EVALUATION OF BEST PARAMETERS IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS FOR MAXIMUM ETHANOL YIELD BY *K. MARXIANUS* IMB3

By

JENNINE B. TERRILL

Bachelor of Science in Agricultural and Biological Engineering
Penn State University
State College, PA
2005

Master of Science in Environmental Engineering
Penn State University
State College, PA
2008

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2011

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Dissertation Approved:

Dr. Mark R. Wilkins

Dissertation Adviser

Dr. Hasan K. Atiyeh

Dr. Danielle D. Bellmer

Dr. Nurhan T. Dunford

Dr. Andrew J. Mort

Outside Committee Member

Dr. Sheryl A. Tucker

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Petroleum is an amazing resource that has allowed for many technological advances in the industrialization of the world, but it has several drawbacks to its future as a commodity. Almost since the advent of black gold and its use in transportation fuel, there has been discussion about the limitations of this resource. The industrial age has served to deplete this resource faster than it could possibly be replenished. Without the option of running vehicles on fossil fuels forever, other avenues need to be explored in order to support the habits that industrialized man has developed. Although the lifetime of current petroleum production is debated, there is no argument that it will run out at some point (Deffeyes, 2001; Hirsch, 2008).

There has also been increased interest in the development of biofuels in the United States to implement energy independence. The volatility of many countries that are top producers of oil has led to the future supply being dictated not only by economics, but also by political turmoil. These issues have led to oil prices fluctuating not only within the terms of supply and demand but also with respect to political events, such as during the Arab oil embargoes of the 1970's. This

uncertainty has made it more important than ever to evaluate sources for domestically meeting energy demands in the US (Demirbas, 2007; Leiby, 2007; Pugliaresi and Montalbano, 2008).

A third issue plaguing the use of fossil fuels is the environmental concern surrounding fossil fuels usage. The measured increase in CO_2, combined with global warming and other greenhouse gases, has sparked an environmental movement that rivals that brought on in the late 1960's by Rachel Carson's "Silent Spring." Although the scientific basis for climate change is still being disputed, most will agree that it is happening and the changes on this planet have been greatly accelerated during the advent of man's industrial age (Tett et al., 2007). This industrial age was fueled by carbonaceous material being mined from both forests and mines (coal, and later oil and natural gas). With less vegetation from deforestation for croplands, cities and goods, there is less of a filter for the greenhouse gases being released into the atmosphere from the anthropogenic load being placed upon it. This cannot be neglected when evaluating climate models (Matthews et al., 2004; Shi et al., 2007). The IPCC (Intergovernmental Panel on Climate Change) issued a statement in its fourth annual report stating that they were "90% confident" that global warming during the last 50 years was due to humans (Keller, 2009).

All of these issues need solutions, and one of the most discussed and debated of the alternate fuels is fuel from biomass. Biofuels, fuels from biomass, are renewable as they are produced directly from crops and do not need millions of years to occur. The advent of such governmental policies as the Biofuels Energy Independence Act (2007) and the goals presented in the aptly nicknamed "Billion Ton Report" (Perlack, 2005) outline the use of biomass to address all three of the above issues. Another benefit stated in these studies would be the improvement of rural economies as the biomass supply would be from these areas and manufacturing facilities would have to be placed relatively close to the production sites. Many methods for reaching these goals have been evaluated and integration of previous research is just as important as developing brand new technology in the realm of biofuel development.

Issues lie within the implementation of biofuels; most notably, the food versus fuel debate, which questions the viability of reducing the supply of food crops (e.g. corn) for production of fuel. Another related argument is the use of cropland for the production of bioenergy crops. In this scenario the actual production crops are not impacted directly, but less land devoted to food crops can mean a decreased supply, increasing costs due to decreased supply. This issue has been addressed by proponents of bioenergy crops with the use of marginal croplands for cellulosic crops (Havlík et al., 2010). Also, an issue lies in the the reduction of biodiversity in the marginal lands that are being repurposed to decrease the impact of biofuel crops on food crops. It has been proposed that better planning and cycling could reduce this impact, perhaps including the incorporation of heterogenous crops (Wiens et al., 2011), but some studies still report a carbon debt (the amount of time the biofuel crop will have to be in place in order to alleviate the carbon released from conversion of the land from unused to agricultural fields) of 50 years for marginal lands (Fargione et al., 2009). A recent modeling study has evaluated the different greenhouse gas evolving scenarios of biofuel crop production including factors such as deforestation, cropland competition between food and energy crops and greenhouse gas emissions. The findings of this study were that the biofuels scenario generated 27% fewer emissions than the conventional fuel scenario, by 2030 (Havlík et al., 2010).

Another very recent study evaluating the ecological implications of biofuels for supplying energy in the world, particularly in the United States, concluded that there was not enough data in regard to management practices and changing landscapes to adequately model how biofuels will impact ecological concerns. This is based on the lack of data currently being correlated to empirical models and the lack of current land use and energy requirements being introduced into these models (Dale et al., 2011).

The different generations of biofuels are denoted by their level of sustainability and technological viability. First generation biofuels include corn ethanol, sugar ethanol and biodiesel from

vegetable oil and animal fat. This generation is already a viable industry. Second generation biofuels refer to cellulosic based fuels, such as lignocellulosic ethanol, bio-oil, butanol and mixed alcohols. Many second generation biofuels are thought to be carbon negative in their implementation, as they theoretically take up more carbon dioxide (CO_2) in their growth than is produced in the production and burning of the fuel. There are still setbacks as to the implementation of second generation biofuels, but the advantages of such fuels seem to far outweigh the negatives (Naik et al., 2010). Additionally, another study using a life cycle analysis of biofuel crop production showed that the benefits of second generation biofuel crops were advantageous over first generation, as long as the second generation crops were not grown in large dedicated plantations (Havlík et al., 2010).

First generation biofuels such as corn ethanol and biodiesel from edible oils have actually been capped to a limit of 15 million gallons a year from corn ethanol set by the Energy Independence and Security Act of 2007 (EISA). This limit was placed due to concerns about impacting the food chain, hypoxia in the Gulf of Mexico and other sustainability and environmental concerns. As for the rest of the 36 billion gallons per year production of biofuels mandated by EISA by 2022, at least 16 billion gallons must be made from lignocellulosic material (Regalbuto, 2010). Due to the lack of commercialization of lignocellulosic and other advanced biofuels at the time of the legislation, the Environmental Protection Agency (EPA) must annually review the goals and set new renewable fuel standards based on the available technology (Carriquiry et al., 2011).

Regardless of the type of biofuel, these fuels will play a large role in supplying the energy necessary for transportation in the near future. The precursor to the EISA of 2007 was the Renewable Fuel Act of 2005. Although EISA expanded upon the 7.5 million gallons of renewable fuels that were to be blended with gasoline by 2012 in this bill, another set of Renewable Fuel Standards is set for public discussion in August 2011. These fuel standards incorporate many of the same standards set previously, but realize that the cellulosic biofuel goals originally set are

not feasible with current technology. The EPA hopes that with setting an aggressive threshold with sizable renewable fuel credits for cellulosic ethanol over corn ethanol amounting to about 2.5 to 1, research and development in this field will be promoted (Carriquiry et al., 2011; EPA, 2011).

Lignocellulosic ethanol is one of the main avenues in which the proposed biofuel legislation will be reached. Conversion of lignocellulosic biomass into ethanol has many steps that are looked at for minimizing the cost and inputs such as maximizing the output of the crop to streamlining the harvest of the biomass in field to biorefinery set-up optimization to maximizing output of microbial catalysts. The cost associated with obtaining the bioenergy crops for refineries is thought to be one of the most economically hindering points of the biorefinery design (Hess et al., 2007). Microbial catalyst optimization becomes extremely important with this in mind as total process efficiency depends on the abilities of fermenting organisms to selectively produce ethanol from the biomass in lieu of other products. Microorganisms able to operate at higher temperatures, such as thermotolerant yeast, e.g. *K. marxianus* species, also improve the process economies by removing costs associated with cooling and reheating product streams for removal of ethanol (Banat et al., 1998). Growth media design and immobilization techniques for such thermotolerant organisms are two ways to selectively encourage ethanol production over other co-products.

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CHAPTER II

OBJECTIVES

The main objective for this project was to increase the ethanol yield from lignocellulosic material using yeast *K. marxianus* IMB3 in an SSF at 45°C. This was intended to be done by reaching several short term project goals:

1) Optimize the media used for glucose fermentation to ethanol and evaluate this media in an SSF for conversion efficiency and minimization of glycerol and other byproducts;

2) Evaluate cell immobilization as a method for increased thermotolerance;

3) Evaluate the ability of cell immobilization to be used in a fermentation of lignocellulosic biomass using *K. marxianus* IMB3

CHAPTER III

LITERATURE REVIEW

3.1 Background

One of the most promising areas in the second generation of biofuel production is the harvesting of lignocellulosic biomass and subsequent processing of this biomass in a biorefinery for ethanol production. Biomass consists of three main components, cellulose, hemicellulose, and lignin. It also contains minor components such as soluble sugars, ash and extractives. The percentages of these components vary depending on the type of lignocellulosic material being looked at, and variations between a particular species can even occur depending on harvest date, year, and any number of physical considerations such as fertilizer application, irrigation, field spacing, etc. (Lewandowski et al., 2003; Nassi o Di Nasso et al., 2010). Mineral composition of biomass can also be important and highly variable as this can cause issues with clogging in thermochemical conversions such as gasification. More generally, high mineral contents in plants mean that the plant is stripping the land of valuable nutrients that may need to be replaced (Monti et al., 2008). Typical carbohydrate composition of cellulosic feedstocks, such as grasses, softwoods and hardwoods, can range from about 40-55% cellulose, 10-30% lignin and 20-50% hemicellulose

(McMillan, 1994; Sun and Cheng, 2002).

There are two main pathways for conversion of lignocellulosic biomass into fuels, particularly ethanol. These two pathways are the thermochemical platform and the sugar (or biochemical) platform (Fernando et al., 2006; Foust et al., 2009; Hess et al., 2007; Wright and Brown, 2007). The biochemical platform has had much more research devoted to its development for ethanol production, as ethanol generating fermentations have been around for millennia in the realm of beverage production, but companies are currently scaling up both of these technologies for commercialization (Fairley, 2011). A general schematic for both of these processes is shown in Figure 3.1

In the sugar platform, the first step is to make the fermentable sugars in the plant accessible to enzymes by breaking down the lignin component of biomass through a pretreatment step. This important step can be done in many ways, such as physical, chemical or biological (da Costa Sousa et al., 2009; Keshwani and Cheng, 2009; Kumar et al., 2009; Sun and Cheng, 2002).

Types of pretreatment include alkali swelling (through the use of ammonia, lime or sodium hydroxide), acid hydrolysis (usually sulfuric acid, sometimes in the form of H_2S), steam explosion, and solvents (such as hydrothermolysis, which uses water at an elevated temperature and pressure to make cellulose accessible, or solvolysis, which uses organic solvents to dissolve lignin). Regardless of which pretreatment method is used, there are four main objectives for pretreatment, firstly, to lose as little fermentable sugar as possible in the pretreatment (good recovery), secondly, to improve the formation of sugars by subsequent enzymatic processing, thirdly, to not form byproducts and finally, to be economically feasible (McMillan, 1994; Sun and Cheng, 2002). The general model used to describe pretreatment is shown in Figure 3.2 as the breakdown of lignin and subsequent liberation of fermentable substrates (Hsu et al., 1980).

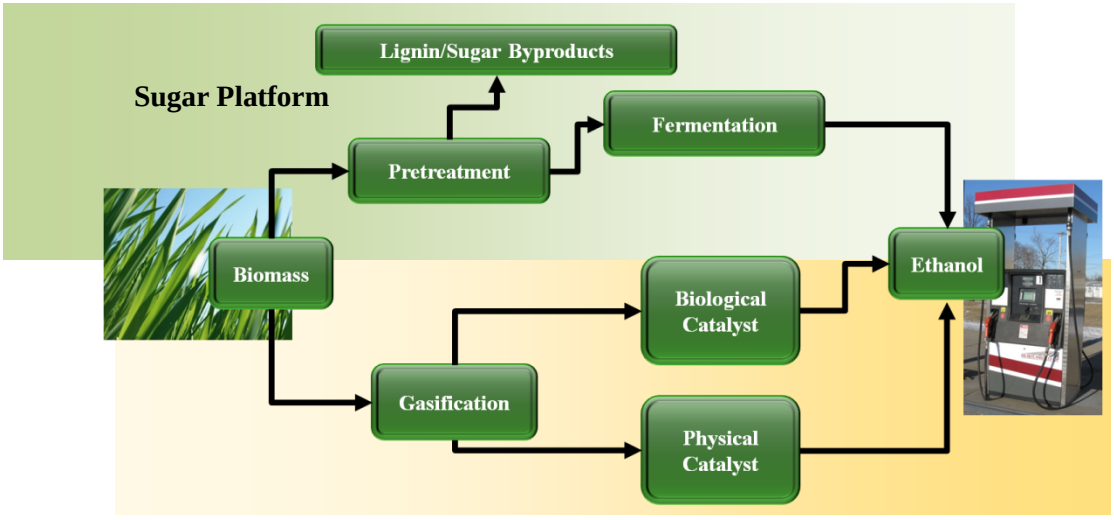

Figure 3.1. Two main platforms for production of ethanol from biomass, modified from (Fernando et al., 2006) and (Foust et al., 2009).

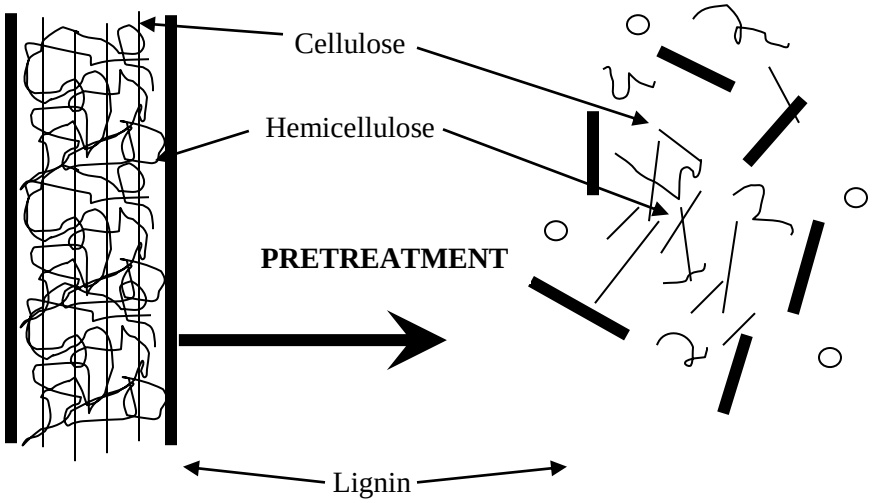

Figure 3.2. Pretreatment breakdown of lignocellulosic biomass, modified from Hsu et al. (1980) and Mosier et al. (2005).

Pretreatment is followed by hydrolysis and fermentation to ethanol in the sugar platform for lignocellulosic fuels, which can be done either in tandem or in a combined method (Olofsson et al., 2008).

3.2 SSF Processing

Simultaneous saccharification and fermentation (SSF) reactions have been heralded as important steps in streamlining and mainstreaming the conversion of lignocellulosic biomass into ethanol (and other products). When compared to separate hydrolysis and fermentation (SHF), SSF has several advantages. A schematic outlining both reactions for ethanol production is illustrated in Figure 3.3. In an economical approach at large scale, byproducts from the pretreatment of the biomass (such as monomeric sugars and cellobiose) can be used in the production of the enzymes (Brethauer and Wyman, 2009; Olofsson et al., 2008; Palmqvist and Hahn-Hagerdal, 2000; Palmqvist et al., 1997; Wingren et al., 2003; Wyman et al., 1992) and lignin remaining at the end of the fermentation could potentially be combusted as another fuel source for the process (Fernando et al., 2006).

The SSF process was first described by Takagi et al. (1977). SSFs have been extensively studied for the yeast *Saccharomyces cerevisiae*, even employing complex models to help predict the outcome of such fermentations (Philippidis and Smith, 1995; Philippidis and Hatzis, 1997; Philippidis et al., 1993; South et al., 1995). *S. cerevisiae* is not the only organism used in SSF reactions, as studies have been done evaluating the use of thermotolerant yeast at temperatures above 37°C in SSFs (Boyle et al., 1997; Faga et al., 2010; Hari Krishna et al., 2001; Suryawati et al., 2008). Bacterial strains such as *Zymomonas mobilis* have also been employed in SSFs with some success (da Silveira dos Santos et al., 2010); however, yeast are still the most widely used organism in SSF reactions and using bacteria requires an even lower temperature (about 30°C).

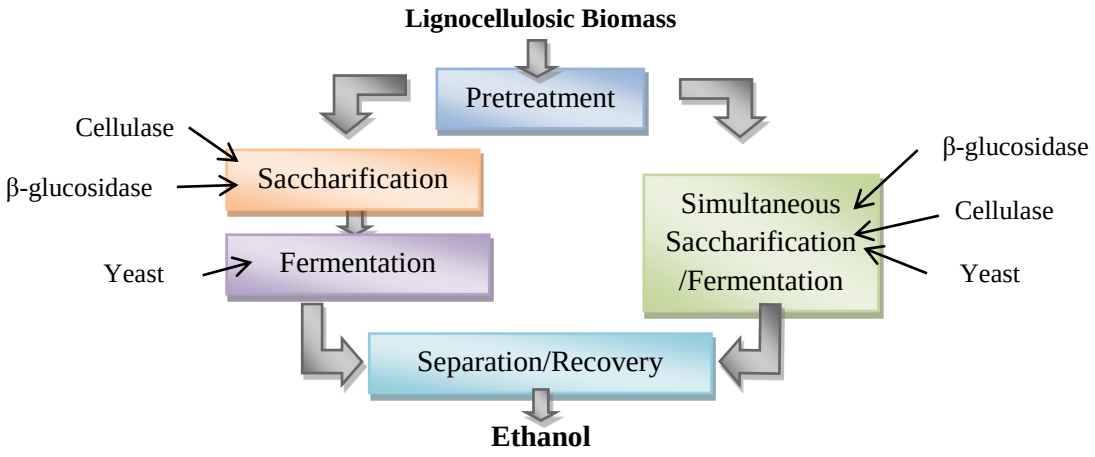

Figure 3.3. Schematic comparing simultaneous saccharification fermentations (SSFs) to separate hydrolysis fermentations (SHFs) modified from da Costa Sousa et al. (2009) and Olofsson et al. (2008).

Using organisms in SSF capable of producing the necessary enzymes for breakdown of lignocellulosic material is a step further than SSF. This type of reaction is known as consolidated bioprocessing (CBP) and has its own set of limitations (Olofsson et al., 2008; Wingren et al., 2003).

3.3 Advantages and Limitations of SSF

Advantages of SSF include the decrease of enzymatic inhibition due to glucose released in the saccharification of biomass and reduction in process steps and reactors (Deshpande et al., 1983). Also, the immediate conversion of sugars produced decreases the instances in which contamination could become an issue within the conversion reaction. This is due to the lack of sufficient time for a contaminating organism to grow to a significant concentration in order to compete with the desired fermenting organism. This reduction in contamination decreases the number of undesirable products, potentially increasing the overall yield with less necessity for sterility. The disadvantages of such reactions include the inability of fermenting organisms to grow and convert glucose to ethanol efficiently at optimal temperatures for enzymatic hydrolysis. Ethanol can also inhibit the hydrolysis of biomass as it can cause uncompetitive inhibition to cellulases (Ghosh et al., 1982; Ooshima et al., 1985; Wu and Lee, 1997). Even with these problems, recent models comparing SSF to SHF have shown SSF reactions to be the superior conversion technology, even at lower temperatures (37°C) (Drissen et al., 2009). Previous work converting complex lignocellulosic materials to ethanol in SSF processes has shown the advantages of using thermotolerant yeast (such as the *K. marxianus* IMB strains) at elevated temperatures when compared to conventional SSF processes at lower temperatures using conventional yeast strains such as *S. cerevisiae* D_5A in both yields (Faga et al., 2010; Suryawati et al., 2008) as well as decreased cost for product removal, cooling and enzyme addition (Abdel-Banat et al., 2010; Suryawati et al., 2008).

3.3.1 Rate Limiting Factors

SSF reactions have limitations to their lignocellulosic biomass conversion abilities. The rate limiting steps in SSF reactions have been found to be dependent on the time of interest in relation to the start of the reaction in batch fermentations. Cell growth is the determining factor in the beginning of SSF batch reactions; whereas, hydrolysis is the rate limiting step in the rest of the fermentation (Philippidis and Smith, 1995). This characterization was completed on yeast that were not thermotolerant; therefore, the rate of cellulose hydrolysis was slower than it would be at the elevated temperature used in thermotolerant SSF reactions. The initial limitation of cell growth could be exacerbated at higher temperatures as the growth rates of thermotolerant yeasts are sometimes much lower than those of mesophillic, conventional yeasts. For example, μ ranges from about 0.18 – 0.19 hr^{-1} for *K. marxianus* strains as compared to 0.3 – 0.4 hr^{-1} for *S. cerevisiae* (Banat and Marchant, 1995). Optimal temperatures for enzyme activity of cellulases (a potential rate limiting factor for SSFs) are usually between 45 and 50°C for the commonly used *Trichoderma reesei* excreted cellulases (Inui et al., 2010), so there is a discrepancy between operating and optimal temperatures for enzymatic hydrolysis in SSF. The increase seen in enzyme activity at higher temperatures should theoretically reduce the amount of enzyme needed to be added in order to secure the same activity level, making the process more cost effective (Kádár et al., 2004). Cellulase enzymes are inhibited by both cellobiose and glucose, but also have some inhibition due to the production of ethanol (Philippidis and Smith, 1995).

3.3.2 Inhibition due to pretreatment conditions

Cellulose availability is identified as the key issue limiting cellulase activity (Gong et al., 1999; Philippidis and Smith, 1995), but this can be alleviated somewhat in the treatment step preceding SSF known as pretreatment. The problem of cellulose accessibility cannot be alleviated by the addition of more enzymes or the increase in physical parameters (such as temperature or pH) to

increase enzyme activity, and is one of the reasons why SSF of lignocellulosic biomass occurs over a longer time period than a comparable starch or even pure cellulose SSF. The mixed substrate of SSFs utilizing pretreated substrates also can cause inhibition through the binding of cellulases to the lignin portion of the pretreated material (Philippidis and Smith, 1995). Choosing a pretreatment method that will minimize the amount of lignin in the fermentation media is possibly the best way to deal with this type of inhibition.

3.3.3 Thermotolerant yeast in SSFs

The use of a thermotolerant yeast, such as the *K. marxianus* IMB strains, in the SSF process has additional problems to the aforementioned well researched and addressed issues. Possibly the most concerning issue of thermotolerant SSFs is that of cessation of glucose conversion to ethanol after 72 to 96 h of fermentation, as seen in previous studies with *K. marxianus* IMB strains (Faga et al., 2010; Suryawati et al., 2008). This issue potentially could relate to the interrelated ethanol and temperature tolerances of the microorganisms. With increasing ethanol concentration (as in a batch SSF), the ability of the microorganism to remain active at higher temperatures is reduced (D'Amore and Stewart, 1987). This is due to the relationship between ethanol tolerance and heat shock protein synthesis (Piper, 1995; Watson and Cavicchioli, 1983).

Methods used to help organisms with ethanol tolerance might also assist microorganisms with thermotolerance if these different stresses bring about similar physiological responses within the cells. One main avenue to address ethanol tolerance of organisms is to introduce the organism into an immobilization matrix (Banat et al., 1998; Ciesarová et al., 1998; Jirků, 1999). Jirků (1999) attributed improved ethanol tolerance to a membrane stabilization of the organism. Such physiological stabilization is akin to the plasma membrane stabilization that an organism might develop when going through heat shock (Piper, 1995), making immobilization an option for increased thermotolerance of organisms. Media optimization is another route in which ethanol

tolerance of microbes has been addressed. Xue et al. (2008) found that three compounds, $CaCl_2$, $ZnSO_4$, and $MgSO_4$, were key to optimizing a media that produced 90.2% viability of heat shocked cells for yeast SPSC01. With the link between ethanol tolerance and thermotolerance, it seems reasonable that both of these avenues would be viable options to improve a sluggish fermentation with thermotolerant yeast.

Increasing the temperature of a fermentation has also been linked to increased glycerol production in wine fermentations, but this is a desirable compound in the production of wine as it is thought to increase fullness of flavor and improve mouth feel (Balli et al., 2003). The generation of coproducts in ethanol production for fuel is not desirable as it reduces the carbon going towards ethanol. The metabolic pathway for the main products of glucose fermentations by yeast is shown in Figure 3.4 with redox equivalents identified. Glycerol is formed as a fermentation product in many yeast strains, such as *S. cerevisiae,* to maintain redox balance in cell mass production (Wang et al., 2001). Research has been done on a molecular level with *S. cerevisiae* with deleting some of the glycerol producing genes in order to reduce the production of this compound (Guo et al., 2010), although many times these genetic manipulations can affect the overall performance of the fermentations. Glycerol production is roughly 1.3 g/L for IMB3 SSFs with *K. marxianus* IMB3 at 45°C with 42 g/L glucan loading (Faga, 2009).

Acetic acid production in fermentations is also of concern in fermentations as it is also produced as part of a redox balance. Faga et al. (2010) identified acetic acid as a major co-product, producing over 1.5 g/L in fermentations with *K. marxianus* IMB3, again using a loading of 42 g/L glucan and 45°C. Acetic acid not only reduced the amount of substrate being converted to ethanol, but also lowered the pH of the media. The citrate buffer was not able to maintain the pH with such a high amount of acetic acid in the fermentation. The role of acetic acid in fermentations is highly debated. Acetic acid is a known component of prehydrolyzate liquids (the liquid portion from pretreatment) and has been shown to exacerbate the effects of other

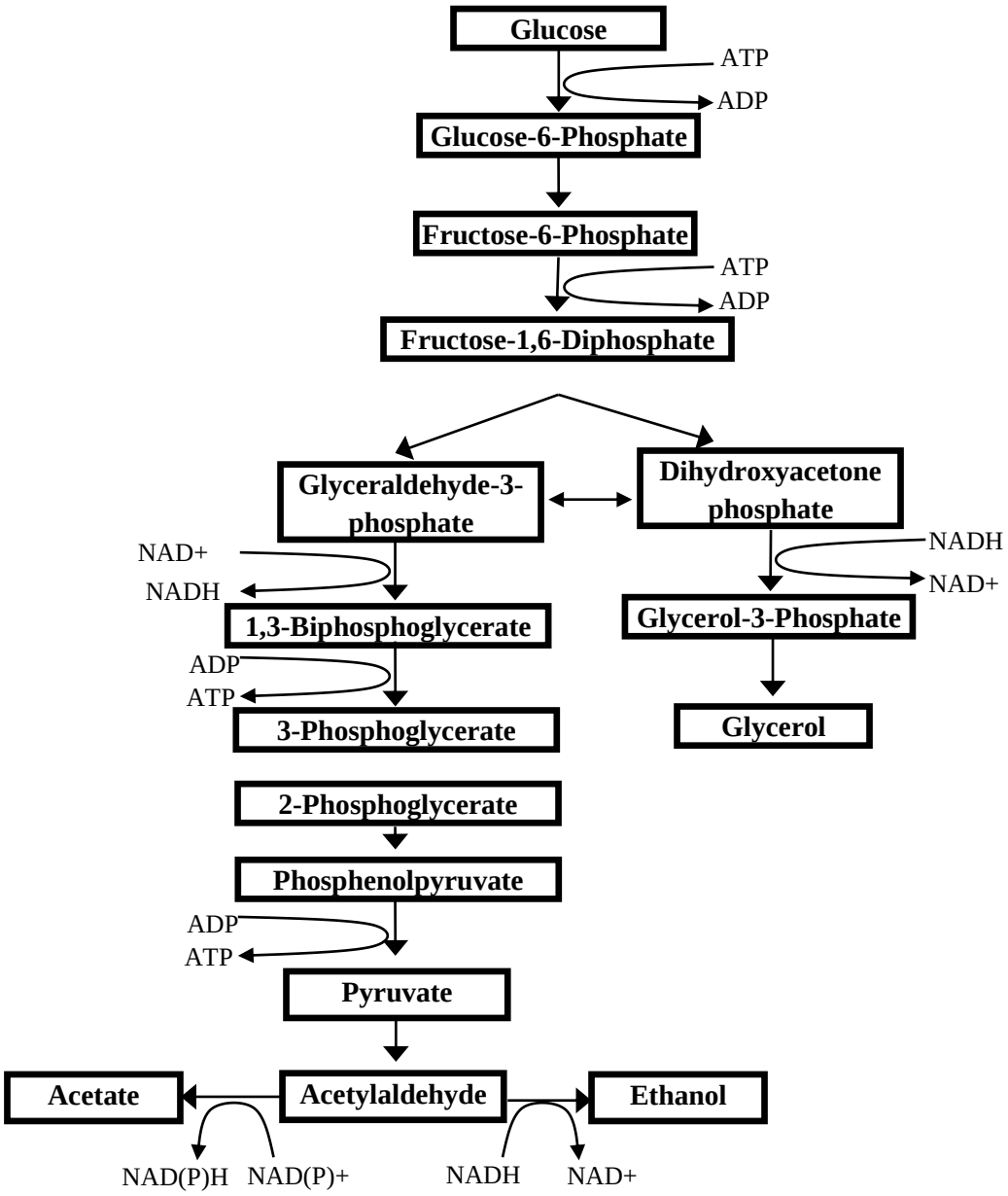

Figure 3.4. Glucose utilization pathway in yeast, specifically *S. cerevisiae*, modified from (Olofsson et al., 2008) and (Berg et al., 2007).

prehydrolyzate constituents (furfural and catechol) in glucose (30 g/L) fermentations using the thermotolerant yeast *K. marxianus* CECT 10875 (Oliva et al., 2006). Weak acids, like acetic acid, are known to reduce microbial growth, although low concentrations of acetic acid (less than 6.005 g/L (100 mM)) were shown to stimulate ethanol production by *S. cerevisiae* (Palmqvist and Hahn-Hagerdal, 2000; Pampulha and Loureiro-Dias, 1989). Conversely, a concentration of 5 g/L (83.26 mM) of acetic acid was found to inhibitory to cell growth in another glucose fermentation with *S. cerevisiae*. An acetic acid concentration of 3.3 g/L was seen to increase ethanol yield by 20% and decrease both glycerol (33%) and biomass (45%) (Taherzadeh et al., 1997).

In summary the reduction of coproducts is another method in which the overall ethanol yield of organisms could be increased. Reduction of both glycerol and cell mass seem to be linked with an increase in ethanol production; although, too much inhibition of cell growth would be detrimental to the conversion rates of the experiments. Media optimization is an excellent way to attempt to reduce coproducts and increase ethanol production. The effect of acetic acid concentration in the fermentation may or may not be positive, depending on whether or not the acid is dissociated, a function of the pH relative to the pKa of the acid (Palmqvist and Hahn-Hagerdal, 2000).

3.4 *Kluyveromyces marxianus* IMB Strains

K. marxianus IMB3 was isolated by Dr. Ibrahim Banat from Indian brewery waste. Identified as one of five different isolates, it has been shown to be the yeast of the group that is best at producing ethanol and growing at elevated temperatures in work done at Oklahoma State University (Faga et al., 2010) and in other international groups (Banat et al., 1996; Barron et al., 1996; Nolan et al., 1994; Singh et al., 1998; Singh et al., 1998). Two reviews have been completed on the potential of *K. marxianus* strains in bioprocessing applications (Fonseca et al., 2008; Lane and Morrissey, 2010) and one has been devoted to this particular strain (IMB3) in

high temperature fermentations (Singh et al., 1998). Also, work has been done on potential genetic manipulations of the organism to identify the alcohol dehydrogenase genes capable of high temperature fermentations (Lertwattanasakul et al., 2007). Further research has been done evaluating the ability of modified strains of *K. marxianus* to use various substrates for ethanol production (Nonklang et al., 2008). Hong et al. (2007) used genetic manipulation on *K. marxianus* to generate a strain capable of converting cellulosic material directly into ethanol. The evaluation of and advancements of *K. marxianus* strains will continue as this organism has great potential for use in SSF, but work is still needed as there is still variability between not only strains, but in the same strain being evaluated by different laboratories (Fonseca et al., 2008). A possible solution to the differences encountered between laboratory results is the use of a defined media for fermentation.

3.5 Optimization of Fermentations

It is important to define the variables of interest when looking at optimization. Since only one product can be evaluated in most types of optimization, it is important to understand the variability of the product profile as it applies to the growth stages of the microorganism because the reactor set-up in which the media is optimized (i.e. batch vs. fed batch vs. continuous) will affect the product yield. Substrate availability, product concentration, temperature, pH, and redox potential can all affect the ability of the organism to produce the product of interest. It is also important to define how this product will be measured, e.g. concentration in the media (g/L), yield based on cell concentration (g/g), yield based on substrate consumed (g/g), yield based on theoretical conversion (%), or enzyme production (Kennedy and Krouse, 1999).

Many studies have looked at optimizing the conditions that organisms are exposed to in order to produce product yields as close as possible to the maximum theoretical yields. These studies usually optimize multiple parameters at once, including temperature and enzyme concentration,

as well as those variables that are more directly related to the media composition, such as the concentration of nitrogen, potassium and other nutrients (Balusu et al., 2005; Dey et al., 2001 Mandenius et al. 2008). Including all of these parameters in a design could serve to confuse the design if the tested organism was much more sensitive to one of the parameters than others. In many ways media optimization refers to not only optimization of the actual media constituents, but also the fermentation conditions of the reactions (Weuster-Botz, 2000). Process boundaries are important in the optimization of bioprocesses as there are usually several steps to a process and inclusion of upstream and downstream variables in an optimization experiment can further muddle responses and exponentially increase the amount of time an optimization experiment takes to complete (Mandenius and Brundin, 2008). Media composition is most usually optimized with the use of undefined compounds such as corn steep liquor, yeast extract, soybean meal, wheat bran, and cotton seed extract (Balusu et al., 2005; Dey et al., 2001; Kundiyana et al., 2010; Singh et al., 2009; Techapun et al., 2002). The problems with using an undefined media in optimization will be discussed later, as these are important considerations in optimization studies (Zhang and Greasham, 1999).

A question can be raised as to whether the best optimization approach is to improve the media or the organism being used for the process (Kennedy and Krouse 1999). This raises an important issue as strain development through both genetic manipulation and biological screening of organisms in challenging environments has given way to more productive microorganisms. Unfortunately, strain development is costly. Insertion of multiple genes of interest for product formation into an organism of interest does not necessarily guarantee the ability of the modified microorganism to make more product due to environmental constraints from the conditions in which it is employed, depending on the method of genetic manipulation and the residual genes present in the modified organism (Hammond, 1995). In fact, such genetic manipulations could affect the ability of the microorganism to reproduce, thereby limiting the population and

decreasing overall yields. Non-targeted genetic manipulation by random mutation and crossing cells has a low probability of working well and being carried over into future generations of the modified microorganism. Targeted genetic manipulation is more effective; although, it has some issues with public perception (Saerens et al., 2010). The genetic manipulation of yeast is still an important research area that has promise in the biofuels arena since ethanol being produced is not for consumption and, therefore, not regulated by stringent food and beverage regulatory agencies (Hammond, 1995).

Even with these modifications, having a media that is optimal for the expression of these genes is necessary in order to allow for the best media to be found, but knowing that the strain being tested is the "best" is not always known. Kennedy and Krouse (1999) defined this phenomenon as the "catch 22" of media development, as it is difficult to determine which parameter is best to start with, but the decision must be made as to the starting point and how many iterations are feasible. Since the term "optimum" implies that all iterations have been looked at and tested, it is important to define the parameters of the optimization (e.g. organism, enzyme loading, fermentation type, etc.) or to just avoid the use of the word "optimum" altogether and go with "best."

Media development can be done by either an open or closed method. In an open method, there are many different iterations of media, as "open" implies that all possible constituents be tested. This is not a realistic methodology as the number of combinations of constituents is practically infinite. In the closed optimization method, one is limited to the constituents that are already present in the media being evaluated. Obviously this has many limitations as well because there is no ability to add possible minerals that may improve performance. A combination of the two methodologies is the best tactic. Such a combination might include using a base media, but then identifying potentially helpful media additives through a review of literature (Kennedy and Krouse, 1999).

This identifies a problem that is not discussed in many optimizations, but is present in all. This is the issue of the starting point for media development. This can be done by reviewing literature to identify an existing media that works well for the process, doing a compositional analysis of the cell itself and mimicking this composition with the media design, or by simply using a media that works as the "if it isn't broken, don't fix it" rule applies (Kennedy and Krouse, 1999).

Optimization of media has been done for different organisms in several ways, but is most easily split into four groups; a classical empirical approach, a statistically designed approach, the use of evolutionary computational methods, or the use of artificial neural networks (Parekh et al., 2000).

The earliest form of media optimization was accomplished purely empirically by the testing of different levels of a component, comparing results and then using the "best" medium out of those tested in subsequent experiments. Many original media were based upon whatever was easily obtained by microbiologists, therefore plant and animal "extracts" are present in many media. As a result there are many interesting components such as soil extract, egg yolk emulsion and sheep blood in media still being used to cultivate organisms (Kennedy and Krouse, 1999).

Classical media development can involve the swapping of one nutrient source for another, component swapping, (e.g. NH_4Cl for $(NH_4)_2.SO_4$ or amino acids) and still has a place in the development of media, as it is straightforward, easily executed and simply analyzed. These benefits and the ability of being able to try many different possible components are far outweighed by the limitations of not being able to evaluate interactions among components and the sheer volume of experiments (Kennedy and Krouse, 1999; Parekh et al., 2000; Pilát et al., 1976).

A method for continuous culture and relatively quick response can be accomplished by growing the organism of interest on a minimal media in a continuous reactor. The fed media can then be "spiked" by injecting known growth-limiting compounds into it and then measuring the impact of

the injection on the growth of the microorganism and substrate depletion and correlating that to a "positive" response (Mateles and Battat, 1974). Steady state is not achieved in this type of experimental set-up and interactions between factors are not able to be quantified due to the nature of the "one by one" experimental procedure. Although the constructed media does not restrict growth, the lack of interactions limits the ability of the procedure to make a "best" media, although a "non-limiting" media could be easily obtained in this manner. Another limitation is that only cell mass can be optimized in this method (Kennedy and Krouse, 1999).

An improvement on this approach is the use of designed experiments in order to optimize media. The easiest of these designed experiments to understand is the full factorial design. This design tests all levels of one factor with each level of all the other components, so for example if three factors were being tested and each factor had three levels, there would be 9 (3x3) possible combinations. The benefits of such a design are that none of the main affects or interactions are confounded with any other. There are enough degrees of freedom to evaluate the whole design with interactions. The disadvantage of such a design is that many experiments are needed in order to fulfill the design, as more than one replication is needed for each combination (Kennedy and Krouse, 1999; Kuehl, 2000).

In order to alleviate the number of experimental runs needed to complete a statistical design, a fractional factorial design can be used. These designs have many different forms, but analysis of such a design is sometimes tricky as there is confounding of higher interactions with other interaction terms and sometimes main effects. One of the most used partial factorial designs is the Plackett-Burman design. This design uses only two levels of each treatment and is only valid when there are no interactions between chemicals, i.e., main effects are additive. This design allows for large numbers of factors to be screened with a minimal amount of runs. Since only two levels of each component are tested in this design, it is difficult to optimize a media from this response. Plackett-Burman is used in order to screen components for further optimization in order

to limit the number of factors in more complete designs. Plackett-Burman designs only have a resolution of 3, meaning that only main effects are evaluated. Increasing the resolution of a design by employing more combinations of treatments can allow for a much broader interpretation of results (Kennedy and Krouse, 1999; Kuehl, 2000)

Response surface optimization is one approach first used for media optimization by Maddox and Richert (1977) for gibberellic acid production. This approach allows for a model to be constructed from the significant compounds. A "best" concentration can then be iteratively calculated, or can be done using computer software capable of this method. Difficulty lies in assigning the correct range of values to the experimental design in order to see significant variances between the treatments. Generating a response surface is helpful as the graphical representation of data can allow researchers to visually evaluate the sensitivity of a response to different levels of factors (Kuehl, 2000).

Response surface optimization is by far the prevailing method for optimizing media and fermentation parameters (Balusu et al., 2005; Bandaru et al., 2006; Dey et al., 2001; Singh et al., 2009; Sreekumar et al., 1999), as the analysis of such experimental designs has become straightforward with the advent of statistical analysis software with user friendly interfaces, such as JMP from SAS (Parekh et al., 2000). Using response surface methodology, Balusu et al. (2005) optimized the concentrations of filter paper, corn steep liquor, cysteine HCl, ferrous sulfate and magnesium chloride in a five factor, five level central composite design with 54 runs for ethanol production from cellulosic biomass by *Clostridium thermocellum* SS19. Using this methodology, the optimized media raised the yield of ethanol from 0.32 g/g to 0.41 g/g, an 18% increase.

3.6 Defined Media Development

Benefits of using a synthetic, defined media include the reproducibility of media and results, the ability to control all media components separately, the elimination of undesirable secondary products that can sometimes result from the incorporation of compounds present in undefined media components such as yeast extract, and the reduction of variability between batches of non-defined chemicals in an industrial application (Yee and Blanch, 1993; Zhang and Greasham, 1999). The defining of a media in an application of fermentation for the production of biofuels can also be advantageous because it allows for control over the chemicals going into the fermentation, as the feedstock composition can vary due to harvest time, pretreatment conditions, field location and growing season. Tools, such as response surface methodology with statistical models, make the process for optimization streamlined with minimal repetition for statistical significance and are sometimes even done in tandem with strain development and screening (Parekh et al., 2000). Such models have given optimized media for fermentations, such as those presented by Sreekumar et al. (1999) for *Z. mobilis*, presenting higher substrate utilization and decrease of byproducts. Media supplements for undefined media can also be optimized as done by Gough et al. (1996) for an undefined molasses fermentation using *K. marxianus* IMB3.

3.7 Nutrients in Yeast Fermentations

A survey of the literature available for media evaluations for fermentations with *K. marxianus* IMB3 (as well as related *Kluyveromyces* species) included the introduction of excess Mn^{+2} into the media to increase ethanol production from lactose (Brady et al., 1995), although the effect of this element on a glucose fermentation was not evaluated. A more recent study found that concentrations of $MnSO_4$ above 0.8 mM inhibited growth of *S. cerevisiae* on glucose in an ethanol fermentation (Stehlik-Tomas et al., 2009). Calcium is also an element of interest that is not present (outside of trace amounts in yeast extract) in the yeast fermentation media (YFM) media for IMB3 (Banat and Marchant, 1995), but is identified as a trace nutrient necessary for cell viability in fermentations (Walker et al., 2004). A study on the effect of calcium

concentration in the media of *S. cerevisiae, Saccharomyces bayanus,* and *K. marxianus* showed that Ca^{+2} concentrations between 0.75 and 2 mM had a positive effect on the organisms' ethanol production and growth (Nabais et al., 1988). Ethanol production has also been found to be increased by the addition of Ca^{2+} and Mg^{2+} to media during the fermentation of glucose to ethanol by *S. cerevisiae* (Ciesarová et al., 1996). Ca^{2+} and Mg^{2+} have also been found to compete with each other and calcium can be seen in some instances to interfere with cellular uptake of magnesium (Walker et al., 2004). The ratio of Mg^{2+}:Mn^{2+} has also been evaluated as a parameter of interest in fermentations. A ratio of Mg^{2+}:Mn^{2} less than two was found to be inhibitory to *S. cerevisiae* cells growing on glucose (Blackwell et al., 1998). Zn^{+2} has also been found to be a cation of interest as fermentations lacking in this micronutrient have been known to not run to completion (Walker et al., 2004). It has also been found that some yeast are able to overcome inhibition due to temperature elevation and ethanol concentration through increased supply of nutrients in the media and higher cell concentration in inoculum (D'Amore et al., 1989). Increasing nutrients in the media has limitations with regard to both solubility and toxicity concerns at high concentrations with most compounds.

3.8 Immobilization

Immobilization technology is of interest in fermentations because it allows for the long-term stabilization of cells (Tampion and Tampion, 1987). Also, the immobilization of some cells allows for stabilization in environments that could potentially be harmful to free cells. Immobilization of cells has been shown to improve the overall thermal stability of cells as compared to free cells (Chang et al., 1996; Williams and Munnecke, 1981), potentially allowing reactors to be operated at higher temperatures in SSF reactions. This stability is possibly due to increased membrane stability in immobilized cells (Chang et al., 1996). Immobilization of fermenting cells for use in SSF reactions has been done previously. Most recently a group from India investigated the immobilization of *Debaromyces hansenii* for use in both SSF reactions and

separate saccharification reactions followed by fermentation. The method of immobilization failed for SSF due to immediate dissolution of the beads within the first reuse (Menon et al., 2010). This problem potentially could be offset by utilizing a media that will not disrupt the stability of the beads, since many buffers (such as phosphate and citrate buffers) are known to disrupt the stability of calcium alginate beads (Smidsrød and Skjak-Brk, 1990). Specifically, *K. marxianus* IMB3 has been used in immobilization and subsequent SSF reactions with some success when grown on pure cellulose, e.g. milled filter paper (Barron et al., 1996; Barron et al., 1996). There is limited information on how immobilized cells would work with a lignocellulosic substrate, but some have been evaluated. Yamashita et al. (2008) evaluated the use of immobilized *Z. mobilis* cells, in both SHF and SSF cultures for the conversion of paper sludge to ethanol at 30°C with a glucan loading of 6.68 g/L. In their study, it was found that SSF without immobilized cells produced no ethanol, but glucose was accumulated up to 6.1 g/L. The SHF reaction using the same inoculum showed only a maximum ethanol accumulation of 2.5 g/L. Using calcium alginate as the immobilization matrix, another SSF was carried out and much better conversion efficiencies were obtained. In this reaction, 47.8% of the theoretical ethanol yield was obtained at 48 h. Recycling of the beads did not work well, as after the fourth cycle only 14.1% of the theoretical yield was obtained; a lower concentration of glucose was also obtained in this SSF, thought to be due to the accumulation of ions inhibitory to the cellulase enzymes on the surfaces of the beads. A further issue could have been with the bead stability if such a phenomenon was occurring since Mg^{+2} ions were present in the sludge ash (Yamashita et al. 2008).

An additional benefit of immobilized cell systems is that the cell growth rate limiting part of the equation becomes moot (as discussed relative to modeling of SSF reactions), as the cells are already fully grown in the immobilization matrix. However, it is still desirable to have fast

growth rates for the establishment of the immobilized system, as well as useful for the comparison of immobilized systems to optimized free cell systems.

There are two main issues with the implementation of immobilized yeast systems in SSF. Both mass transfer issues and the ability of the immobilized cells to be recycled and separated from recalcitrant biomass are of interest. Diffusion of glucose and ethanol within immobilized systems is one key issue. However, the calcium alginate matrix appears to have very high diffusion rates, similar to those present in aqueous solutions. These rates are highly dependent on the concentration of alginate in the system (Hannoun and Stephanopoulos, 1986). Free enzyme is necessary in SSF due to the recalcitrant nature of the solid biomass feedstock. Free enzyme in the solution allows the enzyme to gain access to the solid substrate. When cellulases are immobilized in a matrix, even in optimized saccharification reactions, the hydrolytic efficiency is greatly reduced (Busto et al., 1998). Even the influx of cellobiose into the immobilized matrix appears to be slightly limited (Busto et al., 1995). In summary, the diffusional limitations of glucose and ethanol are not limited by the immobilization of cells in a calcium alginate matrix, so immobilization of fermenting organisms in beads of this make-up should not have any detrimental effects on the process.

Calcium alginate recovery is of interest in a system that cannot use size separation. The insoluble nature of lignocellulosic material means that even with optimal hydrolysis efficiencies, there will still be insoluble particles left because the remaining lignin from the pretreatment will not be broken down by cellulase or β-glucosidase enzymes (Taherzadeh and Karimi, 2007). Possible methods for overcoming this barrier include the incorporation of magnetic particles into the beads to allow for removal due to charge (Brady et al., 1996).

3.9 Concluding Remarks

In conclusion, both media optimization and cell immobilization appear to be two areas where the problem of sustained yeast *K. marxianus* IMB3 conversion during SSFs could be addressed and improved, especially with the lack of previous work done on either of these subjects for SSFs.

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CHAPTER IV

CONSTRUCTING AND TESTING A DEFINED MINIMAL MEDIUM FOR ETHANOL PRODUCTION BY *K. MARXIANUS* IMB3

4.1 Introduction

In the wake of concern over the environmental implications of oil use, rising oil prices and impending scarcity of this commodity, increasing interest has been given to conversion of biomass to ethanol and other fuels that can be used in lieu of gasoline as a transportation fuel (von Sivers and Zacchi, 1996). One of the most common ways that this alternative energy pathway is being approached is by conversion of biomass into sugars, which are then fermented by microbes to ethanol. This process is referred to as the biochemical (sometimes sugar) platform for ethanol production (Wright and Brown, 2007). Using thermotolerant yeasts (yeast capable of converting glucose to ethanol at temperatures higher than 40°C) in sugar fermentations is beneficial by removing costs associated with heating for distillation and also alleviating some of the differing optimal temperature problems (between yeast and enzymes) in simultaneous saccharification and fermentation (SSF) processes. *K. marxianus* IMB3 is one such organism capable of fermentations at 45°C (Banat and Marchant, 1995; Banat et al., 1992; Banat et al., 1998; Banat et al., 1996; Barron et al., 1995; Barron et al., 1996; Barron et al., 1997; Boyle et al., 1997; Faga et al., 2010).

Although many studies have looked at SSF of biomass at higher temperatures (45°C) (Banat et al., 1992; Banat et al., 1998; Banat et al., 1996; Barron et al., 1997; Boyle et al., 1997; Faga, 2009; Suryawati et al., 2009), few have looked at optimizing the parameters within an SSF with respect to media constituents. Most SSFs are supplemented with an undefined additive, whether it is inherent to the substrate (such as switchgrass or wheat straw) or a commercially available complex media additive such as yeast extract. The addition of these components can supply needed nutrients for the fermentations, but may provide nutrients that allow the cells to produce enzymatic machinery tailored to producing products other than ethanol. Also, one of the biggest issues with fermentations is the reproducibility of results. The use of defined media for the purposes of replication has proven to be quite useful, as it eliminates some of the undefined inputs in the fermentation allowing for greater control (Zhang and Greasham, 1999).

Although the substrate of interest in the final application of this media is lignocellulosic material, the innate variability of this substrate as well as the amount of time it takes to complete such fermentations, even on a batch scale, make the use of such substrate unrealistic for initial experimentation in defined media development studies. Harvest date, growth season, soil constraints, species, variety, nutrient application can all affect the elemental composition of the feedstock (Monti et al., 2008; Sassner et al., 2008; Sluiter et al., 2004). Pretreatment methodology can also affect the availability of the glucan content for liberation by cellulases (Laureano-Perez et al., 2005; McMillan, 1994), but this consideration should be insignificant as long as the biomass is treated under the same conditions. Another unknown constituent in SSFs is the cellulase enzyme being added. These enzymes usually are produced by fungi, and depending on the purification processes used, they can contain sugars, cell parts and residual growth media. Even though the addition of this enzyme would be a consistent measure of volume, the innate variability of the enzyme mixture could lead to misleading results. This is due to many statistical analyses having only one trial of certain experimental points and estimating

variance for determination of significance through multiple replications of a chosen point. Time becomes a consideration as running the fermentation trials using glucose only take 48 to 72 h for completion, but using a lignocellulosic feedstock may require up to 168 h for completion. The limitations of doing a statistical analysis also only allow for one point to be evaluated in the fermentation, so the decision has to be made on how to compare the fermentations. Glucose as a substrate allows for both faster throughput of experiments as well as a defined media, but has limitations as production of glucose occurs throughout the course of SSF.

In fulfilling the design requirements of a defined media, the use of screening experiments become necessary. Statistical designs are an excellent and well researched methodology for screening of media components. Full factorials are the best design for evaluating all possible interactions, but become cumbersome in size as the number of factors increase. In lieu of such extensive designs, partial factorials can be used that sacrifice the evaluation of "all possible combinations" for a smaller design size. These statistical tools allow for the evaluation of the significance of many different components in the fewest number of "runs" (in this case, fermentations). The limitations of such a design include the confounding of interaction terms with main effects (factors). This means that the significance of some main effects (factors) may only be significant due to the correlating interaction terms. It is intended through the selected design to minimize these possible erroneous correlations, or to only use this design for factors that will not be confounding (known usually through empirical results) (Kennedy and Krouse, 1999).

In this paper the intent was to design a defined media for the fermentation of glucose. The components of interest were identified in two ways, through identifying the composition of the fermentation medium used in previous *K. marxianus* IMB studies and by completing a literature search of other chemically defined components shown to increase fermentation rates or yields in yeast.

4.2 Materials and Methods

4.2.1 Culture

Cells of *K. marxianus* IMB3 were generously given by Dr. I.M. Banat from the University of Ulster and were maintained on yeast-peptone-dextrose (YPD - 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose (dextrose)) agar, with sub-culturing occurring every three months to prevent contamination. A starter flask was grown aerobically using a 250 ml KIMAX® baffled culture flask (Kimble Chase, Vineland, NJ) and BugStopper® venting closures (Whatman, Inc., Piscataway, NJ) at 45°C and 250 rpm on an orbital shaker incubator from a loopful of cells taken from the stored stock culture slant a week prior to the experiment. The starter flask was stored at 4°C until needed (<1 week). Cells used to inoculate the experiment were grown for 16 h prior to inoculation of the fermentation flasks aerobically at 45°C and 250 rpm on an orbital shaker incubator in 100 ml of YPD medium using a 5% (v/v) inoculation from the starter flask. All inoculations and sampling occurred in a biosafety cabinet under aseptic conditions.

4.2.2 Fermentation Conditions

For inoculation of the fermentation flasks, the method was the NREL procedure for SSF inoculation (Dowe and McMillan, 2001). Optical density (OD) of the cells was checked at 600 nm using a UV-Vis spectrophotometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA, USA) at 16 h. The sample was diluted between 0 and 0.5 OD to ensure the sample was within the linear growth curve of the organism. The amount of culture removed for inoculation followed the equation (Dowe and McMillan, 2001):

$$Volume = \frac{0.5\ OD \times 100\ mL}{OD\ of\ inoculation\ flask} \times number\ of\ experimental\ bottles$$

The volume was removed aseptically in a biosafety cabinet and spun down using a centrifuge for 10 min at 3,600 x g. The supernatant was decanted in the biosafety cabinet and then cells were

resuspended in sterile deionized water. Cells were spun down a second time, supernatant was decanted, and cells were resuspended in sterile deionized water again. Once more the sample was spun down, supernatant was decanted and the cells were resuspended with enough sterile deionized water to result in 1 mL of inoculum having an OD of 50. The washing of the cells served to remove any trace nutrients from the initial rich media used to culture the cells. The concentrated, rinsed inoculum was then added (1 ml) to the fermentation flasks. The dilution of the cells into 100 ml of media resulted in an OD of 0.5, which equals a cell mass concentration of 0.14 g/L (Suryawati et al., 2009). Sterile 250 ml flasks were filled with the designed sterilized media components for the experiment, resulting in a working volume of 100 ml. All glassware, citrate buffer, stoppers, deionized water, and glassware were sterilized at 121°C, 200 kPa and held for 20 min in a steam autoclave (PRIMUS Sterilizer Corporation LLC, Omaha, NE). All other media components were filter sterilized using sterile 0.45 μm polyethersulfone (PES) filters (Corning Inc., Corning, NY). Sterile pipets were used to measure out the needed volume of constituents. All fermentations used glucose at a concentration of 40 g/L in the media and citrate buffer (pH 5.5) at a concentration of 50 mM. Each experiment consisted of three different treatments. Each treatment was done in triplicate, resulting in nine total bottles for each experiment, except for the statistically designed partial factorial screening experiments. Each screening experiment consisted of 16 flasks.

Once inoculated, experimental flasks were sampled, then placed on an orbital shaker/incubator (MaxQ 4450, Thermo Scientific, Waltham, MA) at 130 rpm, 45°C. Samples were taken every 4 to 6 h for the first 12 h and every 6 to 8 h for the next 12 h and then again at 48 h by transferring the flasks into a biosafety cabinet and aseptically transferring 1.5 ml of sample into 1.7 ml centrifuge tubes. Optical density of each sample was measured as described previously and pH was recorded using a pH meter (ORION 310 pH meter, Thermo Electron Corporation, Beverly, MA, USA; VWR symphony probe, West Chester, PA, USA). Samples were immediately spun

down and the supernatant was filtered through 0.45 μm nylon filters into HPLC vials and frozen until further analysis.

Analysis of solvent production and initial glucose was done by first thawing samples, then vortexing the samples to ensure the contents were well mixed. Vials were then placed on an automatic sampler attached to a HPLC (1100 series, Agilent, Santa Clara, CA) and 20 μl of each sample was injected onto an Aminex HPX-87H column (BioRad, Hercules, CA) at 60°C. Peaks were identified using a refractive index detector (RID) (1100 Series). A set of five external standards with known sugar concentrations was prepared and run with each experiment in order to ensure correct quantification of chemicals. Initial samples were diluted with deionized water to minimize peak overlap from high initial concentrations of glucose. Sulfuric acid (0.05M) at a flow rate of 0.6 ml/min was used as the mobile phase (Dowe and McMillan, 2001).

4.2.3 Evaluation of singular media constituents

Three experiments to evaluate the effects of adding $MnSO_4$, $CaCl_2$ and yeast extract to the media were done. $ZnSO_4$ was also evaluated, but was done after the media was defined and screened due to it being a present in yeast extract. The yeast extract experiment was necessary to determine the starting point for a defined media based on a published composition of the yeast extract.

The original yeast fermentation media (YFM) outlined for *K. marxianus* IMB3 contained yeast extract (Banat et al., 1992) and is outlined in Table 4.1. Ethanol yields and by-product concentrations were analyzed for significant differences between treatments in SAS version 9.2 using Tukey's test in proc GLM for comparison of means ($\alpha = 0.05$).

$MnSO_4$ and yeast extract were evaluated at 5 levels in the growth media. $CaCl_2$ was evaluated at three levels. The levels tested are outlined in Table 4.2. $MnSO_4$ was not added in the yeast

Table 4.1. Yeast fermentation media composition for *K. marxianus* (Banat and Marchant, 1995).

Component	Amount (g/L)	mM in Media
$(NH_4)_2SO_4$	2.0	15.14
KH_2PO_4	2.0	14.70
$MgSO_4*7H_2O$	1.0	4.06
Yeast Extract	0.5	--
$MnSO_4$	0.1	0.66

Table 4.2. Levels tested in initial media screens looking at different nutrient levels.

Component Tested	Levels Tested	Manganese Present in Experiment
Calcium	0, 1 (0.111), 2 (0.222) mM (g/L)	No
Manganese	0, 0.05, 0.1, 0.2 and 0.3 g/L	Yes
Yeast Extract	0.1, 0.3, 0.5, 3.0, 5.0 g/L	No
Zinc	0, 0.5 and 50 mg/L	No

extract or $CaCl_2$ experiments as manganese was found to be detrimental to the fermentation efficiency in the first experiment. Table 4.2 also outlines the $ZnSO_4$ levels tested in the defined media. The low level of 0.5 mg/L was the amount present only in yeast extract, and 50 mg/L corresponded to improved fermentation performance with a self-flocculating yeast SPSC01 that was developed in the Department of Bioscience and Bioengineering at the Dalian University of Technology and deposited at Chinese General Microbiological Culture Collection Center (CGMCC) (collection number of 0587) (Xue et al., 2010). Yeast extract was replaced with a defined mineral and vitamin solution in the zinc experiments, hence defined media being the type used. $CaCl_2$ was added to the media at 1 mM in both the yeast extract and $ZnSO_4$ experiments.

4.2.4 Definition of media

Two solutions were prepared to mimic the elemental composition of the yeast extract added to the media when combined in solution,. The composition of the mineral and vitamin solutions were developed from a Difco ® information sheet published online by Voigt Global Distribution Inc. (http://www.voigtglobal.com/Anonymous/DIFCO_Yeast_Agar_Formulations.pdf). The information from this document is shown in the appendices. The formulated mineral and vitamin solutions are presented in Table 4.2 and 4.3, respectively. The 100X solution was used for the vitamins due to the extremely low levels used and the desire to be able to measure this component easily for experimental setup, and still have the components easily dissolve at the concentrations needed. The 20X concentration was used for the mineral replacement as this was how the minerals were initially concentrated in prior experiments in this laboratory.

Three different media were evaluated, each in triplicate in the yeast extract replacement experiment. The first treatment tested the defined mineral solution only as a replacement for

Table 4.3. Composition of 20X concentrated mineral solution for replacement of yeast extract in growth media.

Component	Concentration (mg/L)
$MgSO_4 \cdot 7H_2O$	76
KH_2PO_4	1112
NaCl	379
$ZnSO_4 \cdot 7H_2O$	5
$(NH_4)_2SO_4$	5142

yeast extract, the second treatment used both the vitamin solution and the mineral solution to replace the yeast extract and the third was a control using yeast extract at the original concentration in the media (Table 4.1). The nutrients $MgSO_4 7H_2O$ (1 g/L), $CaCl_2$ (1 mM), $(NH_4)_2SO_4$ (2 g/L) and KH_2PO_4 (2 g/L) were also added to the fermentations in addition to the yeast extract replacement (or control) being tested. $MnSO_4$ was not included in the media.

4.2.5 Screening of media constituents

Two different partial factorial designs were used to evaluate the effects of the components of the vitamin solution (Table 4.4) on the fermentation. The second design was employed in order to make sure that the significance of main effects in the first design was not being confounded in the initial screen with two-way and higher interactions. In the first design, 6 factors were evaluated in 16 runs, with two of the runs being the same. SAS version 9.2 was used to design and evaluate these experiments. The high level (1) of each design was set to the amount present in the designed yeast extract vitamin solution (Table 4.4), the low level (-1) was set to no concentration (0 g/L) to evaluate the necessary components in the media. The confounding rules for the first screening design were: nicotinic acid = P-4-aminobenzoic acid*riboflavin, Ca pantothenate = P-4-aminobenzoic acid*thiamin and pyridoxine = riboflavin*thiamin. The confounding rules were not selected due to knowledge of lack of interactions due to a limited amount of research showing specific confounding of vitamins in yeast fermentations in the literature. The confounding rules used were chosen at random. The first screening experiment design is highlighted in Table 4.5.

The second screening design (Table 4.6) evaluated the four significant components ($p < 0.05$ for increased ethanol production or for reduction of by-products) for ethanol production and added sodium chloride (NaCl) for a total of five factors. Again the experiment used a factorial with 16

Table 4.4. Composition of 100X concentrated vitamin solution for replacement of yeast extract in growth media.

Component	Concentration (mg/L)
P-4-Amino Benzoic Acid	38.2
Riboflavin	5.8
Thiamin	26.5
Nicotinic Acid	29.9
Calcium Pantothenate	14.9
Pyridoxine	2.2

Table 4.5. First screening design for vitamin screening (6 factors, 1/8 factorial done in duplicate).

	Factor						Product
RUN[A]	**p-4-Aminobenzoic Acid**	**Riboflavin**	**Thiamin**	**Nicotinic Acid**	**Calcium Pantothenate**	**Pyridoxine**	**24 h EtOH (g/L)**
1	-1[B]	-1	-1	1[C]	1	1	11.978
2	-1	-1	-1	1	1	1	12.033
3	1	-1	-1	-1	-1	1	4.496
4	1	-1	-1	-1	-1	1	5.150
5	-1	1	-1	-1	1	-1	7.314
6	-1	1	-1	-1	1	-1	7.846
7	1	1	-1	1	-1	-1	5.974
8	1	1	-1	1	-1	-1	10.434
9	-1	-1	1	1	-1	-1	11.844
10	-1	-1	1	1	-1	-1	8.830
11	1	-1	1	-1	1	-1	8.686
12	1	-1	1	-1	1	-1	7.137
13	-1	1	1	-1	-1	1	8.561
14	-1	1	1	-1	-1	1	7.258
15	1	1	1	1	1	1	17.636
16	1	1	1	1	1	1	17.591

[A] Each run is one flask, therefore there is no standard deviation for each run.

[B] (-1) : 0 g/L in the media for the factor in that run

[C] (1): Level in media set to those shown in Table 4.4 for that factor in that run.

Table 4.6. Second screening design (5 factors, ½ factorial) for nutrient screening.

	Factor					
Run[A]	**Thiamin**	**Calcium Pantothenate**	**Pyridoxine**	**Nicotinic Acid**	**NaCl**	**24 h EtOH (g/L)**
1	-1[B]	-1	-1	-1	1	9.524
2	1[C]	-1	-1	-1	-1	8.765
3	-1	1	-1	-1	-1	7.514
4	1	1	-1	-1	1	10.549
5	-1	-1	1	-1	-1	9.919
6	1	-1	1	-1	1	11.388
7	-1	1	1	-1	1	9.354
8	1	1	1	-1	-1	12.072
9	-1	-1	-1	1	-1	14.960
10	1	-1	-1	1	1	15.745
11	-1	1	-1	1	1	15.518
12	1	1	-1	1	-1	15.908
13	-1	-1	1	1	1	14.657
14	1	-1	1	1	-1	15.611
15	-1	1	1	1	-1	14.791
16	1	1	1	1	1	15.940

[A] Each run is one flask, therefore there is no standard deviation for each run.

[B] (-1) : 0 g/L in the media for the factor in that run

[C] (1): Level in media set to those shown in Table 4.4 for that factor in that run.

runs, but the only confounding rule was NaCl = thiamin*Ca pantothenate*pyridoxine*nicotinic acid because a ½ factorial design was used with a resolution of 5. This means that no two way interactions or main effects were confounded with each other. The high values were again set to those in the vitamin solution for the vitamins (Table 4.4) and set to the same as present in the mineral media (Table 4.3) for NaCl.

4.2.6 Yield calculations

For evaluating the data, the actual yield of ethanol (actual/theoretical from glucose consumed) was calculated. This calculation is shown in equation 4.1.

$$Actual\ Yield = \frac{(Ethanol\ concentration\ at\ 24\ hrs)}{0.51\times(Initial\ Glucose\ concentration - Glucose\ concentration\ at\ 24hrs)} \times 100\% \quad (4.1)$$

Ethanol yield based on initial glucose concentration only allowed for a comparison of the extent of glucose conversion to ethanol in 24 h when compared to theoretical complete conversion. This calculation is highlighted in equation 4.2.

$$Total\ \ Yield = \frac{(Ethanol\ concentration\ at\ 24\ hrs)}{0.51\times(Initial\ Glucose\ concentration)} \times 100\% \quad (4.2)$$

The goal of calculating both of these ratios was to allow for there to be a measure of how much glucose went towards ethanol production, even if not all glucose was converted in 24 h. The reasoning behind this was that excess nutrients may promote cell growth, and therefore acid and glycerol production, allowing only for more ethanol to be produced because of a higher cell density. The goal was to be able to sustain cells which promoted ethanol production over acid and glycerol production.

4.3 Results and Discussion

The original media, referred to as YFM (Table 4.1), consisted of 5 components, manganese sulfate ($MnSO_4$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), yeast extract, potassium phosphate monobasic (KH_2PO_4) and ammonia sulfate ($(NH_4)_2SO_4$). Yeast extract provided all trace elements and necessary vitamins for the fermentation media. In addition to the original components, two additional defined minerals were identified as being of interest. These two compounds were $CaCl_2$ and $ZnSO_4$. Since all elements besides $MnSO_4$ and $CaCl_2$ had a significant value in the compositional analysis of yeast extract (appendices), only these two parameters were looked at prior to the screening requirements.

4.3.1 Manganese

Manganese is known as a microelement in yeast fermentations and is usually necessary in the range of 2-4 μM to be used as an enzyme cofactor in cellular functions (Walker et al., 2004). The impetus behind looking at manganese in the media of *K. marxianus* IMB3 was with studies done by Brady et al. (1995), which identified that increased Mn^{+2} ions in the media (1 mM or 0.15 g/L) had a positive correlation with production of ethanol from lactose containing media at 45°C. This increased production of ethanol was suspected to be due to increased thermotolerance of enzymes produced by the organism.

Another study using *S. cerevisiae* found that $MnSO_4$ concentration at 0.8 mM (0.12 g/L) or greater inhibited growth (Stehlik-Tomas et al., 2009). Still another study found that the addition of $MnSO_4$ at 0.1 g/L to molasses media (with $ZnSO_4$ and $CuSO_4$) increased the overall conversion of the sugars in molasses to ethanol with *S. cerevisiae* TVG_4 (Stehlik-Tomas et al., 2004); although, because no trial was done with only manganese addition, the increase may have been due to one of (or both) the other metal additives. Other studies have evaluated the interaction of manganese with magnesium, noting that a critical Mg^{+2}:Mn^{+2} ratio of 2.0 must be achieved in order to negate toxic manganese effects in *S. cerevisiae* NCYC 1383 (Blackwell et

al., 1998). Original concentration in the yeast fermentation media used in the initial evaluation of *K. marxianus* IMB3 was 0.1 g/L (0.6 mM) (Banat and Marchant, 1995).

In the experiments conducted with $MnSO_4$ at 0.0, 0.05, 0.1, 0.2 and 0.3 g/L, the addition of manganese to the fermentation media did not positively affect the conversion of glucose to ethanol by *K. marxianus* IMB3 (Table 4.7), agreeing with the study done by Stehlik-Tomas et al. (2009). The addition of manganese to the media decreased the specific growth rate (data not shown) and the yield based on the ethanol produced/ theoretical conversion of consumed glucose to ethanol. Since no manganese in the media produced the best ethanol yield, manganese was not included in any further experiments.

4.3.2 Yeast extract

Yeast extract is a major constituent of many complex or chemically undefined media. This component serves as the source for trace nutrients and metals in the fermentation (Zhang and Greasham, 1999). The results of the yeast extract testing can be seen in Table 4.8. Increasing yeast extract did not improve the conversion of glucose to ethanol, but decreasing the amount of yeast extract in the media did decrease the rate of glucose consumption and ethanol production. Cell concentration was not significantly different at 24 h among concentration of yeast extract ($p = 0.05$), which indicated that there was a constituent of the yeast extract that aided in alcohol production separate from cell production.

Yeast extract concentration has not been the implicit focus of many studies reviewing nutritional requirements of microorganisms, but it has been used as a factor in many media optimization studies. Skreekumar et al. (1999) used *Z. mobilis* in a response surface optimization study and found that the optimal yeast extract concentration was 6.5 g/L. Glucose, ammonia sulfate,

Table 4.7. Manganese experiment denoting product values at 24 h, different letters denote differences at p = 0.05 level.

Treatment	**Product**				
$MnSO_4$ Concentration (g/L)	Ethanol (g/L)	Total Ethanol Yield (%)	Actual Ethanol Yield (%)	Glycerol (g/L)	Acetate (g/L)
0.00	13.58 ^{A}	68 ^{C}	85 ^{E}	2.74 ^{H}	0.56 ^{J}
0.05	12.61 ^{A}	63 ^{C}	84 ^{E}	2.74 ^{H}	0.56 ^{J}
0.10[a]	10.61 ^{A}	55 ^{C}	85 ^{EF}	2.17 ^{H}	0.53 ^{J}
0.20	2.30 ^{B}	12 ^{D}	73 ^{F}	0.51 ^{I}	0.45 ^{K}
0.30	1.05 ^{B}	6 ^{D}	51 ^{G}	0.34 ^{I}	0.22 ^{L}

a: Level as in Banat and Marchant, 1995 (Table 4.1)

Table 4.8. Yeast extract experiment denoting product values at 24 h, different letters denote differences at p = 0.05 level.

Treatment	**Product**					
Yeast Extract (g/L)	Cell Mass (g/L)	Ethanol (g/L)	Total Ethanol Yield (%)	Actual Ethanol Yield (%)	Glycerol (g/L)	Acetate (g/L)
0.1	1.438[A]	11.29[B]	59.4[E]	89.4[H]	1.527[I]	0.332[K]
0.3	1.598[A]	14.10[C]	75.2[F]	89.7[H]	2.027[I]	0.378[K]
0.5[a]	1.793[A]	16.65[D]	0.86.8[G]	89.3[H]	2.725[J]	0.515[L]
1.5[b]	.	17.34[D]	0.89.1[G]	89.1[H]	3.105[J]	0.780[M]
2.5[b]	.	17.52[D]	0.89.5[G]	89.5[H]	3.152[J]	0.947[N]

a: Level as in Banat and Marchant, 1995 (Table 4.1)
b: Data for cell mass missing because spectrophotometer was misread.

potassium dihydrogen phosphate, magnesium sulfate, and concentration of cell inoculum were all involved in this optimization from literature, providing an increase in conversion to 0.50 g ethanol/g glucose (98% of theoretical conversion). Only glucose and yeast extract were identified as the significant factors for optimization in that study. A concentration of yeast extract that high was not tested in the present study, as the 5 fold (2.5 g/L) increase in yeast extract (over the amount originally present in the 1995 Banat and Marchant medium (Table 4.1)) did not improve performance of the fermentation. In the current study, 0.5 g/L was used in all further experimentation.

4.3.3 Calcium

Calcium is identified as being a micronutrient in the fermentation process. It is thought to only have a minor role in the metabolic pathway as part of a signaling pathway and is thought to only be necessary at a < 1 μM level. This is due to the interaction between calcium and magnesium in fermentations. Calcium present at high amounts can actually inhibit the promoting activities of magnesium due to the deficit between calcium concentration in growth media and intracellular calcium needs (Walker et al., 2004). Birch et al. (2003) saw this phenomenon in an evaluation of grape must nutrient addition where the intrinsic ratio of Mg:Ca is quite low naturally (0.5:1) and the addition of calcium (mM level) further inhibited the fermentation. Rises and Stewart (1997) also observed a decreased fermentation rate and lower ethanol production in high gravity worts (for beer production) using two commercial brewing yeasts, most probably due to competition between magnesium and calcium. In the calcium trials the best ratio of Mg:Ca for ethanol production was 2:1 (on a molar basis). This could explain why the 1 mM concentration of calcium appeared to do better than the higher concentration. There may have been competition between magnesium and calcium at the higher concentration decreasing the benefits of adding calcium to the media (Walker et al., 2004). In fact, Nabais et al. (1988) found that the optimal range of calcium (as $CaCl_2$) in media was between 0.75 to 2.0 mM (0.08 to 0.22 g/L). These

experiments were done on three different strains of yeast, *S. cerevisiae* IGC 3507 III, *S. bayanus* IST 154 and *K. marxianus*, and a positive correlation with fermentation rates was assumed due to calcium helping with cell membrane stability. $MgSO_4 \cdot 7H_2O$ was added at a concentration of 1 g/L (4 mM).

Although no calcium was initially present in the medium described by Banat and Marchant (1995), the decision was made to evaluate this component as some literature supported calcium's ability to increase conversion rates in yeast fermentations (Nabais et al., 1988, Ciesarová et al., 1996). The responses of *K. marxianus* IMB3 in the calcium experiment are shown in Table 4.9. The addition of calcium to the fermentation at a level of 1 mM appeared to be the most productive as this value helped to increase ethanol production rate without decreasing the actual yield of the fermentation. The no calcium addition treatement decreased the amount of glucose consumed and ethanol produced at 24 h. Only about 90% of the ethanol observed in fermentations receiving 1 mM of calcium was produced in fermentations containing no calcium. The results of this experiment do align well with a paper showing a positive correlation between ethanol tolerance and membrane stability (Nabais et al., 1988), as thermotolerance and ethanol toxicity have been shown to exhibit similar physiological responses in yeast (Piper, 1995).

Ciesarová et al. (1996) found that the addition of magnesium and calcium to media did increase the ethanol tolerance of *S. cerevisiae* strains in fermentations with 125 g/L glucose fermentations at 28°C, 0.2M phosphate buffered defined media (pH = 5.8). $CaCl_2$ was tested between 0 and 3 mM, and fermentations containing 3 mM $CaCl_2$ were found to have the highest CO_2 rate of evolution at a 10% ethanol concentration (supplemented concentration, since ethanol tolerance was one of the parameters being tested in the study), indicating that cells were most active in the steady state at this calcium concentration.

4.3.4 Defined Media Comparison

Table 4.9. Calcium experiment denoting product values at 24 h, different letters denote significant differences at p = 0.05 level.

	Product					
Calcium (mM)	Cell Mass (g/L)	Ethanol (g/L)	Total Ethanol Yield (%)	Actual Ethanol Yield (%)	Glycerol (g/L)	Acetate (g/L)
0[a]	1.505 [A]	14.96 [B]	80.1 [D]	89.9 [F]	2.163 [G]	0.458 [H]
1	1.812 [A]	16.98 [C]	90.3 [E]	90.3 [F]	2.486 [G]	0.475 [H]
2	1.604 [A]	16.69 [BC]	89.5 [DE]	89.8 [F]	2.475 [G]	0.481 [H]

a: Level as in Banat and Marchant, 1995 (Table 4.1)

From these initial experiments, a defined media with yeast extract being replaced with the solutions outlined in Tables 4.3 and 4.4 was constructed. These solutions (Mineral solution (M) (Table 4.3) and vitamin solution (V) (Table 4.4)) were tested combined (M+V) or only with minerals present (M) against a control which contained yeast extract (YE) in lieu of the defined media constituents. The consumption of glucose and subsequent fermentative production of ethanol is outlined in Figure 4.1.

The combination of minerals and vitamins was necessary for the fermentation of glucose to ethanol quickly with *K. marxianus* IMB3. The inability of flasks receiving no vitamin addition to ferment glucose to ethanol in a timely conversion was drastic when compared to the YE flasks and the M+V flasks. Over 20 g/l of glucose remained in the fermentation media when no vitamins were added after 48 h; whereas, both the YE and M+V trials ran to completion within 24 h. This resulted in less than 30% yield in these fermentations. This phenomenon agrees with other findings that purport that some of the necessary vitamins for fermentation are not able to be produced in anaerobic sugar fermentations (Dijken et al., 1993). The defined M+V fermentation occurred at a slightly slower rate than the control (YE) fermentation. This phenomenon could easily be explained by the lower amount of cell mass produced in the defined M+V flasks. Flasks not receiving yeast extract reached a maximum cell mass of only 1.5 g/L, as compared to 2.0 g/L in the control flasks with yeast extract.

Yield and other experimental data for the experiment are shown in Table 4.10. Although the initial rate of fermentation was slightly lower, the adjusted ethanol yield for the fermentations given both mineral and vitamin replacement for yeast extract was significantly higher ($p < 0.05$) than that for yeast extract itself. The reason why this comparison is important is that it normalizes the responses based on glucose consumed as the variation between the mineral only media could increase the error term, due to an extremely low growth curve, decreasing the

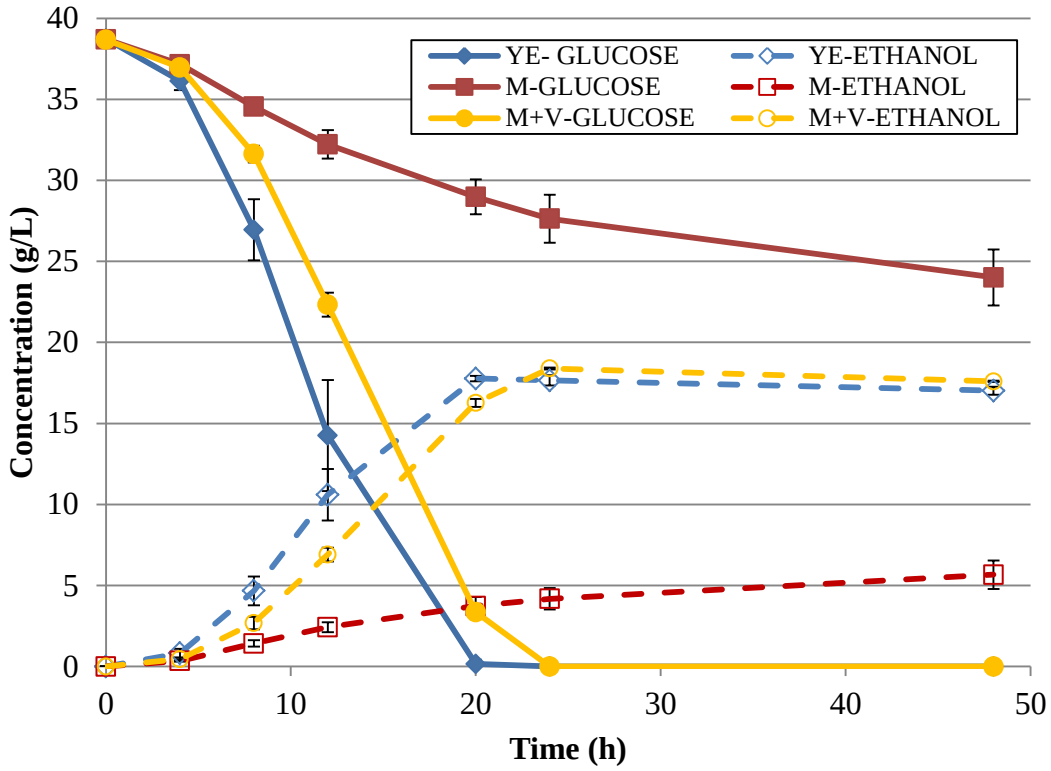

Figure 4.1. Glucose consumption and ethanol production in yeast extract replacement study, M – mineral solution only, M+V – vitamin and mineral solutions, YE – yeast extract only.

Table 4.10. Average values for yeast extract replacement studies at 24 h, Y - yeast extract, M - mineral replacement only, M+V - mineral plus vitamin replacement, different letters denote significant differences at p = 0.05 level.

	Product at 24 h					
	Cell Mass (g/L)	Ethanol (g/L)	Total Ethanol Yield (%)	Actual Ethanol Yield (%)	Glycerol (g/L)	Acetate (g/L)
Y	2.02^{A}	17.66^{D}	89.4^{F}	89.4^{H}	2.69^{K}	0.62^{M}
M	0.56^{B}	4.18^{E}	21.2^{G}	73.9^{I}	0.87^{L}	0.19^{N}
M+V	1.50^{C}	18.39^{D}	93.3^{F}	93.3^{J}	2.54^{K}	0.71^{O}

overall response of the media. The reason why the total ethanol yield was not significant but the adjusted one was can be explained by the calculation of error in the analysis of variance (ANOVA), which is based on the total error of the test based on all observations. Looking at Figure 4.1, it can be seen that the error bars denoting one standard deviation for the M treatment at 24 h are much greater than either of the other two treatments, thus increasing the mean square error for the comparison of means and decreasing the resulting F-values for each treatment. Normalizing the yield calculates the yield based only on consumed sugar removed the differences due to slightly faster growing cultures, which is a distinct possibility if not all vessels were at exactly the same temperature at time of inoculation.

4.3.5 Statistical Screening of Defined Media Components

The first experiment for vitamin screening showed that several components were significant for ethanol production. Calcium pantothenate, pyridoxine, nicotinic acid and thiamin all were shown to be significant using an ANOVA ($p < 0.05$). The statistical values are highlighted in Table 4.11. The issue with this design was that since there were six factors and only eight different runs, the resolution of the design was not high and pyridoxine actually ended up being confounded with the interaction term of thiamin and riboflavin. The model for this test was significant ($p < 0.0004$) and there was no significant lack of fit to the model making it an acceptable model for analysis. The second screening design alleviated this issue by only using five factors and having NaCl as the sole confounded main effect. The addition of NaCl to this test was done in order to be able to remove it from the mineral media in future tests from a statistically relevant standpoint. It was not expected that NaCl would have any effect at the concentration that it was tested, as it was present in similar amounts in the buffer due to pH adjustment. This expectation allowed for the confounding of NaCl to not be of concern. The results of the second screening experiment are highlighted in Table 4.12.

Table 4.11. Results of 1/8 fraction (duplicate) design for first screening design (resolution III).

	Ethanol Concentration at 24 h			Adjusted Ethanol Yield at 24 h		
Factors	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**
P-4-Aminobenzoic Acid	1	0.054025	0.8214	1	1.219506	0.2981
Riboflavin	1	4.049653	0.075	1	0.009571	0.9242
Thiamin*	1	12.98963	0.0057	1	4.799979	0.0562
Nicotinic Acid*	1	41.46537	0.0001	1	0.229233	0.6435
Ca Pantothenate*	1	19.97445	0.0016	1	3.491079	0.0945
Pyridoxine*	1	7.220664	0.0249	1	0.157283	0.7009
Model*	6	14.2923	0.0004	6	1.651109	0.2393
Error	9			9		
(Lack of fit)	1	2.21236	0.1752	1	0.008609	0.9284
(Pure Error)	8			8		
Total	15			15		

***Denotes significance at the α = 0.05 level for this factor**

Table 4.12. Results of two level 1/2 factorial design for second screening design (resolution V).

	Ethanol Concentration at 24 h			Glycerol Concentration at 24 h		
Factors	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**
Thiamin*	1	10.0937	0.0099	1	8.65168	0.0148
Calcium Pantothenate*	1	0.332113	0.5772	1	27.9623	0.0004
Pyridoxine	1	1.595056	0.2353	1	0.71667	0.417
Nicotinic Acid*	1	229.0093	<.0001	1	383.662	<.0001
NaCl	1	0.682012	0.4282	1	1.125667	0.3137
Model	5	48334244	<.0001	5	84.42366	<.0001
Error	10			10	0.031786	
Total	15			15		

***Denotes significance at the α = 0.05 level for this factor**

For the second vitamin screening experiment, there are three factors of significance ($\alpha = 0.05$) in the production of ethanol from glucose and reduction of glycerol. These components are thiamin ($P = 0.0099$ for ethanol), nicotinic acid ($P < 0.0001$ for ethanol production) and calcium pantothenate ($P = 0.0004$ for glycerol production). The negative t-value in the glycerol ANOVA for glycerol (not shown) indicates that the addition of calcium pantothenate to the media actually decreases the amount of glycerol produced, increasing ethanol production slightly, although not to a significant level in this experiment since the main effects of calcium pantothenate were not significant for ethanol production. From the results shown in Table 4.12, both NaCl and pyridoxine can be removed from the media with no significant effect ($\alpha = 0.05$).

Significantly less ethanol was produced in the second screening experiment (Table 4.6) when compared to the first screening design (Table 4.5), as can be seen looking at the control runs (runs 15 and 16 in the first screen and run 16 in the second screen). A possible explanation for this could be that one of the replications in the first experiment was not representative of the experiment and should have been thrown out. Runs 7 and 8 were the same treatment, but resulted in very different ethanol concentrations at 24 h. This could have been due to experimental error in the setup of the test or in contamination somewhere in the fermentation and analyzing process, or, most likely, a failed fermentation due to either a toxin on the glassware or culture issues.

Another explanation for the reduction in these yields from the first screening design to the second could be the fact that riboflavin was not included in the second screening experiment. The reduction in yields from the screening of components might be able to be explained by including riboflavin in the vitamin media as even though it did not have a significant effect on ethanol, it was one of the components that could have made pyridoxine significant due to its' interaction with thiamin in the confounding rules. The removal of riboflavin from the growth medium reduced all of the adjusted ethanol yields from around 89% of theoretical to less than 80%.

Further experimentation would be needed to determine if this is in fact a problem, or if there was another reason for this apparent decrease in yield.

Experiments using defined media for complex substrates have looked at the requirements of certain yeasts with respect to vitamins, but many times these studies are inhibited due to the presence of vitamins at low levels in the substrates (such as grape musts) (Ough et al., 1989). Calcium pantothenate is a precursor to coenzyme A (Taherzadeh et al., 1996), which is used to transfer acyl groups in metabolic processes (Berg et al., 2007). Coenzyme A combines with pyruvate to form acetyl-CoA, a precursor for many cell components as well as acetic acid. The reaction for the function of coenzyme A in yeast metabolism (*S. cerrivisiae*) is highlighted in equation 4.3 (Pronk et al., 1996).

$$pyruvate + coenzyme\ A + NAD^+ \rightarrow acetylCoA + NADH + H^+ + CO_2 \quad (4.3)$$

The addition of calcium pantothenate was found to reduce glycerol production (Table 4.12). This could possibly be due to an increased flux of cellular products, reducing the pyruvate concentration in the cell and decreasing the flux of carbon to dihydroxyacetone phosphate and therefore production of glycerol via that pathway (Berg et al., 2007). This phenomenon could also be partially explained by the concentration of calcium pantothenate in the media. Taherzadeh et al. (1996) found that 30 μg/L of calcium pantothenate was enough to generate a pantothenate deficient media that was found to be beneficial in their fermentations. Only 14.9 μg/L of calcium pantothenate were used in the current vitamin evaluation, less than half of what was found to be beneficial in the Taherzadeh et al. (1996) study. This analysis only shows that the addition of calcium pantothenate was significantly better than not including the compound in the fermentation media with respect to reduction of glycerol production. Even at much higher concentrations (20 μg/l to 40 μg/L), Slaughter and McKernan (1988) found that calcium

pantothenate did not affect the overall quantity of products produced, but did affect the distribution of these products.

Nicotinic acid is a precursor for nicotinamide adenine dinucleotide (NAD^+), which is used in oxidation/reduction processes throughout the cell, but most notably in the glycolysis pathway. It would make sense that by having this compound as part of the media, metabolic reactions would proceed at faster rates since more electron acceptors would be present (Berg et al., 2007). In *S. cerevisiae* it is known that the production of nicotinic acid involves oxygenation reactions, and therefore must be supplied during anaerobic growth conditions (Dijken et al., 1993). This aligns well with this experiment since the removal of this vitamin greatly reduced the efficiencies of the fermentations, as all runs with nicotinic acid converted glucose to ethanol several hours faster than runs without nicotinic acid (Tables 4.5 and 4.6).

Thiamin is a precursor for the coenzyme thiamin pyrophosphate and is used in aldehyde transfer in metabolic processes (Berg et al., 2007). Although many types of yeast, such as *S. cerevisiae*, can produce thiamin, the process is energy intensive and in the presence of thiamin cells will uptake it and store it rather than produce their own (Hohmann and Meacock, 1998). In fact, the elevated uptake of thiamin in contaminated (or mixed) fermentations can lead to stuck or sluggish fermentations because of the lack of thiamin available for other metabolic processes (Bataillon et al., 1996).

As a result of this study, it was decided to employ only the three vitamins proving to be statistically significant in further experimentation.

4.3.6 Zinc

Zinc is a metal that is found in many enzymes that are important to fermentation pathways in yeast cells (De Nicola et al., 2007; Magonet et al., 1992; Satyanarayana et al., 2009). Theoretically, zinc limited growth could be ascertained by removing it from media, but trace

amounts are found in many alloys present in fermentations (such as tanks and glass jars) which prevents the complete removal of this element from the media in trace amounts (De Nicola et al., 2007).

There is a background for the addition of zinc to the fermentation broth. Sequencing research has identified that some of the alcohol dehydrogenase genes found in the *K. marxianus* genome are from the zinc containing family. Determination as to which AdH gene is expressed can be decided by the fermentation stage of the reaction as well as the substrate the yeast is being grown upon (Lertwattanasakul et al., 2007). It is thought that the zinc atom adds to the structural stability of the alcohol dehydrogenases in the yeast (Magonet et al., 1992). In brewing applications, the supplementation of zinc has been done for many years to overcome sluggish fermentations. The range of supplementation varies in literature, but 0.05 to 0.30 ppm covers this range; although, the actual amount is both strain dependent and dependent on the substrate being used (Satyanarayana et al., 2009).

Zhao et al. (1999) found that the addition of zinc to fermentation media for a self-flocculating yeast strain improved both the thermal and ethanol stability of the yeast. Zinc addition at 50 mg/L gave the best results for ethanol yields, but the addition of zinc at even the lowest concentration (10 mg/L) gave improved cell viability from heat shock up to over 82% compared to 60.2% in non-supplemented (control) media (Zhao et al., 2009).

For the zinc evaluation, the high value of zinc used was at 50 mg/L because that was what was seen in literature to give positive results (Xue et al., 2008; Xue et al., 2010; Zhao et al., 2009). The low value (0.5 mg/L) was used because it was close to the concentration already present due to the yeast extract and the amount observed to increase the rate of sluggish fermentations in brewing (Satyanarayana et al., 2009). The control bottles had no zinc added to them. Media also contained calcium pantothenate, thiamin, nicotinic acid (all at the level present in the vitamin

media (Table 4.5), $CaCl_2$ (100 mM), NH_4SO_4, $MgSO_4$, and KH_2PO_4 (all three at the levels in Table 4.1).

The average experimental data for this experiment is shown in Table 4.13. All values are averages of three preparations, except for the 0.5 mg/L value, which only had two replicates due to a glucose loading error. Although results of adding zinc resulted in higher biomass, ethanol, and total yields, none of these values were significantly different from the control (no zinc). The only significant difference ($p = 0.05$) observed at 24 h was the actual ethanol yield, which differed only between the 50 mg/L loading and the control (0 mg/L) loading. Actual ethanol yield did not differ significantly from the low level (0.5 mg/L), so Zn was used at this minimal concentration in further experiments.

Even without a statistically significant benefit in yields, the benefits of defined media, such as ease of use and composition, ability to scale up and ease of sterilization all have positive applications in this research. Further work will be done to optimize the concentrations found in this analysis.

Although others have reported zinc having a positive effect on glycerol inhibition in fermentations (Zhao et al., 2009), no significant effect was seen in this experiment. Possible issues with the lack of differences between the "no zinc" control and the added low concentration could be that some yeast (such as *S. cerevisiae*) cells accumulate zinc heavily during the initial stages of growth (De Nicola et al., 2007) and the flasks may be inoculated with cells that already have enough zinc for their biological processes, although the highest amount of zinc accumulation has been linked to anaerobic growth (Stehlik-Tomas et al., 2004), and these cells were growth aerobically. Since the media used to grow up cells for inoculation was very rich (10 g/L yeast extract), this is a distinct possibility as to why the differences were not more pronounced.

Table 4.13. Zinc addition experimental values at 24 h, different letters denote significant differences at p = 0.05 level.

Treatment	**Product at 24 h**					
Zinc Concentration (mg/L)	Cell Mass (g/L)	Ethanol (g/L)	Total Ethanol Yield (%)	Actual Ethanol Yield (%)	Glycerol (g/L)	Acetate (g/L)
50.0[a]	1.37[A]	15.82[B]	80.1%[C]	80.2%[D]	2.53[F]	0.68[G]
0.5[b]	1.47[A]	15.83[B]	78.8%[C]	78.8%[DE]	2.70[F]	0.72[G]
0.0[a]	1.29[A]	14.77[B]	74.4%[C]	77.9%[E]	2.63[F]	0.71[G]

a: n = 3
b: n = 2

4.3.7 Overall Discussion

The yields obtained in these experiments were comparable to those obtained in other studies using *K. marxianus* IMB 3 with undefined media. The first journal article bringing attention to the IMB isolates and their thermotolerant tendencies, Banat et al. (1992), identified a 0.826 g actual/g theoretical ethanol ratio for IMB3 at 45°C with glucose at a concentration of 140 g/L and 42 h of fermentation (Banat et al., 1992). The media used in this fermentation contained peptone (5.0 g/L), yeast extract (6.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), $(NH_4)_2SO_4$ (2.0 g/L) and KH_2PO_4 (4.0 g/L) (Banat et al., 1992). Another study using this same yeast strain and a modified media, peptone (2.0 g/l), yeast extract (3.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), $(NH_4)_2SO_4$ (2.0 g/L), $MnSO_4$ (0.1 g/L) and KH_2PO_4 (2.0 g/L), again in a batch flask at 45°C, showed ethanol a conversion efficiency of 100% (Banat et al., 1996). With fed batch experiments, Nabais et al. (1988) obtained a 0.735 g actual/g theoretical ethanol yield using 20 g/L glucose at 45°C with this strain after 25 h of fermentation.

4.4 Conclusions

A minimal defined media was constructed for *K. marxianus* IMB3. A defined media did improve the performance of the yeast fermentation over a medium with yeast extract and manganese. Further tests are necessary to optimize the actual composition of the eight components in the media. Table 4.14 outlines the defined media, the only trial implementation of which was done in the low concentration zinc experiment.

Table 4.14. Defined minimal media for ethanol production by *K. marxianus* IMB3.

Component	Amount
KH_2PO_4	2.0556 g/L
$(NH_4)_2SO_4$	2.257 g/L
$MgSO_4 \cdot 7H_2O$	1.0038 g/L
$CaCl_2$	1 mM
Thiamin	0.0265 mg/L
Calcium Pantothenate	0.0149 mg/L
Nicotinic Acid	0.0299 mg/L
$ZnSO_4$	0.5 mg/L

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CHAPTER V

OPTIMIZATION OF A DEFINED MINIMAL MEDIA FOR ETHANOL PRODUCTION BY *K. MARXIANUS* IMB3

5.1 Introduction

The previous media for production of ethanol by *K. marxianus* IMB3 for ethanol production has been stated (Banat et al., 1992; Banat et al., 1996), but not without the use of undefined media components such as yeast extract or with optimization of other parameters, such as temperature, pH, and shaker speed. The previous chapter identifies a statistically meaningful starting point to optimize such a chemically defined medium for optimization. No optimization of a designed media for *K. marxianus* IMB3 has been recorded in the literature. Optimization of both fermentation parameters with media constraints and media optimization alone have been completed in other SSF reactions with increased activity. In this study the defined media identified in the previous study will be optimized using response surface methodology techniques in SAS v. 9.2 Design of Experiments, and the media will be tested in an SSF to evaluate its ability to translate into that application.

5.2 Materials and Methods

5.2.1 Strain

Cells of *K. marxianus* IMB3 were generously given by Dr. I.M. Banat from the University of Ulster and were maintained on yeast-peptone-dextrose (YPD - 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose (dextrose)) agar, with sub-culturing occurring every three months to

prevent contamination. A starter flask was grown aerobically using a 250 ml KIMAX® baffled culture flask (Kimble Chase, Vineland, NJ) and BugStopper® venting closures (Whatman, Inc., Piscataway, NJ) at 45°C and 250 rpm on an orbital shaker incubator from a loopful of cells taken from the stored stock culture slant a week prior to the experiment. The starter flask was stored at 4°C until needed (<1 week). Cells used to inoculate the experiment were grown for 16 h prior to inoculation of the fermentation flasks aerobically at 45°C and 250 rpm on an orbital shaker incubator in 100 ml of YPD medium using a 5% (v/v) inoculation from the starter flask. All inoculations and sampling occurred in a biosafety cabinet under aseptic conditions.

5.2.2 Substrate preparation

There were two distinct substrates that were tested in these experiments. All optimization experiments and initial tests were conducted with glucose (40 g/l) to facilitate throughput of experiments. Results were then tested with hydrothermolysis pretreated switchgrass at a loading of 36 g/L glucan.

Kanlow switchgrass (*Panicum virgatum* var. Kanlow) was obtained from the Oklahoma State University Plant Sciences Research Farm, from the November 2008 harvest. This switchgrass was milled to ~ 2.0 mm using a Thomas-Wiley mill and attached 2.0 mm screen (Model 4, Arthur H Thomas Co., Philadelphia, PA). Milled switchgrass was then pretreated using the hydrothermolysis method. In this method, 540 g of deionized water was added to 60 g (dry weight) milled Kanlow switchgrass in the 1 L steel reactor vessel of a Parr pressure reactor (Parr Series 4520, Parr Instrument Company, Moline, IL). The reactor vessel was sealed completely and heated to 200°C. The heating ramp time took about 34 min. The 200°C temperature was held for 10 min (Suryawati et al., 2009), and then the reactor vessel was removed from the heating coils and immediately placed into an ice bath where the sealed reactor vessel was cooled to less than 30°C (Faga, 2009). Once cooled, the contents were emptied into a VWR ceramic

Buchner filter (89038-130, VWR International, Radnor, PA) lined with Whatman #5 filter paper (Whatman, Inc., Piscataway, NJ) and vacuum filtered. After removing the prehydrolyzate fraction of the pretreatment liquid, the solids were rinsed 5 times with 500 mL of deionized water heated to 60°C. The pH of the last rinse volume was measured to ensure the pH was greater than 4 to ensure removal of most fermentation inhibitors from the pretreatment. After the last rinse, samples were stored in Ziploc® bags at 4°C until use. This pretreatment procedure was repeated 8 times to ensure enough biomass was prepared for SSF reactions.

A sample of biomass (~10 g) was dried to a moisture content of less than 5% in a vacuum drying oven at 40°C. Samples of both vacuum dried and pretreated only biomass were evaluated for moisture content using a drying oven at 105°C (Sluiter et al., 2005). Compositional analysis was completed on the dried, pretreated switchgrass according to the National Renewable Energy Laboratory (NREL) procedure for compositional analysis of biomass (Faga, 2009; Sluiter et al., 2004) to determine the sugar content of the pretreated biomass. Sugar content (in terms of glucan and xylan since this process hydrolyzed all polymeric sugars to monomeric glucose and xylose), was measured by filtering neutralized samples (using 0.45 μm nylon syringe filters) into HPLC vials and then running these samples on an Aminex HPX-87P column (BioRad, Hercules, CA) installed in a HPLC with autosampler and refractive index detector (RID) (1100 Series, Agilent, Santa Clara, CA) using deionized water as the mobile phase. The physical parameters of the HPLC sugar analysis were 85°C and a flow rate of 0.6 mL per minute. Run time of the analysis was 35 min. The sugars determined were cellobiose, glucose, xylose, mannose, galactose and arabinose. Five different dilutions of external standards were run with the neutralized acid hydrolyzed samples in order to obtain a standard curve, which was used in the quantification of the hydrolyzed samples. All quantifications used peak area as the measured response.

Typical composition (on a dry mass basis) of the switchgrass after pretreatment consisted of 55.5 - 59.8% glucan, 3.8 - 4.0% xylan and 32.3 - 32.5% Klasson lignin. This is comparable to other studies using the same type of switchgrass through a similar hydrothermolysis pretreatment. Faga et al. (2010) found there to be 53.2% glucan, 2.6% xylan, and 33.8% lignin after washing. Suryawati et al. (2009) found there to be 56.6% glucan and 2.4% xylan left in the pretreated solids after rinsing.

5.2.3 Enzymes

Two different enzymes were used in the evaluation of the optimized media in the SSF with hydrothermolysis pretreated switchgrass. Cellic® CTec2 cellulase enzyme was obtained from Novozymes Inc. (Bagsvaerd, Denmark). Cellic® CTec2 is a concentrated blend of aggressive cellulases paired with a high level of β-glucosidases as well as hemicellulases to facilitate cellulose conversion. Cellic® CTec2 was used at the highest recommended industrial dosage of 6.0 % (w/w) enzyme/cellulose (glucan) (Novozymes®, 2011). The enzyme was diluted in deionized water 1/4 (v/v) to facilitate addition by pipette.

Accellerase 1500 was obtained from Genencor, Inc. (Danisco Inc., Copenhagen, Denmark) and was also used in separate fermentations to evaluate the ability of this enzyme to degrade the pretreated switchgrass in thermotolerant SSF conditions. This enzyme was loaded at the highest recommended dosage of 0.5 ml enzyme/g cellulose (Genencor®, 2009).

5.2.4 Statistical Optimization

The media components that were optimized in this experiment were identified through a preliminary screen, as discussed in Chapter 4. These components are outlined with their starting concentrations in Table 4.14. The largest blocked design with the design of experiments interface in SAS v. 9.2 only allowed for a maximum of seven compounds that could be optimized at once, so zinc was left out due to the minimal effect it had on the fermentation and it was added at a

known concentration of 0.5 mg/L ($ZnSO_4$), as this was the minimal amount found to increase fermentation yield in the media through initial screening. The first design used was an orthogonal central composite design with 9 blocks and 7 factors, resulting in 90 runs, the first 8 blocks contained 8 runs each with 2 runs being of the center point. The last run contained 4 replications of the central point. The center point was defined as the point at which all concentrations of components were equal to the initial concentrations, shown in Table 4.14. A table of this design is presented in Appendix 1.

For the response surface methodology using SAS vs. 9.0 Design of Experiments Interface, the numerical values for the experiment were found to be: -2.82843, -1, 0, 1, 2.82843. It was decided to use a linear scale in assigning these values to concentrations of chemicals. 2.82843 (the highest value) was set to 2x the initial concentration, 0 was set to the initial concentration, -2.82843 (the lowest value) was set to 0 (no compound added). The coded variables 1 and -1 were algebraically determined to be 1.35x and 0.65x the initial concentrations. In lieu of making up 4 different solutions for each compound, the volume of the sterile stock solution (20x the initial concentration in the media for magnesium sulfate, ammonia sulfate and potassium phosphate, 100x the initial concentration in the media for thiamin, nicotinic acid, calcium pantothenate, zinc sulfate and calcium chloride) added was modified, not the solution itself. This meant that for the 20x solution, 0 mL, 3.25 mL, 5 mL, 6.75 mL, and 10 mL were used for levels -2.82843, -1, 0, 1, and 2.82843, respectively. For the 100x solution, 0 mL, 0.65 mL, 1 mL, 1.35 mL, and 2 mL were used for levels -2.82843, -1, 0, 1, and 2.82843, respectively.

This design was unwieldy in its size and a significant effect due to blocking, as well as a significant lack of fit term in any fitted model parameter, were observed, so it could not be used for further optimization. The design was still included in this chapter as it illustrates the difficulties in optimizing many components that may have significant interactions with one another. This led to the development of two smaller, more manageable, designs, one for vitamins

and one for macronutrients (i.e. nitrogen, potassium and magnesium). Each of these designs was optimized separately and used three factors and 20 runs in an orthogonal central composite design organized into 3 blocks with 6 replications of the center point. This design is shown in Table 5.1.

It was determined that the scale used in the initial optimization experiment was not varied enough in range to allow for optimal values of the $(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_4$. A second optimization was done using 20 runs to predict the best concentrations for these components. All other compounds were set to the initial concentrations and $CaCl_2$ was omitted from the media as it was found to not have any effect on 24 h ethanol yield. Sampling was done at initial inoculation (time = 0 h) and at 20 h since it was expected that the growth rates would be greatly increased due to the increased nitrogen concentrations and it was desired to sample before complete exhaustion of glucose in order to prevent possible consumption of ethanol by the organism or product evaporation. Blocks 1 and 2 for the experiment were completed at the same time, but due to shaker limitations, block 3 for each experimental design was completed directly following the completion of blocks 1 and 2.

For the response surface methodology, using SAS vs. 9.2 Design of Experiments Interface, the numerical coded values for the experiment were found to be: -1.68, -1, 0, 1, 1.68. It was decided to use an exponential scale in assigning these values to concentrations of chemicals in order to cover a larger range of values. The conversions to obtain this scale follow. The values were first shifted (+1) and then 5 was raised to the power of this value. This allowed the central point to be set to the initial concentration as used in all previous experiments. Therefore coded level -1 was set to 0.2x the initial concentration and level 1 be set to 5x the initial concentration. The conversion can be seen in the Table 5.2.

The media for the optimization experiments was constructed using sterile 20x solutions (5 mL of the concentrated stock into 100 mL total volume fermentation flasks for the initial concentration

Table 5.1. Coded values for vitamin and nutrient optimization experiments.

	Vitamin	Calcium Pantothenate	Nicotinic Acid	Thiamin
	Nutrient	$(NH_4)_2SO_4$	$MgSO_4 \cdot 7H_2O$	KH_2PO_4
Run #	Block	Coded Value		
1	1	-1[a]	-1	-1
2	1	-1	1	1
3	1	1[b]	-1	1
4	1	1	1	-1
5	1	0[c]	0	0
6	1	0	0	0
7	2	-1	-1	1
8	2	-1	1	-1
9	2	1	-1	-1
10	2	1	1	1
11	2	0	0	0
12	2	0	0	0
13	3	-1.68[d]	0	0
14	3	1.68[e]	0	0
15	3	0	-1.68	0
16	3	0	1.68	0
17	3	0	0	-1.68
18	3	0	0	1.68
19	3	0	0	0
20	3	0	0	0

a: 1x
b: 25x
c: 5x
d: 0.33x
e: 75x

Table 5.2. Scaling for nutrient addition of nitrogen, magnesium and potassium.

Set Level	+1 shift	=5^power
-1.68[a]	-0.68	0.33
-1[b]	0	1
0[c]	1	5
1[d]	2	25
1.68[e]	2.68	74.9

a: 0.33x
b: 1x
c: 5x
d: 25x
e: 75x

of component) for nitrogen, potassium and magnesium and 100x solutions for the vitamin components, as in the first screening design. The concentrations of each component in the fermentation media at each level is quantified in Tables 5.3 and 5.4 respectively.

5.2.5 Inoculation and Fermentation Conditions

For inoculation of the fermentation flasks, the method was the NREL procedure for SSF inoculation (Dowe and McMillan, 2001). Optical density (OD) of the cells was checked at 600 nm using a UV-Vis spectrophotometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA, USA) at 16 h. The sample was diluted between 0 and 0.5 OD to ensure the sample was within the linear growth curve of the organism. The amount of culture removed for inoculation followed the equation (Dowe and McMillan, 2001):

$$Volume = \frac{0.5\ OD \times 100\ mL}{OD\ of\ inoculation\ flask} \times number\ of\ experimental\ bottles$$

The volume was removed aseptically in a biosafety cabinet and spun down using a centrifuge for 10 min at 3,600 x g. The supernatant was decanted in the biosafety cabinet and then cells were resuspended in sterile deionized water. Cells were spun down a second time, supernatant was decanted, and cells were resuspended in sterile deionized water again. Once more the sample was spun down, supernatant was decanted and the cells were resuspended with enough sterile deionized water to result in 1 mL of inoculum having an OD of 50. The washing of the cells served to remove any trace nutrients from the initial rich media used to culture the cells. The concentrated, rinsed inoculum was then added (1 ml) to the fermentation flasks. The dilution of the cells into 100 ml of media resulted in an OD of 0.5, which equals a cell mass concentration of 0.14 g/L (Suryawati et al., 2009).

Sterile 250 ml flasks were filled with the designed sterilized media components for the experiment, resulting in a working volume of 100 ml. All glassware, citrate buffer, stoppers,

Table 5.3. Coded and real values for central composite design for nitrogen, potassium and magnesium.

	Actual Concentration in the Media (g/L)		
Set Level	**K_2HPO_4**	**$(NH_4)_2SO_4$**	**$MgSO_4 \cdot 7H_2O$**
-1.68[a]	0.137	0.151	0.067
-1[b]	0.411	0.451	0.201
0[c]	2.056	2.257	1.004
1[d]	10.278	11.285	5.019
1.68[e]	30.794	33.811	15.037

a: 0.33x
b: 1x
c: 5x
d: 25x
e: 75x

Table 5.4. Coded and real values for central composite design for vitamins.

	Actual Concentration in the Media (mg/L)		
Set Level	**Calcium Pantothenate**	**Thiamin**	**Nicotinic Acid**
-1.68[a]	0.032	0.057	0.064
-1[b]	0.060	0.106	0.120
0[c]	0.150	0.265	0.300
1[d]	0.375	0.663	0.750
1.68[e]	0.700	1.237	1.401

a: 0.33x
b: 1x
c: 5x
d: 25x
e: 75x

deionized water, and glassware were sterilized at 121°C, 200 kPa and held for 20 min in a steam autoclave (PRIMUS Sterilizer Corporation LLC, Omaha, NE). All other media components were filter sterilized using sterile 0.45 μm polyethersulfone (PES) filters (Corning Inc., Corning, NY). Sterile pipets were used to measure out the needed volume of constituents. All fermentations used glucose at a concentration of 40 g/L in the media and citrate buffer (pH 5.5) at a concentration of 50 mM. Each experiment consisted of three different treatments. Each treatment was done in triplicate, resulting in nine total bottles for each experiment, except for the statistically designed partial factorial screening experiments. Each screening experiment consisted of 16 flasks. All glassware, citrate buffer, stoppers, deionized water, and glassware were sterilized at 121°C, 200 kPa and held for 20 min in a steam autoclave (PRIMUS Sterilizer Corporation LLC, Omaha, NE). All other media components were filter sterilized using sterile 0.45 μm polyethersulfone (PES) filters (Corning Inc., Corning, NY). Sterile pipets were used to measure out the needed volume of constituents.

5.2.6 Glucose Run Fermentations:

Fermentations for statistical analysis used glucose at a concentration of 40 g/L in the media and citrate buffer (pH 5.5) at a concentration of 50 mM. In a biological system, some issues can come from variation in lag time, resulting in lower yields at the prescribed product analysis point. This was variable was minimized by warming media constituents prior to inoculation, but blocking issues remained. All experiments comparing media (optimized vs. original) were evaluated in triplicate, but statistically designed experiments were run exactly as designed, with replication built into the experimental design.

Once inoculated, experimental flasks were sampled then placed on an orbital shaker/incubator at 130 rpm, 45°C. Samples were taken at 20 or 24 h by transferring the flasks into a biosafety cabinet and aseptically transferring 1.5 ml of sample into 1.7 ml centrifuge tubes. Optical density

of each sample was measured as described previously and pH was recorded using a pH meter (ORION 310 pH meter, Thermo Electron Corporation, Beverly, MA, USA; VWR symphony probe, West Chester, PA, USA). Samples were immediately spun down and the supernatant was filtered through 0.45 μm nylon filters into HPLC vials and frozen until further analysis.

5.2.7 SSF Experiments:

For SSF experiments, 36 g/l of glucan was loaded into each flask using a balance to ensure adequate loading. The amount of water needed to ensure a 100 mL working volume was then added using a calibrated burette. Flasks were sealed with BugStopper® venting closures (Whatman, Inc., Piscataway, NJ) and autoclave crepe paper, held in place with autoclave tape. Sealed flasks were first weighed then sterilized at 121°C, 200 kPa for 20 min in a steam autoclave (PRIMUS Sterilizer Corporation LLC, Omaha, NE). Once cooled to room temperature, the flasks were weighed to quantify the amount of sterile deionized water to be added to the fermentations to replace any evaporated water. All other components (buffer, minerals, 1 M NaOH, vitamins) were filter sterilized as explained previously and added in a biosafety cabinet using aseptic techniques. Yeast cells were added from an aerobic fermentation flask as explained above. Enzyme was added lastly and a time 0 sample was taken immediately after the addition of the enzyme to ensure minimal saccharification would occur prior to sampling. Samples were taken at 0, 6, 24, 48, 72, 96, 120, 144 and 168 h in the biosafety cabinet. The pH of each sample was then recorded using a pH meter. Samples were immediately centrifuged and the supernatant was filtered through 0.45 μm nylon filters into HPLC vials and frozen until further analysis.

Analysis of solvent production and initial glucose was done by first thawing samples then vortexing the samples to ensure the contents were well mixed. Vials were then placed on an automatic sampler attached to a HPLC (Agilent 1100 series) and 20 μl of each sample was injected onto an Aminex HPX-87H column at 60°C. Peaks were identified using a refractive

index detector (RID). A set of five external standard solutions was prepared and run with each experiment in order to ensure correct quantification of chemicals. Initial samples were diluted with deionized water to minimize peak overlap from high initial concentrations of glucose. Sulfuric acid (0.05M) at a flow rate of 0.6 ml/min was used as the mobile phase in this analysis (Dowe and McMillan, 2001).

5.3 Results and Discussion

5.3.1 Optimization 1, 90 Runs

The results from the initial screen are shown in Appendix 1. This experiment with 90 runs did not allow for an optimization to take place as the constructed model could not be evaluated with any statistical certainty due to the significant lack of fit term. The reason for this significant lack of fit could be due to the inability of the statistical analysis program to fit response data with a cubic or higher interaction. This was justified by the use of the proposed model to maximize ethanol yield. The yield that was obtained using this iterative program was higher than the maximum theoretical yield, therefore solidifying the impropriety of this model for analysis. The use of this model with the lowest runs removed (due to an outlier probability of < 0.05) was also not helpful as this again generated unrealistic yield values and models with excessively low R^2 and adjusted-R^2 values (less than 0.30). The decision was made to split up the experiment into two separate experiments in order to facilitate timeliness of experimentation as well as changes made to the range of tested values to broaden the range of explored values.

5.3.2 Optimization 2: Minerals Only

The parameter being optimized in the second optimization study was actual ethanol yield. The reason this value was used is because it allowed for the independent blocking variable to be insignificant ($p > 0.05$). The "lack of fit" term was also not significant in the predictive model.

The limitations of using this variable for optimization is that the variable becomes independent of time; therefore, rate of experimental conversion of glucose to ethanol was not maximized.

Significant quadratic terms were observed for magnesium sulfate ($p = 0.0137$) and ammonia sulfate ($p = 0.0209$). Magnesium sulfate as a main effect was significant ($p = 0.0255$). There were no significant two-way interactions observed ($p < 0.05$) (Table 5.5).

Once an acceptable model was fit, this model was used in an iterative optimizing process to maximize the adjusted yield of ethanol. This optimization process (using ten points for each variable and the entire experimental space as the range of allowed values) yielded the "best" mineral media recipe as the one outlined in Table 5.6. The maximum actual ethanol yield was 83.6% according to the optimized values. The optimized model parameters are outlined in Table 5.5.

Table 5.6 outlines the composition of the optimized metal solution. This relationship between the chemicals being optimized is visually represented in Figure 5.1. Mid levels appeared to yield the lowest values, whereas high and low levels were points of maximum actual ethanol yield per mass of consumed glucose. The optimized metal media combination had both the nitrogen and potassium set to the lowest levels that were tested. The reasons for this could be that the low levels limited cell yield, therefore reducing growth-related products, as well as the amount of glucose going towards cells themselves. One problem with the optimized combination of metals was that the combination of KH_2PO_4 and $MgSO_4$ at the highest specified concentration of $MgSO_4$ lowered the pH of the reaction by ~0.5 pH units even with the buffer; the combination of these two chemicals effectively overpowered the buffering capacity of the citrate buffer. Therefore, the decision was made after the testing of the media on a glucose substrate to include another trial, pH adjusted optimized media. In these reactions 2 ml of filter sterilized 1 M NaOH was added to the fermentation vessels to offset the reduction in pH occurring due to this combination. It is not

Table 5.5. Results of optimization experiment for nitrogen, magnesium and potassium .

	Master Model			Predictive Model		
Source	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**
$(NH_4)_2SO_4$	1	0.01	0.93	1	0.01	0.92
$MgSO_4$	1	5.61	0.04	1	6.37	0.02
K_2HPO_4	1	0.03	0.87			
$(NH_4)_2SO_4$* $(NH_4)_2SO_4$[a]	1	5.31	0.04	1	6.71	0.02
$(NH_4)_2SO_4$* $MgSO_4$[b]	1	0.03	0.87			
$(NH_4)_2SO_4$* K_2HPO_4[c]	1	0.63	0.45			
$MgSO_4$* $MgSO_4$[d]	1	6.31	0.03	1	7.91	0.01
$MgSO_4$* K_2HPO_4[e]	1	1.18	0.30			
K_2HPO_4* K_2HPO_4[f]	1	1.34	0.27			
Model	9	2.29	0.11	4	4.95	0.01
(Linear)	3	1.88	0.20			
(Quadratic)	3	4.39	0.03			
(Cross Product)	3	0.61	0.62			
Error	10			15		
(Lack of fit)	5	1.37	0.37	4	0.50	0.73
(Pure Error)	5			11		
Total	19			19		

a: Quadratic term for ammonia sulfate
b: Interaction term between ammonia sulfate and magnesium sulfate
c: Interaction term between ammonia sulfate and potassium phosphate
d: Quadratic term for magnesium sulfate
e: Interaction term between magnesium sulfate and potassium phosphate
f: Quadratic term for potassium phosphate

Table 5.6. "Best" concentration of minerals in media for adjusted ethanol production.

Nutrient	Concentration
KH_2PO_4	**0.137 g /L**
$(NH_4)_2SO_4$	**0.150 g/L**
$MgSO_4 \cdot 7H_2O$	**15.037 g/L**

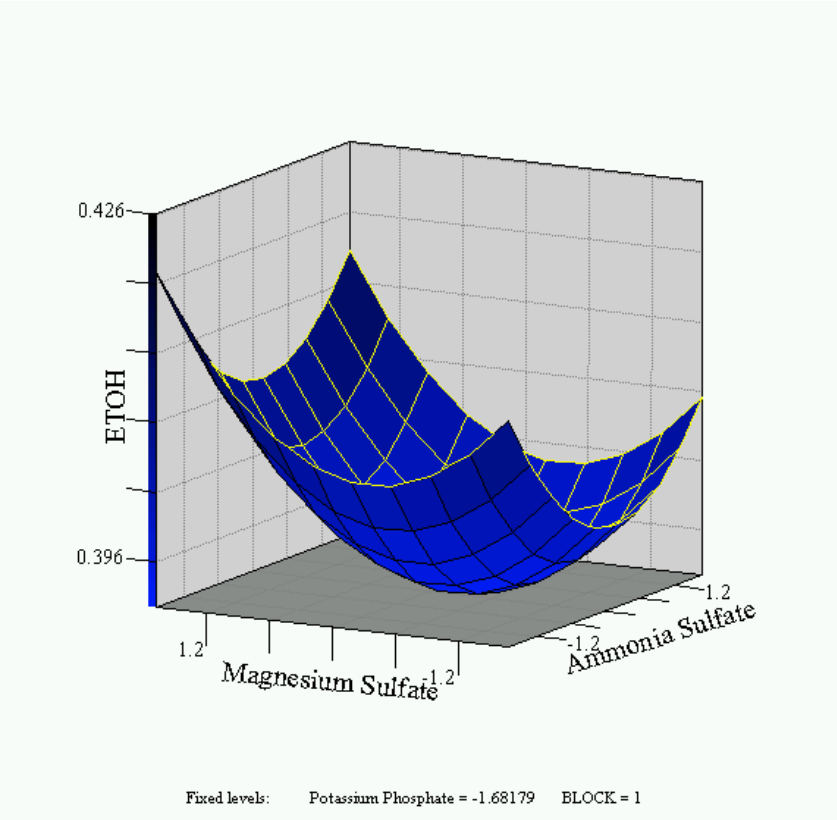

Figure 5.1. Response surface methodology for adjusted ethanol yield for metal experiment with KH_2PO_4 set to the lowest level, since it was not a significant part of the model.

completely unlikely that the drop in pH had an effect on the production of compounds, so a decrease in pH may be helpful in increasing the ethanol yield. Table 5.7 shows the range of starting pH values and the differences between final pH of these fermentations. The very low supply of nitrogen in the optimized media is growth limiting. Although limitation of chemicals in biological fermentations is usually avoided, it was decided to follow these statistical models to see if the end yield would be worth the reduction in initial biomass. If cell biomass is not being produced, it is likely that the extra substrate may go towards the production of ethanol, since no nitrogen is needed to produce this chemical (although trace amounts may be needed in enzymes).

5.3.3 Optimization 3: Vitamins Only

The identified "optimal" mineral solution from the previous section (Table 5.6) was used in the experiments looking at optimization of the three important vitamin components, calcium pantothenate, thiamin and nicotinic acid. Again, the parameter being optimized in the third experiment was actual ethanol yield. This allowed for the blocking effect to be insignificant in the analysis of the experiment, but mainly was used in order to have continuity of the optimization procedure as this was the only effect with insignificant blocking in the previous 20 run optimization for nitrogen, potassium and magnesium.

Three runs were found to be outliers in this statistical design when the studentized residual of each run when in the model was greater than 2 (runs 3, 16, 18). These runs were not considered for the analysis and optimization, so only 17 runs were used. All terms and two way (including quadratic) interactions were significant ($p < 0.05$), except for the quadratic thiamin*thiamin interaction and the Ca pantothenate*thiamin interaction. The resulting full and predicted model (non-significant terms removed) were both significant ($p < 0.05$), and a summary of the significant terms are shown in Table 5.8. The coded values for the optimization of the vitamin

Table 5.7. Cell mass and pH of optimization experiment for nitrogen, magnesium and potassium .

				Cell Mass (g/L)		pH	
Block	**$(NH_4)_2SO_4$**	**$MgSO_4$**	**KH_2PO_4**	**0 h**	**20h**	**0 h**	**20h**
1	-1	-1	-1	0.146	1.308	5.55	5.07
1	-1	1	1	0.140	2.523	5.04	4.63
1	1	-1	1	0.138	2.073	5.35	4.84
1	1	1	-1	0.134	1.759	5.13	4.65
1	0	0	0	0.138	2.166	5.44	4.83
1	0	0	0	0.147	1.861	5.45	4.85
2	-1	-1	1	0.117	1.714	5.44	4.96
2	-1	1	-1	0.115	2.122	5.08	4.6
2	1	-1	-1	0.115	1.288	5.45	4.95
2	1	1	1	0.110	2.261	5.03	4.6
2	0	0	0	0.114	1.813	5.42	4.89
2	0	0	0	0.114	1.926	5.42	4.89
3	-1.68	0	0	0.087	1.120	5.41	5.16
3	1.68	0	0	0.088	1.426	5.17	4.84
3	0	-1.68	0	0.090	1.433	5.46	4.99
3	0	1.68	0	0.087	2.046	4.53	4.31
3	0	0	-1.68	0.091	1.191	5.44	5.07
3	0	0	1.68	0.081	1.794	5.13	4.81
3	0	0	0	0.094	1.859	5.39	4.94
3	0	0	0	0.091	1.565	5.38	4.96

Table 5.8. Results of optimization experiment for calcium pantothenate, nicotinic acid and thiamin.

	Master Model			Predictive Model		
Source	**DF**	**F**	**Pr > F**	**DF**	**F**	**Pr > F**
BLOCK	2	0.944582	0.4488	2	0.785001	0.4925
Calcium Pantothenate	1	13.2501	0.0149	1	4.633347	0.0684
Nicotintic Acid	1	10.64406	0.0224	1	5.018491	0.0601
Thiamin	1	0.0893	0.7771	1	0.110237	0.7496
Calcium Pantothenate*Calcium Pantothenate	1	49.80091	0.0009	1	16.4492	0.0048
Calcium Pantothenate*Nicotinic Acid	1	7.405699	0.0417	1	2.435004	0.1626
Calcium Pantothenate*Thiamin	1	6.379113	0.0528			
Nicotinic Acid*Nicotinic Acid	1	8.58439	0.0326	1	4.279132	0.0774
Nicotinic Acid*Thiamin	1	30.14001	0.0027	1	12.98268	0.0087
Thiamin*Thiamin	1	1.77952	0.2397			
Model	11	13.41345	0.0051	9	5.10981	0.0214
Error	5			7		
(Lack of fit)	2	1.197146	0.4147	4	4.685369	0.1175
(Pure Error)	3			3		
Total	16			16		

experiment were 1.68 for calcium pantothenate, 1 for thiamin and -1.68 for nicotinic acid. The "real" values for these optimizations are shown in Table 5.9.

Figure 5.2 shows the relationship between the vitamins visually in a response surface graph. The maximum value of thiamin was not shown in the analysis as this point in the design was thrown out of the optimization as an outlier. Therefore the optimization could only be done within the range of values actually used in the space.

The fit model did not have a significant lack of fit term, and although the tested values fit well within the model the formulated "best" media gave a high optimized yield parameter, too high for it to make sense in the confines of the experiment as it was greater than any previous yield observed in the experiments.

5.3.4 Glucose test of Optimized Media vs. YFM

Cell mass of the two different compared media, one using the optimized concentrations of vitamin and minerals to replace yeast extract (Tables 5.6 and 5.9, referred to as the optimized medium (OM)), the other the original media used in *K. marxianus* IMB3 fermentations (Table 4.1, referred to as the yeast fermentation medium (YFM)) and the starting point for this optimization, are shown in Figure 5.3. The differences in cell concentration shown in Figure 5.3 are most probably due to the nitrogen limitation in the media itself. The maximum cell mass for OM (Figure 5.3) was similar to the values obtained in the last block of the second optimization experiment when the lowest value of nitrogen was supplied (Table 5.7). About 1/3 of the cell mass obtained in the YFM medium was seen in the OM medium. The time to consume all the supplied glucose was three times longer for the OM medium as compared to the YFM medium. This would imply that there are comparable rates of specific glucose uptake in both treatments on

Table 5.9. "Best" concentration of vitamins in medium for adjusted ethanol production.

Nutrient	Concentration
Calcium Pantothenate	0.6 mg/L
Nicotintic Acid	0.015 mg/L
Thiamin	0.43 mg/L

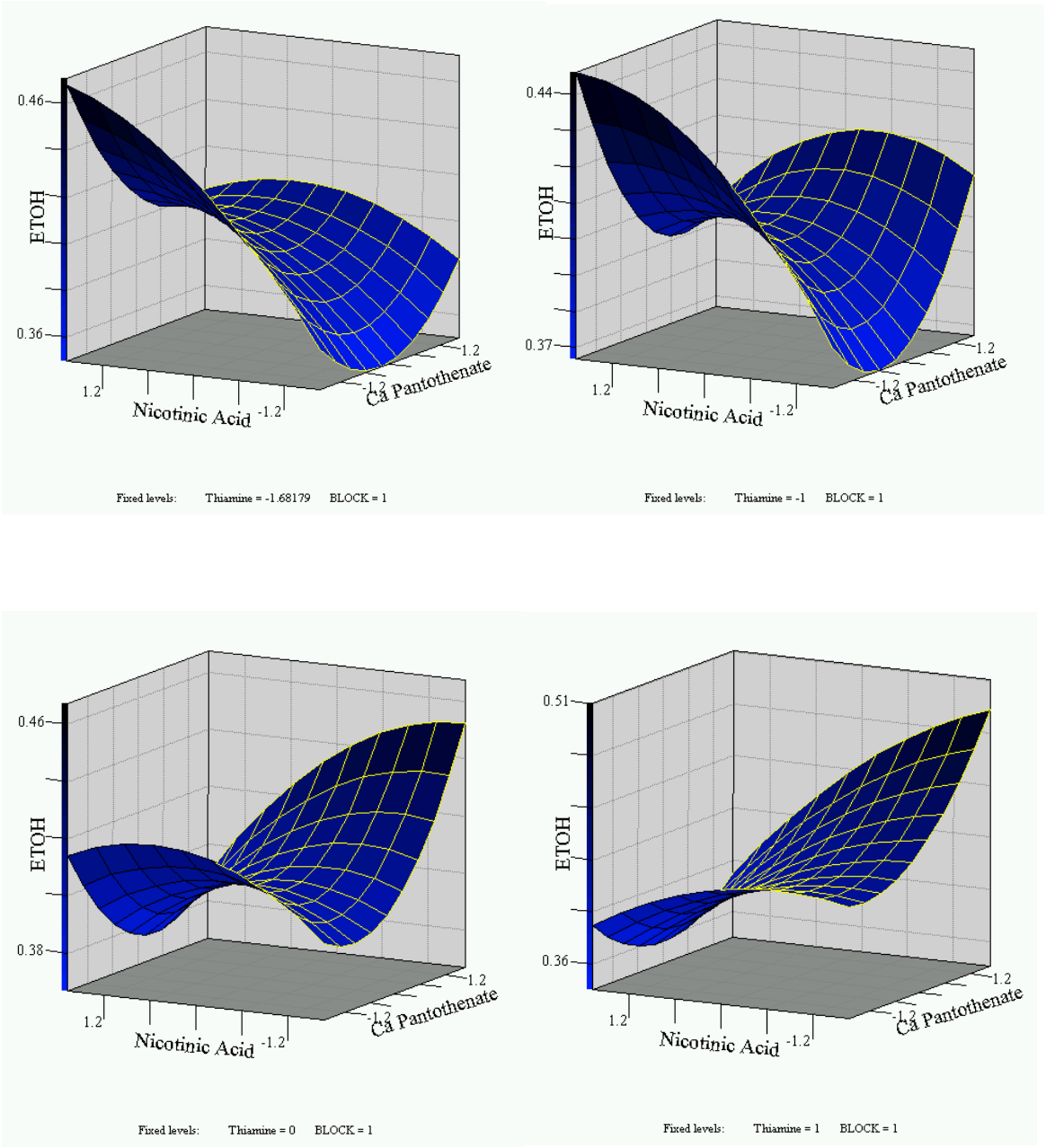

Figure 5.2. Response surface methodology for adjusted ethanol yield for vitamin experiment with thiamin set to all four levels tested, highest level (1.68 coded value, is not shown because this run was thrown out as an outlier).

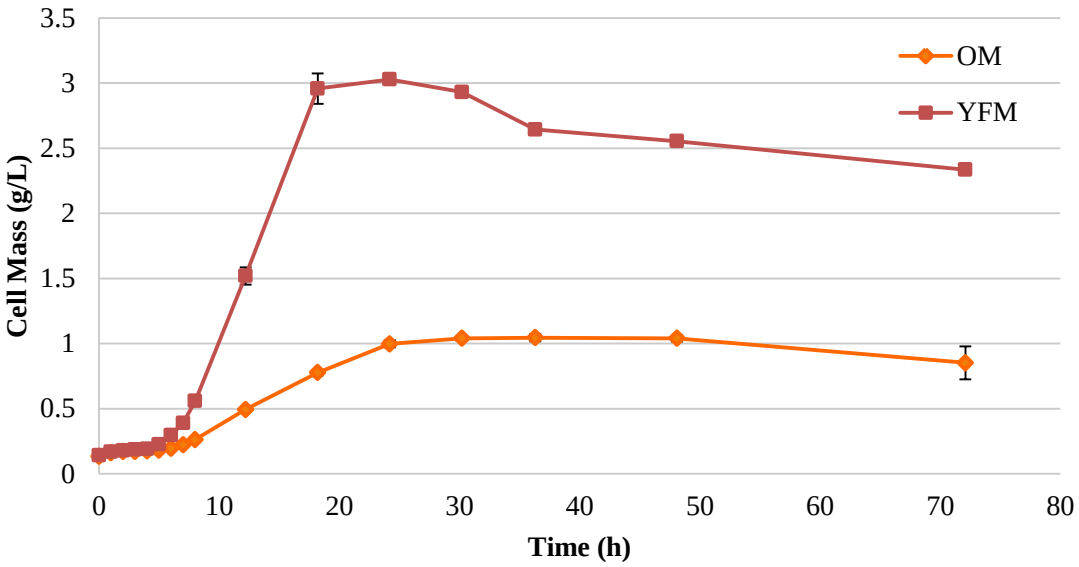

Figure 5.3. Concentration of cell mass in media for optimized synthetic media (OM) versus the original complex yeast fermentation media (YFM), 40 g/l glucose as the substrate.

a per cell mass basis; although, there appeared to be an initial lag phase in both fermentations for the first 6 h after inoculation. This could be explained by the temperature of the fermentation broth, as the reagents were stored at 4°C and were not warmed fully to 45°C or room temperature before inoculation.

Glucose consumption and ethanol production are shown in Figure 5.4. The glucose consumption rate was much slower than the glucose consumption observed in the flasks with the original media, conceivably this would be due to the lower amount of cell mass supported in the optimized medium. The rate of ethanol production was also much less in the optimized medium flasks than in the flasks containing YFM. The final concentration of ethanol produced in the optimized fermentation flasks was greater in than in the YFM, however this final concentration was reached after 72 h in the OM. Figure 5.5 further highlights this yield as it shows ethanol production as a fraction of potential ethanol production from glucose consumed. After 30 h, the adjusted ethanol yield in the optimized media flasks exceeded that in the original media. Figure 5.6 shows that glycerol was most certainly a growth associated fermentation product, as glycerol was only produced while biomass was being produced. Only about 1/3 of the glycerol produced in the YFM medium was produced in the optimized media flasks.

Although the fermentation rate was lower, the constant ethanol production in the optimized medium through 72 h showed that this medium could support fermenting organisms over a long period of time and the overall yields of the OM were greater than in the original media, due most probably to growth limitations.

5.3.5 SSF with Cellulase - CteC2

There was an obvious inability of the CteC2 enzyme to work well at the lower pH conditions (pH = 5.5 and lower) that are apparent in SSFs, even at the highest enzyme loading recommended by the manufacturer. The production of glucose by the enzyme and conversion of glucose to ethanol

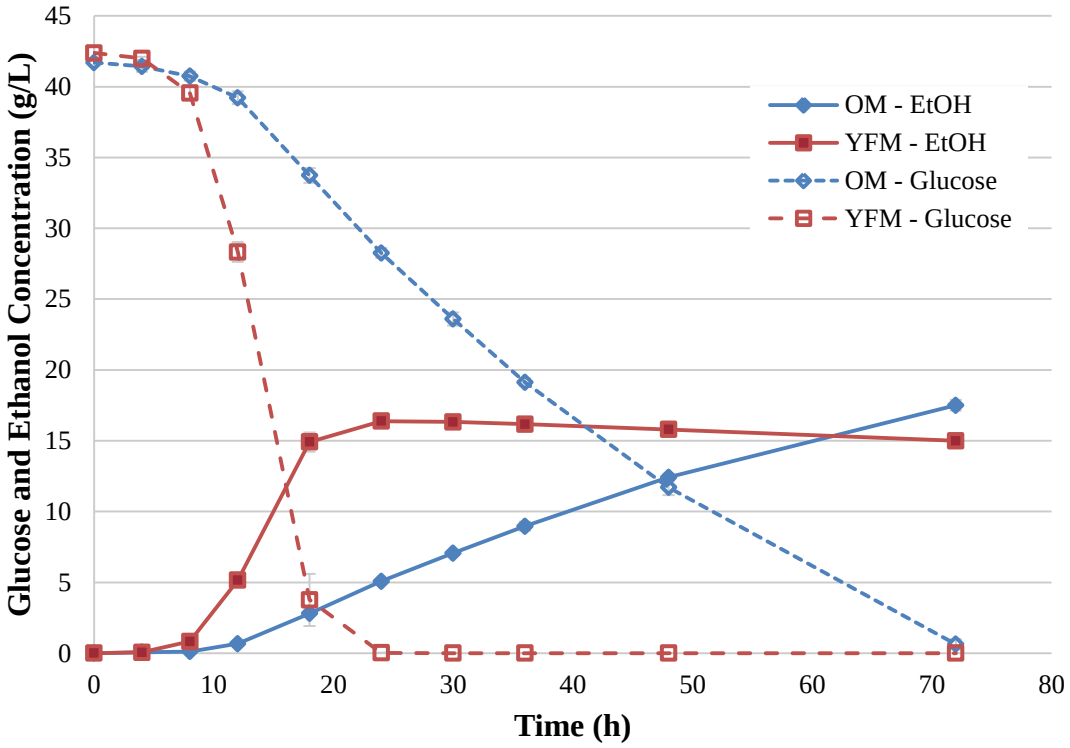

Figure 5.4. Glucose consumption and ethanol production of *K. marxianus* IMB compared over two substrates, the optimized medium (OM) and original yeast fermentation medium (YFM).

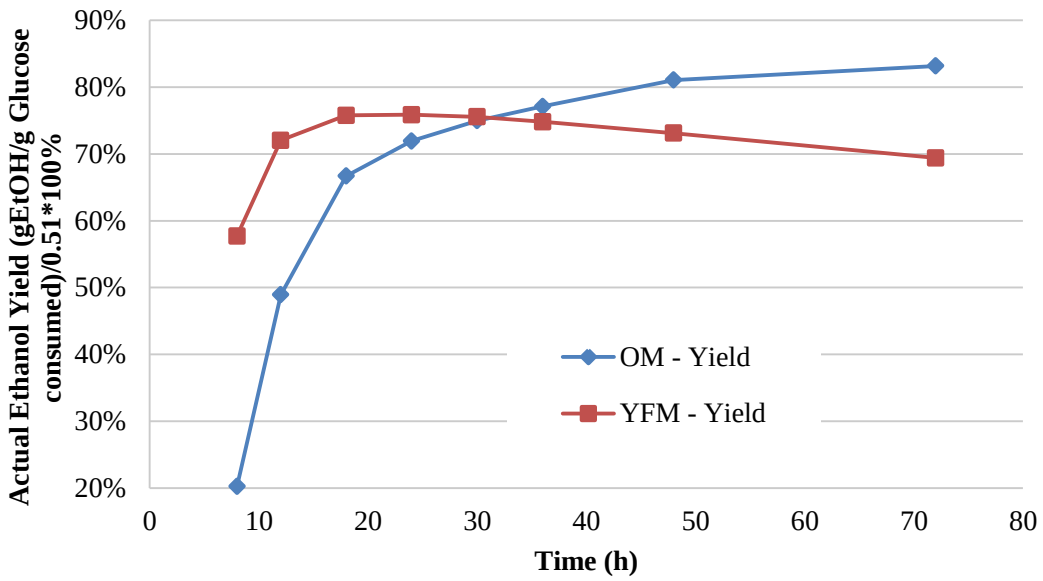

Figure 5.5. Actual ethanol yield of optimized media vs. original YFM media in glucose (40 g/l) fermentation.

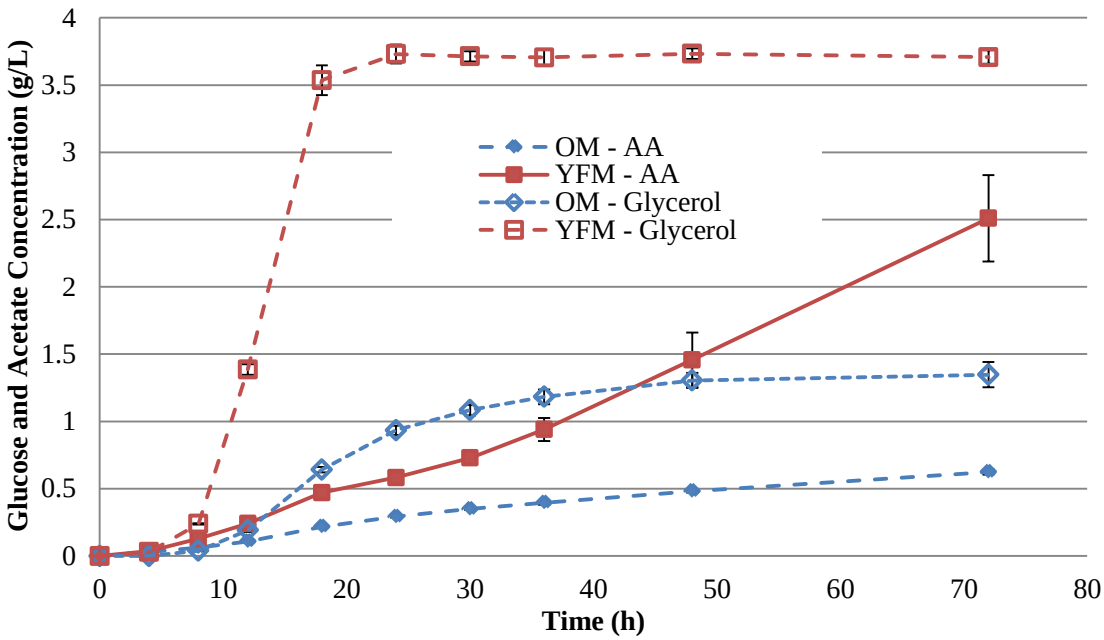

Figure 5.6. Acetic acid and glycerol production for *K. marxianus* IMB3 for optimized medium (OM) vs. original complex media (YFM) (40 g/l glucose substrate).

is shown in Figure 5.7 for this experiment. The optimized medium (represented in Tables 5.7 and 5.9) and optimized medium with addition of initial base for pH adjustment (to an initial pH of ~5.2) represent the same treatment, with pH being the only difference. The pH adjustment in the optimized medium resulted in much better yields when compared to no pH adjustment. Since *K. marxianus* IMB3 has no difficulty fermenting at a lower pH (unbuffered glucose to ethanol fermentation systems utilizing these yeast have final pH values close to 3.1), the inability of the fermentation to reach high yields (Figure 5.8) or to even reach a maximum concentration (Figure 5.7) (the ethanol values of the non-pH adjusted optimized media were increasing through the end of the experiment) was most likely due to enzymatic limitations.

5.3.6 SSF with Cellulase - Accellerase 1500

Ethanol production and glucose concentration for flasks using Accellerase 1500 as the commercial cellulase is shown in Figure 5.9. Maximum ethanol concentration appeared to occur at 72 h in the YFM SSF flasks (16.07 g/L). This was faster than in both the optimized treatment flasks, conceivably due to a slower growth rate of the yeast, as shown in the glucose fermentation with OM versus YFM. The slower production of ethanol in the flasks without pH adjustment appeared to be more due to a lowered enzyme activity, rather than yeast activity as there was no accumulation of glucose in the fermentation before 144 h. Maximum concentration of ethanol in the optimized media flasks occurred at around 120 h. Figure 5.10 shows the actual ethanol yield of the experiment and even though the adjusted yield is lower in the optimized media flasks, the adjusted ethanol yield was statistically the same for pH adjusted media when compared to the YFM after 72 h using the Tukey test for comparison of means ($\alpha = 0.05$).

The pH in the OM pH adjusted flasks was also slightly lower than the YFM media flasks as the calculated base addition was done without the addition of biomass to the flasks, so the amount of

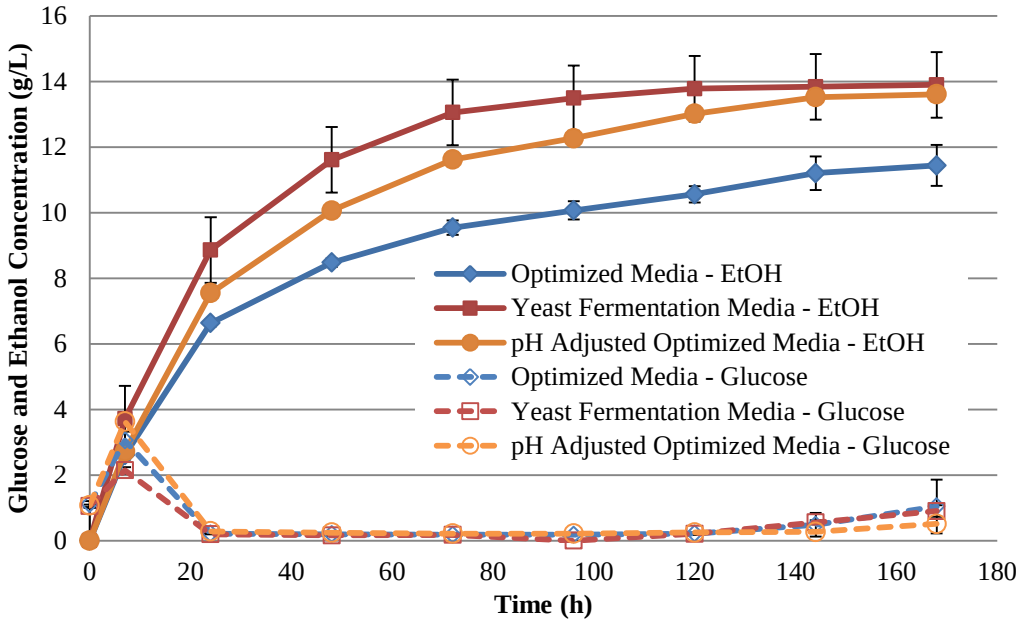

Figure 5.7. Glucose consumption and ethanol production of SSF with CteC2 as the supplied cellulase enzyme (0.06 g enzyme/g glucan, 36 g/L glucan, 45°C), pH denotes pH adjustment for the optimized media in that treatment.

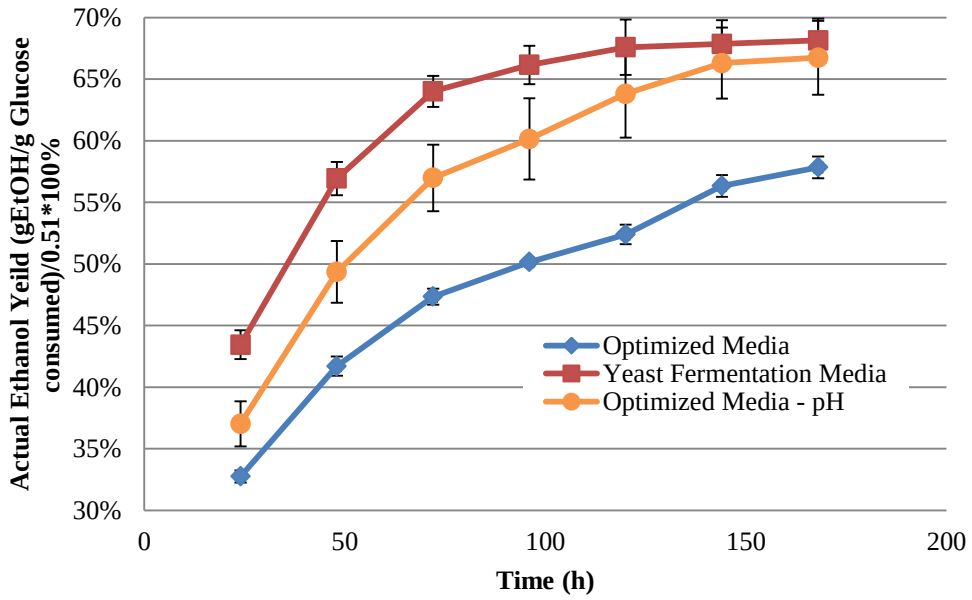

Figure 5.8. Actual ethanol yield of different medias in SSF with CteC2 (0.06 g enzyme/g glucan, 36 g/L glucan, 45°C), pH denotes pH adjustment for the optimized media in that treatment.

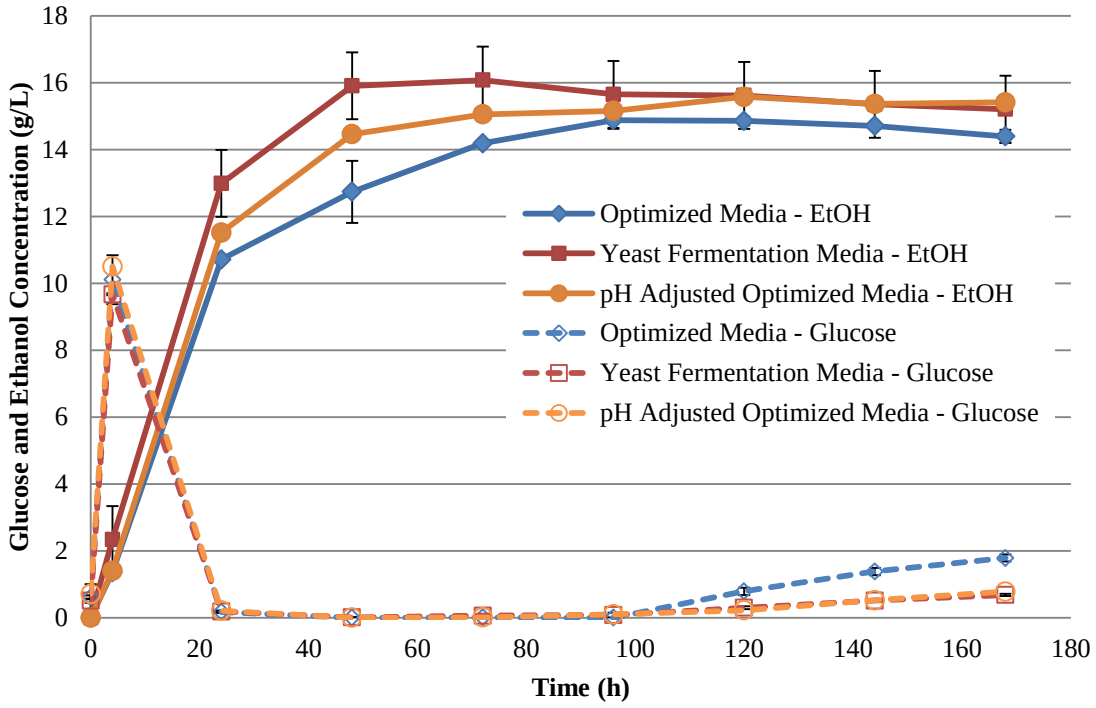

Figure 5.9. Glucose consumption and ethanol production for Accellerase 1500 SSF for media comparison (36 g/L glucan loading, 45°C). pH denotes pH adjustment for the optimized media in that treatment.

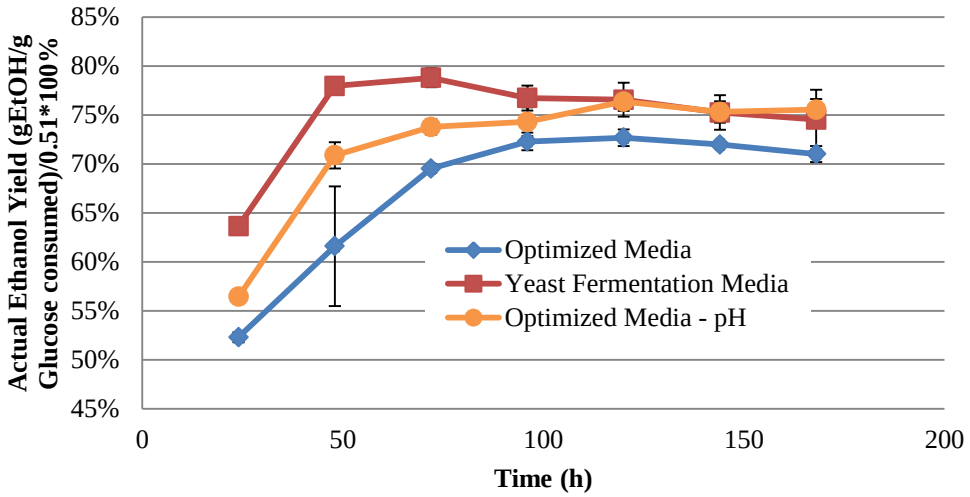

Figure 5.10. Actual ethanol yield for Accellerase 1500 enzyme (36 g/L glucan, 45°C), pH denotes pH adjustment for the optimized media in that treatment.

NaOH needed was underestimated due to the addition of pretreated biomass lowering the pH. Average starting pH for the pH adjusted flasks was 5.2; whereas, the YFM flasks had starting pH values around 5.4.

Figure 5.11 shows the by-product production of *K. marxianus* IMB3 in different media when using Accellerase 1500 as the cellulase in an SSF. Glycerol production was statistically not different for the pH adjusted flasks and the YFM flasks. The optimized medium without pH addition was lower in glycerol and acetic acid production than the other two treatments. This showed that glycerol production appeared to be linked closely to pH. This would make sense from a physiological standpoint as the production of acid would lower the pH increasing the disparity of internal and external pH of the cells. Glycerol is produced, as stated previously, to offset the acetic acid production in the cell. This disparity is thought to disrupt membrane stability, a main component of both temperature and ethanol tolerance (Piper, 1995), and membrane stability issues could result in lower overall yields.

Both the pH-adjusted optimized medium and optimized medium without pH adjustment showed a lower production of acetate than the YFM treatment. The optimized media without pH adjustment showed the lowest concentration of acetic acid. This could be due to the pH effectively turning off the acid production in the cell.

Faga et al. (2010) found that the maximum ethanol yield from hydrothermolysis pretreated switchgrass pretreated using the same conditions as this study (loaded at 42 g/L glucan) with *K. marxianus* IMB3 was 80.7% at 144 h. Although this value is slightly higher than the values obtained in these studies, the discrepancy could possibly be due to the higher solids loading. All other yields observed by Faga et al. (2010) for the IMB strains were less than 80%.

5.3.7 Cost analysis of media

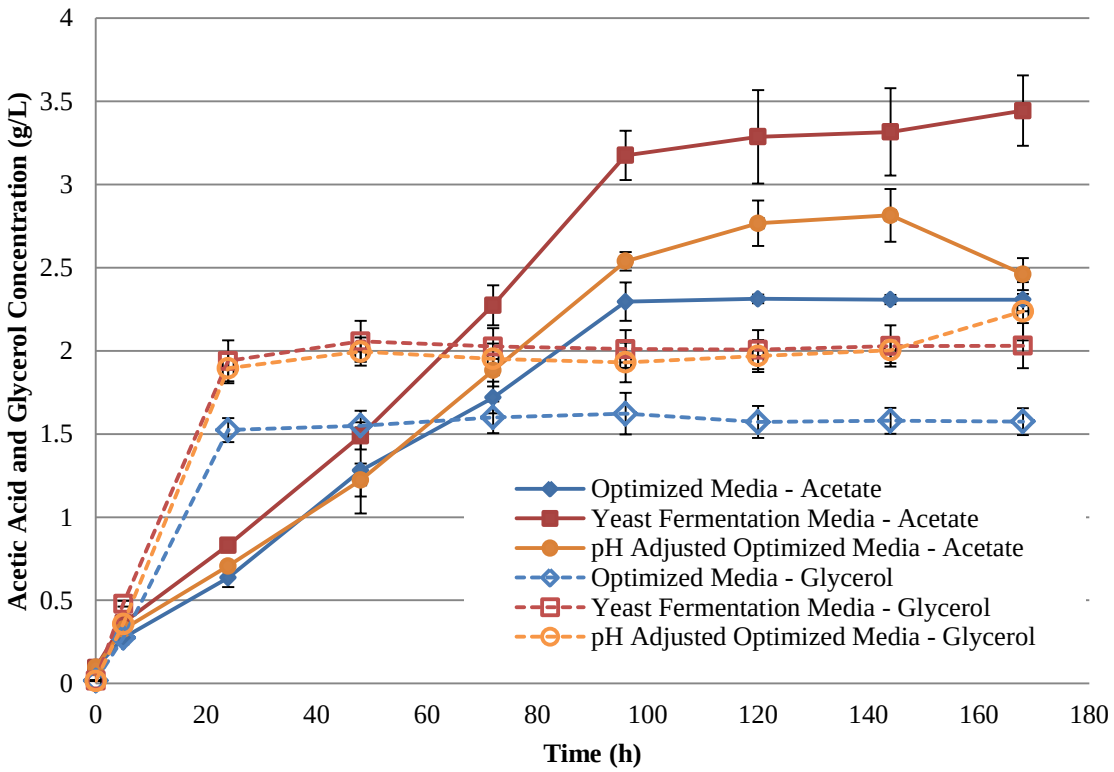

Figure 5.11. Acetic acid and glycerol production for Accellerase SSF (36 g/L glucan, 45°C).

A cost analysis of three media was done looking at the OM, the original YFM medium and the defined medium that was tested with a mineral and vitamin solution to replace the yeast extract in the YFM with a $CaCl_2$ supplement after screening of necessary components. These three media are summarized in Table 5.9. Table 5.10 summarizes the costs for each component of these media. The cost was almost twice as much on a per L basis due to the very high $MgSO_4$ addition to the media. This concentration would probably not be necessary looking at the optimization graph in Figure 5.1. This shows that the highest yields are at the highest and lowest concentrations of $MgSO_4$. Reducing the concentration of $MgSO_4$ to the lowest level given the model should only reduce the adjusted yield by about 2%, but will reduce the cost of the media to 3.7 cents per L versus 74.2 cents per L as the media stands optimized. The table for outlined values of media cost is shown in Table 5.9, with the cost analysis done using prices obtained in August 2011 from Sigma-Aldrich (St. Louis, MO). The cost could potentially be further reduced by the addition of a different nitrogen or potassium source.

Table 5.10. Compositional analysis of media for *K. marxianus* IMB3 fermentations. OM - optimized media, YFM - original yeast fermentation media, DM - defined media after screening experiments.

Component	YFM (mg/L)	OM (mg/L)	DM (mg/L)
$(NH_4)_2SO_4$	2000	150	2257
KH_2PO_4	2000	137	2056
$MgSO_4*7H_2O$	1000	15037	1004
Yeast Extract	500	0	0
$MnSO_4$	100	0	0
$CaCl_2$	111	111	111
Calcium Pantothenate	0	0.6	0.015
Nicotinic Acid	0	0.015	0.027
Thiamin	0	0.43	0.03
$ZnSO_4$	0	0.5	0.5

Table 5.11. Price analysis of media for *K. marxianus* IMB3 fermentations. OM - optimized media, YFM - original yeast fermentation media, DM - defined media after screening experiments.

Component	Price($) /Amount	Supplier	YFM ($/L)	OM ($/L)	DM ($/L)
$(NH_4)_2SO_4$	280.5/ 5 kg	Sigma Aldrich (product # P5655-1KG)	$ 0.11	$ 0.01	$ 0.13
KH_2PO_4	113.5/1 kg	Sigma Aldrich (product # A4418)	$ 0.23	$ 0.02	$ 0.23
$MgSO_4*7H_2O$	565/12 kg	Sigma Aldrich (product # 230391-12KG)	$ 0.05	$ 0.71	$ 0.05
Yeast Extract	197/ 5 kg	Sigma Aldrich (product # 09182-5KG-F)	$ 0.02	$ -	$ -
$MnSO_4$	66.90/ 1 kg	Sigma Aldrich (product # M7634-1KG)	$ 0.01	$ -	$ -
$CaCl_2$	81.50/ 1 kg - dihydrate	Sigma Aldrich (product # C7902-1KG)	$ 0.01	$ 0.01	$ 0.01
Calcium Pantothenate	201/ 500 g	Sigma Aldrich (product # P5155-500G)	$ -	$ <0.01	$ <0.01
Nicotinic Acid	59.80/500 g	Sigma Aldrich (product # N0761-500G)	$ -	$ <0.01	$ <0.01
Thiamin	79.70/100 g	Sigma Aldrich (product # T1270-100G)	$ -	$ <0.01	$ <0.01
$ZnSO_4$	75.90/500 g	Sigma Aldrich (product # Z0251-500G)	$ -	$ <0.01	$ <0.01
Total			$ 0.4217	$ 0.7417	$ 0.4164

August 2011 from Sigma-Aldrich (St.

5.4 Conclusions

The optimized media for the fermentation of hexose based substrates by *K. marxianus* IMB3 was successful in that a media was developed for glucose that worked better on a yield basis than the original media, although it worked much slower. The slow performance would be disadvantageous in an industrial setting as time is money. This optimized media did not perform any better in an SSF set up than the original media, and performed worse statistically than the original media without pH adjustment at the beginning of the fermentation. The media cost of the optimized media was higher than in the original YFM media, but could potentially have similar results with the adjustment of the $MgSO_4$ level to the lowest level with only a slight reduction in yield according to the optimization model for metals.

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CHAPTER VI

CELL IMMOBILIZATION IN SIMULTANEOUS SACCHARIFICATION FERMENTATIONS USING *K. MARXIANUS* IMB3

6.1 Introduction

In a SSF reaction, the only time that the cellular conversion of glucose to ethanol is the limiting step in the combined fermentation/saccharification is in the initial stages of cell growth (Philippidis and Smith, 1995). At all other times, saccharification of the biomass is the rate limiting step. One way to address this initial limitation is to introduce fully grown, immobilized cells into the reaction. With fully grown cells, the glucose needed to propagate new cells should be able to be used for ethanol production instead. Also, if some of the produced by-products are associated with redox balances in propagating cells (like glycerol), then they should not be made in large quantities if the cells are in the stationary phase, perhaps allowing for more ethanol to be produced. Since the optimized medium developed in Chapter 5 was growth limiting, it became of interest to see how fully grown cells would fare on this media.

One of the most researched and evaluated ways of immobilizing living cells is the use of calcium alginate beads for immobilization. Ca-alginate beads are bio-compatible and have the advantage of having very high mass transfer coefficients in aqueous solutions, as well as being physically

strong to endure the conditions in a bioreactor (agitation, flow through, etc) (Cheetham et al., 1979).

Conversion of cellulose to ethanol using *K. marxianus* IMB3 immobilized in calcium alginate has been evaluated previously with purified cellulose (Barron et al., 1996; Barron et al., 1996) and straw-supplemented whiskey distillery spent wash (Barron et al., 1997). These studies showed that immobilized cells had the ability to work well in such systems with a higher ethanol yield than comparable free cell systems. However, calcium alginate systems have encountered issues in their use with solid substrates, as both mass transfer considerations and issues with poisoning of the beads by unidentified inhibitory compounds from substrates accumulating on the beads themselves (Yamashita et al., 2008).

One of the issues with the optimized medium developed in the previous chapter is the high amount of magnesium present in the medium. Magnesium is a known "non-gelling" cation in alginate systems as are many monovalent cations (such as potassium and sodium) (Smidsrød and Skjak-Brk, 1990). Since the original optimized media first developed for *K. marxianus* IMB3 fermentations (as described previously) had a quite high concentration of $MgSO_4$, it would be important to look at the effect of this media on the stability of the beads. Buffers are another known destabilizing agent of calcium alginate beads (Bickerstaff and Fraser, 1997); thus, it becomes important to evaluate other methods of pH control in such experiments. In this chapter, the evaluation of immobilization of *K. marxianus* IMB3 in calcium alginate beads will be evaluated for the fermentation of switchgrass to ethanol.

6.2 Materials and Methods

6.2.1 Cell Culture and Maintenance

Cells of *K. marxianus* IMB3 were generously given by Dr. I.M. Banat from the University of Ulster and were maintained on yeast-peptone-dextrose (YPD - 10 g/L yeast extract, 20 g/L

peptone and 20 g/L glucose (dextrose)) agar, with sub-culturing occurring every three months to prevent contamination. A starter flask was grown aerobically using a 250 ml KIMAX® baffled culture flask (Kimble Chase, Vineland, NJ) and BugStopper® venting closures (Whatman, Inc., Piscataway, NJ) at 45°C and 250 rpm on an orbital shaker incubator from a loopful of cells taken from the stored stock culture slant a week prior to the experiment. The starter flask was stored at 4°C until needed (<1 week). Cells used to inoculate the experiment were grown for 16 h prior to inoculation of the fermentation flasks aerobically at 45°C and 250 rpm on an orbital shaker incubator in 100 ml of YPD medium using a 5% (v/v) inoculation from the starter flask. All inoculations and sampling occurred in a biosafety cabinet under aseptic conditions.

6.2.2 Immobilization of Cells in Calcium Alginate Beads

A 4% (w/v) solution of calcium alginate was prepared. Five flasks of *K. marxianus* IMB3 cells were grown for 18 h aerobically prior to inoculation in 100 ml of YPD media aerobically (using a 250 ml KIMAX® baffled culture flask (Kimble Chase, Vineland, NJ) and BugStopper® venting closures (Whatman, Inc., Piscataway, NJ) at 45°C and 250 rpm on an orbital shaker incubator (MaxQ 4450, Thermo Scientific, Waltham, MA). These flasks were inoculated from the starter flask described in Section 6.2.1 (5% v/v inoculation). After removal from the shaker/incubator, cells were combined in a sterile 1 L beaker in the biosafety cabinet to homogenize the cell density. A volume of cell culture (50 ml) was added to each of ten 60 ml tubes, which were then centrifuged at 3,600 x g with a centrifuge. Media was decanted and cells were resuspended in sterile deionized H_2O. Cells were again spun down and water was decanted. This rinsing happened a third time, but the Ca-alginate solution was added in lieu of deionized water after the last centrifugation. Cells were suspended into the Ca-alginate solution and then transferred into a 3 mL syringe where droplets of the suspended cell/alginate mixture were added to a mixing sterile 50 mM $CaCl_2$ solution as described in other studies (Bickerstaff and Fraser, 1997; Love et al., 1998; Smidsrød and Skjak-Brk, 1990). Beads had an average size of 3 mm. The loading of

yeast cells into the beads was 0.35 g wet cells/g 4% sodium alginate. About 5 g of beads were loaded into each fermentation vessel. After allowing the beads to harden in the $CaCl_2$ solution, beads were stored in a 5 mM $CaCl_2$, 200 mM acetate buffer (pH = 5.0) at 4°C until use in experiments. Cells were stored in this solution as previous literature had shown this to be an effective technique (Love et al., 1998). Cells were stored for less than a week for the first two experiments and stored for almost a month before the SSF was done. Beads were rinsed thoroughly and reused for each experiment.

6.2.3 Glucose Fermentation Conditions

Glucose fermentations were carried out as described previously in Chapters 4 and 5. Glucose was prepared as a 400 g/L solution and sterilized by filtration through sterile 0.45 μm polyethersulfone (PES) filters (Corning Inc., Corning, NY) in the biosafety cabinet and stored at 4°C until use. Ten ml of the sterile glucose solution was added aseptically to each of the fermentation flasks prior to the start of the experiments. This addition was done after aseptic addition of mineral components and concentrated buffer to each of the flasks. The concentration of these materials depended on what was being tested. Samples were taken at every 6 to 12 h starting at time = 0 h.

6.2.4 Reuse of Beads

After beads were used in fermentations, they were rinsed with 50 ml of deionized H_2O and stored in a mixture of $CaCl_2$ (5 mM) and acetate buffer (50 mM) at 4°C until needed for an experiment, which was 1 day between glucose experiments and 2 weeks before the SSF experiments. Beads were not used more than three times, and all fermentations were completed within 5 weeks. Since several sources report beads being used through several cycles of experimentation (Barron et al., 1996), this was not expected to pose a problem. Repeat performance was not compared.

6.2.5 Media Preparation

All media was prepared and filtered through sterile 0.45 μm polyethersulfone (PES) filters (Corning Inc., Corning, NY), except for the two buffer solutions which were sterilized at 121°C, 200 kPa and held for 20 min in a steam autoclave (PRIMUS Sterilizer Corporation LLC, Omaha, NE). For immobilization experiments it was decided to use acetate buffer at the same concentration (50 mM) and the same pH as used in the measurement of enzymatic assays (pH = 5.0). Acetate buffer (1 M) was prepared using glacial acetic acid (5 N) and sodium acetate solution (5 N) so that the diluted (50 mM) solution would have a pH of 5.0. Citric acid buffer (1 M) was prepared using citric acid and pH was adjusted (using 2N NaOH) so that the diluted (50 mM) solution would have a pH of 5.5.

6.2.6 Glucose Experiments

Three experiments were done using a glucose substrate; media comparison (immobilized cells), buffer comparison (free cells) and elevated temperature studies (immobilized cells).

6.2.6.1 Media comparison experiment

The media comparison experiment used nine flasks total, composing of three treatments (one representing a different nutrient media) done in triplicate. The compositions of the three media are highlighted in Table 6.1. All three media were used in the media comparison experiment. All fermentations were run at 45°C using whichever medium specified supplemented with 1 mM of $CaCl_2$ added (to help stabilize the beads) in shaker incubators at 130 rpm.

6.2.6.2 Temperature experiment

The temperature study used yeast fermention media (YFM) in both treatments, but compared two elevated temperatures (48°C and 50°C). 45°C was not included as this had already been done in the previous media comparison experiment. This experiment was an attempt to see if immobilization of the cells could help with their thermal stability. Running an SSF at an elevated

Table 6.1. Table of media compositions used in experiments, all component solutions were prepared, sterilized and stored (at 4°C) until use.

	Yeast Fermentation Media (YFM)	Optimized Media (OM)	Defined Media (DM)
Component	Amount (mg/L)	Amount (mg/L)	Amount (mg/L)
$(NH_4)_2SO_4$	2000	150	2257
KH_2PO_4	2000	137	2056
$MgSO_4*7H_2O$	1000	15037	1004
Yeast Extract	500	0	0
$MnSO_4$	100	0	0
$CaCl_2$	110.984	110.984	110.984
Calcium Pantothenate	0	0.6	0.015
Nicotinic Acid	0	0.015	0.027
Thiamin	0	0.43	0.03
$ZnSO_4$	0	0.5	0.5

temperature would be advantageous as the optimal temperature for enzymatic activity is around 50°C. The 48° and 50° C conditions were run in separate incubators, both rotating at 130 rpm. All treatments were done in triplicate.

6.2.6.3 Buffer comparison experiment

The buffer comparison study had three control flasks using a citrate buffer and three flasks that contained an acetate buffer instead of the citrate buffer. The product characteristics of the two fermentations were compared. The buffers were prepared as described in section 6.2.5. All fermentations were run at 45°C using YFM supplemented with 1 mM of $CaCl_2$ added to help stabilize the beads in shaker incubators at 130 rpm. This $CaCl_2$ was included in the buffer comparison experiment with free cells, as well as immobilized cells, in order to allow for the direct comparison of the results. All treatments were done in triplicate.

6.2.7 SSF Reactions

Biomass was prepared as described previously in section 5.2.2, with slight modifications to the process described in Faga et al. (2010). Biomass was loaded at a rate of 36 g/l glucan, DI water was added to each fermentation so that it would amount to a total mass of 100 g in the fermentation flask after the post sterilization addition of buffer, minerals and vitamins. The flasks with substrate and water were then autoclaved. All other constituents were added aseptically after autoclaving in a biosafety cabinet from sterile stock solutions. Samples were taken at 0, 6, 24, 48, 72, 96, 120, 144 and 168 h.

In all SSF reactions with immobilized cells, Accellerase 1500 (Danisco Inc., Rochester, New York) was used at the highest recommended dosage of 0.5 ml enzyme/g cellulose (Genencor®, 2009) in separate fermentations to evaluate the ability of this enzyme to degrade the pretreated switchgrass in thermotolerant SSF conditions. Two of the SSF treatments were run at 45°C in shaker incubators at 130 rpm (one with immobilized cells and one with free cells), and the third

treatment was run at 50° C with immobilized cells in a separate incubator/shaker, still agitating the flasks at 130 rpm. All treatments were done in triplicate.

6.2.8 Measurements and Analysis

Optical density (OD) of culture was measured at 600 nm using a UV-Vis spectrophotometer (Cary 50 Bio, Varian, Inc., Palo Alto, CA, USA). The sample was diluted between 0 and 0.5 OD to ensure the sample was within the linear growth curve (OD vs. cell mass concentration) of the organism. pH was recorded using a pH meter (ORION 310 pH meter, Thermo Electron Corporation, Beverly, MA, USA; VWR symphony probe, West Chester, PA, USA). Directly following pH and OD measurements, samples were immediately centrifuged and the supernatant was filtered through 0.45 μm nylon syringe filters into HPLC vials. The vials were labeled and frozen at -20°C until further analysis.

HPLC analysis was done by first thawing samples then mixing the samples using a vortexer. Using an auto sampler, 20 μl of each sample was injected onto an Aminex HPX-87H column at 60°C in a HPLC with a refractive index detector (RID) (1100 series, Agilent, Santa Clara, CA). A set of five external standards was prepared (using serial dilutions) and run with each experiment in order to ensure correct quantification of chemicals. Initial glucose experiment samples were diluted 1:1 (v:v) with deionized water to minimize peak overlap from high initial concentrations of glucose. Sulfuric acid (0.05M) at a flow rate of 0.6 ml/min was used as the mobile phase in this analysis (Dowe and McMillan, 2001).

All statistical comparisons between ethanol yields and product comparisons were done using SAS vs. 9.2, Means statement with Post-Hoc comparison done by the Tukey method.

6.3 Results and Discussion

6.3.1 Media Comparison Experiment

Glucose consumption and ethanol production are highlighted in Figure 6.1. No difference in ethanol yield was found to be statistically significant in any time period using a Tukey test for mean comparison of the adjusted experimental yields ($p<0.05$). The YFM media showed the fastest consumption of glucose and production of ethanol (all glucose was consumed within 12 h, whereas glucose in the other two tested media were consumed in under 18 h), so YFM was used in all the following experimental designs. Figure 6.2 shows that there was no statistical difference between the media tested for actual ethanol yield.

This phenomena of minimal differences between immobilized cells used with different media is not novel as Cross et al. (1993) found very little difference in ethanol yields using a defined medium versus a complex medium on glucose using immobilized *Z. mobilis* in a continuous flow bioreactor. Average total ethanol yields were between 86% and 89% of theoretical ethanol yield for the experiment with *Z. mobilis*.

6.3.2 Buffer Comparison Experiment

The comparison of two buffers was done due to the incompatibility of the citrate buffer used in all previous experiments with calcium alginate beads. In the comparison of the two buffers, acetate buffered flasks showed better ethanol production than those flasks using a citrate buffer for pH control (Figure 6.3). Citrate and acetate have similar pKa values, (pKa = 4.77 for acetic acid at 37°C versus 4.75 for citric acid at the same temperature (Biochemistry 221, 2007)), but citrate is not a product of the fermentation. The adjusted ethanol yields were also representative of this phenomenon, as the acetate buffered flasks had ~15% greater actual ethanol yield (Figure 6.4).

Cell mass, as shown in Figure 6.5, was quite stunted by the use of an acetate buffer. This could be due to the fact that acetic acid production was inhibited, which could potentially be growth

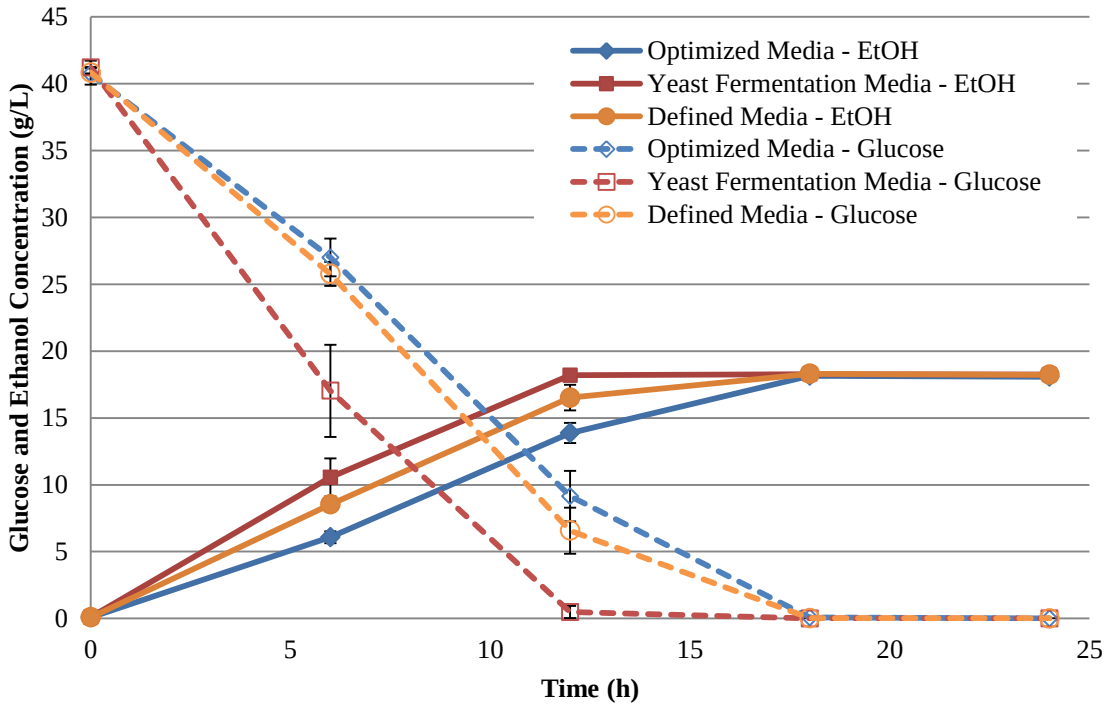

Figure 6.1. Glucose consumption and ethanol production of immobilized *K. marxianus* IMB3 cells in calcium alginate, substrate 40 g/l glucose.

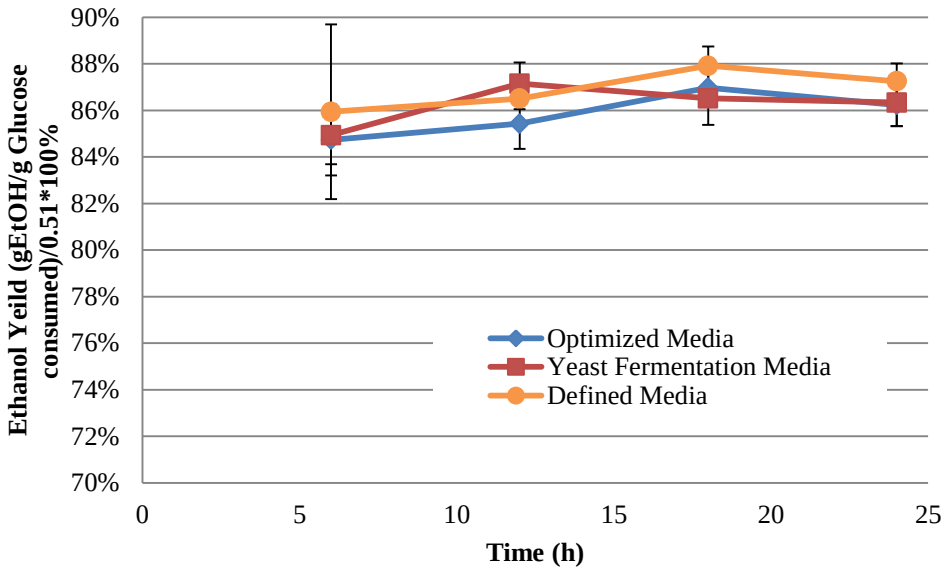

Figure 6.2. Actual ethanol yield for comparison of media using immobilized calcium alginate beads, glucose substrate 40 g/L.

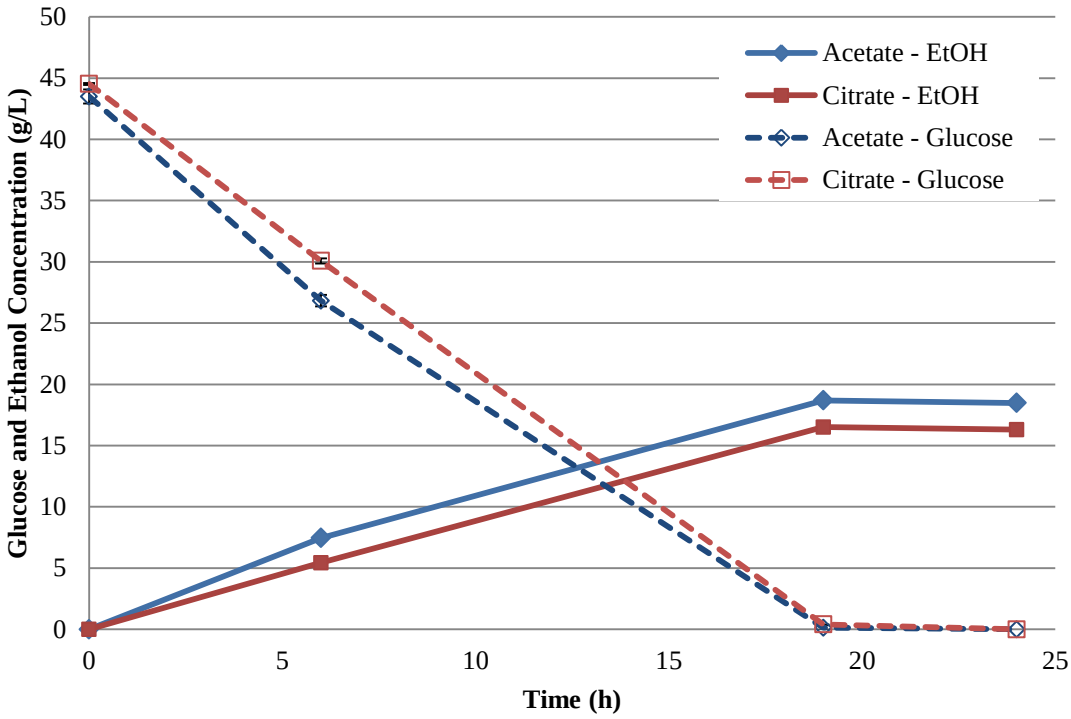

Figure 6.3. Glucose consumption and ethanol (EtOH) production of free *K. marxianus* IMB3 cells in YFM and two different buffers, substrate 40 g/L glucose.

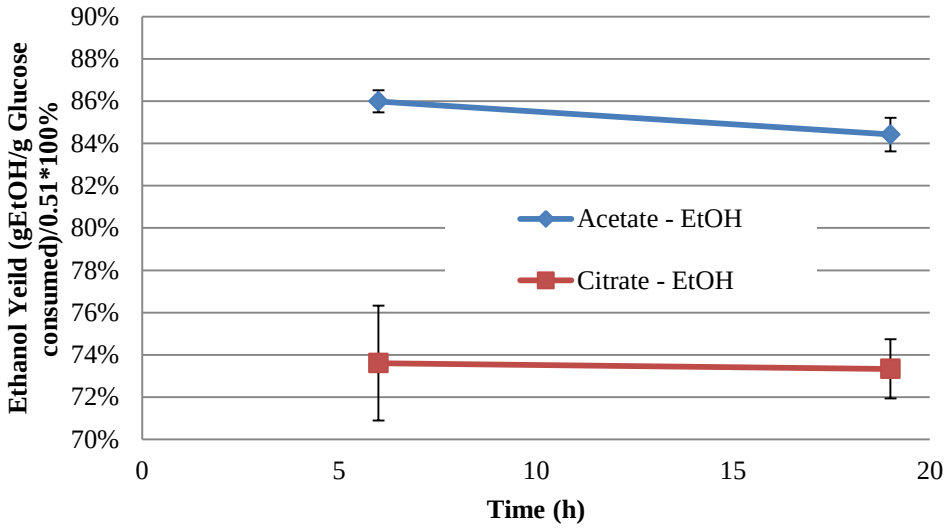

Figure 6.4. Adjusted ethanol yield for comparison of citric acid buffer vs. acetate buffer in YFM media using free *K. marxianus* IMB3, glucose substrate 40 g/L.

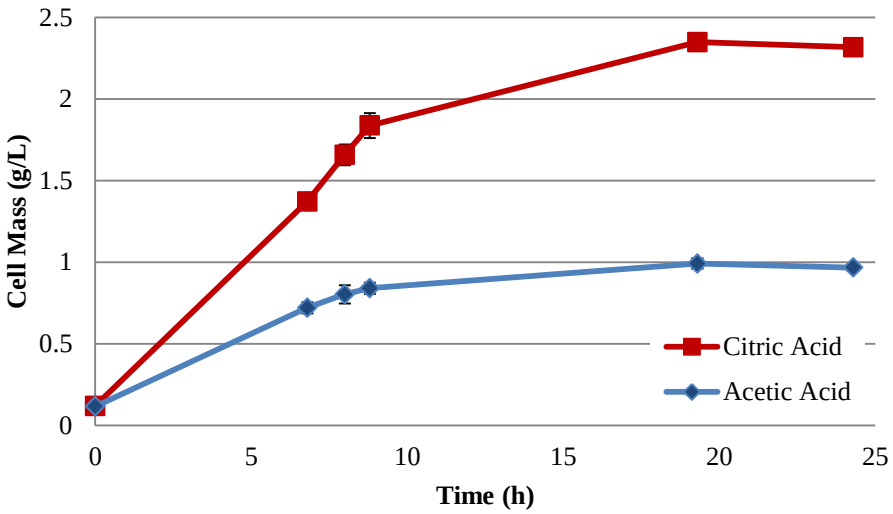

Figure 6.5: Cell mass concentrations in comparison of citric acid buffer vs. acetate buffer in YFM media using free *K. marxianus* IMB3, glucose substrate 40 g/L

related. Cell growth has shown to be inhibited by acetic acid previously in cultures of *S. cerevisiae* when looking at potentially inhibitory compounds from pretreatment methods for lignocellulosic biomass (Oliva et al. 2006). This would mean that the cells have much higher rates of ethanol production and glucose consumption on a per cell basis with this initial concentration of acetic acid in the media.

The pH drop was greater for citric acid buffered fermentations (Figure 6.6). This drop could have been due to the fact that there was a greater difference between starting pH for this fermentation and the pKa of the buffering compound for citric acid, therefore reducing the buffering ability of the compound. The buffering capacity of the chemical should not have been a huge issue as the starting pH (5.3) was still well within the range of the citric acid buffer (3.0 to 6.2).

Figure 6.7 shows that almost no acetate was produced in the fermentations containing acetic acid buffering. This indicates that the presence of acetate in the buffer could be inhibiting the production of acetate by the yeast due to inhibition because of product concentration.

A comparable experiment using immobilized *Z. mobilis* in a continuous flow reactor showed that ethanol production of the fermentation increased to 19 g/l when an acetate buffer was employed over 16 g/l when a succinate buffer was used (Cross et al., 1993). That experiment also saw that the growth rate was substantially decreased, extending the overall life of the reactor. Growth rates were not determined for the current experiment as an excessive lag period hindered this measurement; although, it did appear that citrate buffered fermentations had a faster growth rate. The overall cell mass concentration was higher in the flasks having acetic acid as a buffer for the current experimental conditions.

Acetic acid has also been shown to have an effect on ethanol fermentations with *S. cerevisiae* CBS 8066 , as a concentration of 3.3 g/l in the media increased ethanol yield by 20% and decreased biomass (by 45%) and glycerol yields (by 35%) (Taherzadeh et al., 1997). The

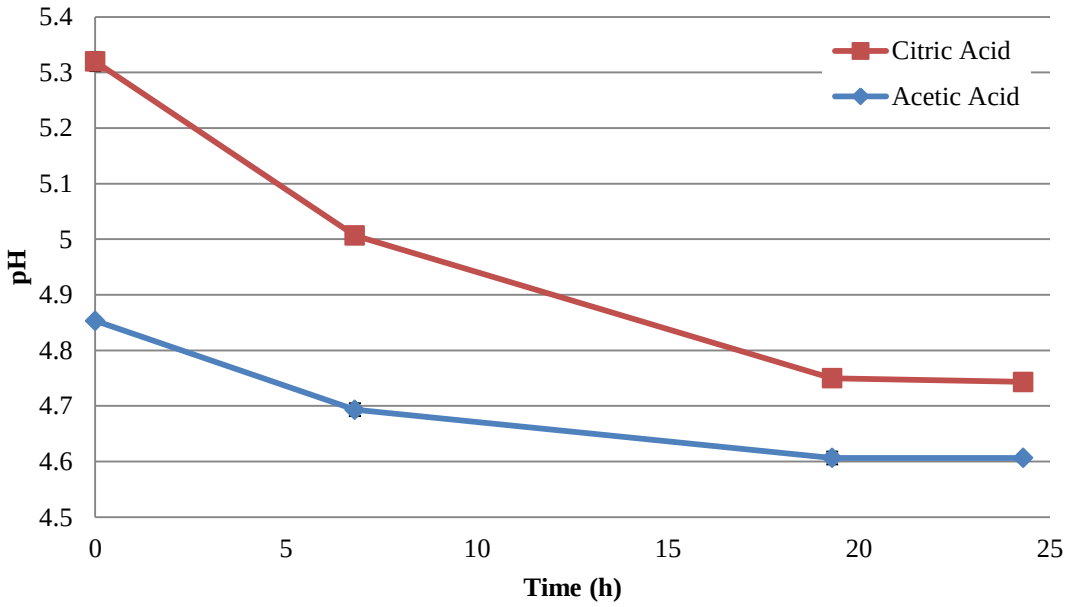

Figure 6.6. pH drop in fermentations comparing citric acid buffer to acetate buffer in YFM media using free *K. marxianus* IMB3, glucose substrate 40 g/L. Acetic acid buffer was used at ~ pH =5 due to this being the pH used in enzymatic assays.

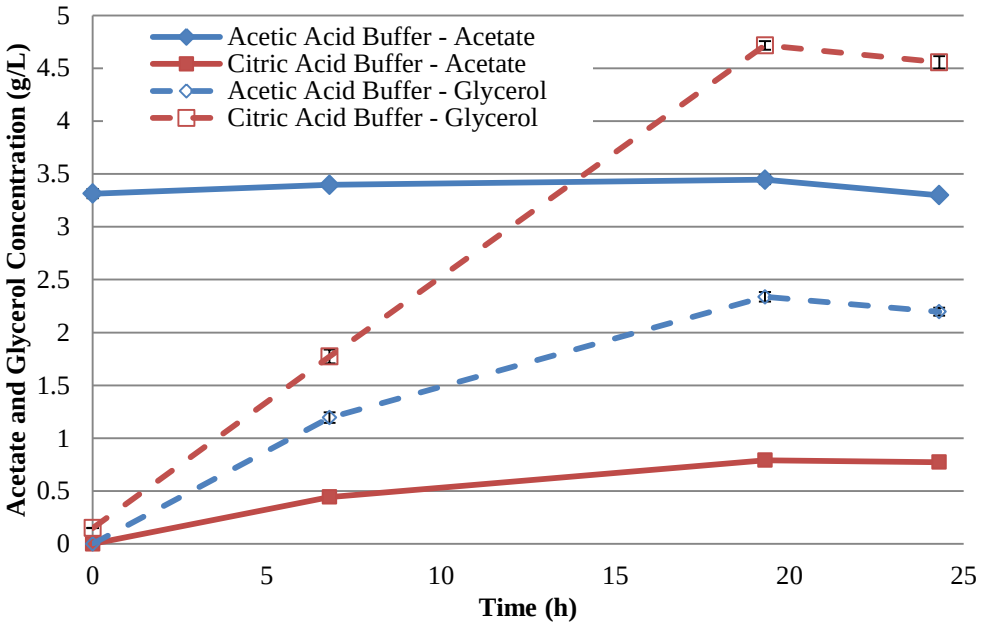

Figure 6.7. Acetate and glycerol production of free *K. marxianus* IMB3 cells in YFM media and two different buffers, substrate 40 g/L glucose.

increase in ethanol yield was only 15% in the current experiment for a comparable amount of supplemented acetic acid, but this is a different organism fermenting a different amount of glucose at a higher temperature.

Oliva et al. (2006) used *K. marxianus* CECT 10875 in glucose fermentations supplemented with acetate concentrations between 2 and 10 g/L in a response surface analysis of soluble inhibitors on cell growth rate, cell yield and ethanol yield. Their models showed that acetic acid negatively impacted biomass yield, but high acetate individually did not cause complete inhibition of ethanol production, although it did lower it. Lower biomass yields were also seen in the current experiments using *K. marxianus* IMB 3 as shown in Figure 6.5. The model presented by Oliva et al. (2006) seems to be somewhat incorrect in the presence of acetate only as acetate did increase ethanol yield at a concentration of about 3 g/L in the current studies. The response surface did not use a design that exhaustively explored the responses of acetate only supplementation, as this was not the goal of the experiment since several components were being concurrently evaluated.

6.3.3 Temperature Studies

Since alginate immobilized beads have been looked at for ethanol tolerance characteristics (Jirků, 1999; Norton et al., 1995) and thermotolerance has been linked to the same genes relating to ethanol tolerance (Piper, 1995), it was necessary to evaluate the use of immobilization to address a problem that may stem from temperature issues. Six flasks were run comparing fermentations at 48°C to 50°C using immobilized cells. As stated in the materials and methods, 45°C was not repeated as this condition was already done in the media comparison experiment. The cells were recycled from the media experiment so as to have one from each media in each temperature setting to minimize carry over effects.

There was no significant difference ($p < 0.05$) between the production of ethanol and consumption of glucose in the experiments (Figure 6.8), although the experiment done at 50°C

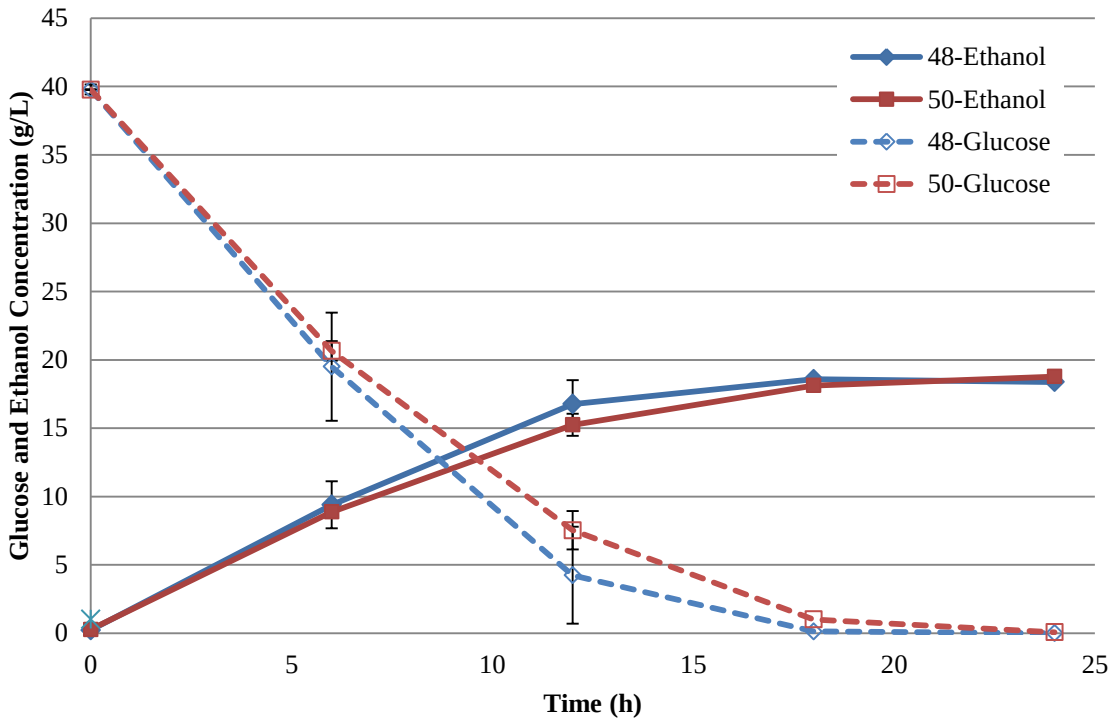

Figure 6.8. Glucose consumption and ethanol (EtOH) production of immobilized *K. marxianus* IMB3 cells in YFM media with acetate buffer and two different temperatures for fermentation (48°C and 50°C), substrate 40 g/L glucose.

appeared to go slower. SSF reactions are usually limited by the activity of the cellulase enzyme, operating at a higher temperature would be beneficial for the overall efficiency of these reactions. This glucose experiment was done to see if higher temperature fermentations could be accomplished, and therefore translate to the SSF without compromising the glucose conversion to ethanol by the yeast catalyst. There was no significant difference when the elevated temperature immobilized fermentations were compared to those fermentations done at 45°C for ethanol yield.

Table 6.2 identifies the yields at 19 h for all the glucose substrate experiments and compares them using a Tukey test on means in SAS vs. 9.2. There was no statistical difference ($p < 0.05$) between the 48 and 50°C fermentations' ethanol yields, but the 48°C fermentation had a higher ethanol yield than the 45°C fermentation. The response at 45°C could have been skewed as it was the first time the beads were used in a fermentation and several studies show a lag in fermentation performance during the initial cycle (Barron et al., 1996; Lee et al., 2011). Liu and Shen (2008) found that increasing the temperature of fermentations using immobilized cultures of *S. cerevisiae* (CICC 1308) actually increased the ethanol yield of sweet sorghum juice from 75.79% to 89.89% when the temperature of the fermentation was raised from 28 °C to 37 °C. Experiments using free cultures ultimately had lower optimal fermentation temperatures (around 30°C). It is important to note that these temperatures are lower than the temperatures used for thermotolerant yeast; therefore, temperature effects may be overwhelmed by the already elevated temperature of the fermentations.

The no buffer experiment did work very well, and from an economic standpoint would be highly advantageous as pH control can be costly due to salt accumulation from adjustment (Lynd et al.,

Table 6.2. Table of ethanol yields (% of theoretical) obtained for different treatments.

Buffer	Immobilized (Y/N)	Medium	Temperature (°C)	Total Ethanol Yield
Acetate	Y	YFM	50	0.903^{AB}
Acetate	Y	YFM	48	0.907^{A}
Acetate	Y	YFM	45	0.865^{BC}
Acetate	N	YFM	45	0.845^{C}
No Buffer	Y	YFM	45	0.903^{AB}
Citrate	N	YFM	45	0.733^{D}

2001; Mielenz, 2001) and materials cost. The problem with such a fermentation is that it is unreasonable to use no pH control in an SSF, since the enzyme activity is highly dependent on the pH of the system. The non-buffered fermentations had a final pH of close to 3.2, much lower than the range (4.0-5.0) specified in the commercial literature for the enzyme used in these experiments, Accellerase 1500 (Genencor®, 2009). This experiment did produce high yields on YFM (>80%) that were very similar to other non-buffered experiments using the same yeast,. Love et al. (1998) obtained an average ethanol yield of 86% of theoretical when 10% (w/v) glucose was in the feed substrate with *K. marxianus* IMB3 immobilized in calcium alginate. An earlier study found that immobilized *K. marxianus* IMB3 cells yielded 80% of the maximum theoretical yield when glucose was used as a substrate (Nolan et al., 1994).

Following these experiments it was decided that three different SSF treatments would be used, one with immobilized cells at 45°C, one immobilized cells at 50°C and one with free cells at 45°C, all with acetic acid buffering the solution at pH 5. Even though the yield was technically slightly greater at 48°C, it was not significantly different than 50°C and the higher temperature should increase the enzymatic activity.

6.3.4 SSF with immobilized *K. marxianus* IMB 3

Figure 6.9 shows the concentrations of glucose and ethanol in the SSFs throughout the course of the fermentation. The accumulation of glucose in the 45°C immobilized flasks indicated that the cells were unable to initiate ethanol production immediately after introduction. The lag time for the immobilized cells could be due to cells not being able to access glucose due to low mass transfer of glucose to the beads due to phenolic compounds and other inhibitors in the substrate,

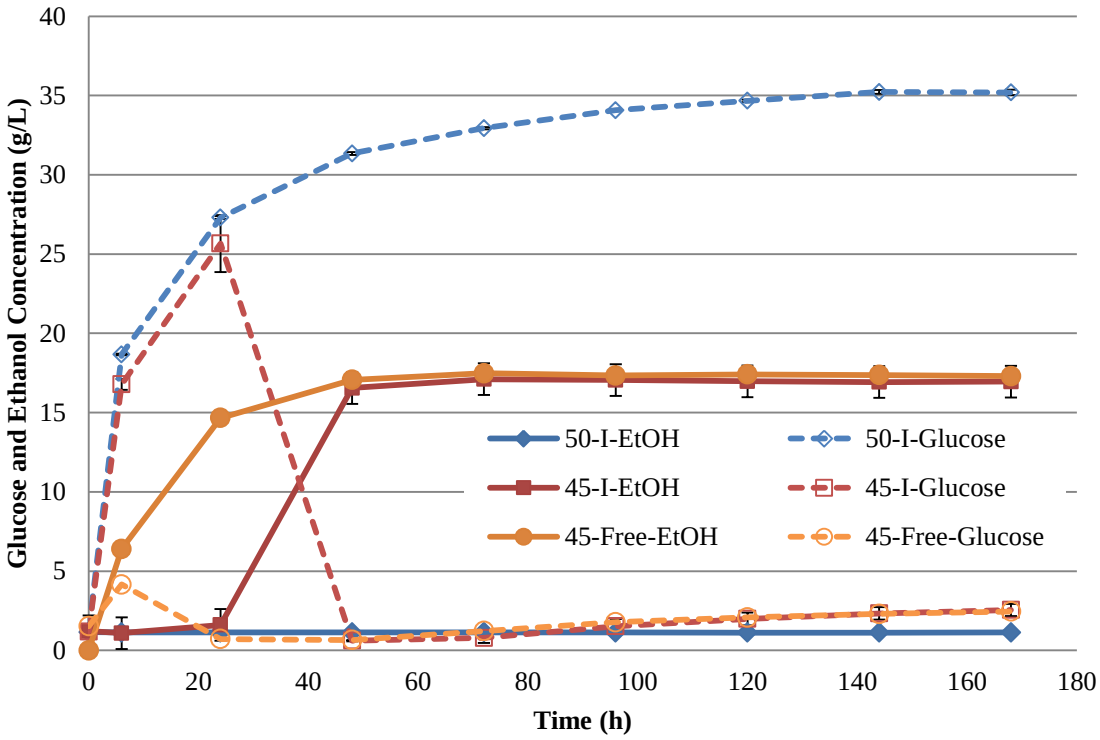

Figure 6.9. Comparison of glucose and ethanol (EtOH) production by *K. marxianus* IMB 3 in immobilized in calcium alginate beads (I) or free (Free) cell configuration at either 45°C or 50°C in SSF.

or that the *K. marxianus* IMB3 cells were adversely affected by the long storage time (~2 weeks) and had to grow up from a minimal concentration outside of the beads. The beads at 50°C showed no ethanol production, which, if the same phenomenon of inhibition due to compounds formed during the pretreatment process occurred at this elevated temperature, the cells may not have been able to recover. The temperature of 50°C has been identified as a condition at which IMB3 is capable of ethanol production, but has very low cell growth rates at this temperature (Banat and Marchant, 1995); hence, the cells were not able to "recover" as the 45°C flasks did by producing cell biomass outside the alginate matrices. Figure 6.10 shows the visual difference between the SSF beads and those used in glucose fermentations.

In addition to the discoloration, the beads were also slightly larger after going through SSF (by about 1 mm). Many beads were split, potentially due to biomass causing shearing of the beads, as well as bead instability due to composition of the medium and CO_2 evolution within the beads. The lower ethanol yields of the immobilized cells at 45°C may be explained by the difference in volume between the immobilized and free cell experiments; although, this was not seen in glucose fed immobilized experiments. The yields were lower for the immobilized cells as the immobilized cells had an initial concentration of ethanol present (< 1 g/L) in the fermentation, potentially due to the prior fermentations that the beads had undergone. Subtracting this initial concentration lowered the yield of the immobilized cells to 77% as compared to 85% for the free cells in fermentation. Maximum concentration of ethanol for both the immobilized cells and the free cells occurred at 72 h for both fermentations, yielding an average ethanol concentration of 17.10 g/L and 17.48 g/L for immobilized cells and free cells, respectively. This was the same time that maximum ethanol concentration occurred in the citric acid buffered SSFs with the same enzyme and solids loading as discussed in Chapter 5, although only an average maximum of 16.07 g/L was reached in those fermentations.

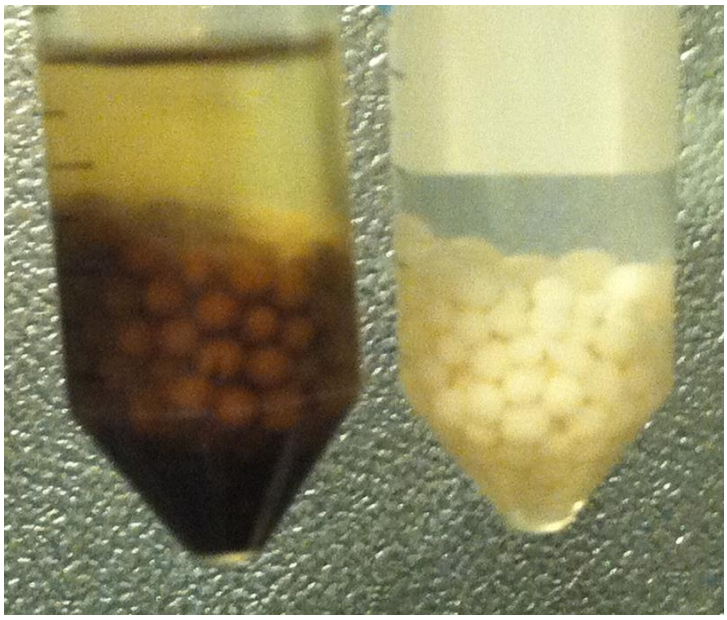

Figure 6.10. Comparison of calcium-alginate immobilized beads from SSF (left) with calcium alginate beads of same age only exposed to glucose substrates (right).

Although experiments have been completed with success using solid substrates with immobilized cells at 45°C, most of these studies used a purified cellulose as the substrate (Barron et al., 1996; Barron et al., 1996); therefore, there was no chance for other inhibitory substances from the hemicellulose and lignin portions of the biomass to interfere with the permeability of the immobilization matrices. Mass flux into the calcium alginate beads for small compounds such as ethanol and glucose is close to those values found in water. More complex substrates have had difficulty in employing immobilized cells in SSF reactions. Yamashita et al. (2008) used *Z. mobilis* in SSF reactions for paper sludge and had good results as the immobilization matrix appeared to actually protect the cells from the high concentrations of inhibitory ions in the paper sludge. Inhibition began after several cycles.

Most fermentations reporting good yields in SSF type fermentations using immobilized cells use a starch or sugar based substrate (Bandaru et al., 2006; Behera et al., 2010), as these substrates do not have the issues with compounds that may negate the ability of these cells to have access to the hydrolyzed glucose from pretreatment that lignocellulosic biomass have.

6.4 Conclusions

Media composition (of the three media tested in this chapter) had no significant effect on the fermentation of glucose by calcium alginate immobilized *K. marxianus* IMB3. Fermentation at higher temperatures (48 and 50°C) was accomplished using immobilized cultures of *K. marxianus* IMB3. Higher temperatures gave higher ethanol yields than fermentations at lower temperatures. Immobilization of *K. marxianus* IMB3increased the ethanol yield of the organism in glucose fermentations slightly. The addition of acetate to the fermentation media in the form of a buffer served to positively affect the production of ethanol in the cells, especially in SSF, increasing final ethanol yields by about 13% (75% of theoretical using a citrate buffer system vs. 85% using an acetate buffered system). *K. marxianus* IMB 3 cells immobilized in calcium

alginate were unable to convert glucose initially in the SSF set-up using hydrothermolysis pretreated switchgrass, possibly due to the blocking of the pores in the beads by compounds formed in the pretreatment of chemicals, although the immobilized cells at 45°C did make ethanol after 24 h.

6.5 Future work

An additional free cell experiment should be done using citric acid buffer at pH = 5.0 to determine if the positive correlation from the buffer is partially due to the lower starting pH or completely due to the back inhibition of acetate production from the presence of the acetate in themedia already. Different methods of immobilization or different pretreatment methods may work better to not cause the inhibition seen in the use of this system.

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APPPENDICES

Table C. Coded values for initial 90 run response surface methodology experiment.

Run	Block	Ca Pantothenate	$(NH4)_2SO4$	KH_2PO_4	$CaCl_2$	Nicotinic Acid	Thiamin	$MgSO_4 \cdot 7H_2O$	Adjusted EtOH Yield (g/g)	EtOH concentration at 24 h (g/L)
1	1	-1	-1	-1	-1	1	-1	-1	0.803	13.970
2	1	-1	-1	1	1	-1	1	-1	0.7935	9.719
3	1	-1	1	-1	1	-1	-1	1	0.8052	15.278
4	1	-1	1	1	-1	1	1	1	0.7955	13.738
5	1	1	-1	-1	1	1	1	1	0.7817	16.102
6	1	1	-1	1	-1	-1	-1	1	0.8102	12.555
7	1	1	1	-1	-1	-1	1	-1	0.805	13.188
8	1	1	1	1	1	1	-1	-1	0.7663	12.393
9	1	0	0	0	0	0	0	0	0.7992	16.403
10	2	-1	-1	-1	1	-1	1	1	0.7941	16.776
11	2	-1	-1	1	-1	1	-1	1	0.767	16.306
12	2	-1	1	-1	-1	1	1	-1	0.7983	16.616
13	2	-1	1	1	1	-1	-1	-1	0.7973	16.731
14	2	1	-1	-1	-1	-1	-1	-1	0.8092	16.888
15	2	1	-1	1	1	1	1	-1	0.7898	16.403
16	2	1	1	-1	1	1	-1	1	0.7835	16.590
17	2	1	1	1	-1	-1	1	1	0.7772	16.218
18	2	0	0	0	0	0	0	0	0.7831	16.417
19	3	-1	-1	-1	1	1	1	-1	0.7922	16.138
20	3	-1	-1	1	-1	-1	-1	-1	0.7595	15.658
21	3	-1	1	-1	-1	-1	1	1	0.7952	16.293
22	3	-1	1	1	1	1	-1	1	0.6658	13.607
23	3	1	-1	-1	-1	1	-1	1	0.785	16.025
24	3	1	-1	1	1	-1	1	1	0.7983	16.396
25	3	1	1	-1	1	-1	-1	-1	0.5628	11.754

26	3	1	1	1	-1	1	1	-1	0.769	15.848
27	3	0	0	0	0	0	0	0	0.7955	16.234
28	4	-1	-1	-1	-1	-1	-1	1	0.7738	16.129
29	4	-1	-1	1	1	1	1	1	0.7906	16.212
30	4	-1	1	-1	1	1	-1	-1	0.7873	16.182
31	4	-1	1	1	-1	-1	1	-1	0.7894	16.327
32	4	1	-1	-1	1	-1	1	-1	0.7902	16.258
33	4	1	-1	1	-1	1	-1	-1	0.7845	16.150
34	4	1	1	-1	-1	1	1	1	0.7751	16.168
35	4	1	1	1	1	-1	-1	1	0.6919	14.417
36	4	0	0	0	0	0	0	0	0.6961	16.442
37	5	-1	-1	-1	-1	-1	1	-1	0.7773	16.607
38	5	-1	-1	1	1	1	-1	-1	0.7786	16.621
39	5	-1	1	-1	1	1	1	1	0.7773	16.321
40	5	-1	1	1	-1	-1	-1	1	0.7559	16.015
41	5	1	-1	-1	1	-1	-1	1	0.7759	16.129
42	5	1	-1	1	-1	1	1	1	0.7755	16.446
43	5	1	1	-1	-1	1	-1	-1	0.7814	16.341
44	5	1	1	1	1	-1	1	-1	0.7616	16.383
45	5	0	0	0	0	0	0	0	0.7767	16.533
46	6	-1	-1	-1	1	1	-1	1	0.7953	16.725
47	6	-1	-1	1	-1	-1	1	1	0.7797	16.336
48	6	-1	1	-1	-1	-1	-1	-1	0.7849	16.259
49	6	-1	1	1	1	1	1	-1	0.7926	16.884
50	6	1	-1	-1	-1	1	1	-1	0.788	16.844
51	6	1	-1	1	1	-1	-1	-1	0.7949	16.934
52	6	1	1	-1	1	-1	1	1	0.8077	16.956
53	6	1	1	1	-1	1	-1	1	0.7813	16.550
54	6	0	0	0	0	0	0	0	0.7869	16.711
55	7	-1	-1	-1	1	-1	-1	-1	0.7661	16.496
56	7	-1	-1	1	-1	1	1	-1	0.7676	16.481
57	7	-1	1	-1	-1	1	-1	1	0.7864	16.494
58	7	-1	1	1	1	-1	1	1	0.6465	11.636
59	7	1	-1	-1	-1	-1	1	1	0.7734	16.175
60	7	1	-1	1	1	1	-1	1	0.7775	16.422
61	7	1	1	-1	1	1	1	-1	0.7734	16.515
62	7	1	1	1	-1	-1	-1	-1	0.7958	16.836
63	7	0	0	0	0	0	0	0	0.7621	16.370
64	8	-1	-1	-1	-1	1	1	1	0.7805	16.781
65	8	-1	-1	1	1	-1	-1	1	0.7942	16.718
66	8	-1	1	-1	1	-1	1	-1	0.7997	16.945

67	8	-1	1	1	-1	1	-1	-1	0.7752	16.385
68	8	1	-1	-1	1	1	-1	-1	0.8102	17.211
69	8	1	-1	1	-1	-1	1	-1	0.8023	17.398
70	8	1	1	-1	-1	-1	-1	1	0.7896	16.747
71	8	1	1	1	1	1	1	1	0.7762	16.529
72	8	0	0	0	0	0	0	0	0.7974	16.770
73	9	-2.828	0	0	0	0	0	0	0.7792	15.840
74	9	2.828	0	0	0	0	0	0	0.7672	16.615
75	9	0	-2.828	0	0	0	0	0	0.4435	0.358
76	9	0	2.828	0	0	0	0	0	0.7876	16.767
77	9	0	0	-2.828	0	0	0	0	0.6328	1.391
78	9	0	0	2.828	0	0	0	0	0.7909	16.762
79	9	0	0	0	-2.828	0	0	0	0.7765	15.187
80	9	0	0	0	2.828	0	0	0	0.7873	17.053
81	9	0	0	0	0	-2.828	0	0	0.7649	9.888
82	9	0	0	0	0	2.828	0	0	0.7561	16.221
83	9	0	0	0	0	0	-2.828	0	0.7497	11.878
84	9	0	0	0	0	0	2.828	0	0.7526	16.566
85	9	0	0	0	0	0	0	-2.828	0.1076	0.098
86	9	0	0	0	0	0	0	2.828	0.7781	16.644
87	9	0	0	0	0	0	0	0	0.7881	16.614
88	9	0	0	0	0	0	0	0	0.7715	16.276
89	9	0	0	0	0	0	0	0	0.7695	16.357
90	9	0	0	0	0	0	0	0	0.7957	16.972

Information from
http://www.voigtglobal.com/Anonymous/DIFCO_Yeast_Agar_Formulations.pdf
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pH, 1% Soln 6.7
Filterability (g/cm2) 2.7
Carbohydrate (%)
Total 17.5
Nitrogen Content (%)
Total Nitrogen 10.9
AN/TN 55.0
Amino Nitrogen 6.0
Amino Acids (%)
Alanine 5.36
Arginine 3.02
Aspartic Acid 6.69
Cystine 0.74
Glutamic Acid 14.20
Glycine 3.25
Histidine 1.20
Isoleucine 3.23
Leucine 4.69
Lysine 5.15
Methionine 1.05
Phenylalanine 2.53
Proline 2.60
Serine 2.84
Threonine 2.95
Tryptophan 1.36
Tyrosine 1.20
Valine 3.79
Inorganics (%)
Calcium 0.013

Chloride 0.380
Cobalt <0.001
Copper <0.001
Iron <0.001
Lead <0.001
Magnesium 0.075
Manganese <0.001
Phosphate 3.270
Potassium 3.195
Sodium 1.490
Sulfate 0.091
Sulfur 0.634
Tin <0.001
Zinc 0.011

Vitamins (µg/g)

Biotin 3.3
Choline (as Choline Chloride) 300.0
Cyanocobalamin <0.1
Folic Acid 1.5
Inositol 1400.0
Nicotinic Acid 597.9
PABA 763.0
Pantothenic Acid 273.7
Pyridoxine 43.2
Riboflavin 116.5
Thiamin 529.9
Thymidine 17.5

Biological Testing (CFU/g)

Coliform negative
Standard Plate Count 60
Salmonella negative
Thermophile Count <5
Spore Count 9

VITA

Jennine B. Terrill

Candidate for the Degree of

Doctor of Philosophy

Thesis: EVALUATION OF BEST PARAMETERS IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS FOR MAXIMUM ETHANOL YIELD BY *K. MARXIANUS* IMB3

Major Field: Biosystems Engineering

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in December, 2011.

Completed the requirements for the Master of Science in Environmental Engineering at The Pennsylvania State University, University Park, PA in 2008.

Completed the requirements for the Bachelor of Science in Agricultural and Biological Engineering The Pennsylvania State University, University Park, PA in 2005.

Professional Memberships:

ASABE member

EIT Certified

Alpha Epsilon Member

Name: Jennine B. Terrill Date of Degree: December, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EVALUATION OF BEST PARAMETERS IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS FOR MAXIMUM ETHANOL YIELD BY *K. MARXIANUS* IMB3

Pages in Study: 151 Candidate for the Degree of Doctor of Philosophy

Major Field: Biosystems Engineering

Scope and Method of Study:

Statistical design and analysis of media and immobilization parameters for yeast *K. marxianus* IMB3.

Findings and Conclusions:

A defined medium was constructed for *K. marxianus* IMB3 with a very similar cost to the original media and a better yield. This media was then screened and optimized to find an minimal media, but significant blocking dictated which parameters could be used in the optimization. The optimized media produced a higher level of ethanol, but only after 72 hours. The increased yield was offset by a lower rate of conversion of glucose to ethanol in the fermentation media. In an SSF the optimized media did not prove to work as well, most probably due to the lower pH of the starting batch fermentation, and the effect of low pH on the cellulase enzyme being used in the reaction. Optimized media with an initial pH adjustment through the addition of a small amount of base, allowed the same overall yield, but still had a lower initial ethanol production. Media composition did not matter for ethanol yield when the yeast cells were immobilized in a calcium alginate matrix. Immobilization decreased the time to completion of a glucose fermentation over that of free cells, but proved to be inadequate in a simultaneous saccharification and fermentation reaction. The best method for increasing ethanol production in an SSF in this study, was the use of an acetate buffer instead of a citrate buffer for pH control. The use of an acetate buffer (50 mM, pH = 5) served to decrease cell mass (g/L) and increase ethanol production in glucose fermentations. SSF using an acetate buffer also showed higher ethanol yields. No acetate production was observed in any of the flasks using an acetate buffer.

ADVISER'S APPROVAL: Mark R. Wilkins