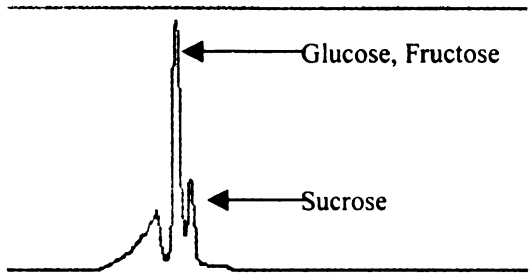


Figure E.3. HPLC Chromatogram showing various sugars: Glucose, Fructose is detected at 6.3 minutes and sucrose at 6.9 minutes following the procedure described in section 3.2 on Methods of Analysis

Appendix F

Parameter Estimation Data Set

F.1. Data for T=11 °C, Viable Yeast Cell Concentration, X_V

Time	Xv cells/mL			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	27000000	23000000	38000000	29000000
0	28333333.3	24000000	40000000	30000000
0	28666666.7	23666666.7	41000000	30000000
76	52000000	61000000	71000000	83000000
76	57333333.3	65666666.7	72000000	82666666.7
76	62666666.7	75333333.3	73000000	89333333.3
103	123000000	128600000	170000000	208000000
103	118000000	132200000	175000000	228000000
103	113000000	135300000	180000000	231000000
210	128000000	177000000	196000000	245000000
210	131000000	168000000	201000000	252000000
210	135000000	174000000	199000000	254000000
330	90000000	125000000	165000000	232000000
330	90000000	121670000	158666667	213000000
330	91000000	123300000	152333333	228000000
500	91000000	105000000	105000000	171000000
500	92413793.1	103448276	106551724	171000000
500	87400000	98214285.7	108214286	172000000

F.2. Data for T=17 °C, Viable Yeast Cell Concentration, X_V

Time	Xv cells/mL			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	31430000.00	55360000.00	55360000.00	28570000.00
0	41790000.00	63930000.00	63930000.00	31430000.00
0	52140000.00	70360000.00	70360000.00	36070000.00
24	62000000.00	207000000.00	207000000.00	290000000.00
24	65330000.00	220330000.00	220330000.00	306000000.00
24	70670000.00	233670000.00	233670000.00	321670000.00
55	133000000.00	280000000.00	280000000.00	390000000.00
55	134670000.00	297330000.00	297330000.00	385000000.00
55	138000000.00	284330000.00	284330000.00	380000000.00
76	241000000.00	317000000.00	317000000.00	330000000.00
76	237670000.00	326330000.00	326330000.00	323000000.00
76	237670000.00	323000000.00	323000000.00	316670000.00
101	310000000.00	353000000.00	353000000.00	375000000.00
101	307000000.00	340000000.00	340000000.00	371330000.00
101	305330000.00	340000000.00	340000000.00	380000000.00
212	299000000.00	325330000.00	325330000.00	322330000.00
212	289000000.00	319670000.00	319670000.00	333330000.00
212	284330000.00	318670000.00	318670000.00	338330000.00
351	167000000.00	288000000.00	288000000.00	325000000.00
351	178670000.00	271330000.00	271330000.00	323330000.00
351	179670000.00	289330000.00	289330000.00	272640000.00
500	155330000.00	247660000.00	247660000.00	307000000.00
500	160000000.00	257330000.00	257330000.00	310690000.00
500	151000000.00	242330000.00	242330000.00	292760000.00

F.3. Data for T=22 °C, Viable Yeast Cell Concentration, X_V

Time	Xv cells/mL			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	18000000.00	23200000.00	37600000	14000000
0	15416666.67	25600000.00	36800000	13600000
0	15652173.91	25200000.00	35600000	15600000
15	126000000.00	64400000.00	124800000	58400000
15	118750000.00	61600000.00	134000000	63200000
15	114782608.70	62400000.00	136800000	67200000
24	149000000.00	230800000.00	403200000	203600000
24	147000000.00	248000000.00	405200000	191600000
24	156500000.00	186000000.00	377200000	171600000
48	203600000.00	283000000.00	388000000	403600000
48	201666666.70	308000000.00	382000000	391600000
48	201739130.40	299200000.00	376000000	371600000
63	210000000.00	294000000.00	424000000	488000000
63	211666666.70	294000000.00	412000000	462000000
63	190000000.00	292000000.00	408000000	412000000
84	234800000.00	265000000.00	340000000	518000000
84	234000000.00	254000000.00	348000000	494000000
84	233300000.00	252000000.00	356000000	466000000
110	201600000.00	234000000.00	352000000	464000000
110	205833333.30	253200000.00	352800000	468400000
110	208260869.60	225000000.00	354000000	370400000
193	185200000.00	212000000.00	316000000	423600000
193	178000000.00	204000000.00	308000000	404000000
193	182000000.00	192000000.00	321500000	402000000
295	162000000.00	156000000.00	242000000	254000000
295	155400000.00	148000000.00	226400000	283300000
295	158000000.00	144000000.00	219100000	278000000
351	150000000.00	130000000.00	164000000	193200000
351	147916666.70	141200000.00	170000000	182400000
351	145652173.90	149200000.00	172000000	176400000
500	47600000.00	43200000.00	78400000	89000000
500	49583333.33	45600000.00	74400000	136400000
500	49130434.78	50400000.00	70800000	147600000

F.4. Data for T=11 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45	64	112	164
0	45	64	112	164
0	45	64	112	164
24	39.7830354	57.60316804	87.24022043	149.6533641
24	39.7830354	57.60316804	87.24022043	149.6533641
24	39.7830354	57.60316804	87.24022043	149.6533641
52	30.3383215	43.04205617	57.72935041	122.2379552
52	30.3383215	43.04205617	57.72935041	122.2379552
52	30.3383215	43.04205617	57.72935041	122.2379552
76	20.6564055	26.0384072	37.11125123	91.30099612
76	20.6564055	26.0384072	37.11125123	91.30099612
76	20.6564055	26.0384072	37.11125123	91.30099612
103	11.3068194	10.80806622	21.34729962	56.80542436
103	11.3068194	10.80806622	21.34729962	56.80542436
103	11.3068194	10.80806622	21.34729962	56.80542436
125	6.33884107	4.603436231	13.43309598	35.27500181
125	6.33884107	4.603436231	13.43309598	35.27500181
125	6.33884107	4.603436231	13.43309598	35.27500181
193	1.01410429	0.339103514	3.486024468	7.093196487
193	1.01410429	0.339103514	3.486024468	7.093196487
193	1.01410429	0.339103514	3.486024468	7.093196487
210	0.66432363	0.189522586	2.571621406	4.835573916
210	0.66432363	0.189522586	2.571621406	4.835573916
210	0.66432363	0.189522586	2.571621406	4.835573916

F.5. Data for T=17 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45	65	127	165
0	45	65	127	165
0	45	65	127	165
24	22.3431924	38.15045585	104.3271583	156.359136
24	22.3431924	38.15045585	104.3271583	156.359136
24	22.3431924	38.15045585	104.3271583	156.359136
42	4.33806745	16.01722754	68.61728958	137.9959603
42	4.33806745	16.01722754	68.61728958	137.9959603
42	4.33806745	16.01722754	68.61728958	137.9959603
55	1.01394586	7.065134792	41.01991383	114.096778
55	1.01394586	7.065134792	41.01991383	114.096778
55	1.01394586	7.065134792	41.01991383	114.096778
76	0.09881884	1.658353357	13.64341851	62.89816682
76	0.09881884	1.658353357	13.64341851	62.89816682
76	0.09881884	1.658353357	13.64341851	62.89816682
101	0.00750595	0.286216576	3.224695001	19.40497316
101	0.00750595	0.286216576	3.224695001	19.40497316
101	0.00750595	0.286216576	3.224695001	19.40497316
125	0.00077222	0.054099652	0.842491706	4.959377215
125	0.00077222	0.054099652	0.842491706	4.959377215
125	0.00077222	0.054099652	0.842491706	4.959377215
193	2.9649E-06	0.000591548	0.030947666	0.081403109
193	2.9649E-06	0.000591548	0.030947666	0.081403109
193	2.9649E-06	0.000591548	0.030947666	0.081403109

F.6. Data for T=22 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45	65	110	165
0	45	65	110	165
0	45	65	110	165
24	21.4270081	54.90532244	97.36440943	156.34515
24	21.4270081	54.90532244	97.36440943	156.34515
24	21.4270081	54.90532244	97.36440943	156.34515
42	4.46851567	43.85542894	84.16440719	143.6076667
42	4.46851567	43.85542894	84.16440719	143.6076667
42	4.46851567	43.85542894	84.16440719	143.6076667
55	1.15086761	30.45588295	66.97349415	121.3479307
55	1.15086761	30.45588295	66.97349415	121.3479307
55	1.15086761	30.45588295	66.97349415	121.3479307
76	0	18.29589934	48.31115162	89.92936739
76	0	18.29589934	48.31115162	89.92936739
76	0	18.29589934	48.31115162	89.92936739
101	0	1.461745516	7.32207622	9.10062081
101	0	1.461745516	7.32207622	9.10062081
101	0	1.461745516	7.32207622	9.10062081
125	0	0.207625667	1.473091544	1.143798166
125	0	0.207625667	1.473091544	1.143798166
125	0	0.207625667	1.473091544	1.143798166
193	0	0.00072462	0.012510934	0.002510864
193	0	0.00072462	0.012510934	0.002510864
193	0	0.00072462	0.012510934	0.002510864

F.7. Data for T=11 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L			
(Hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
52	0.56	0	1.8936	4.4184
52	0.78	0	2.8404	5.6019
52	0.81	0	2.5248	5.2863
92	3.1835	2.7615	7.2588	10.0203
92	2.6569	4.5762	6.4698	10.4937
92	2.789	3.4716	7.6533	9.7836
143	8.523	6.2331	16.8846	21.6975
143	9.5762	7.2588	15.4644	22.3287
143	9.6551	8.2845	17.6736	23.2755
193	16.4955	9.9414	21.8553	25.5636
193	16.2056	10.257	22.2498	26.3526
193	16.679	9.0735	21.9342	24.459
245	19.8625	15.3855	28.7985	34.4793
245	22.4148	15.1488	29.1141	35.8206
245	21.5726	14.5176	30.0609	34.716
330	28.4076	23.3544	34.1637	41.2647
330	27.1452	23.9067	33.138	41.8959
330	26.9085	23.1966	33.2958	41.2647
330	28.4076	23.3544	34.1637	41.2647
330	27.1452	23.9067	33.138	41.8959
330	26.9085	23.1966	33.2958	41.2647
500	30.0609	33.6114	43.395	45.2886
500	28.8774	34.2426	42.4482	45.8409
500	29.193	32.8224	43.6317	45.2097

F.8. Data for T=17 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
48	1.1835	2.7615	7.2588	8.4423
48	1.6569	4.5762	6.4698	8.9157
48	0.789	3.4716	7.6533	9.7836
92	5.523	15.3066	20.1984	21.0663
92	6.6276	14.5965	18.6993	21.6186
92	5.4441	15.1488	20.8296	21.8553
143	16.6479	22.0131	29.5086	31.7178
143	15.5433	23.0388	31.2444	30.2187
143	16.1745	24.0645	28.7196	31.1655
193	23.2755	30.2976	37.6353	39.6867
193	23.9856	30.1398	36.4518	42.9216
193	24.459	29.5875	36.1362	41.2647
500	37.2408	39.6867	45.2886	48.918
500	37.3986	40.3179	44.3418	49.7859
500	36.0573	38.8188	44.6574	47.8134

F.9. Data for T=22 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
48	5.9964	12.1506	11.9928	12.1745
48	6.6276	13.2552	12.7818	15.8057
48	7.2588	13.6497	11.5983	13.6736
92	16.9635	20.0406	24.7746	32.0609
92	16.2534	20.9085	23.8278	33.982
92	15.3855	20.6718	26.5104	31.2444
143	24.5379	24.3801	29.5086	43.2958
143	23.4333	25.4058	31.2444	42.7435
143	23.9856	24.0645	28.7196	42.5857
193	31.9545	36.294	37.6353	47.7142
193	34.716	38.0298	36.4518	48.0298
193	33.2169	34.3215	36.1362	45.8206
500	37.6353	44.3418	46.7088	50.7327
500	37.3197	43.9473	45.8409	49.9437
500	36.294	45.762	45.6042	50.3382

F.10. Data for T=11 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	116	116	116	116
0	116	116	116	116
0	116	116	116	116
52	111.201266	112.4847114	106.0967261	106.3316382
52	111.201266	112.4847114	106.0967261	106.3316382
52	111.201266	112.4847114	106.0967261	106.3316382
92	102.959187	104.6837317	95.03607745	88.98096031
92	102.959187	104.6837317	95.03607745	88.98096031
92	102.959187	104.6837317	95.03607745	88.98096031
143	88.5952773	90.77985775	79.98023197	57.65329257
143	88.5952773	90.77985775	79.98023197	57.65329257
143	88.5952773	90.77985775	79.98023197	57.65329257
193	74.7125694	78.19086024	66.52078682	30.06040873
193	74.7125694	78.19086024	66.52078682	30.06040873
193	74.7125694	78.19086024	66.52078682	30.06040873
245	62.3076042	67.36716282	54.65013978	12.34262544
245	62.3076042	67.36716282	54.65013978	12.34262544
245	62.3076042	67.36716282	54.65013978	12.34262544
330	46.5924344	53.9500996	39.68056648	2.408089893
330	46.5924344	53.9500996	39.68056648	2.408089893
330	46.5924344	53.9500996	39.68056648	2.408089893
405	36.5499563	45.46426771	30.1996127	0.679179924
405	36.5499563	45.46426771	30.1996127	0.679179924
405	36.5499563	45.46426771	30.1996127	0.679179924
500	27.5931765	37.88714237	21.87583241	0.197031307
500	27.5931765	37.88714237	21.87583241	0.197031307
500	27.5931765	37.88714237	21.87583241	0.197031307

F.11. Data for T=17 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	116.00	116.00	116	116
0	116.00	116.00	116	116
0	116.00	116.00	116	116
48	105.47	106.26	102.7333856	106.4694394
48	105.47	106.26	102.7333856	106.4694394
48	105.47	106.26	102.7333856	106.4694394
92	89.19	90.34	68.71753135	62.42043055
92	89.19	90.34	68.71753135	62.42043055
92	89.19	90.34	68.71753135	62.42043055
143	73.32	73.05	35.16326314	15.75405745
143	73.32	73.05	35.16326314	15.75405745
143	73.32	73.05	35.16326314	15.75405745
193	60.58	58.28	16.06795658	2.964851871
193	60.58	58.28	16.06795658	2.964851871
193	60.58	58.28	16.06795658	2.964851871
212.00	56.37	53.24	11.71686095	1.534290748
212.00	56.37	53.24	11.71686095	1.534290748
212.00	56.37	53.24	11.71686095	1.534290748
351.00	33.86	25.43	1.404251642	0.011796163
351.00	33.86	25.43	1.404251642	0.011796163
351.00	33.86	25.43	1.404251642	0.011796163
500	20.82	10.34	0.308015517	6.46724E-05
500	20.82	10.34	0.308015517	6.46724E-05
500	20.82	10.34	0.308015517	6.46724E-05

F.12. Data for T=11 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0.00	116	116	116	116
0.00	116	116	116	116
0.00	116	116	116	116
15.00	114.54387	114.084991	113.0371973	114.1000532
15.00	114.54387	114.084991	113.0371973	114.1000532
15.00	114.54387	114.084991	113.0371973	114.1000532
24.00	112.38142	111.5625198	109.5500385	111.1527944
24.00	112.38142	111.5625198	109.5500385	111.1527944
24.00	112.38142	111.5625198	109.5500385	111.1527944
48.00	102.049076	97.85182154	90.9807048	87.52709999
48.00	102.049076	97.85182154	90.9807048	87.52709999
48.00	102.049076	97.85182154	90.9807048	87.52709999
63.00	94.7545835	86.49300368	74.49529182	62.95072964
63.00	94.7545835	86.49300368	74.49529182	62.95072964
63.00	94.7545835	86.49300368	74.49529182	62.95072964
84.00	85.1170423	71.21066584	52.32328035	34.64154636
84.00	85.1170423	71.21066584	52.32328035	34.64154636
84.00	85.1170423	71.21066584	52.32328035	34.64154636
110.00	74.375303	55.17397849	31.74062889	15.78144938
110.00	74.375303	55.17397849	31.74062889	15.78144938
110.00	74.375303	55.17397849	31.74062889	15.78144938
193.00	47.9677234	23.79321627	6.079927888	1.692738265
193.00	47.9677234	23.79321627	6.079927888	1.692738265
193.00	47.9677234	23.79321627	6.079927888	1.692738265
295.00	28.0740084	9.279564716	1.161020665	0.222923986
295.00	28.0740084	9.279564716	1.161020665	0.222923986
295.00	28.0740084	9.279564716	1.161020665	0.222923986
351.00	21.2436649	6.037606593	0.575723601	0.095897917
351.00	21.2436649	6.037606593	0.575723601	0.095897917
351.00	21.2436649	6.037606593	0.575723601	0.095897917
500.00	11.2510679	2.596126198	0.153309599	0.019685051
500.00	11.2510679	2.596126198	0.153309599	0.019685051
500.00	11.2510679	2.596126198	0.153309599	0.019685051

APPENDIX G
Model Validation Data Set

G.1. Data for T=11 °C, Viable Yeast Cell Concentration, X_V, cells/mL

Time (hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0.00	29555658	24989500	37218413.32	30305000
0.00	26187879.18	25713833.37	40156709.11	31350000
0.00	32353219.25	25189416	36452902.37	31668725
0.00	33950909.05	26284608	38371476.18	32760750
0.00	23923430.77	25919544.04	39330763.08	32760750
76.00	47503594.74	66276500	69539666.99	86735000
76.00	62760162.63	71346833.37	70519098.92	86386666.7
76.00	68598317.37	81849666.63	71498530.85	93353333.3
76.00	43395990.64	66806712	68109370.22	90638075
76.00	68700663.06	71917608.04	72493633.69	90274066.7
76.00	75091422.5	82504463.96	73500489.72	97554233.3
103.00	134642442	139723900	166503428	217360000
103.00	107796618.8	143635300	171400587.7	238260000
103.00	123695902	147003450	176297747.3	221052631.6
103.00	147386887.7	140841691.2	171165524	227141200
103.00	98475517.23	144784382.4	176199804.1	248981700
103.00	135404213.9	148179477.6	181234084.2	211533618.7
210.00	140115712	192310500	191968658.2	234449760.8
210.00	143399674	182532000	196865817.8	241148325.4
210.00	147778290	189051000	194906954	243062201
210.00	153378224.6	193848984	197343780.6	224353838.1
210.00	156973026.7	183992256	192816667.8	230763947.7
210.00	161766096.3	190563408	190898094	232595407.6
330.00	98518860	135812500	161606268.4	222009569.4
330.00	98518860	132194455	155403199.8	203827751.2
330.00	99613514	133965450	149200130.3	218181818.2
330.00	107844064.2	136899000	158282339.2	212449348.7
330.00	107844064.2	133252010.6	152206855.8	195050479.6
330.00	109042331.6	135037173.6	146131371.5	208786428.9
500.00	99613514	114082500	102840352.6	163636363.6
500.00	101161128.3	112396551.9	104360160.6	163636363.6
500.00	95672759.6	106709821.4	105988526.9	164593301.4
500.00	109042331.6	114995160	100725125	156589821.7
500.00	110736433.7	113295724.3	102213673.5	156589821.7
500.00	104728569	107563500	103808547.4	157505551.6

G.2. Data for T=17 °C, Viable Yeast Cell Concentration, X_V

Time	Xv cells/mL			
(Hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0.00	41790000.00	46000000.00	51450000.00	28570000.00
0.00	31430000.00	51000000.00	63930000.00	30450000.00
0.00	41790000.00	39000000.00	70360000.00	32450000.00
0.00	52140000.00	172000000.00		310654000.00
0.00	30400000.00	201000000.00	63930000.00	325767000.00
0.00	31200000.00	188000000.00	70360000.00	340545000.00
24.00	62000000.00	275000000.00	207000000.00	667343000.00
24.00	65330000.00	276000000.00	220330000.00	680450000.00
24.00	70670000.00	289000000.00	233670000.00	615757500.00
24.00	41700000.00	314000000.00	207000000.00	705350000.00
24.00	72100000.00	287000000.00	220330000.00	716354000.00
24.00	65400000.00	262000000.00	233670000.00	669430000.00
55.00	133000000.00	280000000.00	280000000.00	650000000.00
55.00	134670000.00	294000000.00	297330000.00	685000000.00
55.00	138000000.00	277000000.00	284330000.00	701000000.00
55.00	62000000.00	255000000.00	280000000.00	622000000.00
55.00	121000000.00	265000000.00	297330000.00	650000000.00
55.00	148200000.00	262000000.00	284330000.00	612000000.00
76.00	241000000.00	223000000.00	317000000.00	615000000.00
76.00	237670000.00	245000000.00	326330000.00	534000000.00
76.00	237670000.00	248000000.00	323000000.00	598000000.00
76.00	238000000.00	212000000.00	317000000.00	447000000.00
76.00	248500000.00	230000000.00	326330000.00	561000000.00
76.00	211330000.00	234000000.00	323000000.00	521000000.00
101.00	310000000.00	144000000	353000000	40530000
101.00	307000000.00	151000000	340000000	30450000
101.00	305330000.00	155000000	340000000	34330000
101.00	237670000.00	172000000	353000000	333000000
101.00	310000000.00	201000000	340000000	347000000
101.00	310500000.00	222000000	340000000	317000000
212.00	299000000.00	275000000	325330000	456000000
212.00	289000000.00	298000000	319670000	432000000
212.00	284330000.00	297000000	318670000	475000000
212.00	305330000.00	323000000	325330000	662000000
212.00	309330000.00	287000000	319670000	680000000

Time	Xv cells/mL			
(Hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
351.00	178670000.00	294000000	271330000	881000000
351.00	179670000.00	284000000	289330000	795000000
351.00	204330000.00	288000000	288000000	670000000
351.00	182670000.00	265000000	271330000	691000000
351.00	178670000.00	262000000	289330000	703000000
500.00	155330000.00	247000000	247660000	663000000
500.00	160000000.00	245000000	257330000	634000000
500.00	151000000.00	231000000	242330000	647000000
500.00	179670000.00	212000000	247660000	590000000
500.00	155330000.00	242000000	257330000	624000000
500.00	160000000.00	235000000	242330000	612000000
212.00	301300000.00	294000000	318670000	691000000
351.00	167000000.00	340000000	288000000	782000000

G.3. Data for T=22 °C, Viable Yeast Cell Concentration, X_V

Time	Xv cells/mL			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0.00	18000000.00	24000000.00	35460000	14910000
0.00	21000000.00	23600000.00	36000000	14484000
0.00	20000000.00	25300000.00	32000000	16614000
0.00	122000000.00	67700000.00	127000000	15879150
0.00	119000000.00	64750000.00	133000000	15425460
0.00	127000000.00	68674000.00	136000000	17693910
15.00	156000000.00	215640000.00	395000000	62196000
15.00	161000000.00	221546000.00	391000000	67308000
15.00	170000000.00	218454000.00	402000000	63098591.55
15.00	224000000.00	290390000.00	388000000	66238740
15.00	231000000.00	288479000.00	378000000	71683020
15.00	228000000.00	294280000.00	384000000	59247503.8
24.00	225000000.00	304000000.00	395000000	191173708.9
24.00	234000000.00	301000000.00	390000000	179906103.3
24.00	251000000.00	299000000.00	382000000	161126760.6
24.00	210000000.00	301000000.00	364000000	179505830
24.00	235000000.00	293000000.00	380000000	168925918.6
24.00	211000000.00	287000000.00	327000000	151292732.9
48.00	219000000.00	228000000.00	354360000	378967136.2
48.00	214000000.00	251000000.00	354000000	422928000
48.00	211000000.00	234000000.00	326000000	401328000
48.00	201000000.00	226000000.00	341000000	355837686.5
48.00	195000000.00	215000000.00	335000000	456762240
48.00	204000000.00	164000000.00	304000000	433434240
63.00	183000000.00	171000000.00	290000000	527040000
63.00	177000000.00	168000000.00	297000000	498960000
63.00	181000000.00	164000000.00	285000000	438780000
63.00	168000000.00	132000000.00	204000000	569203200
63.00	164000000.00	128000000.00	201000000	538876800
63.00	161000000.00	133000000.00	210000000	467300700
84.00	125000000.00	64000000.00	104000000	551670000
84.00	141000000.00	58000000.00	108000000	526110000
84.00	133000000.00	53000000.00	112000000	496290000
84.00	16000000.00	24240000	35920980	587528550
84.00	15400000.00	23836000	36468000	560307150

Time	Xv cells/mL			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
84.00	17100000.00	25553000	32416000	528548850
110.00	134000000.00	68377000	128651000	435680751.2
110.00	131000000.00	65397500	134729000	439812206.6
110.00	122000000.00	69360740	137768000	347793427.2
110.00	145000000.00	217796400	400135000	409089907.2
110.00	154000000.00	223761460	396083000	412969208
110.00	153000000.00	220638540	407226000	326566598.3
193.00	201000000.00	293293900	393044000	397746478.9
193.00	190000000.00	291363790	382914000	430260000
193.00	185000000.00	297222800	388992000	428130000
193.00	211000000.00	307040000	400135000	373470872.2
193.00	225000000.00	304010000	395070000	458226900
193.00	215000000.00	301990000	386966000	455958450
295.00	245000000.00	304010000	368732000	270510000
295.00	241000000.00	295930000	375123395.9	301714500
295.00	238000000.00	289870000	322803553.8	296070000
295.00	205000000.00	230280000	349812438.3	288093150
295.00	202000000.00	248023715.4	349457058.2	321325942.5
295.00	195000000.00	231225296.4	321816387	315314550
351.00	178000000.00	223320158.1	336623889.4	205758000
351.00	181000000.00	212450592.9	330700888.5	194256000
351.00	183000000.00	162055336	300098716.7	187866000
351.00	155000000.00	168972332	286278381	219132270
351.00	146000000.00	166007905.1	293188548.9	206882640
351.00	145000000.00	162055336	281342546.9	200077290
500.00	103000000.00	130434782.6	201382033.6	94785000
500.00	113000000.00	126482213.4	198420533.1	145266000
500.00	118000000.00	131422924.9	207305034.6	157194000
500.00	65000000.00	63241106.72	102665350.4	100946025
500.00	71000000.00	57312252.96	106614017.8	154708290
500.00	56000000.00	52371541.5	110562685.1	167411610

G.4. Data for T=11 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45.20	64.00	112.00	163.67
0	44.90	63.26	11.45	163.45
0	45.00	64.22	112.30	163.45
0	44.89	63.90	112.03	163.78
0	44.96	64.30	111.89	163.25
0	45.02	64.86	112.00	163.67
24	38.45	58.24	88.46	149.26
24	38.89	57.99	88.56	149.35
24	38.37	58.65	88.66	149.47
24	38.44	58.45	89.01	148.98
24	38.67	58.44	87.99	149.35
24	38.93	57.04	88.03	149.80
52	29.32	43.52	58.54	122.05
52	29.27	42.88	58.70	121.89
52	29.35	43.87	57.13	12.48
52	29.46	43.49	59.35	122.00
52	29.79	43.50	58.75	121.98
52	29.69	42.62	59.36	121.99
76	19.97	26.32	37.63	91.12
76	20.35	26.78	37.55	91.76
76	20.46	26.32	37.63	90.45
76	20.21	25.48	36.38	90.98
76	20.79	25.07	37.47	91.47
76	20.15	25.78	36.38	91.12
103	10.93	10.93	21.65	56.98
103	11.03	11.26	22.02	55.90
103	11.07	10.36	21.98	55.42
103	10.78	10.90	21.14	55.63
103	10.69	11.07	20.90	56.46
103	11.06	10.70	20.92	56.69
125	6.13	4.65	13.62	35.20
125	6.24	4.36	13.44	34.89
125	6.07	4.66	12.57	35.47
125	6.38	4.66	13.46	35.13
125	6.20	4.56	13.99	35.47

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
125	6.20	4.56	13.81	35.20
193	0.98	0.34	3.53	7.08
193	0.90	0.34	3.44	6.98
193	0.98	0.65	3.27	7.13
193	0.98	0.39	3.89	7.25
193	0.94	0.41	3.47	7.05
193	0.99	0.34	3.58	7.08
210	0.64	0.19	2.61	4.83
210	0.63	0.20	2.47	4.57
210	0.65	0.19	2.79	4.68
210	0.63	0.21	2.01	5.03
210	0.68	0.19	2.64	5.10
210	0.65	0.19	2.64	4.88

G.5. Data for T=17 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45.00	65.00	127.00	165.00
0	45.00	65.00	127.00	165.00
0	45.00	65.00	127.00	165.00
0	45.00	65.00	127.00	165.00
0	45.00	65.00	127.00	165.00
0	45.00	65.00	127.00	165.00
24	32.34	28.60	101.34	153.45
24	32.54	39.77	98.54	152.86
24	32.17	39.77	100.40	153.45
24	32.76	40.56	97.45	156.36
24	32.34	40.56	97.33	155.67
24	32.17	42.40	98.74	153.45
42	14.34	16.01	56.43	134.46
42	14.34	16.64	55.34	134.66
42	14.34	16.64	58.43	134.33
42	14.34	20.45	65.82	138.00
42	14.34	20.45	67.49	135.57
42	14.34	22.10	66.53	134.61
55	4.01	7.06	38.82	109.56
55	4.01	8.56	37.45	108.84
55	4.46	8.82	37.91	108.28
55	4.46	8.82	40.31	114.10
55	4.87	9.65	38.45	110.67
55	4.87	10.45	40.10	110.27
76	0.99	1.74	10.56	55.67
76	0.99	1.85	9.44	55.35
76	0.99	1.85	10.34	56.71
76	0.99	1.92	12.56	57.45
76	0.99	1.92	11.49	62.90
76	0.99	2.12	12.48	55.34
101	0.08	0.29	4.55	17.57
101	0.08	0.36	3.19	17.79
101	0.08	0.39	2.60	18.24
101	0.08	0.39	2.33	19.65
101	0.08	0.42	2.84	20.54

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
101	0.08	0.45	2.42	20.05
125	0.00	0.06	0.69	3.77
125	0.00	0.08	0.73	4.12
125	0.00	0.09	0.71	3.95
125	0.00	0.09	0.54	4.55
125	0.00	0.10	0.41	4.62
125	0.00	0.10	0.38	4.49
193	0.00	0.00	0.03	0.06
193	0.00	0.00	0.02	0.07
193	0.00	0.00	0.02	0.04
193	0.00	0.01	0.01	0.04
193	0.00	0.01	0.01	0.04
193	0.00	0.01	0.00	0.04

G.6. Data for T=22 °C, Nitrogen Concentration, *N*

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	45.00	65.00	110.00	165.00
0	45.00	65.00	110.00	165.00
0	45.00	65.00	110.00	165.00
0	45.00	65.00	110.00	164.67
0	45.00	65.00	110.00	164.67
0	45.00	65.00	110.00	164.67
24	24.20	52.36	95.32	156.35
24	24.10	53.15	96.35	156.35
24	23.67	53.86	95.53	156.35
24.00	21.43	54.12	94.47	156.03
24.00	20.54	54.93	95.49	156.03
24.00	23.53	55.67	94.68	156.03
42.00	6.23	41.37	82.33	143.61
42.00	4.47	42.67	81.95	143.61
42.00	4.47	42.74	82.48	143.61
42.00	4.76	42.75	81.60	143.32
42.00	4.56	44.11	81.22	143.32
42.00	4.63	44.17	81.74	143.32
55.00	2.13	28.78	68.00	121.35
55.00	1.89	27.56	68.00	121.35
55.00	2.04	28.35	68.00	121.35
55.00	1.34	29.75	69.05	121.11
55.00	1.36	28.49	69.05	121.11
55.00	1.42	29.30	69.05	121.11
76.00	0.35	17.46	49.06	89.93
76.00	0.26	17.27	49.06	89.93
76.00	0.21	17.37	49.06	89.93
76.00	0.22	18.05	49.81	89.75
76.00	0.23	17.84	49.81	89.75
76.00	0.24	17.95	49.81	89.75
101.00	0.02	2.19	7.43	9.10
101.00	0.04	1.84	7.43	9.10
101.00	0.03	1.98	7.43	9.10
101.00	0.01	2.26	7.55	9.08
101.00	0.01	1.90	7.55	9.08
101.00	0.01	2.05	7.55	9.08

Time	Nitrogen Concentration mg/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
125.00	0.00	0.50	1.50	1.14
125.00	0.00	0.47	1.50	1.14
125.00	0.01	0.35	1.50	1.14
125.00	0.00	0.52	1.52	1.14
125.00	0.01	0.49	1.52	1.14
125.00	0.01	0.36	1.52	1.14
193	0.00	0.00	0.01	0.00
193	0.00	0.00	0.01	0.00
193	0.00	0.00	0.01	0.00
193	0.00	0.00	0.01	0.00
193	0.00	0.00	0.01	0.00
193	0.00	0.00	0.01	0.00

G.7. Data for T=11 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00
52	0.58	2.73	1.94	4.48
52	0.80	4.53	2.90	5.68
52	0.83	3.43	2.58	5.36
52	0.58	6.17	1.96	4.54
52	0.81	7.18	2.94	5.76
52	0.85	8.19	2.61	5.44
92.00	3.27	9.83	7.42	9.81
92.00	2.73	10.37	6.61	10.28
92.00	2.86	9.17	7.82	9.58
92.00	3.33	15.55	7.51	9.61
92.00	2.78	15.32	6.69	10.07
92.00	2.91	14.68	7.92	9.39
143.00	8.75	23.61	17.26	21.25
143.00	9.83	24.17	15.80	21.87
143.00	9.92	23.45	18.06	22.80
143.00	8.90	23.61	17.46	20.81
143.00	10.00	24.17	15.99	21.42
143.00	10.08	23.45	17.50	22.33
193	16.94	33.98	22.34	25.92
193	16.64	34.62	22.74	26.72
193	17.13	33.18	22.42	24.80
193	17.23	0.00	21.64	26.28
193	16.93	0.00	22.03	27.10
193	17.42	0.00	21.72	25.15
245	20.40	0.00	29.43	34.96
245	23.02	0.00	29.75	36.32
245	22.16	0.00	30.72	35.20
245	20.75	2.70	28.52	35.45
245	23.41	4.48	28.83	36.83

Time	Ethanol Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
245	22.53	3.40	29.77	35.69
330	29.17	6.10	34.92	41.84
330	27.88	7.10	33.87	42.48
330	27.64	8.11	34.03	41.84
330	29.17	9.73	34.92	41.84
330	27.88	10.48	33.87	42.48
330	27.64	9.27	34.03	41.84
330	29.67	15.73	33.83	42.43
330	28.35	15.48	32.82	43.08
330	28.10	14.84	32.97	42.43
330	29.67	23.87	33.83	42.43
330	28.35	24.44	32.82	43.08
330	28.10	23.71	32.97	42.43
500	30.87	23.87	44.35	45.92
500	29.66	24.44	43.38	46.48
500	29.98	23.71	44.59	45.84
500	31.40	34.35	42.97	46.57
500	30.16	35.00	42.04	47.13
500	30.49	33.55	43.21	46.48

G.8. Data for T=17 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L				
(hours)	DAP=0 ppm	DAP=300 ppm	DAP=600 ppm	Time (hours)	DAP=100 ppm
0	0.00	0.00	0.00	0	0.00
0	0.00	0.00	0.00	0	0.00
0	0.00	0.00	0.00	0	0.00
0	0.00	0.00	0.00	0	0.00
0	0.00	0.00	0.00	0	0.00
0	0.00	0.00	0.00	0	0.00
48	1.18	7.15	10.04	48	2.70
48	1.34	6.85	9.52	48	2.76
48	1.58	7.05	9.56	48	3.11
48	1.18	7.26	10.06	48	3.15
48	1.66	7.14	10.24	48	3.47
48	0.79	7.04	10.38	48	3.71
92	5.52	21.45	20.47	92	13.24
92	5.13	22.46	21.62	92	14.60
92	4.81	20.20	21.13	92	15.15
92	5.52	22.34	22.87	92	15.86
92	6.63	21.56	23.61	92	16.64
92	5.44	20.20	23.25	92	16.65
143	12.07	31.33	32.45	143	19.33
143	15.54	31.28	33.04	143	19.44
143	14.99	30.88	32.46	143	21.09
143	16.65	31.48	33.45	143	21.44
143	15.54	31.24	32.89	143	22.01
143	16.17	32.94	33.25	143	23.04
193	25.41	39.66	41.46	193	27.23
193	24.62	39.19	42.38	193	28.44
193	24.14	40.60	42.05	193	28.66
193	23.28	38.48	43.67	193	29.59
193	23.99	37.65	44.02	193	30.14
193	24.46	39.56	44.21	193	30.30
500	35.51	47.43	50.06	212	27.22
500	36.53	46.49	49.54	212	27.99
500	34.72	46.23	50.15	212	27.99
500	37.24	47.33	50.56	212	28.10

Time	Ethanol Concentration g/L			Time	
(Hours)	DAP=0 ppm	DAP=300 ppm	DAP=600 ppm	(Hours)	DAP=100 ppm
500	37.40	46.45	51.71	212	28.11
500	36.06	45.61	51.97	212	28.43
				212	29.43
				212	29.43
				212	29.88
				212	30.06
				212	30.61
				212	30.61
				500	35.65
				500	35.65
				500	36.32
				500	38.82
				500	38.82
				500	40.32

G.9. Data for T=22 °C, Ethanol Concentration, *E*

Time	Ethanol Concentration g/L				
(hours)	DAP=0 ppm	DAP=100 ppm	Time (hours)	DAP=300 ppm	DAP=600 ppm
0	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00
48	5.44	10.64	48.00	12.17	12.45
48	5.64	11.46	48.00	12.97	16.17
48	5.63	10.37	48.00	11.77	13.99
48	5.15	10.82	48.00	12.48	12.74
48	5.24	11.66	48.00	13.30	16.54
48	5.75	10.54	48.00	12.07	14.31
92	15.36	18.45	92.00	25.15	32.80
92	15.94	18.46	92.00	23.48	33.22
92	17.01	19.21	92.00	26.12	30.54
92	14.64	18.77	92.00	25.77	33.55
92	16.73	18.78	92.00	24.06	32.47
92	15.17	19.54	92.00	26.77	29.86
143	22.35	22.36	143.00	29.07	42.32
143	23.47	22.75	143.00	31.71	43.73
143	23.92	23.74	143.00	29.15	43.57
143	22.64	22.74	143.00	29.80	41.37
143	23.43	23.14	143.00	32.19	44.73
143	24.19	24.14	143.00	29.59	44.57
193	32.56	35.67	193.00	38.20	48.81
193	34.72	35.83	193.00	37.00	49.13
193	31.05	34.32	193.00	36.68	46.87
193	32.40	36.28	193.00	37.08	49.93
193	32.95	36.44	193.00	35.91	50.26
193	33.22	34.90	193.00	35.60	47.95
500	35.46	45.75	295.00	38.77	50.19
500	36.22	43.95	295.00	37.55	51.44
500	34.57	44.32	295.00	37.23	50.82
500	35.62	46.53	295.00	36.53	51.70
500	35.19	44.69	295.00	35.38	52.99

Time	Ethanol Concentration g/L				
(hours)	DAP=0 ppm	DAP=100 ppm	Time (hours)	DAP=300 ppm	DAP=600 ppm
500	36.29	45.07	295.00	35.08	52.34
			500.00	47.41	52.25
			500.00	46.53	51.44
			500.00	46.29	51.85
			500.00	48.12	53.82
			500.00	47.23	52.99
			500.00	46.98	53.40

G.10. Data for T=11 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	116.03	115.40	116.05	115.46
0	115.69	116.10	116.05	114.57
0	116.10	116.10	116.02	116.23
0	116.12	115.99	113.26	116.25
0	116.24	115.92	116.25	116.03
0	115.99	115.95	116.08	116.11
52	108.81	115.18	100.79	104.45
52	108.98	115.10	100.46	104.37
52	108.57	114.99	100.24	104.31
52	108.13	115.02	101.22	102.37
52	109.65	116.24	100.37	100.36
52	106.47	117.25	100.68	103.47
92	100.74	107.20	90.28	87.41
92	100.59	107.24	90.36	87.27
92	100.34	107.65	90.67	87.15
92	101.24	109.35	92.63	85.86
92	100.47	109.77	92.34	85.12
92	98.57	109.49	92.88	85.97
143	86.69	92.96	75.98	58.11
143	86.14	93.20	76.17	58.49
143	85.99	92.76	75.24	58.60
143	86.13	95.19	74.40	58.58
143	86.98	95.19	75.16	58.21
143	84.82	95.36	75.16	58.35
193	73.10	80.24	66.12	58.13
193	73.13	80.12	64.84	30.30
193	73.57	80.07	64.52	30.47
193	73.55	81.99	63.79	30.54
193	71.53	82.15	64.27	30.34
193	71.94	81.80	64.79	30.98
245	63.68	68.98	51.95	12.12
245	63.81	68.46	51.46	12.22
245	63.49	68.80	51.35	12.46
245	65.08	70.64	51.40	11.95
245	65.54	69.27	51.44	11.91

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
245	65.13	69.27	51.95	11.65
330	47.62	54.71	38.68	2.37
330	47.30	54.71	36.57	2.23
330	47.38	54.71	38.59	2.69
330	48.67	55.47	38.46	2.35
330	48.17	55.18	38.65	2.76
330	48.95	55.24	38.12	2.32
405	37.35	45.65	28.69	0.88
405	37.22	45.88	29.46	0.75
405	37.94	45.24	28.65	0.71
405	38.18	45.27	29.43	0.73
405	38.16	46.20	29.48	0.70
405	38.35	45.36	29.35	0.72
500	28.20	38.04	21.32	0.19
500	28.69	38.02	20.35	0.19
500	28.44	38.13	20.95	0.18
500	28.82	37.90	20.78	0.20
500	28.72	38.13	19.90	0.19
500	28.82	38.19	20.49	0.19

G.11. Data for T=17 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	112.00	116.00	116.00	116.00
0	112.00	116.00	116.00	116.00
0	112.00	116.00	116.00	116.00
0	112.00	116.00	116.00	116.00
0	112.00	116.00	116.00	116.00
0	112.00	116.00	116.00	116.00
48	108.40	108.22	103.45	108.35
48	106.20	106.26	99.56	108.45
48	108.10	110.45	101.45	107.78
48	105.47	108.40	100.98	106.34
48	105.47	110.13	100.30	108.46
48	105.47	109.45	101.46	107.26
92	90.50	91.45	62.33	65.33
92	92.80	94.54	65.35	64.21
92	94.38	95.30	64.75	66.29
92	89.19	90.34	63.57	64.13
92	89.19	92.65	64.22	63.69
92	89.19	94.32	63.46	64.83
143	75.60	76.35	30.44	17.39
143	78.33	75.00	36.34	18.35
143	73.32	75.33	30.79	17.92
143	73.32	76.80	31.07	16.38
143	73.32	74.22	34.25	16.83
143	73.32	73.05	35.76	17.16
193	64.22	60.43	12.68	3.58
193	61.30	61.34	14.66	3.14
193	60.58	59.87	13.98	3.24
193	60.58	61.10	12.36	2.87
193	60.58	61.34	13.64	2.80
193	60.58	59.87	14.15	2.72
212.00	58.40	56.67	9.45	1.84
212.00	60.20	54.12	10.54	1.73
212.00	59.80	55.34	8.67	1.63
212.00	56.37	53.24	8.27	1.84
212.00	56.37	53.24	9.45	1.95

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
212.00	56.37	28.54	9.33	1.93
351.00	31.30	26.34	0.88	0.06
351.00	31.33	25.43	0.56	0.03
351.00	33.86	28.63	0.89	0.08
351.00	33.86	23.45	0.94	0.20
351.00	33.86	25.43	0.65	0.09
351.00	33.86	25.43	0.63	0.11
500	22.40	14.14	0.19	0.00
500	24.60	13.26	0.07	0.00
500	24.20	15.44	0.24	0.00
500	20.82	10.34	0.16	0.03
500	20.82	15.44	0.10	0.03
500	20.82	13.26	0.11	0.02

G.12. Data for T=11 °C, Sugar Concentration, *S*

Time	Sugar Concentration g/L			
(hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
0	116.00	116.00	116.00	116.00
0	116.00	116.00	116.00	116.00
0	116.00	116.00	116.00	116.00
0	116.00	116.00	116.00	116.00
0	116.00	116.00	116.00	116.00
0	116.00	116.00	116.00	116.00
15	115.35	113.34	114.56	112.70
15	113.98	112.95	114.56	111.44
15	114.26	112.48	114.56	112.70
15	115.35	113.34	116.10	111.32
15	115.12	112.95	116.10	111.44
15	115.40	112.48	116.10	111.32
24	111.57	110.87	111.02	109.79
24	112.85	110.65	111.02	108.45
24	111.62	110.48	111.02	109.79
24	112.69	110.87	112.52	108.45
24	113.98	110.65	112.52	108.45
24	112.74	110.48	112.52	108.45
48	100.63	98.38	92.20	86.46
48	100.98	96.94	92.20	87.45
48	101.63	99.92	92.20	86.46
48	101.64	98.38	93.44	85.40
48	101.99	96.94	93.44	87.45
48	102.65	99.92	93.44	85.40
63	92.65	85.32	73.51	62.18
63	93.70	86.90	73.51	67.35
63	92.98	87.34	73.51	62.18
63	93.57	62.38	72.53	61.42
63	94.64	62.38	72.53	67.35
63	93.91	62.38	72.53	61.42
84	81.49	70.32	51.63	34.22
84	82.49	69.12	51.63	33.56
84	82.14	69.34	51.63	34.22
84	82.30	83.17	50.94	33.80
84	83.31	83.17	50.94	33.56
84	83.84	83.17	50.94	33.80

Time	Sugar Concentration g/L			
(Hours)	DAP=0 ppm	DAP=100 ppm	DAP=300 ppm	DAP=600 ppm
110	72.50	52.74	32.17	15.59
110	73.09	53.82	32.17	16.20
110	72.58	52.83	32.17	15.59
110	73.23	52.74	32.60	15.40
110	73.82	53.82	32.60	16.20
110	73.31	52.83	32.60	15.40
193	46.87	24.66	6.16	1.67
193	45.26	23.15	6.16	1.54
193	46.14	22.76	6.16	1.67
193	47.34	23.15	6.24	1.65
193	45.71	23.15	6.24	1.54
193	46.60	22.76	6.24	1.65
295	25.64	10.42	1.18	0.22
295	25.82	11.29	1.18	0.31
295	26.26	10.92	1.18	0.22
295	25.90	10.42	1.19	0.22
295	26.08	11.29	1.19	0.31
295	26.52	10.92	1.19	0.22
351	20.46	5.04	0.57	0.09
351	21.05	5.64	0.57	0.09
351	19.74	5.02	0.57	0.09
351	20.66	5.04	0.56	0.09
351	21.26	5.64	0.56	0.09
351	19.94	5.02	0.56	0.09
500	10.57	2.42	0.15	0.02
500	11.83	2.55	0.15	0.00
500	10.82	2.24	0.15	0.02
500	10.68	2.42	0.15	0.02
500	11.95	2.55	0.15	0.00
500	10.93	2.24	0.15	0.02

NOMENCLATURE

E	Ethanol concentration, g/L
E_T	activation energy for temperature effect, J/gmol
E_N	activation energy for nitrogen effect, J/gmol
K_N	Monod constant for Nitrogen, mg/L
K_S	Monod constant for Sugar, g/L
k_d	ethanol independent death rate constant, L g ethanol^{-1} h^{-1}
k	any non-linearly estimated parameter
k_r	value of the same parameter at reference temperature and initial nitrogen
N_i	nitrogen concentration at t=0, ppm
N	nitrogen concentration, ppm
N_O	nitrogen concentration, ppm
R	Universal gas constant, J/gmol K
S	sugar concentration, g/L
S_O	sugar concentration, g/L
t	time, hour
T	temperature, °C
T_k	temperature, K
T_r	reference temperature, K
X_V	viable yeast cell concentration, cells/L
y	generic dependent variable
Y	generic dependent variable
$Y_{X/N}$	stoichiometric yield coefficient of biomass on nitrogen, no. of cells/g N
$Y_{E/S}$	stoichiometric yield coefficient of ethanol on sugar, g ethanol/g sugar

Greek symbols

β	specific ethanol production per gram of sugar consumed, g ethanol g sugar^{-1} hr^{-1}
β_m	maximum specific ethanol production rate, g ethanol g sugar^{-1} hr^{-1}
μ	specific growth rate hr^{-1}
μ_m	maximum specific growth rate, hr^{-1}

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