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Early-stage techno-economic analysis of renewable diesel production via hydrothermal liquefaction of filamentous fungi

by

Kowshik Eggoni

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Civil Engineering (Environmental Engineering)

Program of Study Committee:
Johannes van Leeuwen, Major Professor
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ABSTRACT

Cultivation of filamentous fungi on corn-ethanol thin stillage has been proved to have a potential to create value-added products while reducing the COD, TSS, and other undesirable compounds in the thin stillage. However, inconsistencies in fungal growth yields hinder the commercial viability of large-scale fungal cultivation. The undesirable morphological growth of fungi overburdens the energy requirement for fungal cultivation while causing a poor mass transfer in the bioreactor. Morphological differences in fungal growth are influenced by various fermentation parameters. Lab-scale experiments on the cultivation of *Rhizopus oligosporus* on thin stillage were carried out to understand the effect of temperature and inoculum concentration in the production of optimum yields with pellet morphology.

Production of renewable diesel from biocrude obtained via hydrothermal liquefaction of fungal biomass cultivated on thin stillage has been demonstrated. While the process seems to be a great way to produce second-generation biofuel, inconsistencies in fungal growth, and high-energy requirement encumbers the economics of the process for large-scale production. An early-stage techno-economic analysis was conducted to understand the key logjams in the commercialization of the process. The price of gasoline gallon equivalent (GGE) of the renewable diesel was obtained in practical and worst-case scenarios. Sensitivity analyses were conducted to understand the effect of various model inputs and model parameters on the price of a GGE of fuel.

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CHAPTER I

INTRODUCTION

Corn-ethanol production in the US has seen tremendous growth in the last few decades. The production volume of corn ethanol has increased to 14.8 billion gallons in 2015, compared to the 1.6 billion gallons in 2000 (RFA, 2016a). Establishment of Energy Independence and Security Act (EISA, 2007) has played a vital role in the increase in annual production volume of corn ethanol along with other renewable fuels (EPA, 2016). EISA of 2007 mandates a production of 15 billion gallons of ethanol through 2022. While contributing to an annual GDP of $44 million in the year 2015, the corn-ethanol industry has created nearly 358,000 jobs directly and indirectly largely contributing to employment in the U.S. (RFA, 2016b). Liska et al. (2009) have reported a net reduction of 54% of greenhouse gas emissions from the use of a gallon of ethanol (including the emissions from agriculture and industrial production of ethanol) replaced with a gallon of gasoline. Despite all these achievements, corn-ethanol industry still faces the criticism where the efficiency of the process is questioned (Runge, 2010; Bhat, 2008).

Renewable Fuels Association (RFA, 2016c) has reported the average energy balance of dry-milling biorefineries producing corn ethanol in the U.S. has reached 2.6-2.8 compared to the 1.9-2.3 in 2008 (Shapouri et al., 2010). However, a significant amount of energy is invested in the conversion of the thin stillage to a syrup that lowers the energy efficiency of the overall process. The van Leeuwen group at Iowa State University has demonstrated the cultivation of fungi on thin stillage to produce value-added products like high-protein animal feed and feedstock for renewable diesel production.

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The fungal cultivation on any complex substrate has to be well understood to ensure optimum productivity of the process. Morphology of fungus plays a vital role in determining the productivity of fungal cultivation process. The growth of filamentous fungi is typically characterized into two morphological types, namely pellet morphology and dispersed or clump morphology (Metz et al., 1977). Pellet morphology is desired in most of the submerged fermentations due to its Newtonian flow, that ensures adequate mass transfer in the fermentation broth. On the other hand, dispersed morphology results in non-Newtonian flow and increased broth viscosity. Increased broth viscosity not only results in increased energy requirement for bulk mixing but also forms clumps around impeller and clogs pipes, resulting in poor bioreactor performance and reduced process efficiency.

Suesse et al. (2016) have demonstrated the conversion of fungal biomass into biocrude via hydrothermal liquefaction process. The biocrude was used in the production of renewable diesel. The research suggests that commercial feasibility of the process can only be achieved with consistent fungal yields during the fermentation process. However, the process involves several other unit operations and the other bottlenecks involved in the process can only be understood through an early-stage techno-economic analysis of the entire process.

Research work in this thesis is divided into two parts. The first part (chapter 3) focusses on developing an early-stage techno-economic analysis to identify the key logjams in the commercial feasibility of renewable diesel production via hydrothermal liquefaction of fungal biomass. Data obtained from pilot-scale experiments was used to compute mass balances and estimate capital and process costs. The gasoline gallon equivalent price of the

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renewable diesel was determined for the practical and worst-case scenarios while the bottlenecks in process commercialization were identified.

The second part (chapter 4) focusses on the cultivation of *Rhizopus oligosporus* to obtain consistent pellet morphology in thin stillage. Lab-scale testing was used to identify the morphological differences in fungal growth. Different temperatures and inoculum concentrations were tested for their efficiency in producing optimum pellet growth.

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CHAPTER 2

LITERATURE REVIEW

Abstract

This literature review consists two parts related to utilization of fungal biomass to produce high-value products. The first part provides an analysis on the importance of different morphological forms of filamentous fungi in submerged fermentations. An overview of submerged fermentations, and their background, the important role of morphological differences in process efficiency, and several factors that play a vital role in determining the fungal morphology were discussed. The second part describes the importance of techno-economic analysis during multiple stages of a product commercialization in biorenewable industry. The significance of an early-stage analysis to ensure process efficiency before pilot-scale or large-scale production will be highlighted.

1. Introduction

The natural metabolism of fungi has been exploited by civilizations in numerous applications ranging from baking and brewing to manufacturing of chemicals and antibiotics. Fungal fermentation has gained immense importance over the past few decades and is practiced by many industries for the production of commercial products. While the commercial production of citric acid in 1919 marked a new beginning for commercial fungal fermentation, mass production of penicillin by US war production board for soldiers in World War II denotes the role fungi played in world history (Bellis, 2015).

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Among the numerous applications of fungi in the food industry, production of blue cheese, soy sauce, tofu, and tempeh are some of the well-known processes used worldwide. Fruiting bodies of several species of the fungal phyla, Ascomycota and Basidiomycota have been consumed as edible food since several centuries. The ability of fungi to grow on a variety of substrates makes them the favorable microbes in various fermentation practices.

Utilization of complex media for fungal fermentation has received continuous attention over the past few years. Several complex substrates such as brewery wastewater, corn-ethanol thin stillage, crop processing wastewaters and sugarcane molasses have been explored for their abilities in supporting fungal growth to produce value added products (van Leeuwen et al., 2012; Das and Brar, 2014). These substrates are high in CBOD, TSS, organics, and other undesirable materials. Treatment of these substrates for the removal of undesirable components and safe disposal is a cost-consuming process.

These substrates are typically treated by activated sludge process, utilizing bacteria to convert organic matter into carbon dioxide and water. This process usually generates 0.4g of bacterial biomass for every gram of COD removed (Metcalf and Eddy, 2003). Handling the bacterial biomass produced in activated sludge process is an expensive option, that accounts for 40-60% of plant operational costs generally (Canales et al., 1994). Cultivation of fungi on these complex substrates not only achieves the desirable treatment, but also produces the biomass that can produce a multitude of high-value products such as chitosan, animal feed, and feedstock for renewable diesel production (van Leeuwen et al., 2012).

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Table 1. Biochemical composition of *R. oligosporus* feedstock.

Biochemical content (wt.%)	
Carbohydrates	34.8
Proteins	34.2
Lipids	22.4
HHV (MJ/kg)	28.3

The biochemical content of *Rhizopus oligosporus* presented by Suesse (2016) is represented in Table 1. Suesse (2016) claims that the high HHV, lipids, and proteins qualify biomass of *R. oligosporus* as a competitive feedstock for production of renewable diesel via hydrothermal liquefaction.

The literature review is divided into two parts. The first part will investigate different morphological growth patterns in filamentous fungi while identifying the conditions that could be adapted for consistent pellet growth. The second part of the review inspects the role of techno-economic analysis in various stages of a product commercialization while attempting to understand the significance of early-stage techno-economic analysis.

2. Morphology of fungus and its importance in industrial operations

Filamentous fungi exhibit two major morphological growth forms in submerged fermentations that are called as dispersed and pellet morphologies usually. The former is ascribed to the biomass growing as freely dispersed hyphae or mycelial clumps. The latter describes biomass growing in dense and highly intertwined spherical aggregates of mycelia with diameters varying between few micrometers to several millimeters (Cox et al., 1998). Both morphologies have their significance in several processes, and the desirability of either

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of the morphology depends on a number factors such as maximum end product concentration, ease in product recovery, purity of the product obtained, and the energy inputs.

No direct relation has been established towards the favorability of a particular morphology in the production of a particular product. Steel et al. (1954) proclaims that pelleted form is most favorable for citric acid production by *Aspergillus niger*, while Kristiansen and Bu'lock (1988) suggests that dispersed morphology achieves maximum production of pectic enzymes by *A. niger*.

Differences in morphology during the growth cycle affects the mass transfer in submerged fermentations (Schugerl et al., 1983). Pellet morphology is often preferred in submerged fermentations, as the pellet growth results in a Newtonian flow of the broth allowing efficient mass transfer and simplified downstream processing (Hille et al., 2005). Dispersed growth, in contrast, causes the increase in broth viscosity resulting in non-Newtonian flows and high viscosity of the broth. Mass transfer in such situations requires increased bulk mixing that accounts for additional energy costs and poor reactor performance (Kristiansen and Bu'lock, 1988). In addition, dispersed morphology often causes clogging around the impeller and in the pipes blocking the flow in both situations.

Morphology of fungus in a submerged fermentation process is controlled by a number of factors. Some of the key factors that are assumed to be more relevant for the present work are discussed below.

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2.1. Dissolved oxygen tension

From the literature review conducted by Tabak and Bridge Cooke (1968), most of the commercial fungal fermentations were observed to be aerobic and require fairly higher dissolved oxygen tension compared to other fermentation processes. Kubicek et al. (1980) have reported a steady increase in citric acid production as they increase dissolved oxygen tension from 40 to 150 mbar with aerobic fermentation of *Aspergillus niger*. Gomez et al. (1988) demonstrated a decrease in citric acid yield with *A. niger* from 48.0 g/L to 30.3 g/L and some variation in pellet sizes with a decrease in the rate of mixing. They also observed an increase in citric yield from 30.3 g/L to 48.7 g/L with the increase in air flow from 0.9 to 1.3 vvm.

While Hemmersdorfer et al. (1987) hypothesize that starvation of any nutrient and oxygen results in pellet morphology, no direct relationship has been established between oxygen tension and morphological changes in fungi. van Suijdam and Metz (1981) observed no influence in fungal morphology to the changes in oxygen tension in the range of 12-300 mm Hg with *Penicillium chrysogenum*. In contrast, Higashiyama et al. (1999) showed a change in morphology from dispersed to pellets with an increase in dissolved oxygen concentration from 7-15 ppm to 20-50 ppm in *Mortierella alpina.*

2.2. Inoculum type and spore concentration

The amount, age and type (vegetative vs. spore) of inoculum are among the prime variables that determine the morphology of biomass in fungal cultures. Especially with filamentous organisms in submerged fermentations, spore concentration is considered as a governing factor for pellet formation (Papagianni, 2004). Higher sizes of inoculum during

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early stages of growth results in more interaction between hyphae resulting in greater possibilities for clump or dispersed morphologies (Liu et al., 2007). However, the optimum inoculum concentration is found to be variable for different strains (Metz and Kossen., 1977).

van Suijdam et al. (1980) reported that *A. niger* would form pellets only with inoculum sizes less than 10^8 spores/mL, while Calam (1987) suggested an inoculum size lower than 10^4 spores/mL to form pellets with *P. chrysogenum*. Calam (1987) also indicated a 10-fold decrease in penicillin production when the inoculum concentration was reduced from 10^4 to 10^2 spores/mL.

Image analysis conducted by Tucker and Thomas (1992) on *P. chrysogenum* batch cultures exhibited a sharp transition from pellets to dispersed morphology as the inoculum size increased to $5*10^5$ spores/mL. Several studies conducted on *Rhizopus* species indicate the use of relatively low inoculum sizes (10^4 to 10^6 spores/mL) is favorable for pellet production compared to other well studied filamentous fungi like *Aspergillus* species (Liu et al., 2007; Znidarsic et al., 2000; Byrne and Ward, 1989).

2.3. Temperature

No direct relation between temperature and fungal morphology has been reported so far. While few studies suggest that increase in temperature induces faster pellet formation compared to lower temperatures (Liu et al., 2007; Nyman et al., 2013), other studies suggest decreasing the temperature as a method to induce pellet formation (Braun and Vecht-Lifshitz, 1991; Das and Brar, 2014).

In support of the latter observations, Schügerl et al. (1998) has demonstrated highest growth rates of *Aspergillus awamori* with complete pellets at 25˚C, in contrast to mixed

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morphology and dispersed morphology at 30˚C and 35˚C respectively, in shake flask cultures. Papagianni (2004) suggests inadequate oxygen supply with an increase in temperature as a possible reason for dispersed morphology. Understanding the growth cycles of fungus at different temperatures could be a helpful approach to optimize temperatures for higher pellet yields with less incubation time.

2.4. Medium composition

Typical media composition should constitute essential elements for growth, which are oxygen, carbon, hydrogen, nitrogen, phosphorus, sulfur, potassium, and some micronutrients. Fermentation media can either be defined or complex. Media used in applications such as antibiotic production might have additional components such as buffers, precursor compounds, minerals or any specified sources for some of the macronutrients.

Variations in growth patterns were rarely observed in studies on the synthetic media (Atkinson and Mavituna, 1991), compared to complex media that are obtained as a coproduct from industrial activities (Das and Brar, 2014; Liu et al., 2007; Papagianni, 2004). A study conducted by Das and Brar (2014) indicates an inversely proportional relation of total solids concentration with pellet formation. The study utilizes brewery wastewater for the production of fumaric acid by the cultivation of *R. oryzae*. The study suggests the high solids concentration leads to high viscosities resulting in poor mixing and dispersed morphology eventually. Similar observations were indicated by Mitra et al. (2012), with *R. oligosporus* culturing in corn-ethanol thin stillage.

Some of the studies suggest the addition of anionic polymers and surfactants increases the pellet number and decreases the pellet density and size (Ryoo and Choi, 1999;

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Metz et al., 1977; Papagianni, 2004). Liu et al. (2007) proclaim that addition of rice to the potato dextrose broth increases the probability of pellet formation with inocula containing high spore concentrations, in the case of *R. oryzae*.

2.5. pH

pH of the media is one of the parameter often neglected in fungal fermentations while it has a pronouncing effect on fungal growth. Most of the fungal species can adapt to a pH range of 4-9 while neutral pH favors sporulation and optimum growth (Cochrane, 1958). Buffering the media based on its composition is required to avoid drastic pH shifts during fungal growth. Inadequately buffered media with ammonia salts shifts to acidic pH during growth, while the media containing excess nitrates likely shifts to alkaline. Gaseous CO_2 could contribute to increasing in dissolved bicarbonates concentration due to pH influences (Papagianni, 2004). Earlier studies suggest that the response to the pH differences varies among different species and the influence of pH on pellet morphology is mainly through a change in surface properties of fungi (Metz et al., 1977).

Liu et al. (2007), indicates that the morphology of *R. oryzae* was invariable to any changes in the pH range of 2.5-7 in potato dextrose broth media. In contrast to this observation, Das and Brar (2014), noticed profound variability in growth patterns of *R. oryzae* in brewery wastewater, within the pH range of 4-9. In the latter study, loose and hairy pellets were observed at pH 5, compared to solid and non-aggregated pellets at pH 6. More aggregation of pellets was observed at pH 7, while no growth was observed beyond pH 9. These observations indicate the variability of growth patterns of same species that can be due to the differences in medium composition (Zhou et al., 2011).

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3. Significance of early-stage techno-economic analysis

The interest in renewable fuels since past few decades grew out of increased concerns regarding the excessive use of petroleum-based fuels and the damage occurring to the environment due to excessive carbon emissions. Biotechnology Energies Office, U. S. Department of Energy (2013) has reported the biofuels contributing to a reduction in reliance on fuel imports from 60% in 2005 to 40% in 2012, with a projection of continuing trend in the future. The corn ethanol industry alone has created 85,967 direct and 271,440 indirect jobs contributing to a $44 billion towards the gross domestic product of the US in the year 2015 (Renewable Fuels Association, 2016). However, bio-based technologies currently are still in their developmental stage and requires financial support in various forms such as tax incentives, research funding, and investments (Biotechnology Industry Organization, 2012).

Commercialization of a bio-based product is influenced by several factors such as feedstock cost and availability, maturity level or efficiency of the process in terms of energy requirement, and reaction rates (United States International Trade Commission, 2011). Economic feasibility of a particular process is determined through techno-economic analysis of the overall process using commercial software tools that are typically expensive and requires a detailed analysis of each unit operation. Also, the analytical information provided as inputs for the techno-economic analysis software tools has to be obtained through several expensive tests that might add up to an unnecessary expense at an early stage of a process.

Center for Biorenewable Chemicals (CBiRC) (2008), at Iowa State University, has developed an index involving Technology Readiness Level (TRL) on a scale of 1-9 to

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indicate different stage gates from innovation research to product commercialization. The TRL concept was originated in aviation, space and defense industries and was developed by NASA to monitor the progress in the technology of a particular project (Mankins, 1995; Weber et al., 2013). Figure 1 indicates the TRL index for typical innovation-based biorenewable companies with estimates for scales and costs for different phases. The information presented in Figure 1 was from the startup companies originated through CBiRC.

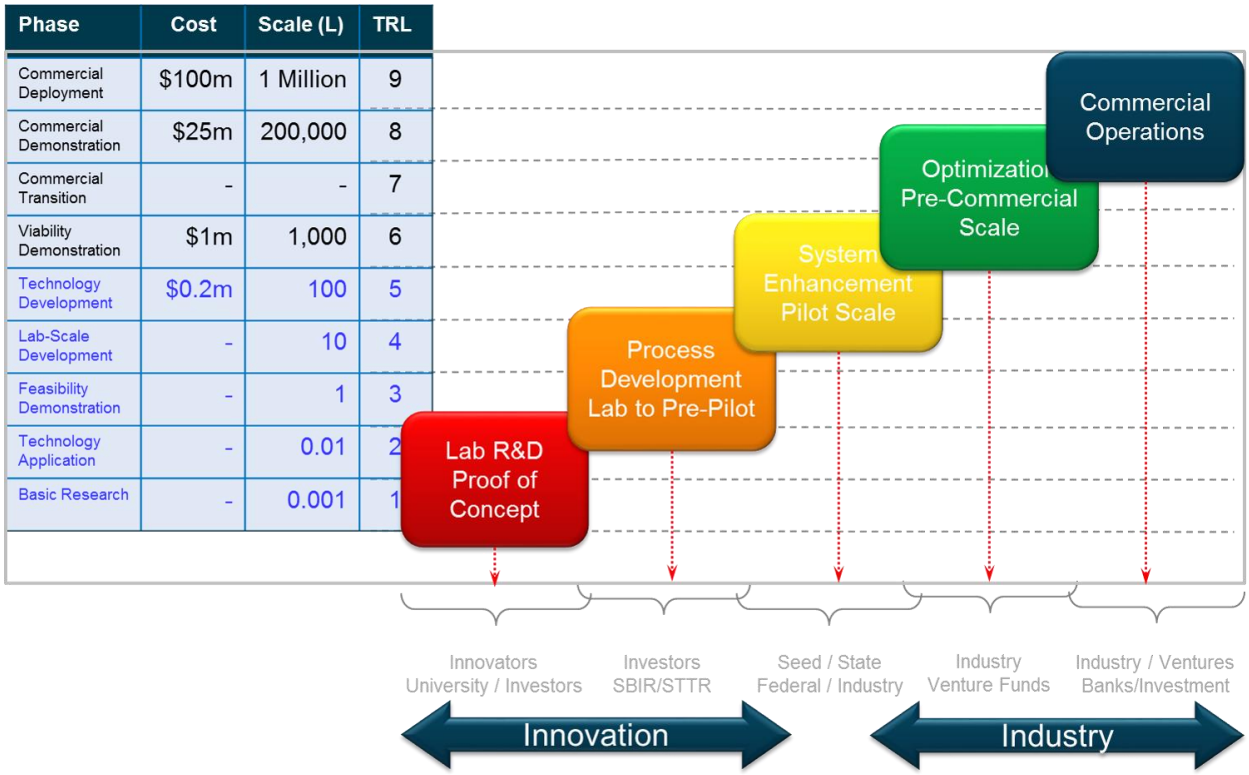

Figure 1 Technology Readiness Level (TRL) based classification for biorenewable industry (Source: Technology Readiness Levels, CBiRC, 2008).

The business evolution process involves conducting a techno-economic analysis at various levels of product/process development. Typically, software tools like SuperPro Designer®, Aspen HYSYS and CHEMCAD are used for intermediate level analysis which is

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conducted during TRL 5. Beyond that level, the techno-economic analysis is performed through direct communication with vendors. However, at TRL 3 and 4, the bottlenecks of the process should be identified from the information available at that point (CBiRC, 2008). Such analysis is typically conducted through mass and energy balance information and estimation of capital and operating costs projected for a larger scale. Viswanathan and Raman (2015) have developed an MS Excel-based tool called ESTEA (Early Stage Techno-Economic Analysis) specific for fermentative-catalytic biorefineries that provide the estimation of early stage cost analysis and greenhouse gas emissions. A modified version of ESTEA is used for the early-stage techno-economic analysis presented in chapter 3.

4. Conclusion

Fungal growth on coproducts of industrial processes is a great way to treat the organics with a potential to produce some value-added products. However, understanding the morphology of fungal growth in a particular substrate is important for process optimization. While the behavior of fungal species on a defined media is predictable to some extent, growth patterns of fungi on industrial co-products can be extremely variable. Also, the influence of fermentation parameters differs among various species, and a thorough understanding of the fungal growth pattern should be developed for a particular substrate to ensure efficacy and efficiency of the process. Also, early-stage techno-economic analysis is crucial to obtain feedback in identifying the bottlenecks of existing process and make necessary changes to ensure commercial viability of the process developed.

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CHAPTER 3
EARLY-STAGE TECHNO-ECONOMIC ANALYSIS OF RENEWABLE DIESEL PRODUCTION FROM *Rhizopus oligosporus* CULTIVATED IN CORN THIN STILLAGE

Abstract

The work investigates the economic feasibility of renewable diesel production via cultivation of fungal biomass in corn thin stillage from ethanol production, followed by hydrothermal liquefaction, and transesterification of biomass. Capital and operating costs were evaluated for all the unit operations to identify the bottlenecks involved in scaling up and commercialization of the process. The minimum selling price of the final product was estimated to be $7.47 in the worst-case scenario and $6.17 for a practical scenario respectively for a gasoline gallon equivalent of diesel at a production rate of 12000 gallons of diesel per day and 10% internal rate of return. Further research into optimization of fermentation and HTL processes was suggested to make the process economically feasible.

1. Introduction

According to the revised Renewable Fuel Standard (RFS 2) by EPA, it is mandated that 16 billion gallons of ethanol can be produced conventionally using any biomass sources through 2022 (EPA, 2010). According to RFA (2015), the annual ethanol production was 14.7 billion gallons that year.

According to RFA (2007), 82 percent of the ethanol in the US is produced from dry-milling ethanol plants. In a typical dry-milling process, six liters of stillage is produced for every liter of ethanol produced. Nearly half of the stillage, after centrifugation to thin stillage,

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is recycled as a backset (Ethanol Producer Magazine, 2006). Currently, the excess thin stillage is evaporated to a syrup, which along with wet distillers' grains is dried to produce dried distillers' grains with solubles (DDGS). The whole process is very energy intensive accounting for additional drying costs while the DDGS is often sold at a very low price within a small market (Johnson, 2013).

Cultivation of fungus on the thin stillage has a potential to produce a wide range of high-value products while removing the COD and suspended solids from the water (Rasmussen et al., 2014). One such high-value product is a biocrude produced through hydrothermal liquefaction (HTL) (McMahon, 2015) that can be turned into renewable diesel through hydrocracking and treating the biocrude. Compared to conventional biofuels like ethanol, a renewable diesel could be much more favorable as it can be used in standard diesel engines without requiring any specific modifications (Nagarajan et al., 2013).

The biocrude produced from the HTL process has significantly lower oxygen content (10-20%) compared to pyrolysis bio-oil (40%). Also, the heating value of HTL bio-oil is higher (35 MJ/kg) compared to pyrolysis bio-oil (16-19 MJ/kg) (Xu and Lad, 2008; Demirbas, 2011). HTL uses wet biomass feed that saves the energy costs in drying the feedstock compared to pyrolysis or gasification. In addition, the hot water stays liquefied inside the reactor saving up the energy penalty for vaporizing water unlike in pyrolysis or gasification (Zhu et al., 2014).

As the feasibility of HTL for commercial applications is being rigorously researched, a minimal amount has been published on techno-economic analysis of HTL feedstocks. Techno-economic analysis of liquid fuel production from woody biomass via HTL by Zhu et al. (2014) reports a minimum fuel selling price of 4.44 for a gasoline gallon equivalent

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(GGE) of the fuel with an annual production rate of 42.9 million gallons of final hydrocarbon product. Zhu et al. (2013) conducted a techno-economic analysis on liquid fuel (mainly alkanes) production using HTL and further upgrading (hydrotreating and hydrocracking) of a lipid-extracted algae feedstock. The minimum selling price range estimated was $2.07/GGE to $7.11/GGE for a feed rate of 608 metric tons/day. Jones et al. (2014) presented design and economics for the conversion of algal biomass to hydrocarbons through HTL and upgrading in which the minimum fuel selling price reported was $4.49/GGE assuming a feed consumption of 1340 U.S. tons per day of algae available at $430 per ton.

There has not been any work published reporting the costs involved in renewable diesel production using a fungal biomass feedstock. As with most of the ventures in chemical industries, a coarse assessment of costs is always necessary to assess the viability of the process and identify the limiting steps in the process to guide the development process. This study aims to model the process and evaluate the economic feasibility at an early stage. In this study, the costs involved in the production of biocrude and further upgrading to a renewable diesel product was investigated. The feedstock for the HTL was obtained through fermentation of fungus on the corn ethanol thin stillage which was accounted for in the techno-economic analysis conducted. The HTL process was coupled with a thermal cracking approach called renewable fuel production (RFP) to convert the bio-oil into a drop-in diesel fuel.

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2. Methods

The methods followed for techno-economic analysis are presented in this section. Materials and technologies are adapted from the pilot-scale work carried out in the van Leeuwen research group. A modified version of the spreadsheet-based tool called ESTEA (Early-Stage Techno-Economic Analysis) developed by Dr. Raj Raman's research group was used in developing the detailed process model. Capital and operation costs of the plant are evaluated using the available literature data.

2.1 Materials and Technologies

A variety of the key inputs provided for economic analysis and process design were obtained from pilot-scale operation of the fermentation and hydrothermal liquefaction processes. Reasonable assumptions and best design practices were incorporated to characterize the whole process. The model evaluates a practical scenario and a worst-case scenario to provide insights into the key logjams in production process proposed. By doing so, an early-stage feedback could be obtained which guides towards future research and design of the process.

The feedstock for the process is corn-ethanol thin stillage obtained as a coproduct of a typical dry-grind corn-ethanol production. Iowa being the largest producer of corn ethanol in the US has the highest number of ethanol production facilities compared to any other state (Ethanol Producer Magazine, 2016). A small-sized ethanol plant with a production capacity of 20 million gallons of ethanol per year would produce nearly 60 million gallons of thin stillage per year (Ethanol Producer Magazine, 2006). Considering this, the plant production

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capacity was assumed to be 300,000 gallons (of renewable diesel production) per year that requires nearly 58 million gallons of thin stillage as the feedstock per year.

2.2 Processes

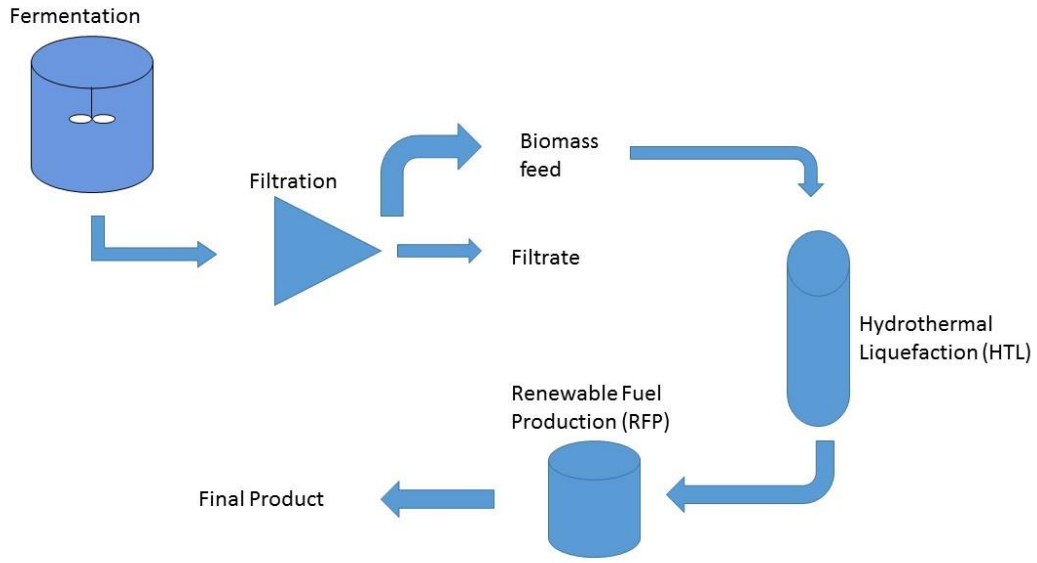

Figure 1: Generalized process flow diagram

The process model was developed using the pilot-scale fungal cultivation and the HTL operations conducted by the van Leeuwen research group at Iowa State University. Feasible design assumptions from the literature were made along with the original process for scaling up. A schematic of the generalized process flow diagram is presented in Figure 1 above. The process assumes that fermentation of thin stillage is the first step of the production process.

The biomass from the fermentation is harvested by filtration to obtain a 10% solids biomass solution. The feed should be homogenized using a high-shear mixer to achieve a desirable particle size before feeding into the HTL reactor. Later on, the feed is pumped

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through a preheater for initial heating of the feed to nearly 133°C. The feed is then pumped into the HTL reactor where it undergoes liquefaction under supercritical conditions. Under these conditions, the feed undergoes a series of chemical reactions producing an output called biocrude once filtered for solids (biochar). The filtrate is extracted using an organic solvent to produce bio-oil. This oil is supplied to the RFP reactor to undergo thermal cracking and produce renewable diesel that can be used as a drop-in fuel product.

Mass balance and volume balance tables were created for all the processes listed, and a final mass balance was made combining all the mass balance tables of individual processes.

Fermentation

The process parameters for fermentation process were obtained from pilot-scale cultivation tests carried out with a 1600L airlift bioreactor at BECON facility. The strain *Rhizopus microsporus* var. *oligosporus* was used for fungal biomass production. The yield was determined to be 10 g/L (Suesse, 2016). The productivity and titer were calculated and found to be 0.21 g/L/h and 0.35 $g_{biomass}/g_{TS}$ respectively. The inoculum for the fermentation was prepared through the cultivation of spores at a concentration of 0.5 v/v% on Yeast Malt (YM) Broth (Suesse, 2016). The assumptions considered for the cost analysis of fermentation process are presented in Table 1 below.

Table 1: Fermentation process and cost assumptions

Maximum Size of the Fermenter	3785	m^3	(Humbird et al., 2011)
Usable Percentage	80%	m^3used/m^3purchased	(Cysewski & Wilke, 1978)
Base Size	757	m^3	(Humbird et al., 2011)
Base Cost	$ 590,000	USD	
Scaling Exponent	0.7	Dimensionless	
Fermenter Downtime	10%	h/h	Assumption

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Fermentation time was determined to be 48 h through the lab-scale and pilot-scale experiments. The productivity was amended considering a down-time percentage of 10% between two fermentation batches. Fermenter working volume was assumed to be 80%. Although CO_2 and filtrate from the process could be valuable, by-product economic value is not considered in the model.

Since the biomass production is the key requirement for the whole process, a total number of annual batches were calculated using the equation 1.

$$N_b = \frac{N_d}{t_{fm}} \quad (1)$$

Where,

N_b – Number of annual batches produced

N_d – Number of days of plant operation

t_{fm} – Total time to complete a fermentation batch (days)

From the number of annual batches, the overall plant productivity, the annual thin stillage required, and the size of the fermenter required were computed.

Filtration

Filtration is carried out for the biomass recovery from the fermentation broth. According to Koza (2012), a custom-built tangential filter screen can achieve up to 15% suspended solids concentration through simple gravity filtration. Since the maximum requirement for the HTL feed assumed in the study was only 15% suspended solids, gravity filtration was assumed to be the cheapest possible method to achieve the desired percentage suspended solids. Filtration calculations and assumptions are listed in Table 2 below.

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Table 2: Filtration process and cost assumptions

Cake thickness	0.05	m	Assumptions
Residence time per batch	1	min	
Filtration time	8	h	
Total operation time	9	h	
Total backwash time	1	h	
Backwash & cleaning time	0.1	h BW/h Fltr.	
Base Cost	$ 50		twpinc.com

A one m^2 filter mesh was assumed as the standard size of each filter unit, and the number of units was computed using the total filtration area required and the area of each unit. Filtration time for a micro batch is assumed to be one minute to achieve 10% suspended solids while the filtrate water can be mixed back with the filter cake to make up for 10% suspended solids in the case of excessive filtration. Average cake thickness per each micro batch was assumed to be five cm.

Operation time for each filtration batch was assumed to be 8 h. An additional down-time percentage of 10% between two filtration batches was assumed. From the volumetric production per batch of fermentation and the operation time (filtration) assumed, the filtration rate ($m^3/m^2/hr$) and the volumetric flow rate (m^3/hr) were computed.

Hydrothermal Liquefaction (HTL)

A continuous liquefaction process was assumed for the HTL process design. The optimum temperature and retention time were determined to be 350°C and 15 minutes

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respectively. Biocrude yield at the given conditions was reported as 55 $g_{biocrude}/g_{biomass}$% (Suesse, 2016). The process components were considered as individual unit operations occurring simultaneously as a continuous process. The HTL process has not been demonstrated at industrial-scale yet. Therefore, the costs of individual components of HTL process were compiled to compute the cost of the HTL reactor. The process flow is represented in figure 2 below.

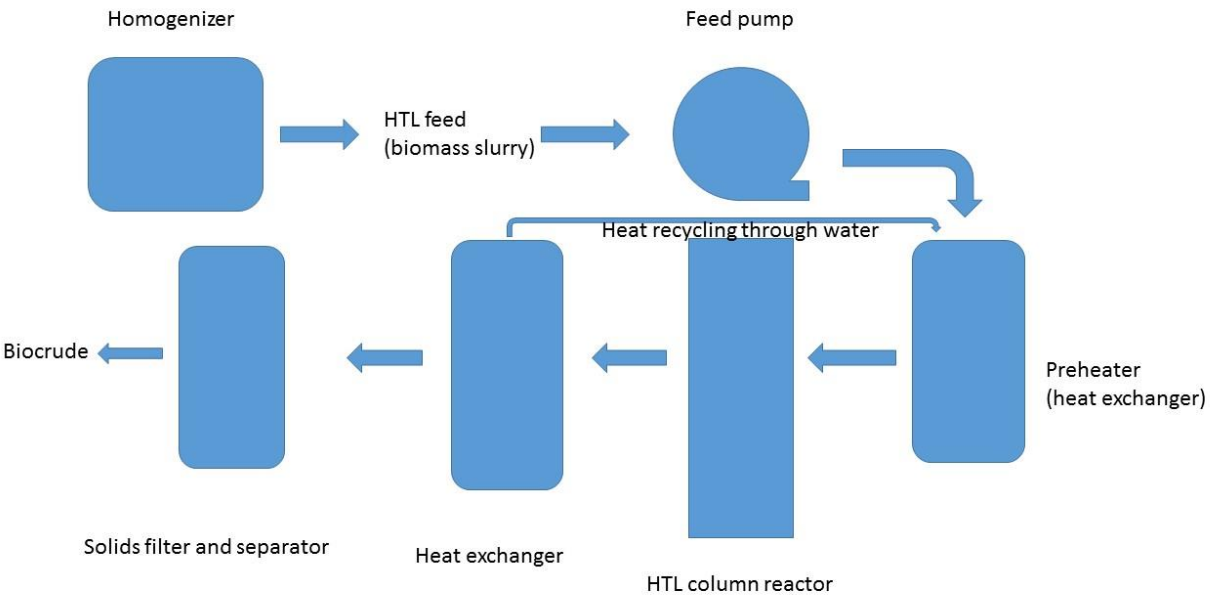

Figure 2: HTL process flow diagram

The feed enters the homogenizer which is a high-shear mixer that produces a finely ground liquid feed. A 20 gpm pump is used to account for the pressure requirement to maintain, near supercritical conditions along with required flow rate. The feed is pumped through a recycle heater which is used as a preheater to increase the temperature of the feed to about 133°C. Preheat temperatures above 133°C were not recommended by Elliot et al. (2013) to avoid baking of fungal slurry onto the walls of the preheater which could possibly

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decrease the flow diameter over the time. Process parameters and the assumptions for the HTL process are enlisted in Table 3 below.

Table 3: HTL process assumptions

Solids percentage	10%		Assumption
pump base size	190	gpm	**Knorr et al. (2013)**
Pump base cost	$ 364,700.00		
preheater base size	3000	ft^2	
preheater base cost	$ 1,965,333.00		
Reactor base size	480	ft	
Reactor base cost	$ 272,788.00		
Heater base size	6032	ft^2	
Heater base cost	$ 998,850.00		
Heat exchanger base size	3255	ft^2	
Heat exchanger base cost	$ 63,900.00		
Separator base size	3689	gpm	
Separator base cost	$ 3,565,000.00		
Solids filter base size	3689	gpm	
Solids filter base cost	$ 1,311,000.00		

The feed is then pumped into the HTL reactor where the biomass feed is converted to biocrude under near supercritical conditions (350°C temperature and 4000 psi pressure). The reactor design was adopted from the Knorr et al. (2013) where the reactor length is computed to be 76 ft. that consists of seven 8 ft. long tubes connected by hairpin turns. The output produced at 350°C goes through a heat exchanger which decreases the solution temperature nearly to room temperature. The solution further goes through a solids filter followed by a separator to extract biocrude, filtering the undissolved solids and water. The solvent used for the extraction process is methylene chloride which is recycled through evaporation.

The water from the heat exchanger is recycled through the preheater to save up on energy costs involved. However, the biogas and biochar produced during the process were

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not considered of any economic value. All the equipment sizes considered were based on the required flow rate which is 13.5 gpm approximately.

Renewable Fuel Production (RFP)

The Renewable Fuel Production (RFP) is the final step of the process that involves the production of renewable diesel using the bio-oil from the HTL process. The design considered in this step was adopted from the full-scale RFP reactor developed by the company, RFP Inc. based in Huntington Beach, CA. The biocrude undergoes a process of thermal cracking producing renewable diesel with 80 v/v% yields. The solid wastes produced from the process are recycled as fuel for the burners used in the operation. The key process parameters and assumptions that were considered are presented in Table 4 below.

Table 4: RFP process assumptions

Base Size	0.06	m^3	Bell (2016)
Scaling Component	1		
Base Cost	$ 25,000.00		
Lang Factor	3		

The process was designed to be continuous with a residence time of 1 h approximately (typically lesser) and a throughput rate of 0.16 m^3/h.

3. Economic Analysis

Literature data and company quotes were utilized to estimate the equipment costs and the other capital costs involved throughout the operation. Unit costs for the equipment were scaled using standard scaling laws that correlate equipment cost to equipment size through equation (2).

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$$Cost_n = Cost_0 \times \left(\frac{Size_n}{Size_0}\right)^x \quad (2)$$

Where,

$Cost_n$ - Cost of newly sized equipment

$Cost_0$ – Cost of the base size equipment

$Size_n$ – New size of the equipment

$Size_0$ – Base size of the equipment

x – Scaling factor

The scaling factor values specific to a particular type of equipment and a range of values from 0.6 to 1 has been utilized in the current analysis. The capital cost estimation approach in the model computed major equipment costs (*i.e.,* purchasing cost of the piece of equipment) for every unit operation and then converted the total equipment cost into the total plant cost by the use of a Lang factor (Peters et al., 2003).

Lang factor accounts for cost factors such as piping costs, labor costs for installation, auxiliary costs, engineering expenses and construction overhead. Such an estimation incorporates the total construction costs with all major and minor facilities on previously undeveloped land. Alternative estimation methods incorporate a set of installation factors and define the capital expenditure directed towards individual plant construction processes. Lang factor is a simple aggregation of all the individual processes involved. Lang factors 6 and 5 were chosen for the worst-case scenario and the practical scenario respectively.

As the capital costs were computed as described above, the total annual capital payments were computed assuming a 10-year payout period, and 10% internal rate of return (IRR). The total plant operating time was required for computation of process flows and was

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assumed as presented in Table 5 below. Labor costs were estimated based on the unit operations and the equipment involved in the processes (Peters et al., 2003).

Table 5: Process Assumptions

Lang Factor	6	Dimensionless	Assumptions
Operating Days (OpDays)	300	days	
Mass Ratio	1.4	Dimensionless	
IRR (%)	10%	Dimensionless	
Plant Life	10	Years	

The annual production and the plant operating time directed the sizing of the constituent unit operations. The data was utilized for identification of baseline equipment costs that were used to compute overall capital requirements as explained before. The annual costs were allocated through amortization of the overall production costs at the IRR assumed and a 10-year payout period.

Operating costs

There is always some degree of fluctuation in operating costs which is mainly due to factors like oil prices, annual corn crop yields, and solvent prices. However, the feedstock considered in this model has a very low demand, and the price was assumed to be $0.01 per gallon of thin stillage, which is still considered higher taking into account the drying costs involved in DDGS production (Ethanol Producer Magazine, 2006). All the other operating costs were obtained from ICIS and literature and are listed in Table 6.

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Table 6: Cost Assumptions

Water ($/kg)	0.004	EIA Monthly Energy Review (March 2016)
Electricity ($/kWh)	0.06	
Wastewater ($/kg)	0.00053	
Steam ($/kg)	0.005	
Thin stillage ($/kg)	0.0026	Assumption
Methylene Chloride ($/kg)	0.97	ICIS

4. Results and Analysis

4.1. Process modeling

The HTL process used in the model was not demonstrated at the industrial-scale, as the fermentation and the filtration. Therefore, a feasible process design was assumed for the HTL process. RFP design was adopted from the 60 L capacity continuous process developed by RFP Inc., Huntington Beach, CA, USA. All the HTL capital costs were estimated as a sum of the unit process equipment used in the reactor (Knorr et al., 2013). The base size and cost for the RFP process were quoted by RFP Inc., Huntington Beach, CA, USA.

4.2. Economics Results

Since the analysis aims towards providing insights and early stage feedback on the plausibility of technology at commercial scale, three cases were studied to understand the variation in MSP with annual production change. The three cases selected were based on the production capacities of corn-ethanol plants in Iowa (Ethanol Producer Magazine, 2016). The

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smallest production capacity considered was 20 MGPY, followed by a medium and large production facilities with 55 and 240 MGPY capacities respectively.

These three cases were estimated and compared in a worst-case scenario and a practical scenario. The worst-case scenario assumes all the design and cost factors as discussed in the earlier sections while the practical scenario makes a few reasonable assumptions that are achievable with large-scale production.

4.2.1. Worst-case scenario

The total installed equipment cost was estimated to be $10 million for a 1000 gal d^{-1} production facility. The total annual costs were $5 million which were computed through amortization as mentioned in Economic Analysis section. The Minimum Selling Price (MSP) of the product was observed to be $10.76/GGE for a 1000 gal d^{-1} production plant using worst-case scenario. Figure 3 represents the cost distribution of the constituent processes in a worst-case scenario. The three bars correspond to production capacities of 1000 gal d^{-1}, 2750 gal d^{-1}, and 12000 gal d^{-1} respectively.

Figure 4 represents the variation in MSP with respect to change in annual production capacities. A clear decreasing trend could be observed for MSP with increasing annual production. At a fixed IRR of 10%, there was a steep decrease in MSP from $10.4 to $7.47 per gallon product, as the plant size is increased from 1 to 10 kTA.

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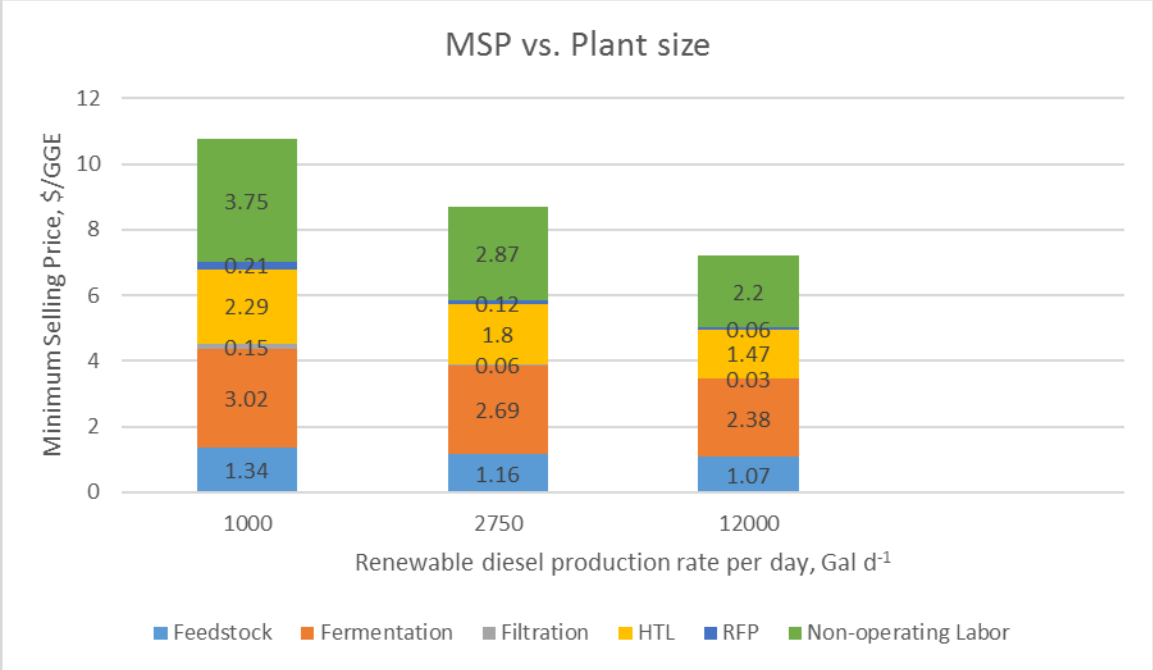

Figure 3: Cost contribution of various components for MSP for different production scales in worst-case scenario

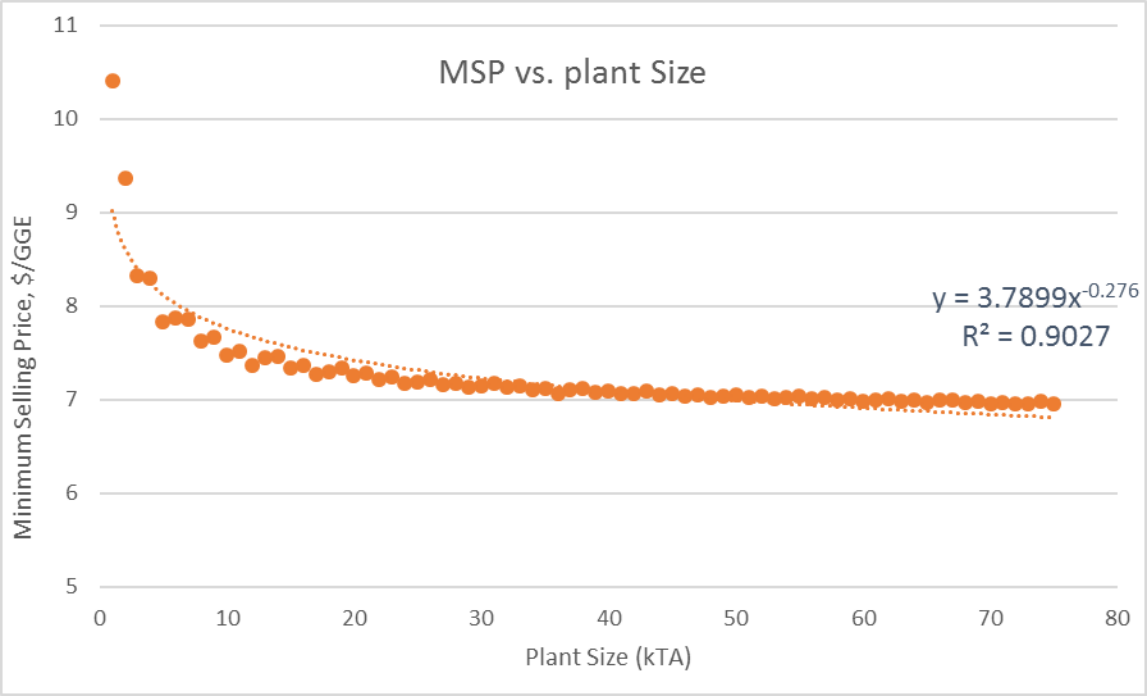

Figure 4: Variation of MSP with plant size in worst-case scenario

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4.2.2. Practical scenario

For a 1000 gal d^{-1} production capacity, the installed equipment cost was estimated to be $9 million with an MSP of $9.02/GGE. The total annual costs were computed to be $5 million. Figure 5 depicted below represents the distribution of costs for three production capacities, *i.e.*, 1000, 2750, and 12000 gal d^{-1} respectively. The MSP computed for the three cases was observed to be $9.02, $7.26, and $5.98 per gallon product respectively.

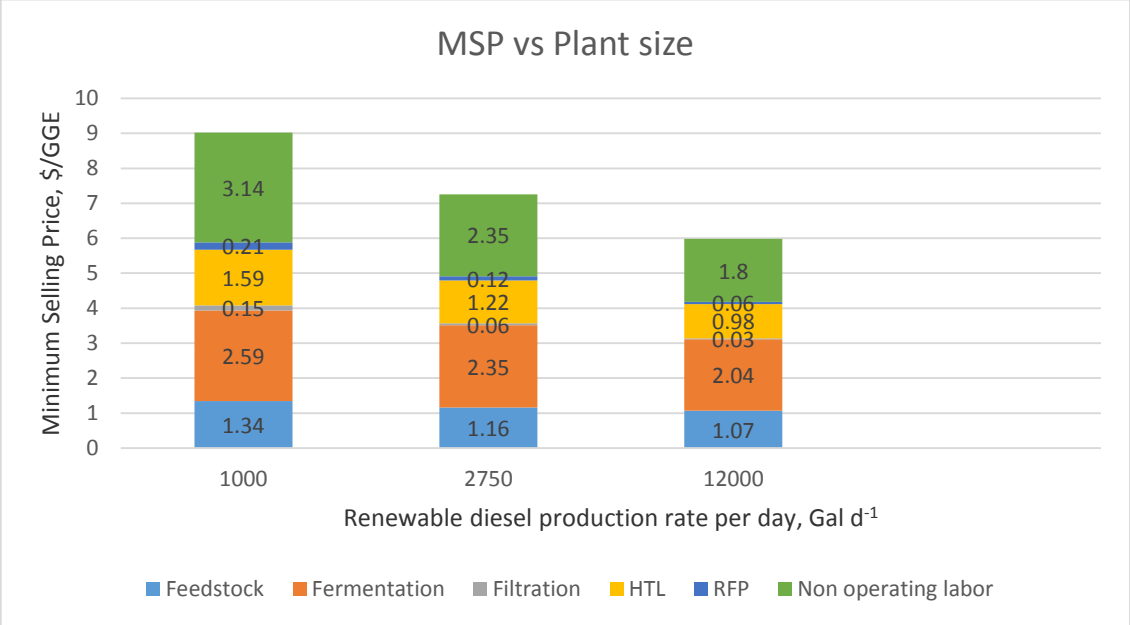

Figure 5: Cost contribution of various components for MSP for different production scales in practical scenario

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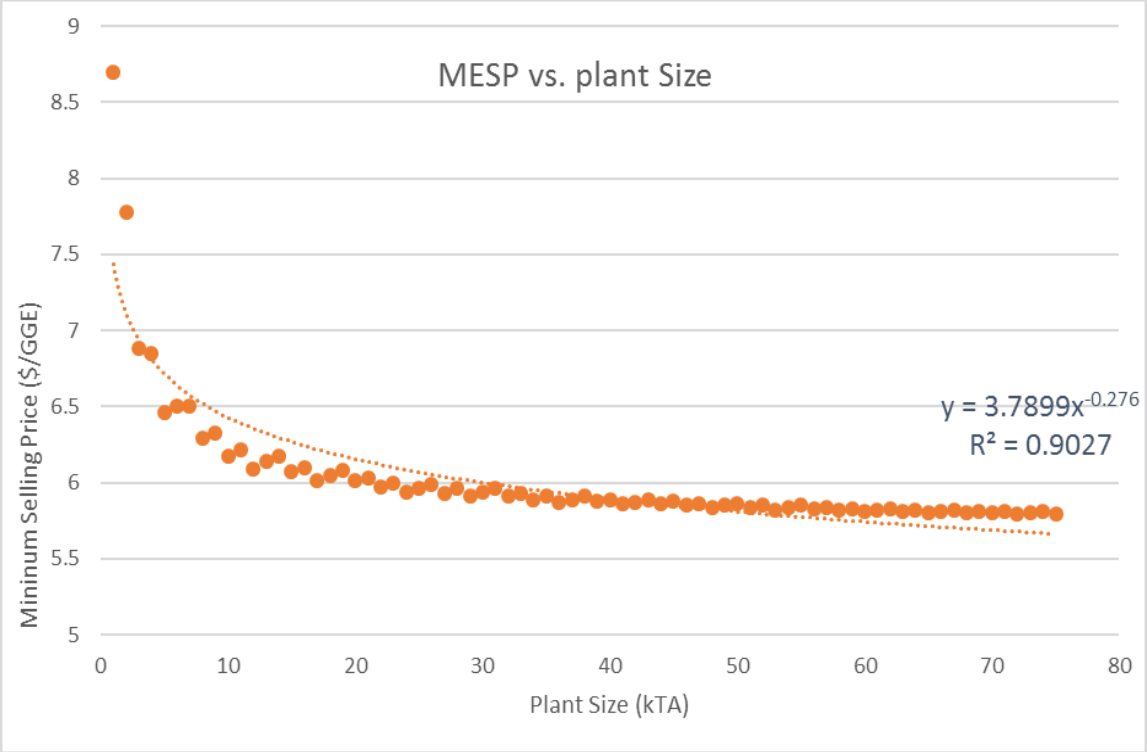

Figure 6: Variation of MSP with plant size in practical scenario

Figure 6 shown above exhibits a similar trend as observed in the worst case scenario as there was a steep decrease in MSP from $8.70 to $6.17 per gallon when the plant size was increased from 1 to 10 kTA.

4.2.3. Uncertainty Analysis

Sensitivity analyses are presented in figures 7&8 to determine the sensitivity of MSP to changes in parameters and process inputs respectively. The parameters considered for sensitivity analysis were the number of operating days, IRR, plant operating life and Lang Factor. From the figures 3&5 fermentation and HTL were observed to be highest contributors towards the MSP. Therefore, the model inputs considered were productivity, titer, yield, and percentage solids in HTL feed. The model inputs selected for the analysis were based on the figures 3&5 where fermentation and HTL were observed to be the major contributors for the

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MSP. The sensitivity was computed at ±10% change in all the parameters and inputs, and the results were presented in terms of the percentage increase or decrease in costs.

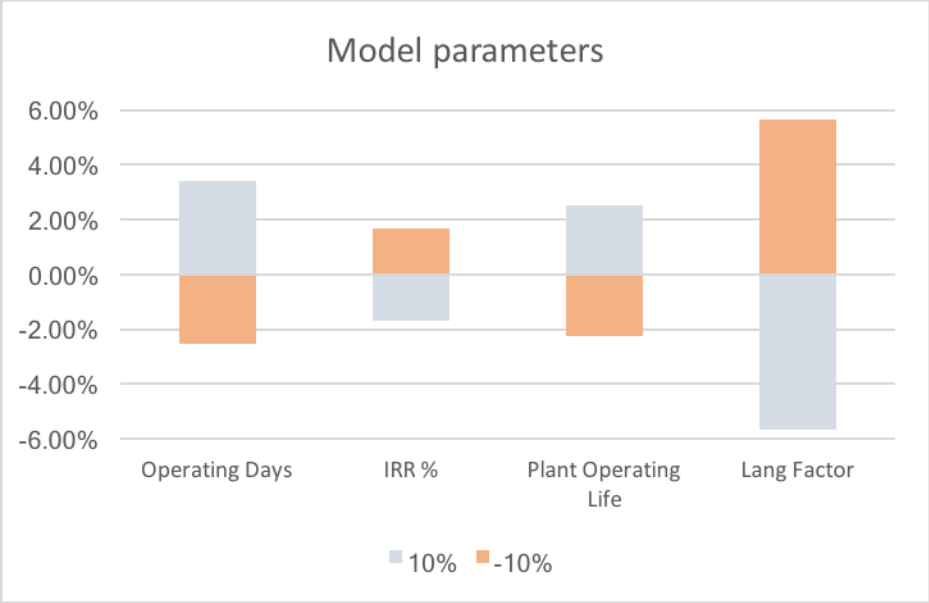

Figure 7: Percentage variation of MSP with ±10% variation in selected model parameters

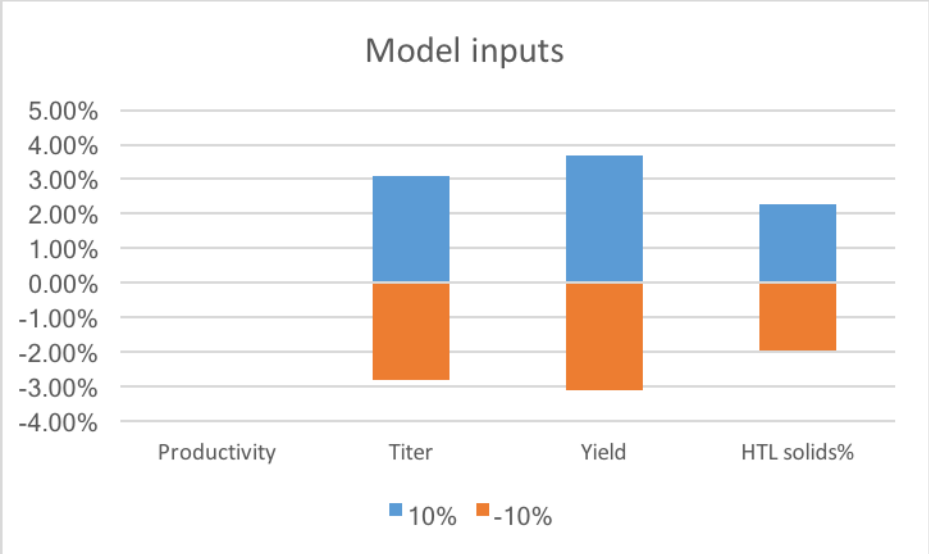

Figure 8: Percentage variation of MSP with ±10% variation in selected model inputs

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From the figures 7&8 presented above, it can be construed that the MSP is more sensitive to Lang factor and is influenced very least by IRR among the parameters studied. Also, both the fermentation titer and yield were observed to be more influential in determining the MSP compared to productivity.

The sensitivity analyses study the effect of one parameter on the MSP while assuming all the other parameters to be constant. In retrospect, several parameters could be dynamic, simultaneously in reality. Much more sophisticated uncertainty analyses such as Monte-Carlo simulations are to be used to understand the effect of a set of key parameters interacting at the same time.

5. Discussion

Renewable diesel production through hydrothermal liquefaction (HTL) of biomass is a process that's being rigorously researched. Several feedstocks are being tested while the commercial production of fuels through HTL process has not been achieved yet. Many studies have suggested the HTL process to be capital intensive, requiring the production facility to be on a fairly high scale for the minimum feasibility of process commercialization (Zhu et al., 2014; Liu et al., 2013; Zhu at al., 2013; Jones et al., 2012). Results from the current study suggest that the HTL costs could account for 15-25% of the MSP, contributing within a range of $0.98 to $2.29 for a gallon diesel. While the capital costs being unavoidable, electricity costs for HTL were found to be accountable for 45-70% of total HTL costs involved. This could be reduced by encompassing temperature and pressure recovery strategies.

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Several studies considered the solids percentage of the HTL feed to be between 15-25% (Zhu et al., 2014; Zhu et al., 2013; Knorr et al., 2013; Jones et al., 2012). However, pilot-scale studies conducted by the van Leeuwen group supported only 4% solids due to piping constraints. The highest solids percentage assumed in current analysis was 15% which is in the practical scenario. From the literature data, it can be claimed that the solids percentage can be improved beyond 15% with further research focusing on the issue. Nonetheless, the filter screens are limited for the dewatering up to 15%, and the additional dewatering attempts could potentially vary the MSP, due to additional dewatering costs and the resultant low percentage solids.

Fermentation was observed to be another major roadblock in the path of process commercialization. Results from the current study suggest that fermentation process solely is contributing for about 25-35% of the MSP. The uncertainty analysis was used to test the claim and both the yield and titer were observed to be the most sensitive model inputs among the model inputs tested. The yield and titer (35% and 10 gL^{-1} respectively) were obtained from the pilot-scale cultivation studies. However, the average titer of about 20 gL^{-1} was observed during the lab scale cultivation studies in the past (Suesse, 2016). Investigation on improving the growth yields and titer could certainly decrease the fermentation costs to a considerable degree.

A steep decrease was observed in a variation of the MSP with an increase in production capacity up to 10 kilotons per annum (kTA) of renewable diesel production, while the increase in plant capacity showed a nearly negligible decrease in MSP after 20 kTA production. These observations are in agreement with economies of scale where larger capacities would be in favor. The largest corn-ethanol plant in Iowa can achieve nearly 10

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kTA production. To adapt this technology for various plant capacities, establishing a centralized facility within the nearest vicinity of multiple corn-ethanol plants, would be a possible approach. However, the transportation costs could be mounting in that scenario. Another possible approach could be establishing decentralized fermentation and filtration facilities along with a centralized HTL and RFP facility. However, the feasibility of these approaches should be tested through more extensive techno-economic analysis.

Conclusion

While the current production process is far from commercialization, improvements to the fermentation and HTL processes could improve the feasibility of the commercialization path. The study could be considered a first pass analysis of the project providing early feedback and insights on the plausibility of the process. On the other hand, several uncertainties are present in the study that needs to be addressed for a better techno-economic analysis. Use of techniques like Monte-Carlo simulations and other advanced uncertainty analysis tools is required to foresee the risks upon the process developments suggested. Economic analysis is not a single pass process but an iterative process that should undergo revision as the research progresses.

General Conclusion

With current diesel prices around $2.2 per gallon, our process is far from commercialization at this point. Provided the research strategies become successful, the prices of around $4 could be projected with a practical scenario and 240 MGPY ethanol facility. Making use of tax credits on biodiesel could bring the MSP further down. Non-

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operating labor contributes for a major fraction of the MSP. With fluctuating diesel prices and limited fossil fuels, this process could be commercialized in the future.

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CHAPTER 4

EFFECT OF FERMENTATION PARAMETERS ON GROWTH RATE AND PELLET MORPHOLOGY IN *Rhizopus microsporus* var. *oligosporus*

Abstract

The study examined the feasibility of cultivation of *Rhizopus microsporus* var. *oligosporus* with pellet morphology on thin stillage (TS) from dry-grind ethanol production. Cultivation of fungus on this corn-ethanol coproduct can produce a range of high-value products such as chitosan, high-protein animal feed, nutraceuticals and renewable fuel feedstock. However, the growth of fungi as dispersed mycelia adds up to downstream processing costs and makes the dewatering process energy-intensive. Dispersed growth also affects process efficiency by clogging the pipes and causing impaired functioning of bioreactors. Lab-scale testing with 100 mL cultures indicated that high temperatures and low oxygen availability could be the reasons for deviation from pellet morphology. Also, storage of TS for longer than three weeks was observed to be contributing to the decline in biomass yields. While both 25°C and 30°C were observed to be desirable temperatures for pellet growth, a maximum yield of 17.44 g/L was obtained with 30°C temperature and 10% inoculum concentration with pellet morphology.

1. Introduction

The Renewable Fuels Association (RFA, 2016) reported an annual production of 14.7 billion gallons of corn ethanol in the US in 2014. The Renewable Fuel Standard (RFS2) set up by EPA mandates a minimum annual production of 15 billion gallons of corn ethanol by

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2022. RFA (2016) has reported an improvement in net energy balance ratio of corn ethanol from 1.9-2.3 (Shapouri et al., 2010) in 2008 to an average of 2.6-2.8 with the highest efficiencies of 3.4 in the case of dry-milling biorefineries and 4.0 in the case of wet-milling biorefineries in 2016. Considering the new plants being constructed and the retrofits made to the existing facilities, further increases in production volume and energy efficiencies can be projected (RFA, 2016). An increase in the corn-ethanol coproducts can be expected as a consequence of developments reported.

Conventional dry-grind ethanol plants produce 5-6 gallons of stillage per gallon of ethanol produced. The stillage is centrifuged to produce the top liquid phase (thin stillage) and the denser bottom phase (wet distillers' grains). Less than half of the thin stillage is recycled directly as backset (Ethanol Producer Magazine, 2006). The other half goes through several energy-intensive evaporators to produce a condensed syrup that is mixed with wet distillers' grains and dried into dried distillers' grains with solubles (also called DDGS) (Kim et al., 2008). The DDGS is typically sold at low margins as animal feed (Moreau et al., 2011).

The composition of thin stillage mainly constitutes sugars, oils, protein, corn fiber and the unfermented components of grain (Kim et al., 2008). With all these constituents, thin stillage can be qualified as a complex media for biomass cultivation. Cultivation of fungi on food processing wastewater has been demonstrated to produce valuable biomass, with a multitude of applications, along with water reclamation (Jin et al., 1998). Rasmussen et al. (2014) have demonstrated a removal of 80% COD, 98% suspended solids and 100% organic acids and glycerol with fungal cultivation in thin stillage, which makes it suitable for in-plant reuse.

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Rhizopus. microsporus var. oligosporus has been one of the widely used microbes in the food industry as tempeh starter (Shurtleff and Aoyagi, 2011) and classified as a domesticated variant that is isolated only from food sources (Samson, 1985). Successful cultivation of *R. microsporus var. oligosporus* on corn wet-milling streams was demonstrated for 80-90% COD reduction (Gautam et al., 2002; Sankaran et al., 2008). Cultivation of *R. microsporus* var. *oligosporus* on low-value thin stillage has the potential for production of a variety of high-value products such as chitosan, bio-crude, animal feed, enzymes and nutraceuticals (Ravi, 2014; Erickson, 2012).

The growth of filamentous fungi like *R. microsporus* var. *oligosporus* is observed in three major morphological forms *viz.*, suspended mycelia, clumps and pellets in submerged cultures (Metz et al., 1977). In bioreactors, suspended mycelia and clump morphologies not only increase the broth viscosity but also blocks the liquid flow channels and wrap around impellers preventing nutrient transport (van Suijdam et al., 1980). All these result in reduced bioreactor performance and eventually account for excessive maintenance costs. Pellet morphology enables decreased broth viscosity, which provides improved aeration, agitation, and mass and heat transfer. Also, pellet morphology facilitates easier cell harvesting, minimizing the energy costs for dewatering and cell recovery (Gibbs et al., 2000). Sparringa and Owens (1999) have reported that *R. microsporus var. oligosporus* can be grown as fungal pellets under controlled conditions. While most of the past research has reported morphology of fungus in synthetic media, much less research has been published on growth morphology of *R. microsporus* var. *oligosporus* in complex media like thin stillage.

The purpose of this study is to observe the effect of fermentation conditions, specifically the temperature and inoculum size on fungal growth rate and morphology. All

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the growth studies were conducted in shake-flask cultures and triplicates for statistical analysis.

2. Materials and Methods

2.1. Thin stillage (TS)

Thin stillage (TS) was obtained from Golden Grain Energy LLC., a dry-grind ethanol plant located in Mason City, Iowa. TS was drawn from the industrial scale collection tank into 50 L carboys that were steam sterilized for 15 minutes at 121°C and 15 psi pressure before collection. TS was collected at 80°C into the 50 L steam sterilized carboys and cooled down to less than 40°C. TS was stored in refrigerators at a temperature of 10°C upon cooling down to less than 40°C. The composition of typical thin stillage is represented in Table 1 (Rasmussen et al., 2014). The exact concentrations might vary in different batches of TS.

Table 1: Composition of typical thin stillage (Rasmussen et al., 2014)

Component in thin stillage	Concentration
COD	90 g/L
Total Sugars	17 g/L
Reducing Sugars	6 g/L
Suspended solids	20-30 g/L
Nitrogen	0.6 wt. %

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2.2. Cultivation

2.2.1. Fungal strain

R. oligosporus was obtained from the American type culture collection (ATCC 22959, Rockville, MD, USA) as a freeze-dried culture. Methods for cultivation, harvesting, and storage of spores was adopted from the methods used by Ozsoy et al. (2008). Spores were cultured on HIMEDIA™ RM301 agar plates at 30˚C for 36-48 hours under aseptic conditions.

A solution of 0.85% sodium chloride and 0.05% polysorbate 80 with distilled and deionized water was used to collect the spores. The solution with spores was passed through a 50 mL syringe filled with glass wool to separate mycelia from spores. The filtrate was mixed with yeast maltose (YM) broth (DIFCO Laboratories, Detroit, MI) in 1:1 ratio and a 20% glycerin was added to the final solution for cryo-preservation. The spore stock solution was preserved in 2 mL cryo-vials at -80˚C. The average spore count obtained through dilution plating was 12330 CFU/ mL spore suspension (colony forming units, CFU).

2.2.2. Inoculum preparation

Mycelial seed cultures of *R. microsporus* var. *oligosporus* were prepared in 2-L Erlenmeyer flasks. One L of culture media was prepared in a 2-L Erlenmeyer flask by dissolving 21g of dry yeast maltose extract solution in distilled and deionized water. The flasks containing media were covered with Kimberly-Clark KimGuard™ KC200 sterilization wrap and steam sterilized at 121°C and 15 psi for 15 minutes. Sterilized Erlenmeyer flasks were cooled down to lukewarm temperature and were inoculated with 2 mL of spore

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suspension under the biosafety hood. Flasks were incubated in a shaker incubator (Lab Companion™ IS 971) at 35°C and 200 rpm for 24 h.

Fungal spores germinated on YM broth grew out into miniature pellets or filamentous flocs. The culture broth is well mixed, pipetted and added to the thin stillage as inoculum at desired concentrations (in v/v %).

2.2.3. Fungal cultivation on thin stillage

Thin stillage stored at 10°C was used as media for cultivation of *R. oligosporus* in 250 mL Erlenmeyer flasks. All the cultivation flasks were steam sterilized at 121°C and 15 psi (100kPa) for 15 minutes by covering with Kimberly-Clark KimGuard™ KC200 sterilization wrap. After cooling down to lukewarm temperature, 100 mL of thin stillage was transferred to each flask under a biohood. Four different inoculum concentrations (2.5, 5, 7.5, and 10 v/v%) and three different temperatures (25°C, 30°C and 35°C) were tested for their influence on fungal growth and morphology. Sterilized pipette tips were used to transfer the inoculum to the cultivation flasks. The shaker was maintained at a constant speed of 200 rpm in a Lab Companion™ IS 971 shaker incubator, throughout the experiment. All flasks were incubated for about 48 hours.

2.2.4. Harvesting and Yield determination

Fungal biomass was harvested by pouring the contents of 250 mL culture flasks into a fine mesh strainer. Upon dewatering, fungal biomass was collected into pre-weighed drying dishes. All the dishes with harvested fungus were oven dried at 70°C for 48 hours for complete evaporation of moisture. The temperature was chosen to allow complete drying of

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the biomass without causing Maillard reactions. The dishes were weighed after 48 hours, and the dry fungal yield was determined in g/L. All the tests were performed in triplicate. Averages were reported in all the tests. The standard deviation was used to measure variability and the significance of results.

2.2.5. Statistical Analysis

Statistical analyses were conducted to find the significance of biomass yields and the effect of inoculum concentration, temperature, and age of thin stillage. A Tukey's honest significant difference (HSD) test was conducted using the JMP software (SAS Institute, Cary, NC, USA). P values greater than 0.05 were determined to show the significance of results.

3. Results

Growth tests were conducted in unopened one-week old thin stillage, three-weeks-old thin stillage that was opened once to compare the effect of age of the thin stillage on fungal morphology. Fungal morphology and biomass yields were compared for four different inoculum concentrations, at three different temperatures. All the data was obtained in three replicates, and the averages were represented as bar graphs for ease in comparison. The standard deviation was represented in the error bars to test the significance of the data.

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3.1. One-week-old thin stillage

On one-week old thin stillage, pellet morphology was observed in all the cultures at both 25°C and 30°C irrespective of the inoculum concentration. Dispersed morphology was observed in all the cultures at 35°C. Fungal morphology was completely dispersed in all inoculum concentrations at 35°C. The highest growth was observed at 7.5% inoculum and 35°C (17.89 g/L). Highest growth with pellet morphology was observed at 10% inoculum and 30°C (17.44 g/L).

3.2. Three-week-old thin stillage

On three-week-old thin stillage, dispersed morphology was observed at all temperatures and inoculum concentrations. The decrease in yield was seen in all the growth cultures compared to one-week old thin stillage. The highest yield was observed to be 12.03 g/L at 7.5% inoculum and 35°C.

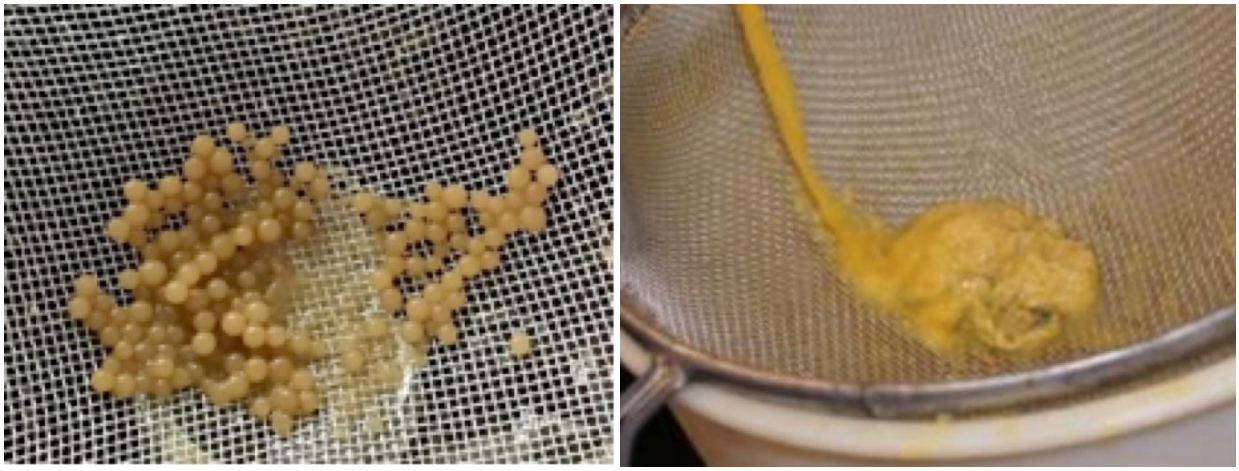

Figure 1. *Rhizopus microsporus* var. *oligosporus* with (a) pellet morphology and (b) dispersed morphology.

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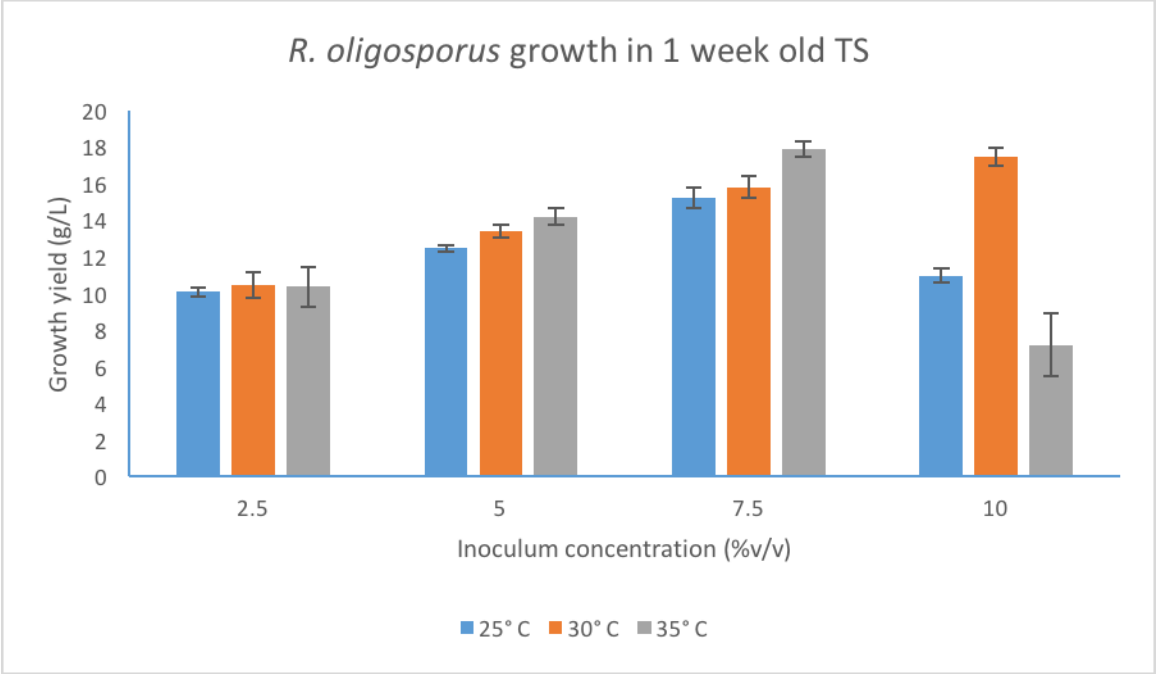
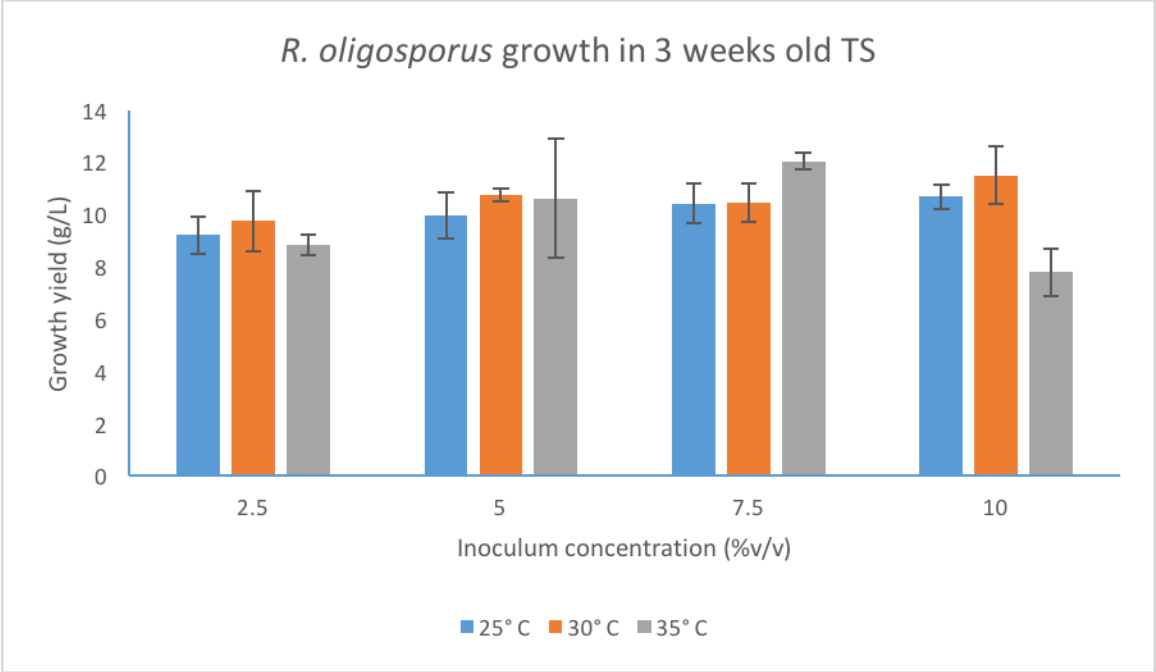


Graph 1. Growth yields at different temperatures and different inoculum concentrations in 1-week old thin stillage inoculated with *Rhizopus oligosporus*



Graph 2. Growth yields at different temperatures and different inoculum concentrations in 3-weeks old thin stillage inoculated with *Rhizopus oligosporus*

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4. Discussion

Pellet morphology was observed with fresh thin stillage while the same fermentation conditions used on three-weeks old thin stillage resulted in dispersed growth. The biomass yield in three-week-old thin stillage decreased to 12.03 g/L from 17.89 g/L in one-week old thin stillage with the same cultivation conditions. Studies (Suesse, 2016; McMahon, 2015; Erickson, 2012) with similar findings have indicated the increase in the COD of thin stillage with increased storage time in the previously opened carboys. Previous studies (Tabak and Bridge Cooke, 1968; Kubicek et al., 1980; Vardar and Lilly, 1982; Papagianni, 2004) indicate that increased demand for oxygen has a deteriorating effect on biomass yields and pellet morphology in filamentous fungi.

From Figure 1, an increasing trend can be observed in biomass yields with increasing inoculum concentrations indicating the high nutritional value of thin stillage. However, decreased growth yields were observed at 35°C with 10% inoculum rate. In addition, pellet formation was not observed in all inoculum concentrations at 35°C. Das and Brar (2014) explained that the inadequate supply of oxygen results in the transformation of pellets into dispersed mycelia at higher temperatures in *Rhizopus* species. Also, the biomass yields were comparatively higher at high temperature with the highest growth rate observed at 35°C and 7.5% inoculum. Similarly, Tung et al. (2004) reported that at higher growth rates, due to high temperatures, the presence of shorter and branched hyphae might lead to less interaction between hyphae, which eventually results in the formation of dispersed morphology.

Wucherpfennig et al. (2010), suggests that the three mechanisms responsible for pellet formation in filamentous fungi are “germination of a fraction of initial aggregated

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spores, growth of an individual spore into a bigger pellet, and the agglomeration of hyphal elements", which could be observed during the fungal cultivation process in this study. Microscopic analysis of biomass at different growth stages can provide better insights into pellet formation, especially at high temperatures with high biomass yields.

Formation of fungal pellets is a complex process controlled by a broad range of factors including hyphal growth and concentration, hydrodynamic factors, cultivation parameters, and nature of the growth medium (Metz et al., 1997). Also, the mechanism of pellet formation is variable among different fungal species (Dynesen and Nielsen, 2003).

5. Recommendations

As contamination was indicated as the reason for inconsistent growth with increased storage of TS, techniques like spread plate culturing of thin stillage can be used to identify the organisms responsible for competing growth. Application of molecular biology techniques could be used to identify the species and determine possible pathways to inhibit its growth.

In addition, microscopic analysis of dispersed and pelleted morphology could provide some insights on the ambient conditions needed for the consistent production of the desired pellet morphology.

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6. Conclusion

Temperature and inoculum concentration substantially influence pellet formation efficiency in *R. microsporus var. oligosporus*. A 10% inoculum concentration at the temperature of 30°C was observed to be optimum for pellet formation. While future research directions are being determined, use of fresh thin stillage at the specified optimum conditions could be used to achieve consistent pellet morphology with an average biomass yield of 17 g/L.

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CHAPTER 5

CONCLUSIONS

Production of pellets with *Rhizopus oligosporus* was successfully achieved on the corn-ethanol thin stillage medium. Biomass yields above 17 g/L with pellet morphology can be achieved at 30˚C and 10% inoculum concentration on the fresh thin stillage. While these results are demonstrated at the lab scale, pilot-scale experiments with similar conditions and improved aeration rates can be tested for their effect on yields. While pellet morphology was not observed in three-weeks old thin stillage with same cultivation conditions, the yields were consistently low compared to the growth in fresh thin stillage. This was speculated due to competitive growth of other microorganisms in the thin stillage. Microbial isolation and molecular biology techniques can be used to identify the microbes competing for growth.

An early-stage techno-economic analysis was conducted for the production of renewable diesel from biocrude produced via hydrothermal liquefaction of fungal biomass cultivated on thin stillage. The minimum selling price (MSP) of a gasoline gallon equivalent (GGE) of the fuel was observed to be $9.02 at the diesel production rate of 1000 gallons of diesel per day. Increasing the production scale from 1000 to 12000 gallons of diesel production per day would decrease the price to $5.98/GGE due to economies of scale. While the tax incentives can further reduce the minimum selling price of the diesel, the final prices are still not considered to be competitive with current diesel prices. Since the fermentation and the HTL processes contribute to the maximum of the diesel price, improvements in the efficiency of these processes such as improved growth yields, and heat and pressure recovery options can contribute towards the commercialization of the process.

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