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Enhancing dry-grind corn ethanol production with fungal cultivation and ozonation

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Enhancing dry-grind corn ethanol production with fungal cultivation and ozonation

by

Mary Lynn Rasmussen

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Civil Engineering (Environmental Engineering); Biorenewable
Resources and Technology

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Ames, Iowa

2009

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Public opinion of the U.S. fuel ethanol industry has suffered in recent years despite record ethanol production. Debates sparked over the perceived environmental impact, high ratio of fossil fuel inputs to ethanol output, and competition with food production (RFA, 2009a). In addition to actively refuting these concerns, the industry continues to seek innovations that will enhance process efficiency and sustainability, as well as produce more nutritious coproducts to expand inclusion in new and existing feed markets.

Research on fungal cultivation of thin stillage, a byproduct stream of dry-grind corn ethanol production, helps address many of the claims waged against the fuel ethanol industry. Fungal treatment offers the potential to recover water and enzymes for in-plant reuse, to save energy by eliminating water evaporation from thin stillage, and to produce value-added animal feed (high-protein fungal biomass). The fungal feed coproduct could command an increased market value, while improving profits and minimizing environmental impacts of fuel ethanol production.

Research on POET's no-cook (BPX™) corn ethanol process investigates an alternative method to antibiotics addition for controlling contamination by lactic acid bacteria during fermentation. The use of antibiotics is problematic because of rising concerns about the emergence of antibiotic-resistant bacteria. This research seeks to replace antibiotics with a disinfection method, ozonation of the corn mash, and to enable operation at a higher pH more conducive to ethanol fermentation. Operating at a higher fermentation pH also reduces sulfuric acid requirements for pH

adjustment and the resulting sulfur content in the distillers grains, an improvement for cattle feed. The enhanced feed coproduct could be marketed as antibiotic-free and reduced-sulfur distillers grains. Ozonation, in place of antibiotic addition and lower fermentation pH, could, therefore, save money by reducing operational expenses (i.e., antibiotic and chemical costs) and improve the feed quality of the distillers grains.

Thesis Organization

The thesis is organized into four main parts, a literature review and three research papers. The review outlines the benefits, challenges, current production, and projections for the U.S. dry-grind corn ethanol industry. Descriptions of the conventional and no-cook (BPX™) processes are provided, as well as detailed background on the production, current feed uses, and opportunities for the principal coproduct, distillers grains. Feed inclusion limitations for distiller grains in swine and poultry diets are discussed since hog and chicken production is prevalent in the U.S. Corn Belt. Recent advances implemented on commercial scale in a few ethanol production facilities, such as corn fractionation, back-end oil recovery, and alternative process energy sources, are mentioned as options to improve the efficiency, sustainability, and array of coproducts of corn ethanol production. This review of today's corn ethanol industry sets the stage for the motivation behind the research projects reported in this thesis.

The research reported in Chapters 3 and 4 investigated fungal cultivation of thin stillage in stirred and airlift bioreactors, respectively, and the composition of the fungal biomass product. Chapter 5 presents research on reducing bacterial

competition in the no-cook corn ethanol process by ozone treatment of corn mash prior to fermentation. Both fungal and ozone applications in the corn ethanol process aim to enhance the efficiency, sustainability, and coproducts of the modern corn ethanol industry. I wrote the research papers (Chapter 3–5) and was principally involved in the data collection, analysis, and interpretation, in collaboration with my major professors.

CHAPTER 2. LITERATURE REVIEW

Corn Ethanol Motivation

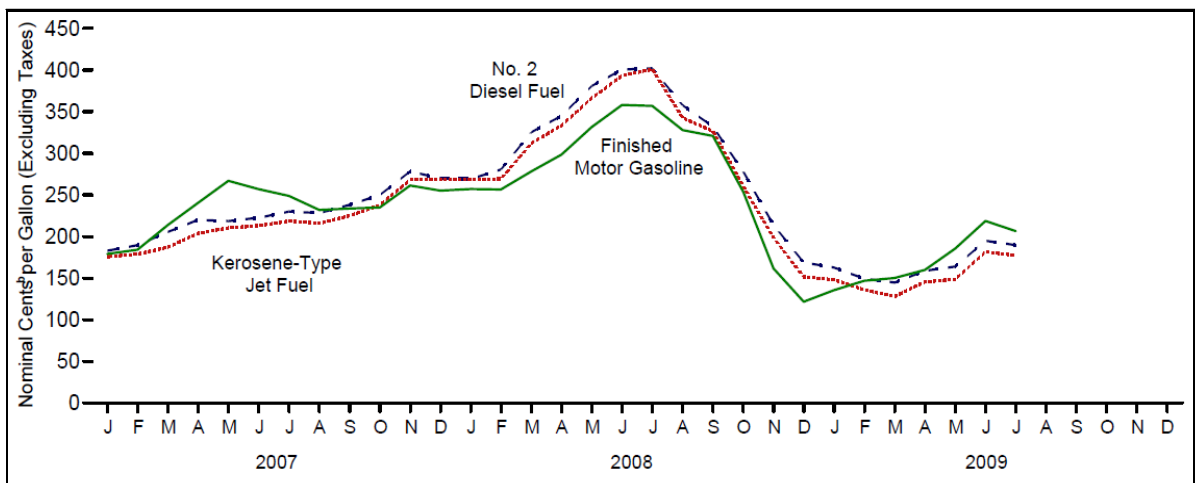
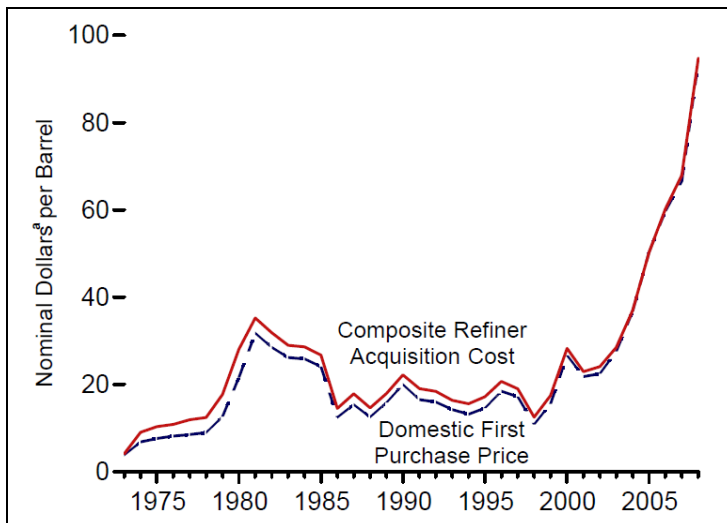
The benefits of biofuels, such as corn ethanol, address a range of national and global challenges, from the economy and national security to the environment. Biofuels not only help alleviate foreign oil dependence and improve national security, they boost rural economies and reduce greenhouse gas emissions.

Economy

In 2008, the fuel ethanol industry supported more than 494,000 jobs in all sectors of the economy, added nearly \$66 billion in spending to the nation's Gross Domestic Product (GDP), and provided an additional \$20 billion in income for American households (Urbanchuk, 2009). The increase in GDP and higher household incomes provided almost \$9 billion and \$12 billion in state/local government and federal tax revenues, respectively. The major federal incentives for the year were the Volumetric Ethanol Excise Tax Credit (VEETC), or the blender's credit, and the Small Ethanol Producer Tax Credit. The estimated cost of these credits totaled less than \$5 billion. The \$12 billion in new tax revenue, thus, yielded a direct return on investment to the federal government of 2.5 to 1.

The blender's credit was created in the American Jobs Creation Act of 2004 and authorized through 2010 (NCGA, 2009b). It provides oil companies with a tax credit of \$0.51 per gallon pure ethanol to blend ethanol with gasoline. The blender's credit ensures ethanol has access to the fuel market. Fuel ethanol has the disadvantage of competing with gasoline, a heavily-subsidized fuel product, and depending on that competitor to reach the consumer (NCGA, 2009a). All fuel

ethanol blended with gasoline in the U.S. qualifies for the blender's tax credit, no matter the country of origin. To ensure that U.S. tax dollars do not subsidize foreign ethanol production, non-Caribbean ethanol imports are subject to a tariff of \$0.54 per gallon (RFA, 2009c).



^a Nominal dollars: a measure used to indicate nominal price.

^b Nominal price: the price paid for a product or service at the time of the transaction. Price has not been adjusted to remove the effect of changes in the purchasing power of the dollar.

Figure 2.1. Petroleum prices: trends in annual crude oil prices (1973–2008); monthly fluctuations in refiner prices to end users (2007–2009) in the U.S. by fuel type (EIA, 2009c).

Domestic ethanol production has additional contributions to the U.S. economy by increasing crop market opportunities for farmers, lowering gasoline prices, and expanding gasoline supplies, which in turn reduces expensive imports (RFA, 2009a). Petroleum prices fluctuated in the range of \$10 to \$30 per barrel from the 1970s to 2000 (Figure 2.1; EIA, 2009c). Since 2001, the average annual cost has jumped from approximately \$20 to \$95 per barrel in 2008. The monthly average peaked at \$129 in July 2008. Recent consumer prices for motor gasoline, in turn, have fluctuated between \$1.84 and \$4.14 dollars per gallon from 2007 to 2009 (Figure 2.1). Francisco Blanch, a Merrill Lynch commodity strategist, noted that gasoline prices would have been \$21/bbl higher in June 2008 (approximately 15%) if biofuels production stopped growing (Global Energy Weekly, 2008).

National security

Simply stated, biofuels are needed for our long-term security – economic, energy, and defense. As President Barack Obama asserted on January 26, 2009, *“America’s dependence on oil is one of the most serious threats that our nation has faced. It bankrolls dictators, pays for nuclear proliferation, and funds both sides of our struggle against terrorism. It puts the American people at the mercy of shifting gas prices, stifles innovation, and sets back our ability to compete”* (RFA, 2009a).

Domestic renewable energy is essential to diversifying our national energy strategy. Total renewable energy usage, including biomass, wind, hydroelectric, geothermal, and solar, has risen over the last 35 years from 4.4 quadrillion BTU (quad) in 1973 to 7.3 quad in 2008 (Figure 2.2; EIA, 2009c); however, it still represents a small fraction

of U.S. primary energy consumption (99.4 quad in 2008). Fossil fuel consumption is a huge fraction (83.5 quad in 2008).

U.S. petroleum consumption in 2008 accounted for nearly half of fossil fuel usage (EIA, 2009c). Since 1973, U.S. petroleum production has decreased by nearly 40% (Figure 2.3) despite high consumption of petroleum products. Consequently, petroleum imports have doubled (Figure 2.3). The transportation sector is by far the largest consumer of petroleum in the U.S., as shown in Figure 2.4. The fraction of oil purchased from OPEC and Persian Gulf nations has fluctuated over the last 35 years but is now comparable to that of 1973 (Figure 2.5). The 9 billion gallons of fuel ethanol produced in 2008 displaced 321 million barrels of oil, the equivalent of over one month's worth of crude oil imports to the U.S. from often hostile nations (Figures 2.5 and 2.6) (RFA, 2009a).

Americans are not alone. The world is addicted to oil – a very unhealthy habit. The world consumed 86 million b/d of oil in 2007, of which the U.S. consumed nearly a quarter (EIA, 2009b). The world is projected to consume 116 million b/d by 2030 (EIA, 2009a). The oil market is very volatile – reaching a record \$140/barrel in 2008 – and conventional oil supplies are dwindling (NYMEX). Other fuel options are a must to divert and still meet our energy addiction. Currently, biofuels are the only non-fossil fuel helping to reduce oil demand. Booming world biofuels production has prevented oil consumption from expanding an additional 1 million b/d (EIA, 2009a).

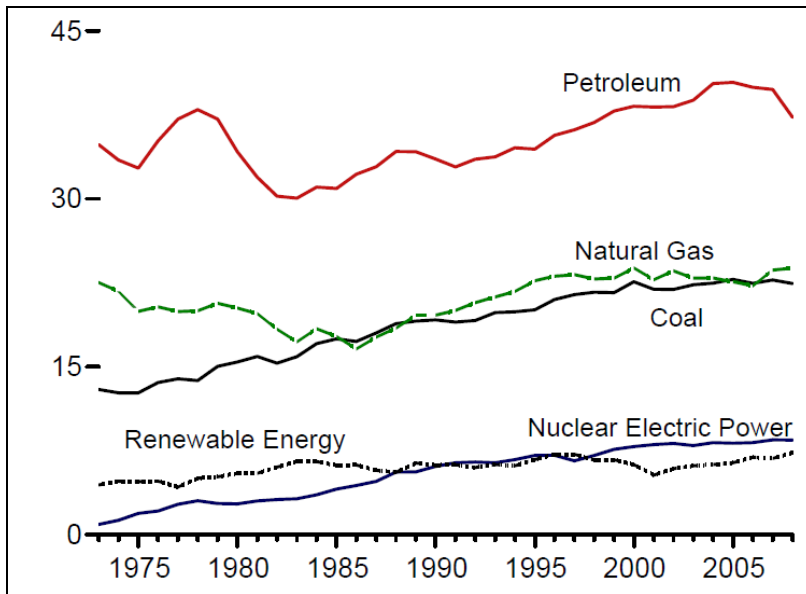
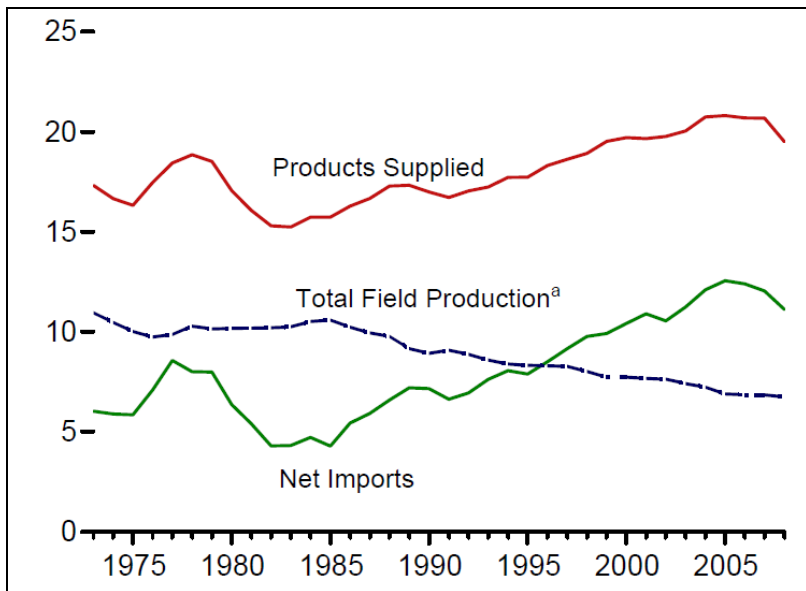
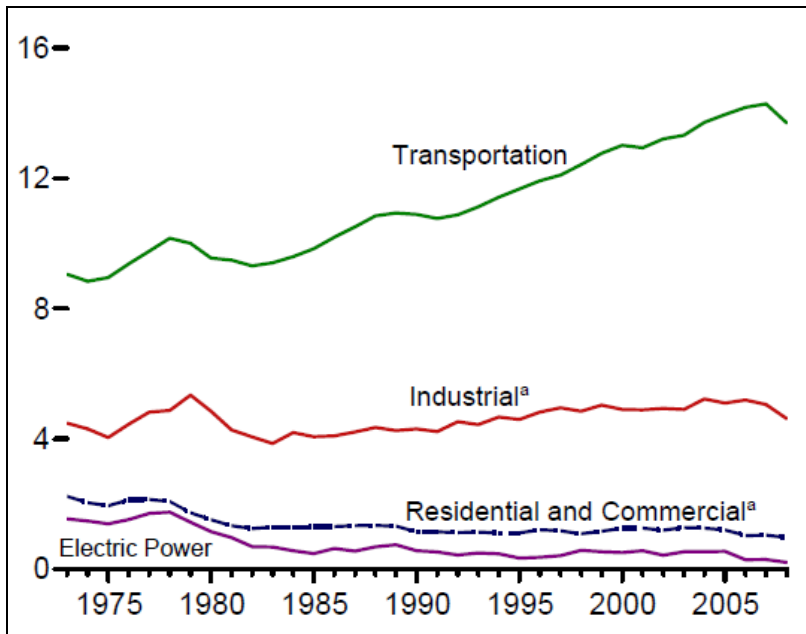


Figure 2.2. Primary energy consumption (quadrillion BTU) by source in the U.S. from 1973 to 2008. (EIA, 2009c).



^a Crude oil, including lease condensate, and natural gas plant liquids field production.

Figure 2.3. Petroleum overview (million barrels per day) in the U.S. from 1973 to 2008 (EIA, 2009c).



^a Includes combined-heat-and-power plants and a small number of electricity-only plants.

Figure 2.4. Petroleum consumption (million barrels per day) by sector in the U.S. from 1973 to 2008. (EIA, 2009c).

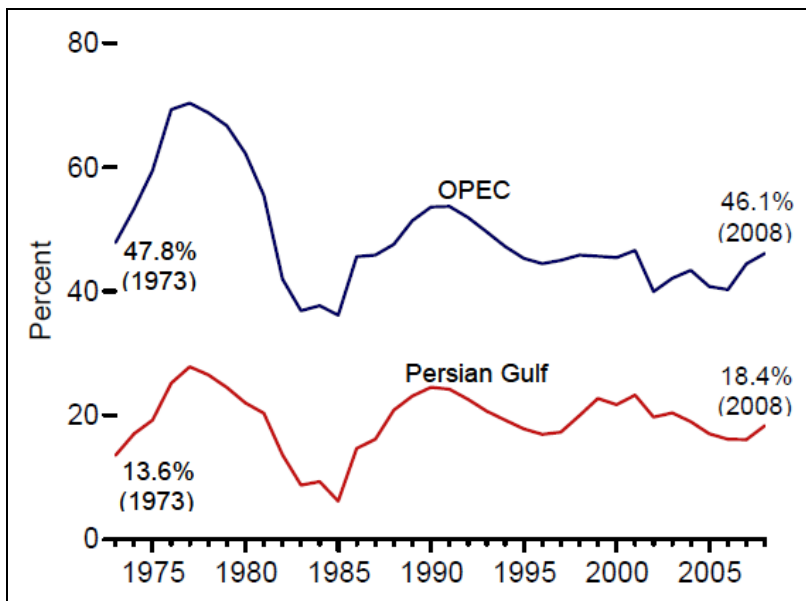


Figure 2.5. Petroleum trade: imports from the Organization of the Petroleum Exporting Countries (OPEC) and Persian Gulf as share of total imports to the U.S. from 1973 to 2008. (EIA, 2009c).

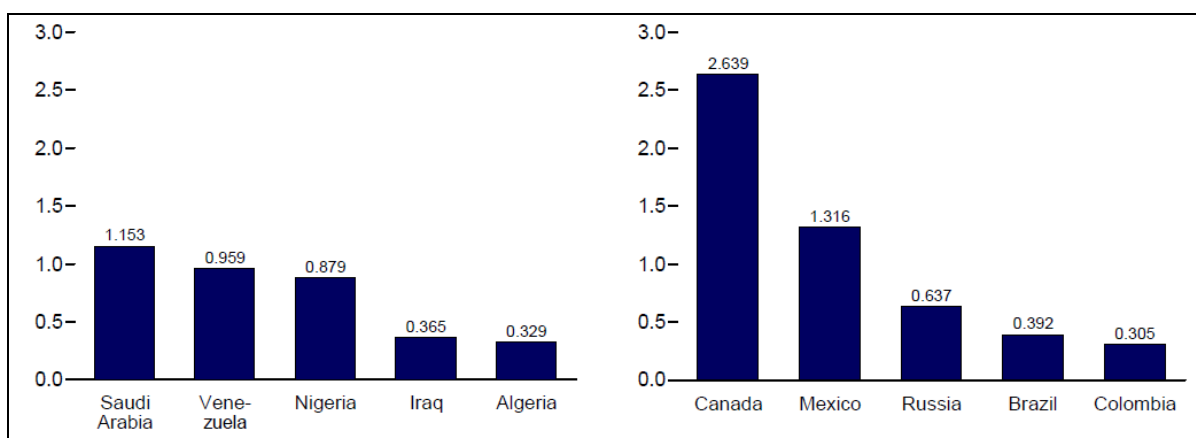


Figure 2.6. Petroleum trade: imports (million barrels per day) from select OPEC and non-OPEC countries to the U.S. in July 2009. (EIA, 2009c).

Environment

Fuel ethanol usage benefits the local and global environment by reducing emissions of harmful air pollutants and greenhouse gases (GHG) (Liska et al., 2009; RFA, 2009c). Carbon dioxide is the main GHG of concern in global warming, accounting for nearly 85% of U.S. emissions (EIA, 2009b); combustion of fossil fuels (petroleum, coal, and natural gas) is the major culprit. The carbon footprint of ethanol, however, is improving and clearly better than its competitor in transportation fuels, gasoline. Ethanol is made from renewable, plant-based feedstocks, primarily the starch in corn grain in the U.S. The carbon dioxide released during fuel ethanol production and combustion is absorbed by photosynthesis into the growth of future feedstock crops. The U.S. emitted 2 billion metric tons of carbon dioxide in 2007 from petroleum used for transportation, of which 1.2 billion was from motor gasoline, as reported by the U.S. Energy Information Administration (EIA) (2009b). The 9 billion gallons of U.S. ethanol produced in 2008 reduced GHG emissions, from crop to ethanol production and use, by 14 million tons – the equivalent of emissions from

more than 2.1 million vehicles (RFA, 2009a). A recent lifecycle analysis shows that fuel ethanol reduces direct GHG emissions by 48–59% compared to gasoline (Liska et al., 2009).

Ethanol producers are continually working to improve process energy efficiencies and sustainability by developing and implementing innovative technologies. Cellulosic feedstocks and higher blends of ethanol (>E10) will contribute to enhancing ethanol's GHG benefits well into the future. Cellulosic ethanol is expected to reduce carbon dioxide emissions by more than 86% compared to gasoline, according to the U.S. Department of Energy (DOE) Argonne National Laboratory GREET model (Wang et al., 2007). The fossil energy use and carbon dioxide emissions are much lower for cellulosic crops, which are not as chemical- and energy-intensive. In addition, the leftover lignin can displace fossil fuels for generating the steam for cellulosic ethanol production. On the contrary, the production of oil from non-conventional Canadian tar sands emits up to three times more GHG than conventional oil production (Woynillowicz et al., 2005). Oil from tar sands is also more difficult to refine, resulting in up to an additional 40% increase in emissions from Midwest oil refineries (RFA, 2009a).

Corn Ethanol Challenges

Despite the positive impact of biofuels on the economy, national/energy security, and the environment, corn ethanol suffered more than its share of challenges and controversies in 2008. Ethanol producers were hit with record input costs (corn prices over \$7 per bushel in June 2008), lower ethanol prices, and credit shortages; some faced bankruptcy (RFA, 2009a). The corn ethanol industry

struggles too with its public perception. The industry and its advocates continue today to contest the food versus fuel and indirect land use change controversies, which are often misinformed.

Food, feed, and fuel

The dramatic increase in U.S. fuel ethanol production – tripling since 2003 – and rising food prices sparked recent controversy about the relationship between corn as feedstock for fuel ethanol and corn for feed/food. Critics attacked the corn ethanol industry; some claimed ethanol's impact on the price of corn was solely responsible for the higher cost of all groceries, from staples like milk and meat to treats (RFA, 2009a). The “food vs. fuel” debate was, and still is, highly emotional and sensationalized, with ethanol production pitted against food prices and feeding the world. The ethanol industry and advocates are actively disputing all of these claims.

The reality is that other factors, namely energy costs and inflation (historically, an annual 2.9% for food), affect food prices much more than the price of corn (RFA, 2009c). Ethanol production has a very minimal impact. Less than 20 cents of every dollar spent on food in 2006 went to farmers; more than 80 cents was for marketing, packaging, and transportation costs to get the food products to the grocery shelves and restaurants (USDA ERS, 2008). The second half of 2008 proved the fallacy of the debate. Although ethanol production continued to increase, crop (e.g., corn) prices dropped by 60% from the record highs in June 2008, and still retail food prices were rising (RFA, 2009a).

There is no need for Americans to decide between growing corn for feed and food or for fuel ethanol. Both can be done in the U.S. The National Corn Growers Association (NCGA) estimates that 15 billion gallons of corn ethanol can be produced without upsetting the existing domestic and export markets for food and feed. Substantial yield increases – 72 bushels of corn per acre in 1974 up to 154 bushels per acre in 2008 – ensure sufficient supplies of U.S. corn grain will be available (NCGA, 2009c; RFA, 2009c). In addition, one-third of the corn processed into ethanol becomes distillers grains, corn gluten feed, or corn gluten meal, which is sold on the animal feed market. These coproducts displace more than one billion bushels of corn per year for feed.

Corn ethanol is only the first step to fuel security. The next step is cellulosic ethanol, which is aggressively being pursued. By 2030, the U.S. could produce a combined 60 billion gallons of grain and cellulosic ethanol without disturbing food, feed or fiber production, according to a joint study by the U.S. DOE and Department of Agriculture (USDA) (Perlack et al., 2005).

Land use change

The analysis of carbon emissions from indirect land use change are one of the latest debates that has serious implications for the future of the U.S. fuel ethanol industry (RFA, 2009a). Indirect land use change refers to the impact of rapidly increasing biofuels production (e.g., in the U.S.) on land use decisions by people around the world. The debate is whether U.S. grain ethanol production is responsible for any of the destruction of rain forests and virgin lands in foreign nations. Clearing forests and land for cultivation results in carbon emissions by

releasing stored carbon from the soil and vegetation to the atmosphere. The 2007 Energy Bill mandates the U.S. Environmental Protection Agency (EPA) to perform lifecycle GHG analyses on biofuels production, including the contributions of indirect land use change. The challenge is that the causes and effects of land use decisions have numerous contributing factors. Attributing the resulting GHG emissions elsewhere in the world to the American ethanol industry is very imprecise.

The ethanol industry argues three main points on indirect land use change as applied to grain ethanol's lifecycle GHG analysis (RFA, 2009a). First and foremost, the science is too new to use as a basis for critical policy decisions. The findings of earlier research varied dramatically and depended highly on the assumptions of the researchers. These studies often failed to account for the rising GHG emissions from non-traditional oil sources (e.g., Canadian tar sands) and the rapidly improving environmental footprint of the agricultural and ethanol industries (Liska et al., 2009; Woynillowicz et al., 2005). Second, minimal use of farmland is required for biofuels production. The net area of cropland required for American ethanol production in 2007 was only 0.6% of total global cropland (RFA, 2009a). The land needed to produce 15 billion gallons of grain ethanol by 2015 to meet the Renewable Fuels Standard (RFS) should be less than 1% of total global cropland. Global agriculture productivity continues to increase to meet the demand of food, feed, and fuel. Third, feed coproducts from ethanol production should be assigned an up-to-date credit to reflect the increasing quantities of corn and soybeans displaced in animal rations.

The bottom line, according to Tom Darlington of Air Improvement Resource, Inc., is that *“based on available projections, we think crop yields will continue to*

improve (especially in the U.S., but also elsewhere), and that this should be properly incorporated into the land use change analysis. Secondly, we believe new government analysis shows that the land use credit for feed coproducts coming from corn-based ethanol production is much higher than 33%. When these factors are incorporated into land use change analysis, as we have done in this study, no new pasture or forest land will need to be converted in the U.S. or outside the U.S. to meet the 15 bgy RFS requirement for corn ethanol in 2015. Thus, the GHG emissions from land use change due to expansion of U.S. corn-based ethanol to meet the RFS are likely zero” (Darlington, 2009).

Ethanol blend wall

Although ethanol is a superior fuel product, blending with gasoline is capped at 10% for most vehicles. Not only clean-burning and renewable, ethanol is a high-quality, high-octane, high-performance fuel for vehicles (RFA, 2009c). Most drivers assume that ethanol blends, though less expensive, reduce gas mileage compared to regular unleaded gasoline. The opposite was true in a recent research study by the University of North Dakota Energy and Environmental Research Center and the Minnesota Center for Automotive Research (Shockey et al., 2007). The study found that mid-range blends (E20 and E30) may improve the gas mileage of cars. Ethanol has a lower energy content (fewer BTU per gallon) but can make up for it by improved combustion efficiency; ethanol’s octane rating is 113–115 compared to 87 for unleaded gasoline. The octane number indicates the fuel’s tendency to detonate prematurely in an internal combustion engine, which reduces fuel economy; a higher value means it is less likely to detonate until ignited by electrical spark (Brown,

2003). The most common ethanol blends sold at pumps today are E10 – 10% ethanol, 90% gasoline – and E85 – 85% ethanol, 15% gasoline. E10 can power any standard car engine or small engines (e.g., motorcycles and lawn mowers).

The U.S. ethanol industry will soon hit a blend wall as ethanol approaches 10% of the nation's gasoline production (Sept. 2009 average of 8.8 million b/d) (EIA, 2009c). This cap on ethanol blending of 10% (E10) was set in 1979 by the federal government for use in all motor vehicles (RFA, 2009a). Comprehensive scientific research is needed to break through the blend wall. Increasing the volume of ethanol blended (13, 15, 20% or higher) with gasoline is essential to expanding the market for advanced biofuels, e.g., cellulosic ethanol.

Ramping production of flex-fuel vehicles (FFVs) is one solution to this limit on ethanol usage that should be promoted (RFA, 2009a). FFVs are manufactured and warranted to run on all ethanol blends from 0 to 85%. Today only 7 million FFVs drive American roads – 3% of the total 220 million vehicles (U.S. DOE, 2009). The number of FFVs continues to increase each year. General Motors, Ford, Chrysler, Toyota, Izuzu, Mercedes, Mazda, and Nissan manufacture flex-fuel models. Investments in America's fueling infrastructure (flex and E85 pumps) will be necessary to meet the rising demand for ethanol. Flex, or blender, pumps, for example, have been installed throughout Midwestern states and are gaining popularity. Flex pumps allow the consumer to choose the gasoline-to-ethanol ratio of their fuel. The most cost-efficient fuel blend depends on the vehicle type and the ethanol/gasoline economics at the time. Consumers may also choose to purchase higher volumes of domestically-produced biofuels regardless of economics.

Current U.S. Ethanol Production

A record 9.2 billion gallons of fuel ethanol was produced in the U.S. in 2008, up from 4.9 and 6.5 billion gallons in 2006 and 2007, respectively (Figure 2.7; EIA, 2009b). The industry is on pace to produce more than 10 billion gallons in 2009 – accounting for 9% of America’s gasoline supply. According to the EIA in a recent Renewable Fuels Association (RFA) press release, American ethanol facilities produced 728,000 barrels per day (b/d; 30.6 million gal/d) in July 2009, up from 614,000 b/d a year ago.

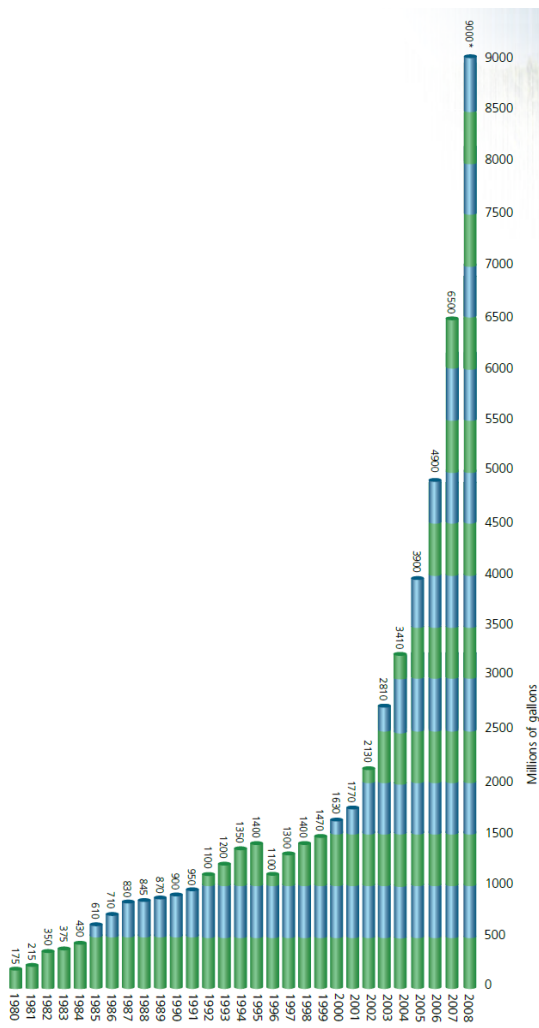


Figure 2.7. Historic U.S. fuel ethanol production (RFA, 2009a).

Nonetheless, ethanol demand continues to outpace production as demand for renewable alternatives to gasoline grows. In 2008, the U.S. fuel ethanol demand was 9.6 billion gallons; U.S. imports were 0.5 billion gallons (EIA, 2009b). The trend of increasing demand continues in 2009. The RFA calculated an ethanol demand of 748,000 b/d in July 2009, up from 635,000 b/d a year ago. U.S. ethanol production in 2030 is projected to be 1.6 million b/d (EIA, 2009a). As of January 2009, there were 170 operating plants across 26 states (Figure 2.8) to meet this demand, providing a production capacity of 10.6 billion gallons and up to 12.5 billion gallons including idled capacity (RFA, 2009a). In addition, 24 plants were under construction/expansion – potentially 2.1 billion gallons more of capacity.

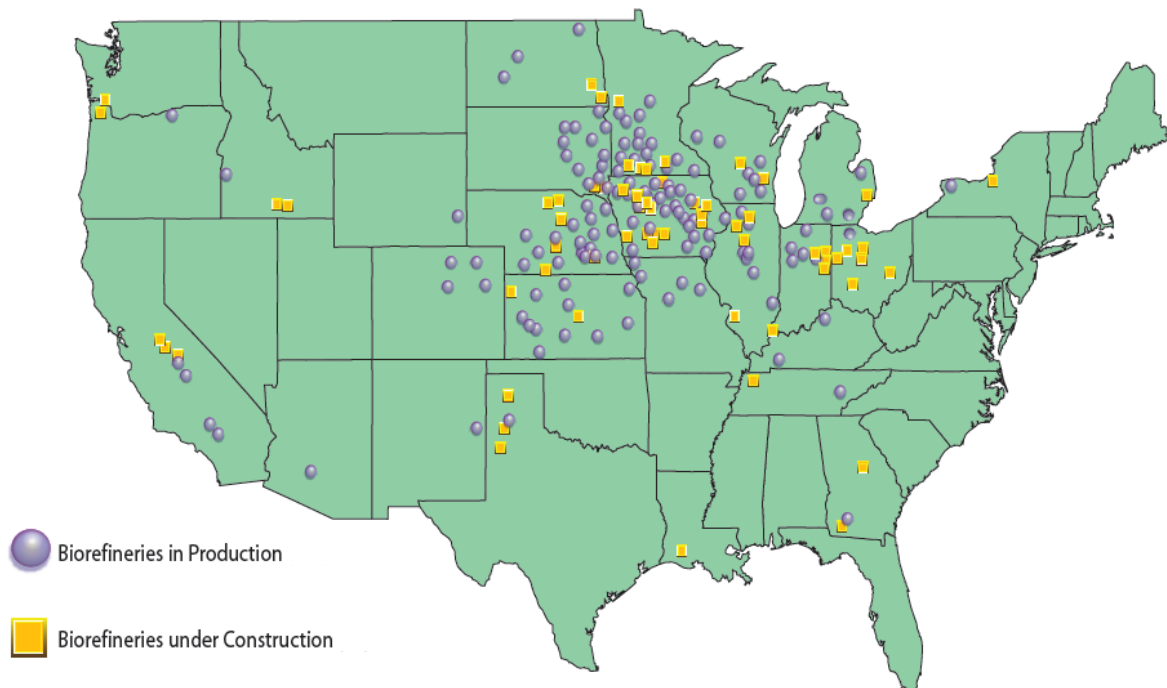


Figure 2.8. U.S. ethanol biorefinery locations in production and under construction (RFA, 2008).

Long-term U.S. Ethanol Production

The Energy Independence and Security Act (EISA) of 2007 created the RFS to establish a schedule for renewable fuel production and usage in the motor transportation sector. The American ethanol industry ramped production and met the 9 billion gallons per year (bgy) renewable fuel standard in 2008 (EIA, 2009b). At current production rates (avg. 845 million gallons per month), which increase each month, the industry is on track to meet and possibly exceed 10 bgy – very close to the 10.5 bgy target for 2009. The RFS calls for a total 11.1 bgy in 2009, including advanced biofuels and biodiesel. The RFS progressively increases total production of renewable biofuels to 36.0 billion gallons by 2022. The contributions of grain-based biofuels, however, level off at 15.0 bgy by 2015. Advanced biofuels, including cellulosic ethanol, are scheduled to contribute 21 bgy, or nearly 60% of the total RFS, by 2022.

The RFS is important to ensure that grain and cellulosic ethanol continue to have market access since fuel ethanol relies on competitors – the petroleum industry – to get to the market. Cellulosic ethanol already faces significant challenges to commercialization. It is essential for cellulosic ethanol to have market prospects while in the early development stages for commercialization.

Dry-grind Corn Ethanol Process Descriptions

Conventional process

A modern, conventional dry-grind corn ethanol plant produces 2.8 gallons of ethanol, 17 pounds of carbon dioxide, and 17 pounds of distillers grains per bushel of corn (RFA, 2009b). Each of the three products accounts for approximately one-

third of the initial corn weight. The exact procedures for ethanol production and coproduct processing vary from plant to plant. The following are the typical process steps at a conventional dry-grind corn ethanol plant as illustrated in Figure 2.9 (Brown, 2003; RFA 2009d):

Milling: the corn kernels are processed without separating out the various grain components (e.g., protein, fat, starch, and fiber). The entire kernel is ground by hammer mills to grain meal consistency to expose the starch.

Slurry: the corn meal is mixed with fresh and recycled process water streams (e.g., thin stillage and evaporator distillate) in the slurry tanks to form a mash. Alpha-amylase enzyme is added to the mash to break down the starch to dextrins (long chain sugars). Ammonia is added to raise the pH and as a nitrogen-source for the yeast. Urea may also be used for nitrogen.

Jet cooking/liquefaction: the mash is steam-cooked in high-temperature hydroheaters to maximize the thermostable α -amylase activity, kill unwanted lactic acid bacteria, and to gelatinize the starch granules for enhanced hydrolysis. The cooking process is completed in the liquefaction tanks. The mash is cooled and fed to the fermentors.

Fermentation: glucoamylase enzyme and yeast are added to the mash to perform two simultaneous processes – saccharification and fermentation. Glucoamylase enzyme converts the starch fragments (dextrins) into simple sugars – glucose (dextrose) and maltose. Yeast ferments the simple sugars to ethanol and carbon dioxide. The mash is continually agitated and cooled to facilitate yeast activity and starch hydrolysis; it is fermented for 40–60 h.

Distillation: the fermented mash, now called beer, contains ethanol plus all the non-fermentable solids (protein, fat, fiber, and residual sugars/starch) from the corn and yeast cells. The beer is pumped to the beerwell to feed the beer column, where the ethanol is recovered from the solids and the water. The residual mash, called whole stillage, is transferred from the base of the column to the coproduct processing area. The rectifier column, the second distillation step, concentrates the ethanol to 95% (190 proof).

Dehydration: the ethanol passes through a molecular sieve dehydration system where the remaining water is removed. The alcohol product at this stage is called anhydrous ethanol (without water) and is approximately 200 proof (100% concentration). Gasoline is added (up to 5%) to denature the ethanol for shipment by truck or rail car to gasoline terminals/retailers. The denaturant renders the final ethanol product undrinkable and, thus, not subject to beverage alcohol tax.

Stillage processing: the whole stillage is centrifuged to physically separate most of the solids – wet grain – from the liquid stream – thin stillage. The thin stillage passes through evaporators to remove water (the distillate); the condensed thin stillage is called syrup (about 30% solids). The wet grain and syrup are dried together to produce distillers dried grains with solubles (DDGS), which is 8–10% moisture. Dryer exhaust is sent to a regenerative thermal oxidizer to remove any odor and particulates.

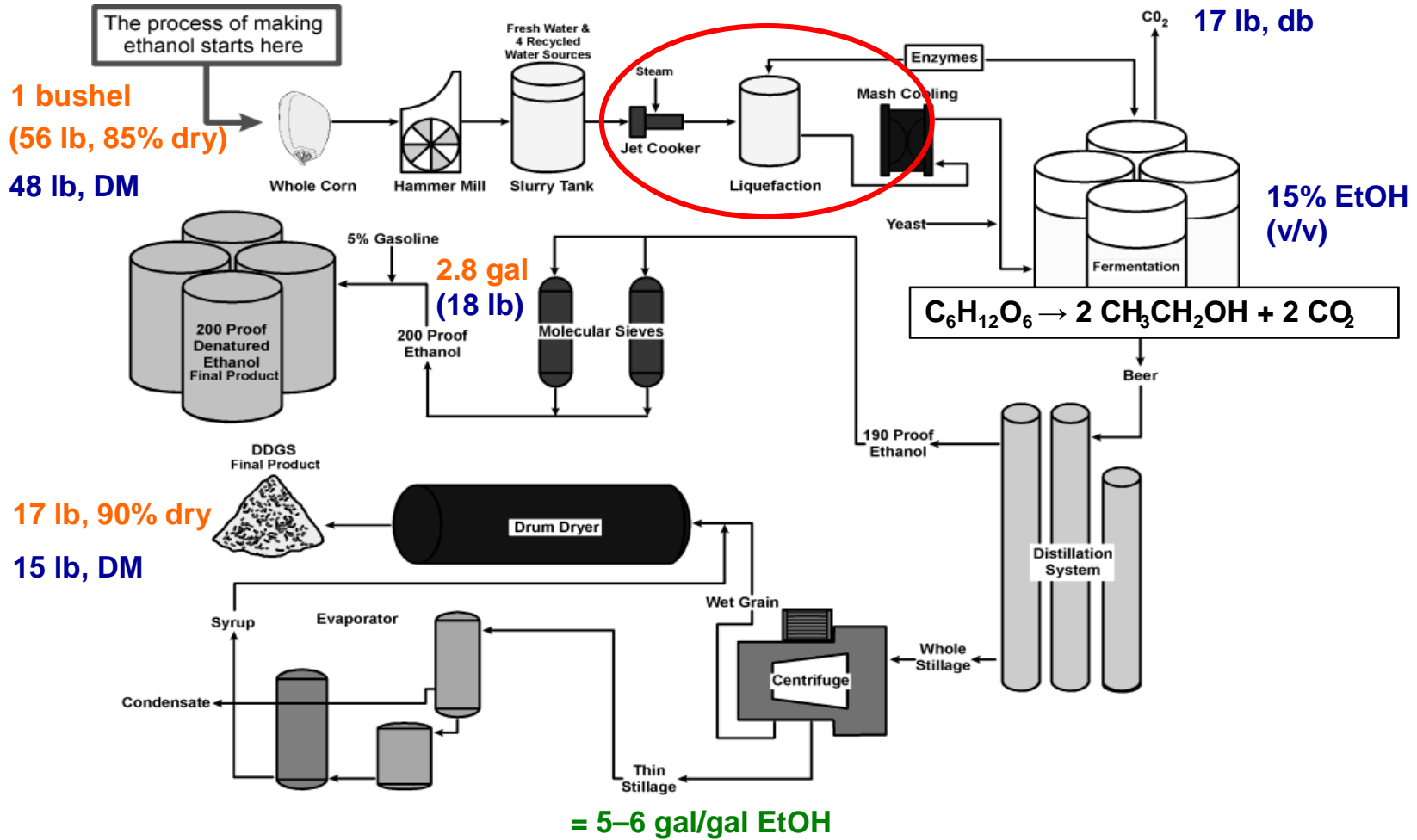


Figure 2.9. Diagram of conventional dry-grind corn ethanol production. The BPX™ process skips the jet-cooking, liquefaction, and mash cooling steps in the red circle (POET, 2009; RFA, 2009b).

Coproducts: two main coproducts are created in dry-grind ethanol production, distillers grains (DDGS) and carbon dioxide. Distillers grains are marketed as animal feed. The carbon dioxide may be captured and sold for use in carbonating beverages and manufacturing dry ice. The carbon dioxide produced is ultimately reabsorbed by crops, such as the corn feedstock for future ethanol production.

No-cook (BPXTM) process

POET leads the corn ethanol industry in streamlining the dry milling process to improve production efficiencies, as well as the resulting distillers grains feed coproduct, Dakota Gold. In addition to incorporating small innovations, POET invested years of development to take the patent-pending BPXTM process to commercial scale in 2004 (POET, 2009). The BPXTM process is now employed in 24 of the 26 POET biorefining plants. POET produces over 1.5 billion gallons of ethanol annually in a network spanning seven states – IA (7), SD (6), MN (4), IN (3), OH (3), MO (2), and MI (1). POET's corporate office is located in Sioux Falls, SD, and the POET Research Center is in Scotland, SD. The research center, in addition to producing 11 MGY ethanol, is used as a R&D facility for continually testing new technologies. Once innovations, such as the BPXTM process, are proven successful at the research center and at a higher-volume commercial facility, they can be quickly implemented across the 26-plant network. Rapid adoption at commercial-scale facilities is possible because POET “delivers a turn-key development, design, engineering, construction, management and marketing services for [its] plant

network.” POET is proud of not only the ethanol volume they produce but the production efficiencies of their plants.

BPX™ is an efficient raw starch hydrolysis (no-heat) process that, like the conventional process, adds enzymes to convert starch to sugars and adds yeast to ferment sugars to ethanol. The fundamental difference is elimination of the energy-intensive jet-cooking and liquefaction steps. The BPX™ process reduces energy use by 8–15% in the ethanol plant (POET, 2009). Incorporating BPX™ technology, in addition to lowering energy costs, has the following advantages: higher ethanol yields by improving starch accessibility, substantially lower volatile organic carbon (VOC) emissions, reduced cooling water needs, and increased DDGS nutrient quality, flowability and anti-caking properties.

The nutritional value of DDGS, specifically the digestibility, is improved by skipping the jet-cooking step. Amino acid digestibility in DDGS varies significantly among ethanol plants and may differ from batch to batch within an ethanol plant (Batal and Dale, 2006; Fastinger et al., 2006). The DDGS drying process primarily causes this variation (Fontaine et al., 2007; Bregendahl, 2008). Heat damage also occurs by cooking the corn mash. These heat sources are responsible for the lower amino acid digestibility in DDGS than in corn grain. Lysine is especially susceptible to heat damage and is highly variable in digestibility among DDGS samples (Fontaine et al., 2007; Stein et al., 2006). Thus, omitting the cooking step improves the feed quality of the Dakota Gold-brand DDGS over conventional DDGS by increasing protein digestibility (POET Nutrition, 2009b).

The major steps in the POET BPX™ process are described on the POET website (POET, 2009) as follows:

Milling: the corn feedstock is finely ground into a powder, called flour, by passing through hammer mills.

Slurry: the corn flour is mixed with fresh and recycled water in the slurry tank – without heat – to create mash.

Fermentation: raw starch hydrolyzing enzymes are added to the mash to convert starch into simple sugars – glucose and maltose. Yeast is added to ferment the sugars to ethanol and carbon dioxide. The mash is continually agitated, cooled, and fermented until the ethanol content is maximized (20% [v/v] ethanol – significantly higher than the conventional industry standard of 16–18%[v/v]).

The distillation, dehydration, and stillage processing steps are similar to the conventional process described in the previous section. The primary feed coproduct of the BPX™ process is known as Dakota Gold-brand DDGS. Dakota Gold DDGS meets a higher level of nutritional content and physical consistency than is possible with the conventional process. It is in demand all around the world and is shipped from the plants via truck and rail car. In many POET plants, carbon dioxide is also collected, compressed, and marketed for use in other industries.

Dry-grind Coproducts

Distillers grains importance

Record corn ethanol production means record volumes of the feed coproducts on the market. The growth rate of distillers grains is the fastest of all feed ingredients (DGTC, 2009a). Today over 98% of the fermentation coproducts

available are from fuel corn ethanol production (University of Minnesota, 2008a). In 2008, the U.S. ethanol industry produced 23 million metric tons of distillers grains (Figure 2.10) (RFA, 2009b). In addition, 3 million metric tons of corn gluten feed and 0.6 million metric tons of corn gluten meal were produced from corn wet milling. Annual U.S. production of distillers grains is projected to increase to nearly 40 million metric tons by 2015, assuming the RFS of 15 bgy is met and based on the current DDGS-to-ethanol ratio. The profitability of dry-grind ethanol plants depends on the sale of both the ethanol and the coproducts. A quarter of the distillers grains was sold wet locally in 2008, reducing energy inputs for drying and transportation costs. The industry had record exports as well. In 2008, the value of coproducts from corn ethanol production was \$3 billion in the feed market. Wholesale prices for DDGS, however, are down. DDGS sold for approximately \$100/ton in Sept. 2009 as compared to \$150/ton in Sept. 2008. (USDA, 2009).

Distillers grains are not only important to the global feed market and ethanol profitability, but also as a land use credit in lifecycle GHG analysis of corn ethanol. A hectare of corn generates over 1000 gallons of fuel ethanol plus the feed equivalent of the corn from 30% of a hectare of corn and the soybean meal from 50% of a hectare of soybeans (RFA, 2009a). New and expanded feed markets will be critical to avert lower DDGS prices and to offset ethanol processing costs as production continues to increase. Ethanol coproducts tend to be priced at a discount to the feed ingredients they replace, corn and soybean meal (SBM). Livestock and poultry feeders often take advantage of these products up to the recommended inclusion rates in least-cost ration formulations (Bregendahl, 2008).

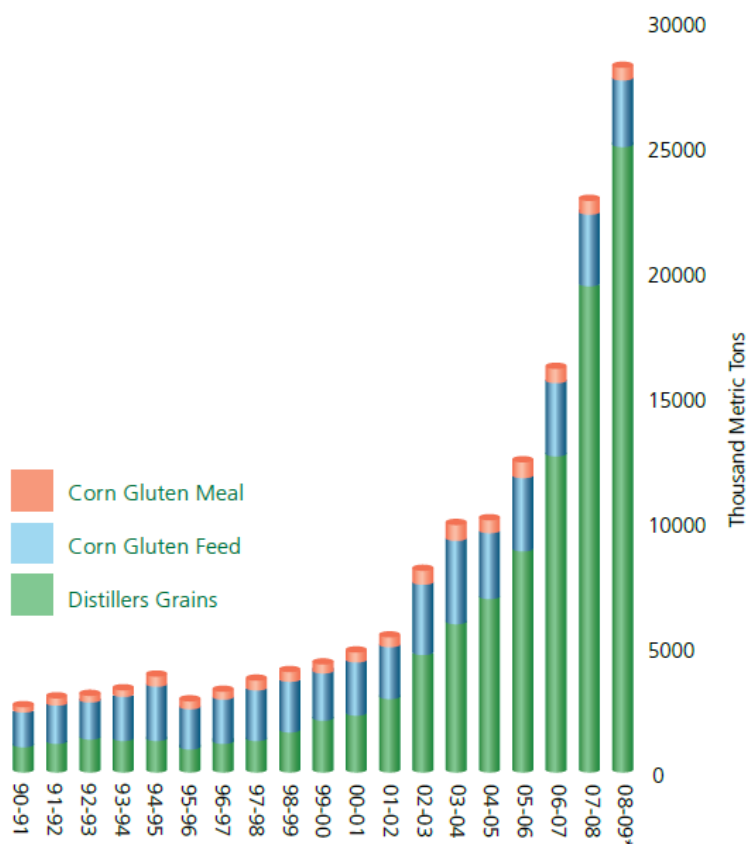


Figure 2.10. Historic production of U.S. corn ethanol coproducts (RFA, 2009a).

Only 5 and 11% of distillers grains were consumed by the nonruminants poultry and swine, respectively, in 2007 (RFA, 2008). The ruminant livestock, cattle and dairy cows, consumed 42% each. The poultry and swine industries are predominant in Iowa, the top ethanol-producing state. Iowa leads the nation in production of eggs and pork, accounting for 15 and 30% of total U.S. production, respectively (Iowa Egg Council; Iowa State University Extension). The low content of digestible amino acids limits the use of DDGS for swine and poultry, which are typically fed corn-SBM diets; corn is the energy source and soybean meal is the protein supplement. A typical swine grower diet (16% crude protein), for example,

contains 74% corn, 21% soybean meal, and 5% salts, minerals, and vitamins (Cheeke, 2005). Common inclusion rates of DDGS used by nonruminant feeders are only up to 20% for swine and 15% for poultry (DGTC, 2009a, 2009b). In addition to expanding use in traditional markets, animal scientists and nutritionists are increasingly studying the impact of feeding ethanol coproducts to other animals, such as goats, sheep, and fish. Human food applications are also being explored.

Distillers grains composition

The corn ethanol coproduct distillers grains returns protein and other non-starch nutrients in the corn grain back to feed markets. The corn grain, or kernel, contains approximately 72% starch, 10% protein, 10% fiber, and 4% fat, on a dry basis (White and Johnson, 2003). The distillers grains composition depends on the corn ethanol process employed – conventional, BPXTM, or BFRACTM – as shown in Table 2.1. POET's BFRACTM process is a dry fractionation approach that separates the corn grain into endosperm (for ethanol fermentation), germ, and fiber, resulting in the feed coproducts Dakota Gold HPTM, Dakota GermTM, and Dakota BranTM, respectively (POET, 2009). Variations in nutrient concentrations are also observed for similar processes among ethanol plants, and some differences occur from batch to batch within a plant even using the same grain (Spiehs et al., 2002). Nutrient digestibility varies among sources, as will be discussed in the next sections; accurate *in vitro* methods are needed to estimate amino acid digestibility (Stein and Shurson, 2009). The industry is working to address issues raised by livestock and poultry feeders with compositional and nutritional consistency of DDGS. As

discussed previously, the processing and heating procedures used to produce DDGS affect its nutritional quality (Cromwell et al., 1993).

Table 2.1. Compositional comparison of corn, soybean meal, and dry-grind corn ethanol coproducts.

Composition (% as fed basis)	Corn^a	Soybean meal^a	Conventional DDGS^b	Dakota Gold BPX^c	Dakota Gold HP^d	Dakota Germ^e
Dry matter	89.0	90.0	89.0	91.6	92.5	90.5
Crude protein	8.3	47.5	26.5	26.1	38.0	14.8
Crude fat	3.9	3.0	10.1	10.1	3.6	16.5
NDF	9.6	8.9	32.2 ^f	24.1	22.1	21.1
ADF	2.8	5.4	11.9 ^f	8.7	11.5	7.0
Lysine	0.26	3.02	0.76 ^g	0.94	1.26	0.83
Methionine	0.17	0.67	0.54 ^g	0.58	0.94	0.25
Tryptophan	0.06	0.65	0.21 ^g	0.24	0.33	0.20
Threonine	0.29	1.85	1.04 ^g	1.05	1.53	0.59
Phosphorus	0.28	0.69	0.77	0.89	0.41	1.43
Sulfur	0.13	0.44	0.84 ^f	0.91	0.78	0.20

^a Data from NRC (1998).

^b Data from Waldroup et al. (2007) except as noted.

^c Data from Poet Nutrition (2009b).

^d Data from Poet Nutrition (2009c).

^e Data from Poet Nutrition (2009a).

^f Mean of 5 samples reported by Fastinger, Latshaw, and Mahan (2006) (Bregendahl, 2008).

^g Mean of 39 samples reported by Stein et al. (2005, 2006), Fastinger and Mahan (2006), Urriola et al. (2007), and Pahm et al. (2008); data compiled by Stein and Shurson (2009).

The protein and fat contents of DDGS are important nutritionally as protein and energy sources for animal diets. Conventional- and BPXTM-based DDGS contain similar amounts of crude protein and crude fat, approximately 26 and 10%, respectively (Table 2.1). DDGS has less protein than soybean meal (48%), but more than corn (8%). The Dakota Gold HPTM product, leftovers from separating and fermenting the starch in the endosperm fraction only, offers an improved protein

supplement (38%). The fat content of DDGS is roughly three times higher than that of corn or soybean meal. Fractionation results in a higher fat fraction in the Dakota Germ™ product; Dakota Gold HP™ has similar fat contents to corn and soybean meal.

The fiber fraction of feed ingredients is typically measured as neutral detergent fiber (NDF) and acid detergent fiber (ADF). To separate the carbohydrate fraction into the fiber and non-fiber components, Van Soest (1963, 1994) developed this detergent system for forage analysis. The cell contents – protein, fat, starch, sugar, and minerals – are first solubilized by boiling in a neutral-detergent solution (sodium lauryl sulfate and ethylenediaminetetraacetate [EDTA]). The insoluble residue is called NDF. The NDF is refluxed in an acid detergent solution (cetyl trimethylammounium bromide in 1 N sulfuric acid), which dissolves hemicellulose. The residue is called ADF. Hemicellulose is quantified by NDF minus ADF. The ADF fraction is primarily cellulose and lignin. The fiber fraction of traditional feed ingredients corn (9.6% NDF) and soybean meal (8.9% NDF) are considerably lower than that of corn ethanol coproducts (32.2% NDF for conventional DDGS). The POET coproducts have lower NDF values of 21–24%.

Knowledge of the amino acid contents and digestibility of DDGS from different sources is critical to formulating feed rations for optimal performance. Lysine, methionine, tryptophan, and threonine are the main amino acids of concern for nonruminants (Cheeke, 2005). Lysine and methionine are usually the first-limiting amino acids in corn-SBM diets for swine and poultry, respectively. Corn grain and conventional DDGS are low in essential amino acids, particularly lysine (0.26 and

0.76%, respectively) (Table 2.1). POET's coproducts have somewhat higher lysine contents of 0.83, 0.94, and 1.26% for Dakota Germ™, Dakota Gold BPX™, and Dakota Gold HP™, respectively. Soybean meal is the standard to which other protein supplements are compared. Dehulled soybean meal marketed at a protein content of 48% has 3.0% lysine. Like lysine, the methionine concentration is low for corn, intermediate for DDGS, and high for soybean meal (Table 2.1). Dakota Gold HP™, moreover, has 40% higher methionine content than soybean meal.

The sulfur and phosphorus composition of DDGS are other important parameters to monitor for livestock feed. The sulfur content in corn grain is fairly low (0.13%; Table 2.1). Fermentation of the starch fraction of corn during ethanol production results in a three times concentrating effect; thus, the sulfur content of DDGS, if only the corn grain contributed sulfur, would be approximately 0.4% on as-fed basis. The actual contents of sulfur in conventional DDGS and Dakota Gold™ are greater (0.84 and 0.91%, respectively) and can vary considerably from 0.3% to more than 1% (Batal and Dale, 2003; Spiehs et al., 2002; University of Minnesota, 2008b). The additional sources of sulfur are the yeast biomass, well water, and sulfuric acid added during ethanol production (Bregendahl, 2008). Sulfuric acid is used to adjust the pH to optimal levels for the enzymes (liquefaction and saccharification) and the yeast. Sulfur concentrations of 0.4% of the diet can be toxic to cattle by causing polioencephalomalacia (NRC, 1980). The sulfur content can, therefore, limit the inclusion rate of DDGS as a feed ingredient. Reducing the sulfuric acid requirements of the ethanol production process could improve the feed

quality of the DDGS. The highly-digestible phosphorus content of DDGS relative to corn is an advantage for swine and poultry feeds.

Distillers grains for swine

Distillers grains are a great source of energy and digestible phosphorus in weanling, growing, and reproducing swine diets (Stein and Shurson, 2009). Utilization of organic phosphorus is increased and inorganic phosphorus supplementation is reduced by using DDGS as a feed ingredient. Lysine content, digestibility, and bioavailability, however, limit feeding DDGS to pigs (Stein and Shurson, 2009). Variation was found in the amino acid digestibility of 39 sources of DDGS, even when produced from the same corn grain (Fastinger and Mahan, 2006; Pahm et al., 2008; Stein et al., 2005, 2006; Urriola et al., 2007). Lysine digestibility varied more than all other indispensable amino acids, likely because of heat-damaged samples (Cromwell et al., 1993; Fastinger and Mahan, 2006; Pahm et al., 2008; Stein et al., 2006). The variation among different DDGS samples was within the normal range observed for other feed ingredients, except for lysine. Most amino acids in DDGS have a digestibility that is around 10 percentage units less than in corn, which may be attributed to higher dietary fiber contents in DDGS. DDGS-based diets can be supplemented with crystalline lysine (Cromwell et al., 1983; Wahlstrom and Libal, 1980). With crystalline lysine added (0.10%), 10% DDGS can displace 4.3% soybean meal and 5.7% corn.

Table 2.2. Summary of the benefits and drawbacks of DDGS in swine diets (Stein and Shurson, 2009).

DDGS inclusion	Benefits	Drawbacks
Nursery diet (up to 30%)	Acceptable performance	
Grower-finisher diet (up to 30%)	May enhance gut health	
Gestation diet (up to 50%)	Replace all dietary SBM; may increase litter size; may improve reproduction	
Lactation diet (up to 30%)	Acceptable performance	
High linoleic acid content of DDGS – finisher diet (more than 20%)		Softens pork fat (higher carcass fat iodine values)
High in digestible phosphorus content	Excellent source for swine diets; reduce inorganic P supplementation (expensive)	
Formulating diet based on digestible P basis	Reduces manure P concentration; minimal impact on gas/odor emissions	Increases manure volume
Swine diets of all production phases		Reduces dry matter and energy digestibility; reduces diet palatability; decreases dressing percentage by increased gut fill

According to the 2007 recommendations of the Distillers Grains Technology Council (DGTC, 2009b), DDGS can be used a feed ingredient in nursery pig, grower-finisher pig, and sow diets at an inclusion rate of up to 20% and in gestating sow diets up to 40%. Excellent performance is achievable as long as diets are formulated based on digestible amino acid and digestible phosphorus contents.

Recent studies focused on using higher inclusion rates in swine diets in all production phases as summarized in Table 2.2. Nutrient concentration/digestibility, feeding value, and unique responses to feeding DDGS to pigs were investigated. Growth performance was acceptable with up to 30% DDGS in nursery pig and grower-finisher diets (Stein and Shurson, 2009). The high linoleic acid content in DDGS oils, however, may soften pork fat unacceptably (high iodine values) at inclusion rates of more than 20% for finisher pigs. High iodine values in feed result in soft and less valuable bellies and loins in swine. Withdrawing DDGS in late finisher diets is one promising solution to this problem. DDGS inclusion rates of up to 50 and 30% in gestation and lactation diets, respectively, did not adversely affect sow and litter performance, but rather tended to increase litter size and boost reproductive performance.

Inclusion of DDGS in diets of growing pigs may enhance gut health; more research is needed to confirm repeatability (Stein and Shurson, 2009). DDGS dry matter contains 4% residual yeast cells and components (Ingledew, 1999). The β -glucans, mannan-oligosaccharides, chitin, and proteins present in yeast cell walls are important to the immune system and capable of stimulating phagocytosis – a major mechanism for removing pathogens and cell debris (Stone, 1998). Yeast cells also contribute nucleotides, glutamate, and other amino acids, vitamins, and trace minerals to DDGS-based swine diets, which may enhance the immune system (Stone, 1998). Feed trial results indicate that dietary inclusion of DDGS is as effective in improving resistance to moderate ileitis as approved antimicrobial agents, but DDGS may not be effective in more severe cases.

Diet formulation on a digestible P basis reduces manure P content, but increases manure volume because of the lower digestibility of DDGS dry matter (Stein and Shurson, 2009). It has minimal effect on gas and odor emissions from manure, and the manure chemical composition remains the same, except for the P content. Research is needed to find economical methods to improve DDGS dry matter and energy digestibility. The feeding value of DDGS could be significantly improved by increasing dry matter digestibility, in particular the digestibility of the insoluble-fiber fraction of DDGS. A drawback of inclusion of high-fiber ingredients in swine diets is a potential reduction in the dressing percentage as a result of increased gut fill and intestinal mass (Kass et al., 1980). The dressing percentage is the carcass-to-live weight ratio and is an economically important trait.

Distillers grains for poultry

Distillers grains are an alternative to corn and soybean meal in poultry rations up to inclusion limits. The most important benefit of feeding DDGS is the potential for reducing ration cost by displacing portions of expensive corn, soybean meal, and dicalcium phosphate (Bregendahl, 2008; DGTC, 2009a). DDGS offers a number of additional advantages for poultry diets as summarized in Table 2.3, many of which were mentioned for swine diets. DDGS is high in protein and energy, including corn gluten and associated pigment factors, and yeast cells (DGTC, 2009a). Dried yeast cells provide B vitamins, promote palatability, and increase feed consumption by poultry. The phosphorus in DDGS is highly bioavailable and could reduce dicalcium phosphate supplementation, thus lowering ration cost. The fat in DDGS (8–10%, as fed) is an excellent source of energy and linoleic acid – an advantage – for poultry.

An added benefit for layer diets is the increase in yolk color with inclusion of DDGS (15%) and no negative impact on egg production, weight, or specific gravity.

Table 2.3. Summary of the benefits and drawbacks of DDGS in poultry diets (Bregendahl, 2008; DGTC, 2009a).

DDGS inclusion	Benefits	Drawbacks
Starter chick diet (up to 8%)	Acceptable performance	
Grower-finisher diet (up to 15%)	Acceptable performance	
Layer diet (up to 15%)	Increases yolk color	
High digestible/bioavailable phosphorus content	Excellent source for poultry diets; reduces inorganic P supplementation (expensive)	
Yeast biomass content (4% dry matter basis)	Provides B vitamins; more palatable; improves feed consumption	
Poultry diets of all production phases	Displaces corn & SBM; reduces ration costs; contains fat (energy) & linoleic acid	High variability in amino acid digestibility, in particular for lysine, and energy content affects inclusion rates; gut-fill limits feed consumption

The primary drawbacks of DDGS for poultry feed is variability and the challenge it poses for optimizing feed formulations, in addition to the high-fiber content. Batal and Dale (2006) determined the lysine digestibility in eight DDGS samples using the cecectomized rooster assay and obtained a range from 46 to

78%, with an average of 70%. Lysine is especially susceptible to heat damage occurring in the jet-cooking and distillers-grains drying steps (Fontaine et al. 2007; Stein et al., 2006). Maillard reactions between the ϵ -amino group on lysine and reducing sugars affects digestibility (Bregendahl, 2008). Poultry do not produce enzymes able to break bonds in the Maillard-reaction product in order to use lysine for protein synthesis; the Maillard product is either not absorbed and excreted into the feces (not digested) or is absorbed and excreted into the urine (not bioavailable).

The digestibility of a feed component, such as amino acids, is important as an estimate for bioavailability – the fraction of the consumed amino acid that can be used for protein synthesis (Bregendahl, 2008). Bioavailability of an amino acid is determined by the slope-ratio method, which compares the relative bioavailability of the amino acid between two feed ingredients (Batterham et al., 1979). Body weight gain of broiler chicks, for example, is used as the measured response. Crystalline L-lysine·HCl is 100% bioavailable and used for comparison (Izquierdo et al., 1988). Lumpkins and Batal (2005) estimated the lysine bioavailability in DDGS samples relative to crystalline lysine. The relative bioavailability of digestible lysine in DDGS was found to be 80% for poultry. Based on an average lysine digestibility of 70% in DDGS (Lumpkins and Batal, 2005), the bioavailable lysine content was thus 56% of the total lysine content. Reactive lysine is another estimate for bioavailability. Fontaine et al. (2007) determined the contents of reactive lysine in 80 DDGS samples and found that 10 to 40% of the lysine was heat damaged, with some batches of DDGS losing up to 59% of the lysine.

Although heat damage reduces lysine bioavailability, it increases phosphorus bioavailability by heat destruction of phytate during drying (Bregendahl, 2008).

Poultry lack the enzyme phytase to release the phosphorus from phytate in corn. The majority of the phosphorus in DDGS is bioavailable (62%) compared to corn (30%), which reduces inorganic phosphorus addition to feed and saves money.

The recommended inclusion rates by the DGTC (2009a) are as follows: 5% for starter chicks, 10% for chicken/turkey grower-finisher and broilers, and 15% for laying hens. DDGS usage at these levels result in comparable performance, as measured by body weight and feed conversion, relative to the control corn-SBM diet. DDGS, like corn, is low in lysine and tryptophan; supplementation may be required with higher DDGS inclusion rates, especially in grower-finisher rations. The feed formulation must be adjusted based on amino acids to optimize performance.

Recent studies showed that somewhat higher proportions of DDGS can be used in poultry feed, such as 6% for starter chicks and up to 12% for grower chickens (Dale and Batal, 2003). Inclusion of 5–8% DDGS in starter diets and 12–15% DDGS in grower-finisher diets for broiler, layers, and turkey resulted in acceptable performance in studies by Swiatkiewicz and Koreleski (2008). Pineda et al. (2008) demonstrated that as much as 70% DDGS could be included in laying-hen diets if feed cost is not considered. This high inclusion is possible by supplementing the relatively-low DDGS energy content with oil, which increases diet cost and decreases flowability. The practical limit is 20–25% and would require pelleting, flow agents, or antioxidants (Bregendahl, 2008). Poultry diets are ultimately formulated on a least-cost basis provided that performance is acceptable.

Non-feed opportunities

Bio-oil: pyrolysis is thermal cracking in the absence of oxygen to produce solid and gas products (Briens et al., 2008). Liquid bio-oil, typically the most valuable product, is recovered by rapid cooling of the condensable vapors in the gas product stream. The endothermic pyrolysis process requires a continuous, high rate of heat transfer to the ground biomass feed. Product gas is usually burned for energy to drive the reaction. Conventional pyrolysis is used for making charcoal. Fast pyrolysis, on the other hand, achieves high yields of bio-oil by rapid heating to around 500°C and short residence times for the product gas (Bridgwater and Peacocke, 2000). The vapors produced condense to form bio-oil, a dark brown mobile liquid; the higher heating value (17 MJ/kg) is less than half that of conventional fuel oil (42–44 MJ/kg). Bio-oil yields of up to 80% on a dry feed basis have been reported.

Generating energy from biofuel residues, such as distillers grains, would greatly improve the energy efficiency of ethanol production (Briens et al., 2008). Pyrolysis of distillers grains to bio-oil may be an appealing option, particularly if bio-oil fractions can be converted to transportation fuels and used as an energy source for ethanol plant operation. Direct combustion of distiller grains to produce heat is more energy efficient, but produces twice the heat requirements of ethanol production. Most existing plants, which are set up for natural gas combustion, would need a major retrofit to directly combust distillers grains. Natural gas burners, however, can be easily retrofitted to use bio-oil. Preliminary pilot-plant pyrolysis tests of distillers grains demonstrated large yields of bio-oil containing aqueous and

oily (organic) phases. Distillers grains pyrolysis at 10% moisture resulted in approximately 42.0% organics, 22.4% water, 19.3% solids, and 16.3% losses/gases. After valuable chemicals are extracted, the aqueous phase could be fermented to produce more ethanol. The oily phase could be burned to fuel the ethanol process or converted to a syngas.

Biobased products: DDGS is an inexpensive, abundant coproduct that contains valuable corn components, but is not currently used for industrial applications. Therefore, in addition to increasing consumption among ruminant and nonruminant animals, new markets and uses (fertilizer, pet litter, packaging materials, food, etc) are under investigation (RFA, 2009a). The high fiber levels in DDGS may enable incorporation into biopolymers, an option that could earn greater economic returns. Tatara et al. (2009) evaluated the feasibility of using DDGS as a biofiller in molded, phenolic resins to produce a new biomaterial. DDGS was blended with phenolic resin at four levels (0-75%, by weight) and compression molded at different pressures and temperatures. Pressure and temperature had little effect on the mechanical and physical properties of the molded specimens. DDGS content greatly affected all of the properties, including tensile yield strengths, water absorption, biodegradability, and surface hardness. Results were similar to those obtained in other studies on biofillers. Cellulose was extracted by Xu et al. (2009) from corn kernels and DDGS to evaluate its suitability for films and absorbents. DDGS contains about 9–16% cellulose. Alkali and enzymes were used to extract cellulose, with resulting minimum yields of 1.7 and 7.2% and crude cellulose contents of 72 and 81% from corn kernels and DDGS, respectively. The

crude cellulose obtained after extraction were made into films using water only. The cellulose recovered could also be used as an absorbent; it was found to hold water up to 9 times its weight. Other potential applications include paper, composites, lubricants, and nutritional supplements.

Other Corn Ethanol Process Innovations

The corn ethanol industry has developed and continues to pursue innovative process technologies. Goals include recovering new value-added products, enhancing the protein content and quality of feed coproducts, reducing the environmental footprint (e.g., energy and water efficiencies), and improving process economics. Raw starch fermentation (the BPXTM process), corn fractionation, and back-end corn oil extraction are a few of the non-conventional processing options utilized by select ethanol plants (RFA, 2008).

Corn fractionation

Both dry and wet methods have been investigated to recover the germ and fiber prior to fermentation of the starch in the endosperm fraction at the front-end of dry-grind corn ethanol production (Murthy et al., 2009). This separation provides more flexibility, enabling the production of feed to better meet the specific nutritional needs of the consumer (e.g., swine and poultry). In dry fractionation, the main unit operations added are corn tempering, size reduction, sieving, aspiration, and density separation, similar to conventional corn dry milling. Wet fractionation methods resemble conventional corn wet milling. An aqueous medium is used to separate and recover the corn germ, pericarp fiber, and/or endosperm fiber prior to fermentation. The main unit operations are soaking/steeping, size reduction, density

separation, and sieving. Corn fractionation prior to fermentation helps reduce the amount of DDGS produced by up to 66% depending on the fractionation method (Wang et al., 2005).

The POET BFRAC™ process is a corn dry fractionation technology that is operating on commercial-scale (Clark, 2006; Nilles, 2006). Low lysine content in corn and corn fermentation coproducts has always been a great concern in animal nutrition. One way of increasing the lysine content is to increase the protein fraction of coproducts like DDGS. POET's BFRAC™ process separates corn into fiber, germ, and endosperm. Liquefaction and fermentation of endosperm produces ethanol and high-protein residue, called high protein DDGS (HP-DDGS). High protein DDGS has higher amino acid contents than conventional DDGS and Dakota Gold, (e.g., the lysine content of soybean meal [2.76%], DDGS [0.88%], and HP-DDGS [1.11%]).

Back-end corn oil recovery

Technology is also developed to remove corn oil from the stillage prior to mixing with wet distillers grains in the dryer (RFA, 2007). The crude oil recovered can serve, for instance, as a feedstock for biodiesel production. It can also be sold as an energy source in the poultry feed market or be further refined for the human food market. Removing the oil enhances the value of distillers grains as a feed coproduct by concentrating the protein.

Alternative processing power options

Another processing option is to use ethanol coproducts to supply part of the energy demands of the ethanol plant. Corn Plus, a Minnesota-based ethanol

producer, installed a fluidized bed reactor to generate steam for running the facility from biomass, such as syrup (RFA, 2007). The resulting reduction in natural gas usage was more than 50%. In addition, Corn Plus has two wind turbines to provide about 45% of the electric needs. At POET, the latest technology advancement in this area, waste-powered ethanol production using methane gas from a local landfill and wood waste, completely eliminates the natural gas used at POET Biorefining – Chancellor (POET, 2009).

Cellulosic corn ethanol

The production of more ethanol from corn by hydrolysis and fermentation of agricultural/ethanol processing residues, like corn stover, corn cobs, corn fiber, and distillers grains, is an attractive possibility; it will help bridge the industry to the conversion of other cellulosic feedstocks and energy crops, such as switchgrass and miscanthus (RFA, 2008). Many research project have studied this area. POET's Project Liberty focuses on utilizing corn cobs.

Motivation for Fungal Cultivation of Stillage Research

As discussed earlier in this chapter, U.S. fuel ethanol production is booming, despite the public perception and economic challenges encountered in recent years. Biofuels, such as corn ethanol, are in high demand; they help alleviate oil dependence, improve energy security, boost the economy, and reduce greenhouse gas emissions. Although the corn ethanol industry is advancing in process efficiencies, there is ample room to improve. Innovations currently implemented on commercial scale include the no-cook process (POET BPX™), back-end crude corn oil recovery, and corn fractionation (e.g., POET BFRAC™) (RFA, 2009a). The use

of renewable energy sources, such as biomass gasification and methane digesters, are displacing traditional natural gas and coal energy in select ethanol plants. To ensure sustainable corn ethanol processing, research is needed to continue to reduce process energy inputs, reuse water, and create value-added coproducts. Such innovations also help to tackle public-opinion issues, such as debates on food vs. feed and indirect land use change.

Research motivation

Our research on fungal cultivation of thin stillage obtained from conventional dry-grind corn ethanol production is reported in Chapters 2 and 3. Fungal cultivation offers potential benefits that address each of these sustainability areas, including: water and enzyme recovery for in-plant reuse, energy savings by eliminating water evaporation from thin stillage, and high-quality animal feed production (high-protein fungal biomass). The fungal feed coproduct could command an increased market value, while improving profits and minimizing environmental impacts at the ethanol production facility.

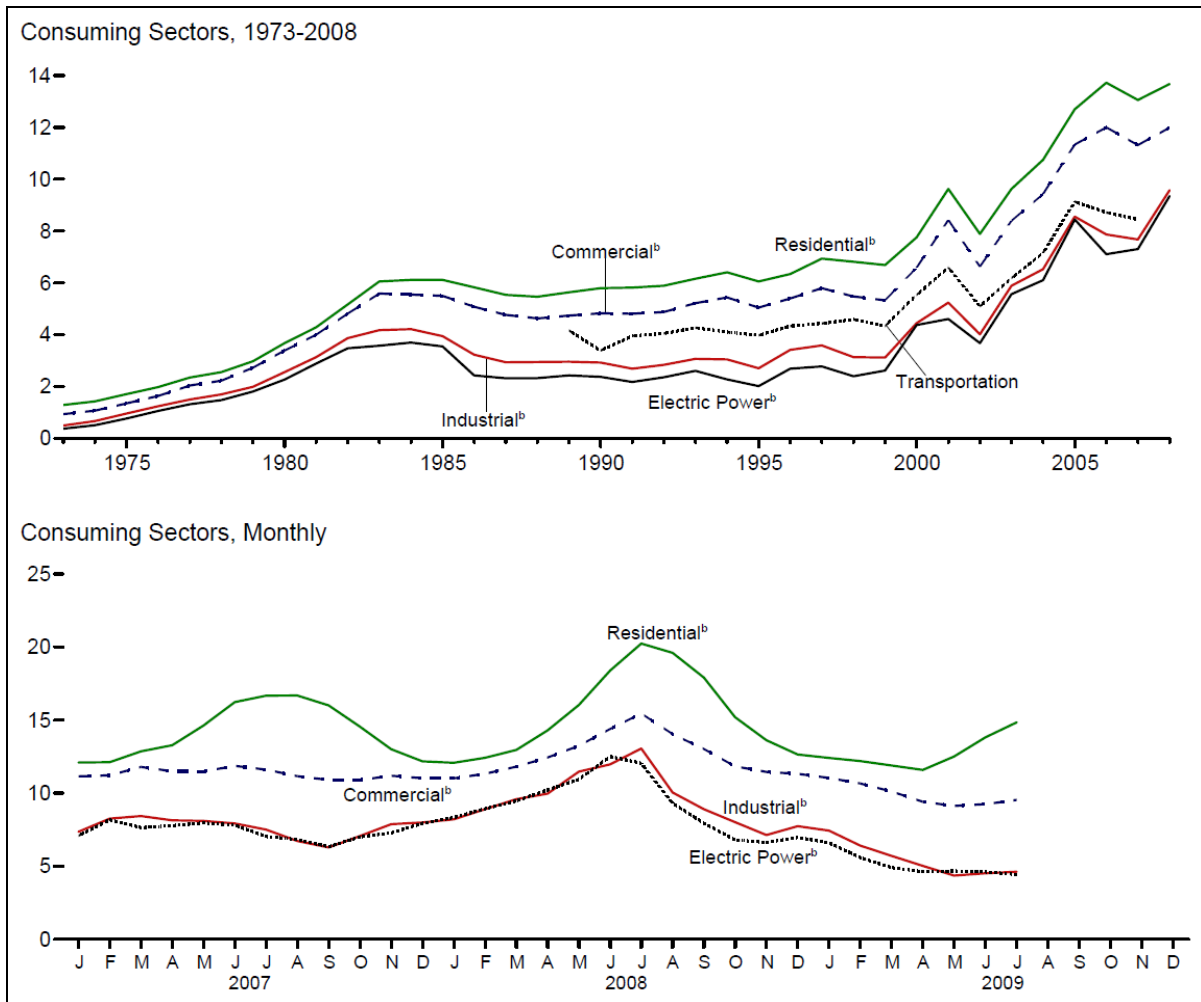
Water recycling

Conventional dry-grind corn ethanol plants use on average 3.5 gal of fresh water (Wu, 2009) and generate 5–6 gal stillage per gal ethanol, of which up to half is recycled directly as backset (Dunn, 2008). The beer from a finished fermentation essentially consists of ethanol and stillage. Minimizing the stillage fraction in the beer saves on process water requirements, but is limited by increasing ethanol concentrations and toxicity to the yeast. Recycling water is important to enhance the environmental footprint and image of the industry, in addition to saving money.

There are limits to water supply, and ethanol plants are not permitted to discharge processing water. Reusing the fungal-treated thin stillage also enables the reuse of enzymes present initially in the thin stillage, as well as those potentially produced by the fungus. Based on 5 gal stillage per gal ethanol and recycling 50% as backset, there is 2.5 gal thin stillage per gal ethanol available for fungal treatment.

Energy savings

A typical ethanol plant evaporates water to condense thin stillage from approximately 7% solids to a syrup of 30% solids using multiple effect evaporators (RFA, 2009d). The syrup is dried with wet grains to 90% solids in drum or ring dryers. A 50 MGY ethanol plant – condensing 125 MGY of thin stillage to syrup for drying with wet grains – would thus evaporate 96 MGY of water from the thin stillage fraction only, not including water evaporated from the syrup in the dryers. The total energy and economic savings associated with evaporating water highly depends on the equipment efficiencies, in-plant heat reuse, and energy source used to fuel the plant. Nominal natural gas prices have increased dramatically from an annual average of \$0.5 in 1973 to \$4.5 in 2000 and \$9.6 in 2008 (per thousand cubic feet) for the industrial sector (Figure 2.11). The monthly average prices delivered to industrial consumers from 2007–2009 had a huge fluctuation from \$13.1 in July 2008 to \$4.3 in August 2009 per thousand cubic feet (Figure 2.11; EIA, 2009c). Our fungal process would save energy by replacing the multiple effect evaporators. Water would be recovered from the thin stillage for recycling to the front of the ethanol production process (i.e., the slurry tank), while simultaneously producing high-quality, high-protein fungal biomass.



^b Includes taxes.

Figure 2.11. Trends and fluctuations in average annual (1973–2008) and monthly (2007–2009) consumer prices per thousand cubic feet (MBTU) of natural gas in the U.S. by consuming sectors (EIA, 2009c).

Value-added feed

The profitability of dry-grind ethanol production depends on the sale of both the ethanol and the coproducts from stillage. Approximately one-third of the corn is converted to ethanol, one-third to carbon dioxide, and one-third remains as dissolved and suspended organics in the stillage. As described previously, the primary coproduct from conventional stillage processing is distiller grains, which is

sold mainly as livestock feed for cattle and dairy. The low-digestible amino acid and high fiber composition of DDGS limits its use for nonruminants, swine and poultry. The demand for syrup has declined as it is not a nutritionally-beneficial feed additive; syrup is sold for very low prices and is even given away (Wicking, 2009).

Fungal biomass production from thin stillage provides an additional feed product that is higher than DDGS and corn in protein and indispensable amino acids, in particular lysine. The fungal biomass may be a great alternative feed ingredient for nonruminants, which is advantageous because of the higher proportions of hogs and chickens produced in the U.S. Corn Belt. Feeding the fungal biomass to local livestock and poultry has the added benefits of reducing transportation costs and keeping the minerals (P and N) in the proximity of the feedstock corn, which require them for growth.

Fungal cultivation

A soon-to-be-published review by Sankaran et al. (2010) discusses the extensive use of fungi for multiple purposes, including wastewater treatment and production of high-value byproducts. Filamentous fungi and yeast are currently cultivated in industry on relatively expensive substrates, e.g., starch and molasses, compared to the organic matter present in wastewater streams (Barbesgaard et al., 1992). Food-processing wastewater has great potential as substrate for fungal growth on the abundant, often readily-biodegradable organic materials and macro/micronutrients (Sankaran et al., 2010). Diverse fungal species have been studied for the aseptic and non-aseptic treatment of food-processing wastewater, ranging from apple distillery, olive oil mill, and sugar refinery, to starch production

from potato, cassava, wheat, and corn wet milling (Friedrich, 1987; Guimaraes et al., 2005; Huang et al., 2003; Jasti et al., 2006, 2008, 2009; Jin et al., 1998, 1999 a–f, 2001a,b, 2002, 2009; Mishra and Arora, 2004; Tung et al., 2004; van Leeuwen et al., 2009; Yesilada et al., 1999). *Rhizopus microsporus* var. *oligosporus* (shortened *R. oligosporus*), for example, has been successfully cultivated on both wheat milling and corn wet-milling streams, achieving significant reductions in COD of up to 80–90% (Jasti et al., 2006, 2008, 2009; Jin et al., 1998, 1999 a–f, 2001a,b, 2002, 2009; Sankaran et al., 2008). Dry-grind corn ethanol byproduct streams offer similar advantages. The low initial pH of 4, high organic content, and pasteurized condition of thin stillage make it an ideal feedstock for fungal cultivation.

Both yeast-like and filamentous fungi have been considered for the production of microbial protein and other byproducts, in addition to wastewater remediation. Yeast species are attractive because of the ease of cultivation and faster growth rates than those of filamentous fungi (Sankaran et al., 2010). The filamentous nature of fungal mycelia and potential for pellet formation, however, simplify separation of the biomass from the liquid substrate (Jin et al., 1999a, 1999b; Nigam, 1994). Unlike bacterial cells, the highly-dewaterable fungal biomass has commercial value as a protein source suitable for animal feed and even for human food, depending on the substrate and processing steps (Stevens and Gregory, 1987).

The primary byproducts of interest in these research studies were fungal biomass (e.g., protein), extracellular enzymes and biochemicals (e.g., lactic acid), and chitin/chitosan. In sterile nutrient medium, the fungal biomass of *R. oligosporus*

consisted of 3% chitosan and 40% protein; moreover, the main amino acid of concern for animal feed, lysine, was 4% of the biomass (Rhodes et al., 1961; Tan et al., 1996). In addition to growing biomass, fungi make and excrete a wide array of biochemicals and enzymes, which are effective in degrading complex carbohydrates (van Leeuwen et al., 2003). The following are a few of the enzymes produced by the food-grade fungus *R. oligosporus*: β -glucosidase, glucoamylase, lipase, phytases, and chitinases (Dhiraj et al., 2002; Gautam et al., 2002; Jin et al., 1999a; Nahas, 1988; Sutardi and Buckle, 1988; Yanai et al., 1992).

The research presented in this thesis is the first study of fungal cultivation on corn ethanol stillage. Chapters 2 and 3 report the bench- and small pilot-scale experiments performed using *R. oligosporus*. Fungal treatment of thin stillage was investigated for fungal biomass production and organics removal to recycle the water for in-plant reuse.

Fungal biomass for food and feed

Fungi are micro- and macroscopic, eukaryotic organisms consisting of proteins, nucleic acids, carbohydrates, and lipids. Fungal proteins include structural components, enzymes, nucleoproteins, and glycoproteins. Crude protein measurements (total nitrogen x 6.25) on fungal biomass overestimate the protein content; two main sources of non-protein nitrogen are nucleic acids and chitin, which are nutritionally unavailable for nonruminants (Sadler, 1994). Fungi can have relatively high DNA and nucleic acid contents because of rapid rates of cell division. Kendrick (2000), however, found the DNA content of fungi to be as low as 0.15 to 0.30%. Chitin – a cellulose-like polysaccharide containing nitrogen – and β -glucan

are fungal cell wall materials that are of low digestibility for nonruminants and serve as dietary fiber.

Research on microbial cultivation of a variety of substrates in order to produce protein specifically for animal feed or human consumption began in the 1960's (Casas, 1993). The research on new protein sources was motivated by concerns over the booming human population and the potential for shortages in animal protein sources. The biomass product has been called single cell protein (SCP) and microbial biomass protein (MBP) in the literature. Table 2.4 provides a list of filamentous fungi and yeast that have been studied for SCP production. Diverse fungal species and substrates, such as wastewaters, have been evaluated in lab-scale, batch studies to produce fungal biomass for animal feed (Hiremath et al., 1985; Thanh and Simard, 1973a, b). As discussed previously, fungal wastewater treatment is a low-cost, technically-simple option for producing this alternative protein source (Stevens and Gregory, 1987).

Fungi in the form of mushrooms and fermented food, such as yeast in bread and beverages, have been consumed by humans for thousands of years. Filamentous fungi are used to make blue and white molded cheese, tempeh, and miso. Microbial protein from filamentous fungi is generically named mycoprotein. The only commercial mycoprotein product available for human consumption, Quorn[®], is biomass of the filamentous fungus *Fusarium venenatum* A3/5 (previously identified as *F. graminearum*) (Thrane, 2007). The fungus is cultivated aseptically in a 50-m tall, 150-m³ airlift (compressed-air) bioreactor started with culture broth in batch mode and moved to continuous mode with glucose, biotin, mineral salts, and

ammonia feeds. The airlift design is advantageous for viscous fungal growth due to improved oxygen and nutrient transfer, efficient carbon dioxide removal, and reduced heat generation (Trinci, 1992). In a glucose (100 g) medium, 54 g of fungal biomass (dry wt), containing 45% protein, is produced at a rate of 300–350 kg/h (Kavanagh, 2005; Wiebe, 2002).

Table 2.4. Fungal species evaluated for single cell protein (SCP) production.

Filamentous fungi and yeast	Reference^a
<i>Actinomucor elegans</i>	Hang, 1976
<i>Aspergillus fumigatus</i>	Khor et al., 1977; Reade and Gregory, 1975
<i>Aspergillus niger</i>	Christias et al., 1975; Hang, 1976; Oboh et al., 2002; Singh et al., 1991
<i>Aspergillus oryzae</i>	Hang, 1976
<i>Candida lipolytica</i>	Achermowicz et al., 1977
<i>Candida tropicalis</i>	Achermowicz et al., 1977; Christias et al., 1975
<i>Candida utilis</i>	Villas-Boas et al., 2003
<i>Chaetomium globosum</i>	Hang, 1976
<i>Fusarium moniliforme</i>	Christias et al., 1975; Drouliscos et al., 1976
<i>Fusarium oxysporum</i>	Sukara and Doelle, 1989
<i>Fusarium venenatum</i>	Anderson and Solomons, 1984; Trinci, 1992
<i>Geotrichum candidum</i>	Robinson and Smith, 1984; Ziino et al., 1999
<i>Pestalotiopsis westerdijkii</i>	Hang, 1976
<i>Phanerochaete chrysosporium</i>	Cardoso and Nicoli, 1981
<i>Rhizopus oligosporus</i>	Sukara and Doelle, 1989
<i>Thielavia terrestris</i>	Bajon et al., 1985; Stevens and Gregory, 1987
<i>Trichoerma viride</i>	Hang, 1976; Youssef and Aziz, 1999

^a References collected by Thrane (2007).

The fungal biomass in the broth is heat-shocked (64°C for 20 min) to reduce the RNA content from 10% to below the recommended maximum of 2% for humans (Thrane, 2007). High RNA intake can lead to uric acid accumulation and deposition in the kidneys and joints, known as gout. The thermal treatment results in protein and cell component losses for a net yield reduction of approximately one-third. The mycelia is subsequently harvested by filtration and concentrated from 1.5% (w/v) to 25–30% (w/v). The mycoprotein undergoes further product-dependent processing (e.g., the addition of egg albumin as a binder and other colors/flavors) and is frozen for storage.

The mycoprotein product Quorn[®] has a protein content of 12% (w/w, wet basis), comparable essential amino acid composition to eggs, and comparable protein digestibility to beef and soybeans (Miller and Dwyer, 2001; Rodger, 2001). The product is 45% protein, 14% fat, and 26% fiber, on a dry basis, and provides the range of B vitamins. Mycoprotein has a net protein utilization (NPU) of 75, compared to 80 for beef and 83 for fish (Kavanagh, 2005). The fungal cell wall materials, namely chitin and β -glucan, contribute the dietary fiber. Monitoring mycotoxin concentrations is a crucial part of quality control; the *F. venenatum* strain produces mycotoxins, such as diacetoxyscirpenol, in small quantities under optimal conditions (Miller and MacKenzie, 2000; O'Donnell et al., 1998).

In addition to mycoprotein, there are numerous food products across the world fermented by filamentous fungi, such as koji and katsuobushi (Japan), tempeh and oncom (Indonesia), chu, sufu, and pehtze (China), marcha (India), and Camembert, Roquefort, and Brie (France) (Nout, 2007). The fungus *R. microsporus*

var. *oligosporus* (shortened *R. oligosporus*) is known best for tempeh fermentation. Originating in Indonesia, tempeh is the solid-substrate fungal fermentation of cooked seeds (e.g., soybeans and cereals) (Nout and Kiers, 2005). Fungal spore inoculum for starting tempeh fermentations is prepared by growing *R. oligosporus* on cooked rice and storing as dehydrated powders. *R. microsporus* spp. prefer fermentations at temperature ranges of 30–40°C. The growing fungal mycelium binds the bean cake and constitutes an estimated 6% (dry wt) of the tempeh (Sparringa and Owens, 1999). Table 2.5 provides the functional contributions of the fungus to the tempeh food product. Freshly fermented tempeh, for instance, has a mushroom flavor, in addition to a number of health benefits. *R. microsporus* strains can form rhizoxins and rhizonins; however, *R. oligosporus* does not produce these toxic metabolites.

Table 2.5. Tempeh functional properties contributed by fungal colonization of soybeans by *Rhizopus oligosporus*.

Functionality	Example(s)	Reference
Texture	Bean cake solidity; gradual softening during storage	Ariffin et al., 1994; Reu et al., 1997
Flavor	Mushroom	Nout, 2007
Enzymatic transformations	Carbohydrases degrade dietary fiber; proteases improve digestibility	Sarrette et al., 1992; Kiers et al., 2003
Antioxidants	Phenolic antioxidants released from soybean isoflavones	McCue and Shetty, 2003
Vitamins	Folates	Nout and Kiers, 2005; Ginting and Arcot, 2004
Health-promoting unsaturated fatty acids	γ -linolenic acid	Liu et al., 2004

Similar to mycoprotein and fermented food products, fungi are already used in animal feed. Fungal yeast cells are a rich protein source (40–60% protein) fed to poultry and livestock as part of distillers grains and in the form of brewer's dehydrated yeast (Walker, 1998). According to the Nutrient Requirements of Swine (NRC, 1998), brewer's dehydrated yeast (7% moisture) contain 46% crude protein on an as-fed basis, with a high lysine content of 3.2% comparable to SBM (3.0%). Lysine digestibility is 76% for yeast and 85% for SBM; whereas, it is only 66% for corn grain and 57% for DDGS. The metabolizable energy of this yeast source for swine is 3,025 kcal/kg (SBM – 3,380; corn – 3,420; DDGS – 2,820 kcal/kg). In addition to yeast cells as a protein feed additive, many fungal enzymes are being used in the feed industry. Cellulase, mannase, phytase, proteolytic enzymes, and xylanolytic enzymes are added to improve feed digestibility (Whitaker et al., 2003).

The nutritional value of fungal biomass, such as *R. oligosporus*, for nonruminants is best assessed using feed studies; digestibility and bioavailability are two critical factors (Bregendahl, 2008). Chitin and chitosan, present in fungal cell walls, have a high viscosity, which may increase the water fraction of poultry gut contents and reduce digestibility by impeding mucosal nutrient uptake (Cheeke, 2005). *In vitro* digestibility of *R. oligosporus* biomass is reported to average 84% (Jin et al., 2002), which is comparable to soybean meal (85%). *In vivo* studies on the digestibility of individual amino acids, especially lysine, and energy bioavailability in the fungal biomass from thin stillage cultivation will enable more accurate comparisons with commercial feed products (Bregendahl, 2008). Results of broiler chick studies are applicable to laying hens, turkeys, and pigs because of similar

digestive physiology. Feed studies are an important next step to the research on fungal treatment of thin stillage. Large volumes of fungal biomass are required and should be prepared, ideally, using the same processing procedures (collection, dewatering, and drying) that will be employed in full-scale production.

Motivation for Corn Mash Ozonation Research

BPX™ process benefits

As discussed earlier in this chapter, the POET BPX™, or no-cook, process has a number of important advantages over the conventional process. The BPX™ process omits the energy-intensive liquefaction/cooking step, thus reducing plant energy requirements by 8–15% (POET, 2009). The BPX™ process results in higher ethanol yields by improving starch accessibility, lowers VOC emissions, cuts cooling water needs, and increases DDGS quality (e.g., protein/amino acid digestibility).

BPX™ process drawbacks

Fuel ethanol fermentations are not conducted under pure culture conditions. The high-temperature jet-cooking step (90° to 165°C) in the conventional process not only gelatinizes the corn starch, it also reduces contamination and yield losses caused by unwanted lactic acid bacteria (LAB) (U.S. Grains Council, 2007). To compensate for not cooking in the BPX™ process, POET ethanol plants add antibiotics, namely virginiamycin (brand-name Lactrol®), and antimicrobials to the mash prior to fermentation. The fermentations are also performed at a lower pH, which is less conducive to ethanol fermentation but favors yeast over LAB activity. The use of antibiotics, in addition to the added cost, is problematic because of the rising concerns about the emergence of antibiotic-resistant bacteria. All antibiotic

applications are essentially selection experiments for resistant microorganisms. It should be noted that conventional ethanol plants, despite jet cooking, dose with antibiotics on a consistent basis or as LAB-contamination issues arise (Bayrock and Ingledew, 2003; Day et al., 1954; Narendranath and Power, 2005; Skinner and Leathers, 2004; Stroppa et al., 2000).

Research motivation

The objective of the research discussed in Chapter 5 was to investigate an alternative method to controlling LAB contamination in the BPX™ process. This research sought to replace antibiotics with a disinfection method, ozonation of the corn mash, and to enable operation at a higher pH more conducive to ethanol fermentation. Operating at a higher fermentation pH may also reduce sulfuric acid requirements for pH adjustment and the resulting sulfur content in the distillers grains. The enhanced coproduct could be marketed as antibiotic-free and reduced-sulfur distillers grains. Ozonation, in place of antibiotic addition and lower fermentation pH, could therefore save money by reducing operational expenses (i.e., antibiotic and chemical costs) and improve the feed quality of the DDGS.

Bacterial contamination

Bacteria in fuel ethanol fermentations generally originate from the corn grains, which carry them in from the field (Bryan, 2006). Bacteria compete with yeast for nutrients – glucose, trace minerals, vitamins, and free amino nitrogen – and lower ethanol yields by diverting glucose to lactic and acetic acids, which are inhibitory to yeast (Barbour and Priest, 1988; Maiorella, 1983; Mankanjuola et al., 1992). Bacterial contamination affects yeast growth, yeast viability, and ethanol production. Gram-

positive and gram-negative bacterial isolates from fuel ethanol plants include species of *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, *Clostridium*, and *Lactobacillus* (Lushia and Heist, 2005; Skinner and Leathers, 2004).

Lactobacilli sp. are the most prevalent in the industry. Gram-positive LAB are ethanol tolerant, grow faster than yeast, and produce organic acids. A number of commercially-isolated lactobacilli are able to grow in ethanol concentrations exceeding 10% (v/v) (Hynes et al, 1997; Narendranath et al., 1997).

In a study of two commercial dry-grind ethanol plants by Skinner and Leathers (2004), the genus *Lactobacillus* was isolated most frequently, representing 38 and 77% of total bacterial isolates from the first and second plants, respectively. Samples were taken at different process times, dates, and locations over a 9-month period. The results showed characteristic bacterial flora at each plant and low variability for a given sampling time and season, indicating the occurrence of chronic infections. The antibiotic dosing procedures differed between the two ethanol plants; one added antibiotics to the yeast propagation tank only, and the other dosed with antibiotics every four hours of the fermentation. Neither procedure reliably reduced bacterial contamination. Less contaminant diversity, however, was observed in the second plant, which dosed with antibiotics more frequently. In addition to yield losses by chronic contamination, acute infections arise unpredictably and can result in plant shut-downs for cleaning – an expensive loss in productivity (Makanjuola et al., 1992; Narendranath et al., 1997; Skinner and Leathers, 2004). If no antibiotics are used, however, it is common for a plant to lose 1–5 % of its potential ethanol yield (Bryan, 2006).

Current antibacterial control approaches

In addition to steam cooking the corn mash, cleaning and sanitation are important microbial control measures in the corn ethanol industry. Infections in process tanks and continuous yeast propagators, as well as resistant biofilms within the process train, can continually reintroduce bacterial contaminants (Bischoff et al., 2009; Skinner and Leathers, 2004). Large yeast inoculum helps control contamination during fermentations; however, yeast growth and fermentation rates are still reduced by high lactobacilli concentrations (Thomas et al., 2001).

Penicillin and virginiamycin are the antibiotics available commercially for fuel ethanol production (Connolly, 1997; Lushia and Heist, 2005); the antibiotics tetracycline and monensin have also been investigated (Aquarone, 1960; Hynes, 1997; Stroppa, 2000). Virginiamycin, an antibiotic produced by *Streptomyces virginiae*, has had limited use in human medicine but extensive use as a growth-promoting additive in animal feed (Cocito, 1979). The recommended dose of virginiamycin in fuel ethanol fermentations is 0.25–2.0 ppm (Hynes et al., 1997).

The use of antibiotics, in addition to being controversial, has limitations. Hynes et al. (1997) observed reductions in the effectiveness of virginiamycin after extended fermentations, possibly resulting from its breakdown by lactobacilli (Dutta, 1981). *Lactobacillus* sp. with higher tolerance to virginiamycin have been isolated from fuel ethanol plants that dose with virginiamycin. Moreover, the emergence of bacterial isolates with resistance to both virginiamycin and penicillin has been reported (Bischoff et al., 2007; Lushia and Heist, 2005). New antibacterial agents and procedures should be investigated. The emergence of antibiotic-resistant

bacteria may further limit the effectiveness of commercial antibiotics to control bacterial contamination in fuel ethanol plants (Bischoff et al., 2009). Antibiotic use in the industry could also potentially be prohibited in the future, as has occurred in Europe.

Research on alternative antibacterial agents

Hop acids offer a natural alternative to antibiotics for fuel ethanol plants (Bryan, 2006). Many of the organic acids found in hops exhibit antimicrobial activities. Hop-compounds inhibit growth by dissipating the trans-membrane pH gradient of microbes (Haakenson et al., 2009; Simpson 1993). Hops have long been used in the brewing industry to contribute to the flavor of beer, by adding bitterness and aroma, and to improve product shelf-life. IsoStab™, a liquid hop extract, is a commercially-available BetaTec product with fuel ethanol applications. As observed by Liliith Ruckle, BetaTec's European ethanol project manager, "*in the European Union, for example, if fermentation coproducts are used as animal feed, they must not contain residues of antibiotics. This law became effective at the end of 2005. Other nations have implemented similar laws and some believe the United States could eventually follow suit*" (Bryan, 2006). Several U.S. ethanol producers are using IsoStab™ and obtaining higher ethanol yields with less fluctuation.

A variety of disinfectants have been investigated on lab scale for use in fuel ethanol fermentations, including hydrogen peroxide, chlorine dioxide, potassium metabisulfite, and 3,4,4'-trichlorocarbanilide (Chang et al., 1997; Gibbons and Westby, 1986; Meneghin et al., 2008; Narendranath, 2000; Oliva-Neto and Yokoya, 1998; Skinner and Leathers, 2004). Bacteria were inhibited over yeast by all of

these agents, but with variations in sensitivities among different strains (Skinner and Leathers, 2004). A recent study also evaluated the addition of lactate (1–2%) as a bacterial inhibitor by utilizing a lactate-tolerant yeast strain (Watanabe et al., 2008).

Narendranath et al. (2000) investigated the use of urea hydrogen peroxide (UHP) to control the growth of lactobacilli strains during the fermentation of wheat mash. Lactobacilli lack the enzyme catalase, which decomposes hydrogen peroxide, and are more susceptible than yeast to its toxic effect. Hydrogen peroxide dosages of 1–10 mM were studied previously by Chang et al. (1997) for ethanol fermentations. UHP has the additional benefit of providing the yeast with assimilable nitrogen (urea) and oxygen. The yeast strains employed in alcohol production can only use low-molecular-weight nitrogen sources, such as ammonium ion, urea, amino acids, or dipeptides (Ingledew, 1993; Patterson and Ingledew, 1999). Urea and liquid ammonia are added in the fuel alcohol industry as nitrogen sources (Ingledew, 1995). High concentrations of 30–32 mmol UHP/L (approximately 3 g/L) effectively controlled bacterial competition in this research, with no significant difference in ethanol yields between UHP-treated, spiked samples and non-treated, non-spiked samples. The wheat mash particulate matter greatly reduced the effectiveness of UHP as a disinfectant. Only 2 mmol UHP/L (~190 mg/L) was required for disinfection in mash with little or no particulate matter present. Hydrogen peroxide (H_2O_2) and UHP exhibited similar H_2O_2 -decomposition in wheat mash at 30°C.

Meneghan et al. (2008) assessed the effect of chlorine dioxide, a common disinfectant in water treatment applications, against bacteria prevalent in ethanol

fermentations (e.g., *Lactobacillus plantarum* and *L. fermentum*). The minimum inhibitory concentrations (MIC) of chlorine dioxide on the bacterial stains and on the yeast inoculum were determined using nutrient media. The effectiveness in nutrient media will likely be different than in the high-suspended-solids mash used at fuel ethanol plants, as observed by Narendranath et al. (2000). The MICs for chlorine dioxide ranged from 10–125 ppm for the species of bacteria tested; *L. plantarum* had the highest MIC of 125 ppm. Yeast growth was also affected at chlorine dioxide concentrations over 50 ppm. The authors advised using dosages less than 50 ppm, but admitted that this may not be effective against *Lactobacillus* sp.

Ozonation

Chlorine dioxide and ozone are common alternatives to chlorine for drinking water and advanced wastewater treatment. Disinfection by chlorination has become controversial because chlorine reacts with organic materials found in natural water sources to form trace amounts of trihalomethanes (THMs), such as chloroform (CHCl_3) (Bellar et al., 1974; Rook, 1974). THMs are low-molecular-weight, chlorinated hydrocarbons which are suspected carcinogens (Pieterse, 1988). The alternative disinfectants – chloramines, chlorine dioxide, and ozone – do not form or form little THMs. Ozone (O_3) is a highly-reactive gas, which must be generated on-site, and is formed artificially from oxygen molecules by energy introduced using electrical discharge. Ozone is a powerful disinfectant and strong oxidant, with an oxidation potential ($E^\circ=2.08$ V) higher than that of chlorine dioxide ($E^\circ=0.95$ V) and hydrogen peroxide ($E^\circ=1.78$ V) (AWWA, 1999). The hydroxyl radicals formed by the breakdown of ozone to oxygen have an even higher E° value of 2.80 V.

Physicochemical damage to DNA is the mechanism implicated for ozone inactivation of bacterial cells (Hamelin and Chung, 1978). In modern water treatment applications, ozone is used not only as a disinfectant, but also as a pretreatment to aid coagulation, to promote the removal of organic and inorganic contaminants, and to eliminate taste, odor, and color (AWWA, 1999; Beltran et al., 1999; Langlais et al., 1991).

Ozone has a short half-life (20 min only in pure water) and leaves no residual as it reverts back to oxygen. The addition of oxygen in small amounts is important for ethanol production. Yeast require oxygen to synthesize unsaturated fatty acids and sterols for the cell membrane (Andreasen and Stier, 1954). Typically, oxygen is not available at optimal levels in industrial fermentations because of operation procedures and low solubility in mash (Ingledew, 1995). The addition of oxygen by ozonation of corn mash, thus, offers a benefit for yeast performance.

In addition to municipal water/wastewater applications, recent studies related to fuel ethanol production investigated the use of ozone treatment to accomplish the following: to purify fuel ethanol (Onuki et al., 2007), to reduce the organic content of thin stillage from corn dry-grind ethanol production (Singh et al., 2007), and to selectively control bacterial growth during fungal cultivation of corn wet-milling wastewater (Sankaran et al., 2008). In research by Onuki et al. (2007), fuel-grade ethanol was ozonated to oxidize impurities that impart unpleasant taste and odor, in order to upgrade the ethanol to pharmaceutical and beverage grades. Ozone dosages of up to 160 mg/L were reported. The estimated treatment cost was less

than a penny per gallon ethanol, as compared to the multiple distillation steps typically employed at a cost of at least \$0.30 per gallon.

The research by Singh et al. (2007) focused on the thin stillage fraction obtained from fuel ethanol production, similar to our fungal research. The objective was to use ozonation to lower the organic content, measured as chemical oxygen demand (COD), of thin stillage so that a larger fraction could be recycled as backset. Thin stillage typically has a COD of 75 g/L. A COD removal of 85% was achieved with a high ozone dosage of 4,000 mg/L into a 40x-diluted sample. Complete oxidation of the high organic content of thin stillage is uneconomical because of the huge ozone demand; it is also impractical to dilute thin stillage. The next research step would be to use the ozone-treated thin stillage as process water for ethanol fermentations in order to demonstrate that a larger portion can be reused as backset.

Similar to our fungal research conducted on thin stillage, Sankaran et al. (2008) investigated non-aseptic, fungal treatment of wastewater from the corn wet-milling process. Bacterial contamination is a major concern in fungal wastewater treatment. Selective disinfection with ozone was employed to reduce bacterial competition during filamentous-fungal cultivation; a low ozone dosage of 57 mg/L was found to be most effective for improving both fungal biomass production and COD removal. Fortunately, thin stillage is essentially pasteurized during the distillation step. Bacterial competition is not an issue for fungal cultivation on fresh thin stillage samples, which were 80°C when collected from the ethanol plant.

CHAPTER 3. WATER RELAMATION AND VALUE-ADDED ANIMAL FEED FROM CORN ETHANOL STILLAGE BY FUNGAL PROCESSING

A paper prepared for submission to *Biotechnology and Bioengineering*

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Abstract

Dry-grind corn ethanol plants, the backbone of a rapidly expanding biofuel industry, generate copious amounts of stillage, the leftovers from fermentation followed by distillation. Most thin stillage is currently concentrated by flash evaporation – an energy-intensive process – blended with distillers grains, and dried to produce distillers dried grains with solubles (DDGS). Thin stillage is generated in pasteurized condition and is rich in nutrients, with a chemical oxygen demand (COD) up to 100 g/L. The initial pH of 4 and high organic content make it an ideal feedstock for fungal cultivation. This research cultivated the food-grade fungus *Rhizopus microsporus* var. *oligosporus* on thin stillage from a local dry-grind corn ethanol plant. Batch experiments in 5- and 50-L stirred bioreactors showed prolific fungal growth under non-sterile conditions. COD, glycerol, and organic acids

removals, critical for in-plant water reuse, reached 80, 100, and 100%, respectively, within 5 d of fungal inoculation. The initial 20–30 g/L suspended solids decreased to nearly non-detectable levels, enabling effluent recycle as process water. The fungus contains 2% lysine and 2% methionine (corn contains 0.3% and 0.2%, respectively) and 43% crude protein, enhancing the nutritional value as a poultry and livestock feed. With minimal pretreatment, the fungal biomass could be co-fed with distillers grains to nonruminants – swine and poultry. Avoiding water evaporation from thin stillage would save substantial energy inputs in corn ethanol plants.

Keywords: Thin stillage; fungal process; animal feed; water reuse; dry-grind ethanol plant; distillers dried grains with solubles (DDGS)

Introduction

Fuel ethanol production in the U.S. is booming with 9.0 billion gallons produced in 2008, up from 4.9 and 6.5 billion gallons in 2006 and 2007, respectively (RFA, 2009a). The industry is on pace to produce more than 10 billion gallons in 2009 – 9% of America's gasoline supply. Biofuels, such as corn ethanol, help alleviate oil dependence and improve energy security, while boosting the rural economy and reducing greenhouse gas emissions. The corn ethanol industry has advanced in efficiency, but still has considerable room for improvement. Innovations to date include no cook – raw starch hydrolysis (POET BPX™ process), back-end crude corn oil recovery, and corn fractionation (e.g., POET BFRAC™ process). Fuel ethanol companies are also pursuing renewable energy sources in order to reduce natural gas consumption, for instance, by integrating biomass gasification and methane digesters.

Reducing energy inputs, creating value-added coproducts from stillage, and recycling water are important to sustainable corn ethanol production. Innovations for improved sustainability help to contest recent ethanol issues, such as indirect land use changes and food versus fuel, which affect public perception of corn ethanol. Most conventional dry-grind corn ethanol plants use on average 3.5 gal of fresh water (RFA, 2009a) and generate 5–6 gal stillage per gal ethanol after distillation, of which up to half is recycled directly as backset (Dunn, 2008). The ethanol-to-stillage ratio in the beer from a finished fermentation is limited by ethanol toxicity to the yeast. The profitability of dry-grind ethanol plants depends on the sale of both the ethanol and the coproducts from stillage. Recycling water is important as there are limits to supply, and the ethanol plants are not permitted to discharge processing water.

Dry-grind processing produces ethanol from corn by milling, hydrolyzing with enzymes (with or without cooking), fermentation, and distillation. Approximately one-third of the corn is converted to ethanol, one-third to carbon dioxide, and one-third remains as dissolved and suspended organics in the stillage. Most suspended solids – wet distillers grains – are removed by centrifugation. The liquid centrate – thin stillage – contains mainly dissolved organic materials (solubles) of 75–100 g/L as chemical oxygen demand (COD) (Schaefer et al., 2008; Singh et al., 2007). Up to half of the thin stillage is directly recycled as process water, and the remainder is evaporated to produce a syrup of 30% solids (RFA, 2009c), requiring a substantial part of plant energy inputs. The syrup is blended with wet grains and dried to produce distillers dried grains with solubles (DDGS). Dry-grind corn ethanol plants

produced 23.0 million metric tons of distillers grains in 2008, a significant increase from the 14.6 million metric tons in 2007 (RFA, 2009b). Distillers grains is sold primarily as livestock feed for cattle and dairy. The low content of digestible amino acids limits its use for nonruminants. Only 11 and 5% of distillers grains was consumed by swine and poultry, respectively, in 2007, in spite of higher proportions of hog and chicken production in the U.S. Corn Belt. The syrup has suffered a decline in demand as it is not nutritionally expedient to include syrup; it is sold for very low prices and is even given away (Wicking, 2009).

The low initial pH of 4 and high organic content make thin stillage an ideal feedstock for fungal cultivation. Fungal treatment of thin stillage has the potential to recover water and enzymes for in-plant reuse and to produce a high-quality animal feed (distillers dried grains with fungal protein or fungal protein only). The fungal coproduct could command an increased market value, while improving profits and minimizing environmental impacts. Based on 6 gal stillage per gal ethanol and recycling 50% as backset, there is 3 gal thin stillage available for fungal treatment.

The use of fungi is advantageous as they produce a wide array of biochemicals and enzymes, which tend to be more effective in degrading complex carbohydrates than bacteria (Sankaran, 2010; van Leeuwen et al., 2003, 2009). The food-grade fungus *Rhizopus microsporus* var. *oligosporus* (shortened *R. oligosporus*) produces numerous enzymes, including β -glucosidase, glucoamylase, lipase, phytases, and chitinases (Dhiraj et al., 2002; Gautam et al., 2002; Jin et al., 1999a; Nahas, 1988; Sutardi and Buckle, 1988; Yanai et al., 1992). *Rhizopus* sp. are also known to produce α -amylase (Jin et al., 1998). Under aseptic conditions in

a nutrient medium, *R. oligosporus* was reported to produce a 3% yield of chitosan (Tan et al., 1996), as well as 4 and 40% yields of lysine and protein, respectively (Rhodes et al., 1961). *R. oligosporus* was successfully cultivated on both wheat milling and corn wet-milling streams, achieving significant reductions in COD of up to 80–90% (Jasti et al., 2006, 2008, 2009; Jin et al., 1998, 1999 a–f, 2001a, b, 2002, 2009; Rasmussen et al., 2007; Sankaran et al., 2008). This research is the first study of fungal cultivation on dry-grind corn ethanol stillage. The filamentous nature of fungal mycelia and potential for pellet formation aid in the recovery of fungal biomass (Jin et al., 1999a, 1999b; Nigam, 1994).

Based on this rationale, bench- (5 L) and pilot-scale (50 L) experiments were conducted to evaluate fungal treatment of thin stillage for fungal biomass production and organics removal to obtain recyclable effluent for in-plant use. Aeration rates in stirred bioreactors were varied from 0.2 to 1.0 L air/L working volume/min (vvm). Feed stillage and bioreactor samples were analyzed to determine the removal of total and soluble COD, total and volatile suspended solids (TSS, VSS), glycerol, and lactic and acetic acids, critical for recycling the effluent as process water. Fungal biomass production was quantified, and samples were analyzed for protein and amino acid contents.

Materials and Methods

Thin stillage

Thin stillage samples were obtained from Lincolnway Energy (Nevada, IA, USA), a local dry-grind corn ethanol plant. Samples were collected in sterile 10- and 20-L carboys and stored at 4°C prior to use. The pH of fresh thin stillage was acidic

(pH 3.8 to 4.7), and the COD averaged 90 g/L, of which 55 g COD/L was dissolved solids. The total and reducing sugar contents averaged 17 and 6 g/L, respectively. Suspended solids were 20 to 30 g/L, and the total nitrogen content was 6 g/L. Thin stillage suspended solids settled during storage at 4°C. Supernatant from settled thin stillage (0.2 g/L suspended solids) was used as substrate for preparation of fungal mycelia inoculum and to determine the effect of thin stillage particles on fungal growth and morphology.

Fungal culture

Freeze-dried culture of the fungus *Rhizopus microsporus* var. *oligosporus* was obtained from the American Type Culture Collection (ATCC 22959, Rockville, MD, USA). The culture was revived in yeast mold (YM) broth (Difco Laboratories, Sparks, MD, USA) at 25°C. Plates of potato dextrose agar (Difco Lab) were inoculated with the revived culture and incubated at 25°C for 5–7 d. Fungal spores were harvested from the plates using sterile deionized water containing 0.1% (w/v) peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). Glycerin (20% [v/v]) was added to the spore suspension prior to ultra-low freezer (-75°C) storage in sterile 2-ml cryovials. The harvested spore count of 5×10^6 spores per ml was determined by haemocytometer. Mycelia inoculum was prepared by heat sterilization (121°C for 20 min) of YM broth or settled thin-stillage supernatant (1 L), inoculation with 1 vial (2 ml) of spore suspension, and incubation at 150 rpm shaking and 37°C for 3 d (Figure 3.1).

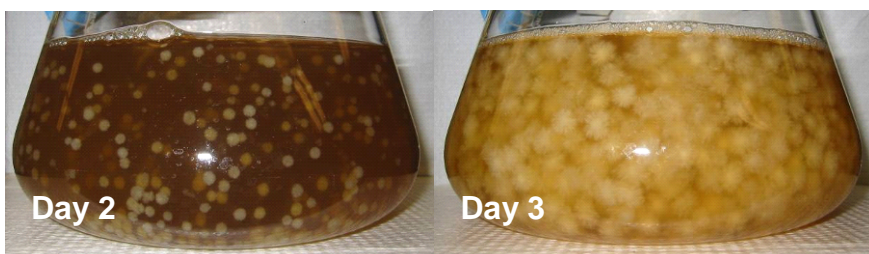


Figure 3.1. Mycelia inoculum: fungal pellet formation from spores on days 2 and 3 in sterile settled thin-stillage supernatant (1 L) with shaking at 150 rpm and 37°C.

Bioreactor set-up and operation

Batch fungal cultivations on thin stillage were performed in bench-top stirred bioreactors with 5-L working volumes (Figure 3.2). Bench-top bioreactors were sterilized with water by autoclaving for 45 min at 121°C, drained, and filled with thin stillage aseptically using a peristaltic pump. Culture conditions were chosen based on previous fungal research treating corn ethanol wet-milling wastewater (Jasti et al., 2009). Once culture conditions stabilized at pH 4, 250 rpm agitation, 37°C, and the filter-sterilized aeration rate chosen for the experiment, the dissolved oxygen (DO) probe was calibrated to 100%. The thin stillage was then inoculated with fungal spore suspension (1×10^7 spores). The pH was controlled using a pH probe, acid pump, and hydrochloric acid (3 N). An external water jacket maintained the temperature at 37°C. Aeration rates were increased from 0.2 to 1.0 L air/L thin stillage/min (vvm) over successive experiments. Evaporative losses were quantified on a daily basis to compensate for evaporation in sample analyses calculations.

Batch stirred fungal experiments were scaled up to 50-L working volumes. These experiments were operated with similar culture conditions. The bioreactor

was steam sterilized, filled aseptically with thin stillage, and inoculated with fungal mycelia (2% [v/v]).

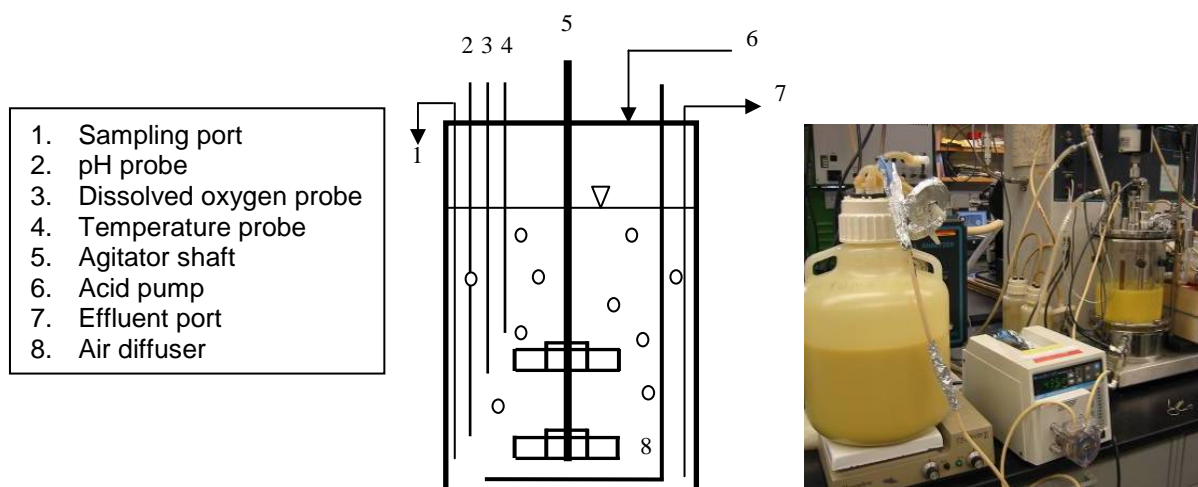


Figure 3.2. Schematic of stirred bioreactor (5 L) (left); photo of stirred bioreactor set-up and aseptic addition of thin stillage (right).

Sample analyses

Feed thin stillage and/or daily bioreactor samples were analyzed for total and soluble chemical oxygen demand (TCOD, SCOD), total and volatile suspended solids (TSS, VSS), nitrogen content, total and reducing sugars, lactic and acetic acids, and glycerol. The COD and suspended solids analyses were performed as described in Standard Methods (APHA/AWWA/WEF, 2005). The nitrogen content of fresh thin stillage was determined according to the Dumas method (AOAC, 1995). Reducing and total sugars in the thin stillage were measured by using the Somogyi-Nelson carbohydrate assay (Antai and Crawford, 1981) and the phenol-sulfuric method (Dubois et al., 1956), respectively.

The organic acid and glycerol contents were determined with a Waters high pressure liquid chromatograph (Millipore Corporation, Milford, MA, USA). The system was equipped with a Waters Model 401 refractive index detector, column heater, autosampler, and computer controller (Kunduru and Pometto, 1996). The sample components were separated on a Bio-Rad Aminex HPX-8711 column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) using 0.012 N sulfuric acid mobile phase at 0.8 ml/min, an injection volume of 20 μ l, and a column temperature of 65°C.

Effluent and biomass collection

Fungal process effluent was pumped from the bioreactors through the effluent port. Fungal biomass was collected from the bioreactor vessel wall, agitator blades, and probes/ports. Suspended biomass was recovered from the effluent by centrifuging for 20 min at 7,277 x *g* and decanting the supernatant. Attached and suspended biomass was freeze-dried to quantify fungal biomass production and to avoid the potential for heat-drying effects on feed quality for research purposes.

Results and Discussion

Visual observations

Fungal growth and morphology in 5-L experiments depended on the aeration rate and initial suspended solids concentration. With 0.2-vvm aeration, suspended fungal growth was visible within 4 d of spore inoculation. Accumulation of mycelia was observed on day 4 between the pH and DO probes and the vessel wall. The fungus continued to agglomerate and grow, with considerable clarification of the liquid on day 5. By day 8, the vessel was filled with fungal biomass. Increased

aeration of 0.4 vvm triggered biomass agglomeration and liquid clarification on day 4, a day earlier than observed with 0.2-vvm aeration. The fungal mycelia transitioned from suspended growth to attachment on the agitator blades and between the probes and vessel wall (Figure 3.3). Liquid collected from the effluent port was well-clarified with a yellow tint.

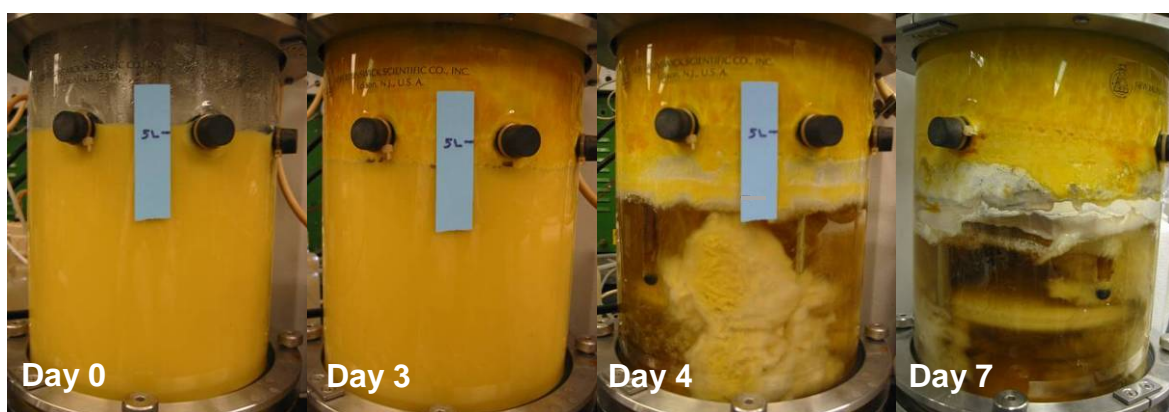


Figure 3.3. Fungal cultivation of thin stillage (5 L) in a batch stirred bioreactor with 0.4-vvm aeration on the day of spore inoculation (day 0) to day 7.

Higher aeration rates of 0.8 and 1.0 vvm promoted a different fungal morphology; fungal mycelia became filamentous flocs on day 4, and some mycelia remained in suspension throughout the 5-L experiments (Figure 3.4). The liquid remained in suspension throughout the 5-L experiments (Figure 3.4). The liquid began to clarify between days 3 and 4. In 50-L experiments, most fungal mycelia and suspended solids remained in suspension. Solids also accumulated above the liquid level on the vessel wall with both 0.2- and 1.0-vvm aeration rates.

A bench-scale experiment with settled thin-stillage supernatant, 0.1 g/L suspended solids, as compared to 20–30 g/L in thin stillage, was performed to determine the effects of thin stillage particles on fungal cultivation. The bioreactor

was filled with small fungal pellets within 2 d of spore inoculation (Figure 3.5).

Mycelia continued to grow in suspension as pellets and began to attach to the vessel wall, reducing the liquid volume (Figure 3.5, day 6). The pellets were smaller and more compact, or less filamentous, than fungal flocs formed in the presence of thin stillage particles with the same aeration rate (Figure 3.4, day 4). Fungal mycelia and sporangiospores were observed under the microscope on days 1 and 4, respectively (Figure 3.6).

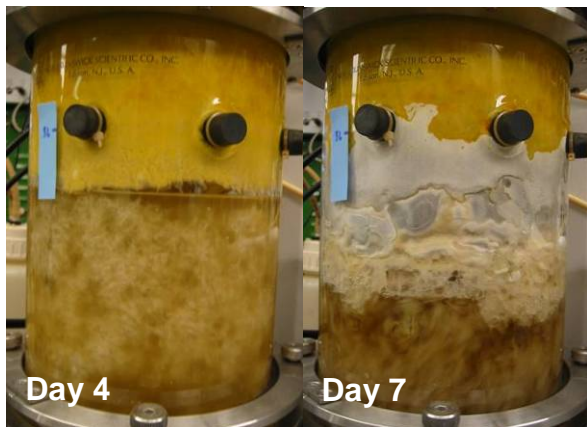


Figure 3.4. Fungal cultivation of thin stillage (5 L) in a batch stirred bioreactor with 0.8-vvm aeration on days 4 and 7.

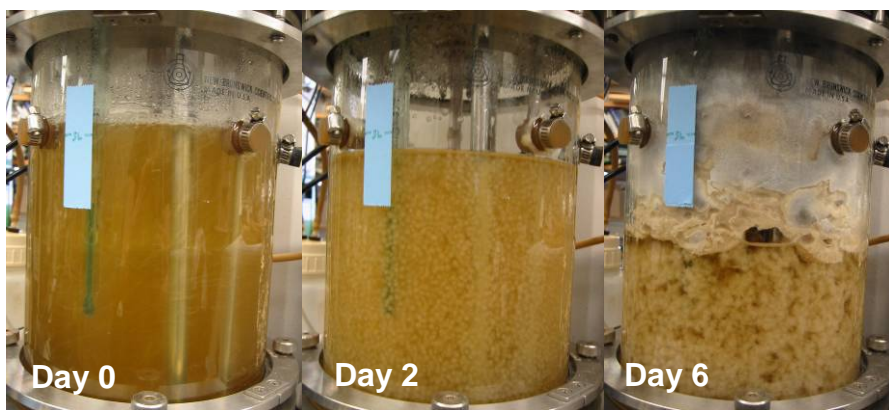


Figure 3.5. Fungal cultivation of settled thin-stillage supernatant (5 L) in a batch stirred bioreactor with 0.8-vvm aeration on day of spore inoculation (day 0), day 2, and day 6.

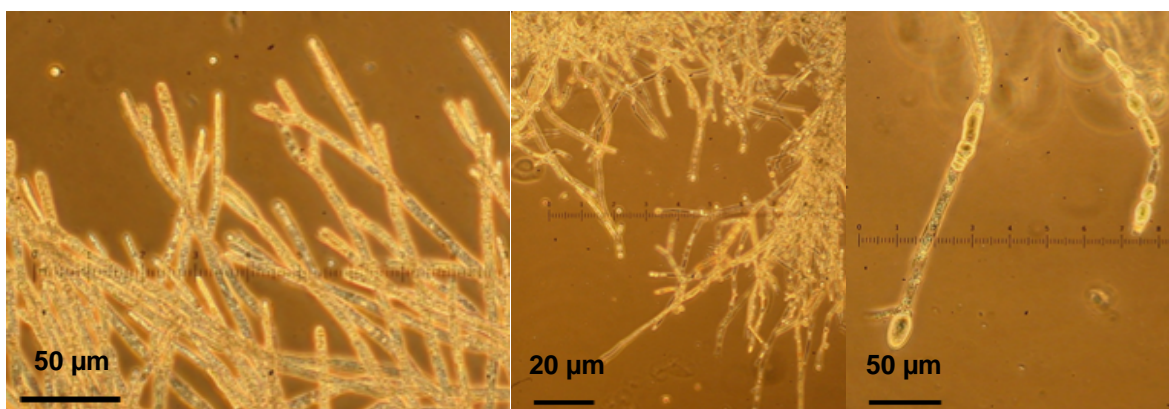


Figure 3.6. Microscopic observations of fungal mycelia and sporangiospores grown in settled thin-stillage supernatant on day 1 (left photo) and day 4 (right photos).

Organics (COD) removal

The total and soluble organic content of fresh thin stillage batches, as measured by COD, ranged from 81–98 g TCOD/L and 49–60 g SCOD/L, respectively. Reductions in SCOD tended to improve with increasing aeration rates up to 0.8 vvm in bench-top stirred bioreactors (Figure 3.7). The SCOD was reduced by 17, 36, and 46% in 5 d with aeration rates of 0.2, 0.4, and 0.8 vvm, respectively. Increasing the aeration rate to 1.0 vvm had a slightly lower SCOD removal in 5 d (42%) than obtained with 0.8 vvm (46%) (data not shown). This outcome may be attributed to the concentrating effect of rapid reduction in liquid volume with 1.0-vvm aeration, due to evaporation and suspended solids accumulating on the vessel wall. The DO level with 0.8-vvm aeration, the optimal rate for SCOD reduction in 5-L bioreactors, dropped rapidly from 100 to 75% of saturation in 1 d and to 3% in 2 d (Figure 3.8). The DO averaged 4% from day 2 to day 5.

The initial SCOD of settled thin-stillage supernatant (57 g/L) was within the range of SCOD values for fresh thin stillage with suspended solids as expected.

The SCOD in the soluble thin stillage fraction was reduced by 41% in 5 d with 0.8-vvm aeration. The higher reduction (46%) with thin stillage suspended solids may indicate that the particles provided a nutrient that enhanced fungal growth. It may also be a result of the different fungal morphologies, filamentous flocs versus compact pellets, observed with and without thin stillage particles, respectively.

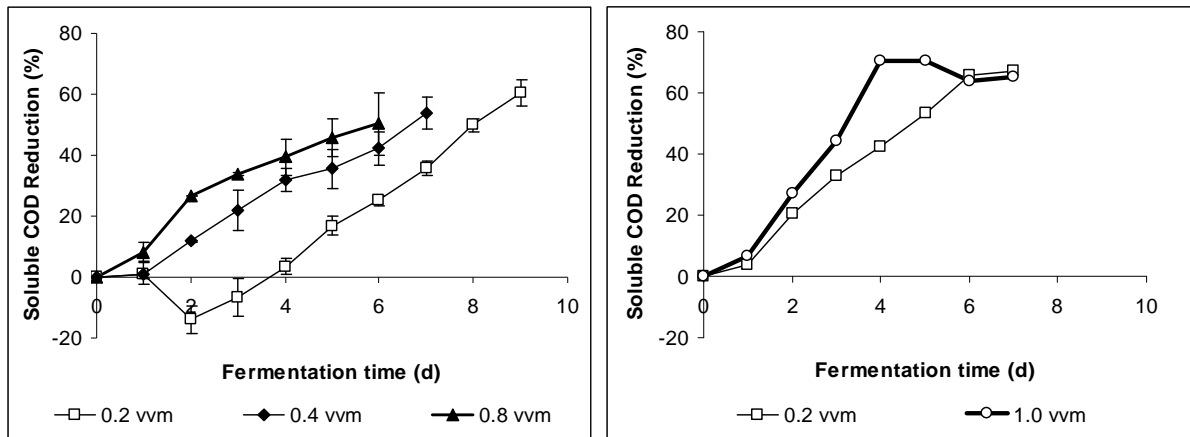


Figure 3.7. Soluble COD reductions during fungal cultivation of thin stillage in batch stirred bioreactors, 5 L on left (n=2) and 50 L on right (n=1), with increasing aeration rates of 0.2 to 1.0 vvm.

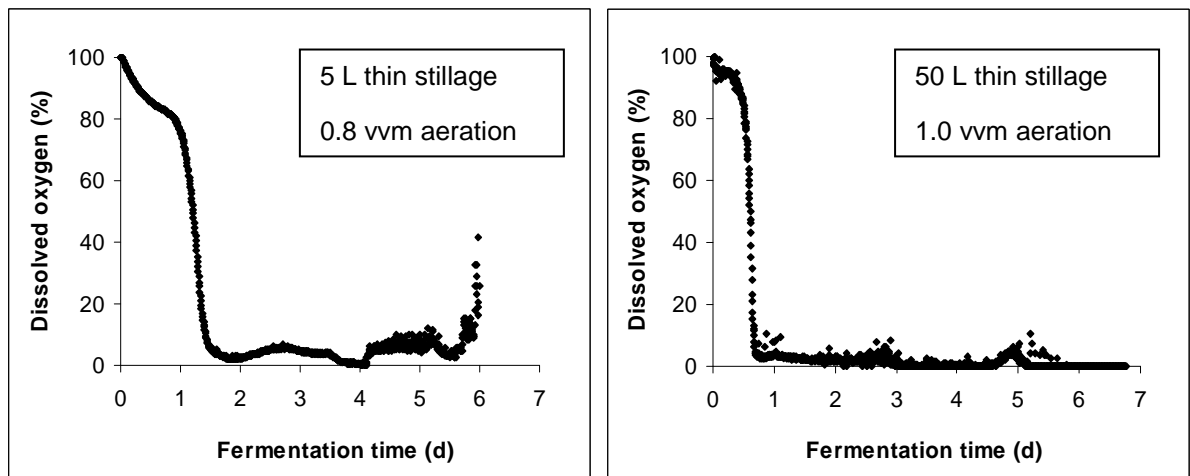


Figure 3.8. Dissolved oxygen reductions during batch stirred fungal cultivations of thin stillage, 5 L with 0.8-vvm (left) and 50 L with 1.0-vvm aeration rates (right). (n=1)

The scaled-up batch fungal bioreactor (50 L) demonstrated a more rapid reduction in SCOD (Figure 3.7). The SCOD decreased by 53% in 5 d with 0.2-vvm aeration and by 71% in 4 d with 1.0-vvm aeration; corresponding results for the bench-top experiments were 17 and 39%, respectively. Improved SCOD removals were expected due to more rapid start-up from mycelia inoculum, better oxygen transfer, and less wall effect in the larger bioreactor. The fermentation broth was dense with fungal growth by day 4 with 1.0-vvm aeration. The decrease in SCOD reduction after day 5 may be the result of fungal cell lysis. DO levels during the 50-L fungal cultivation with 1.0-vvm aeration dropped from 100% to 5% of saturation in 1 d (Figure 3.8). The DO remained low, averaging only 1%, from day 1 to the end of the experiment.

The removal of total COD (TCOD) was less straight-forward; it depended on the harvesting method, such as settling, centrifugation, or filtration, and the amounts of suspended solids removed by attachment to the fungal biomass. In the 0.2- and 0.4-vvm aeration bench-top experiments, the effluent was well-clarified with almost no suspended solids. The TCOD reductions for the 0.2-vvm aeration effluent reached 55 and 79% in 5 and 9 d, respectively. For 0.4-vvm aeration effluent, TCOD reduction was slightly higher (56% in 5 d). In bench-top experiments with 0.8- and 1.0-vvm aeration and the suspended mycelia separated from the effluent, the TCOD decreased by 58 and 63% in 5 d, respectively. Most of the thin stillage suspended solids and mycelia remained in solution during the 50-L fungal cultivations. Effective separation of these suspended solids from the 5-d effluent

would result in TCOD removals of 75 and 84% for 0.2- and 1.0-vvm aeration rates, respectively.

Suspended solids removal

The initial suspended solids were between 20 and 30 g/L in fresh thin stillage batches. Total and volatile suspended solids contents were similar in all experimental samples, indicating low levels of fixed, inorganic suspended solids. Reductions in suspended solids in daily bioreactor samples differed based on the aeration rate (Figure 3.9). Gradual removal of suspended solids was observed with 0.2-vvm aeration from day 0 through day 4 (up to 39%), in part due to accumulation on the bioreactor wall above the liquid level and between the probes and vessel wall. The suspended solids concentration decreased rapidly after day 4 reaching 89 and 99% removals by days 5 and 6, respectively; this coincided with the liquid clarification observed on day 5 (TCOD and SCOD became equal). Thin stillage particles were removed by attachment to mycelia and by biological mineralization.

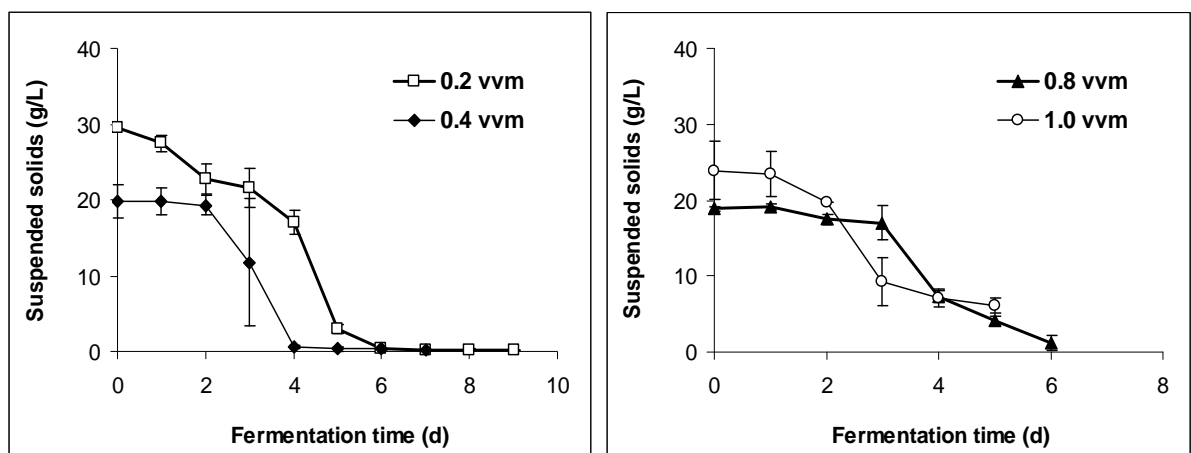


Figure 3.9. Suspended solids reductions during fungal cultivation of thin stillage in batch stirred bioreactors (5 L) with increasing aeration rates of 0.2 to 1.0 vvm. (n=2)

Liquid collected through the effluent port was well-clarified, as low as 20 mg/L suspended solids, with a yellow tint (Figure 3.10). Solids separation before returning the water to the corn fermentation process is very important to avoid the build-up of non-biodegradable substances. The fungal process effluent could potentially be recycled with minimal further treatment. An increased aeration rate of 0.4 vvm resulted in more rapid clarification of the bioreactor samples on days 3 and 4 (Figure 3.9). The average removals with 0.4-vvm aeration were 45 and 97% in 3 and 4 d, respectively.



Figure 3.10. Clarified effluent and bioreactor vessel filled with biomass after fungal cultivation in thin stillage (5 L) for 7 days with 0.4-vvm aeration.

Suspended solids contents in bioreactor samples with 0.8- and 1.0-vvm aeration were higher than observed with lower aeration rates because fungal mycelia remained in suspension throughout the experiment (Figure 3.9). Clarification of the bioreactor samples, due to thin stillage solids removal by degradation and attachment, occurred on day 4 with aeration of 0.8 vvm and day 3 with 1.0 vvm, as compared to days 5 and 4 with 0.2 and 0.4 vvm, respectively.

Organic acids and glycerol removal

Initial lactic and acetic acids concentrations in fresh thin stillage ranged from 1.5–3.1 g/L and 1.0–1.5 g/L, respectively. The glycerol content in thin stillage was high, between 12.8 and 15.9 g/L. Organic acid production is primarily from bacterial contamination. Glycerol accumulation is a byproduct of yeast fermentation under stressed conditions (Walker, 1998). In bench- and pilot-scale bioreactors, fungal removal of lactic acid, acetic acid, and glycerol tended to improve with increasing aeration rates. Lactic acid contents in bench-top bioreactor samples increased during the first 2 d of fungal cultivation (Figure 3.11). This trend may indicate that the fungus was producing lactic acid initially. Several *Rhizopus* strains are known to produce lactic acid in submerged cultures (Zhang et al., 2007). From day 2 onward, lactic acid concentrations decreased, with reductions of 14, 31, 54, and 71% with aeration of 0.2, 0.4, 0.8, and 1.0 vvm, respectively, by day 5. Fermentation of soybeans with *R. oligosporus* for tempeh production confirms that this fungal species is able to degrade lactic acid (Sparringa and Owen, 1999).

Fungal cultivation of settled thin-stillage supernatant improved lactic acid biodegradation, with 60% removal in 5 d with 0.8-vvm aeration (as compared to 54% with thin stillage particles). Lactic acid reductions were substantially higher in the 50-L bioreactor, reaching 100% in 6 d with 0.2-vvm aeration and in 4 d with 1.0-vvm aeration, which may be related to better DO transfer.

Acetic acid removals also improved with increasing aeration rates (Figure 3.11). Complete removal was achieved in 4 d with aeration of 0.2 vvm, in 3 d with 0.4 vvm, and in 2 d with 0.8 and 1.0 vvm. Similar results were obtained using the

settled thin-stillage supernatant with 100% removal in 2 d with 0.8-vvm aeration. In the 50-L fungal cultivations, the acetic acid removal was more rapid with 100% removal by day 2 with 0.2-vvm aeration and by day 1 with 1.0-vvm aeration.

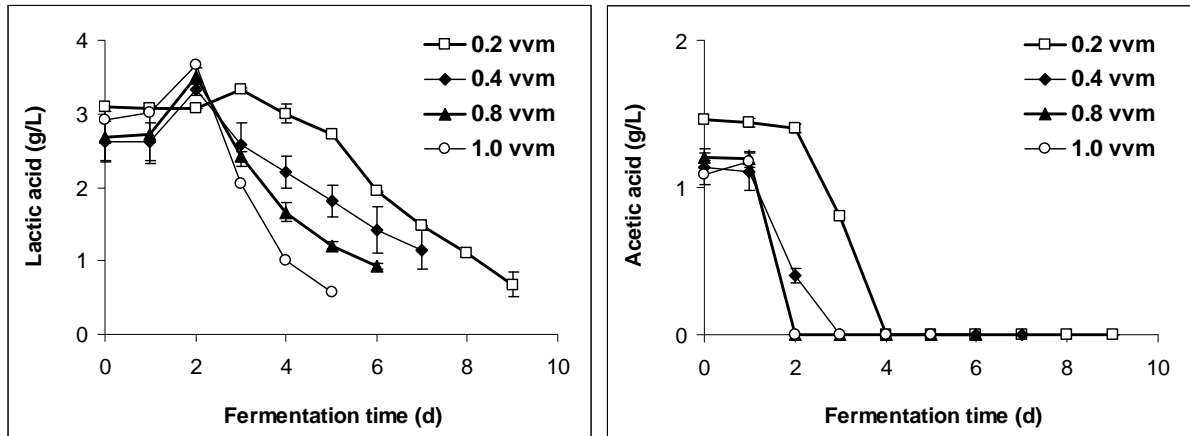


Figure 3.11. Lactic and acetic acid reductions during fungal cultivation of thin stillage in batch stirred bioreactors (5 L) with increasing aeration rates of 0.2 to 1.0 vvm. (n=2)

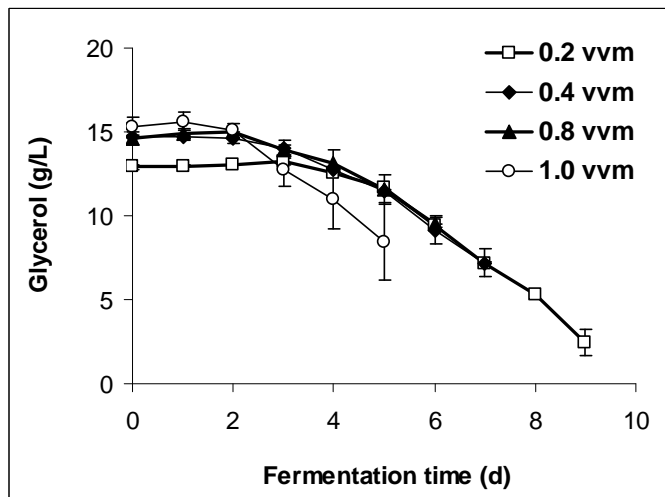


Figure 3.12. Glycerol reductions during fungal cultivation of thin stillage in batch stirred bioreactors (5 L) with increasing aeration rates of 0.2 to 1.0 vvm. (n=2)

Glycerol biodegradation in the thin stillage began 2 d after fungal inoculation (Figure 3.12). This delay may be associated with the time required for the fungal culture to grow from spores. Reductions in glycerol concentrations by day 5 reached 11% with aeration of 0.2 vvm, 22% with 0.4 and 0.8 vvm, and 45% with 1.0 vvm. Degradation of glycerol increased to 33% in 5 d with settled thin-stillage supernatant (0.8-vvm aeration), compared to 22% with thin stillage particles present. Glycerol reduction in 50-L bioreactors was significantly better with 100% removal achieved in 6 d with 0.2-vvm aeration and 5 d with 1.0-vvm aeration, which again suggests better oxygen transfer in the 50-L compared to the 5-L experiments.

Fungal biomass production

Quantifying fungal biomass production was complicated by the removal of thin stillage solids via attachment to the vessel wall above the liquid level and to the fungal mycelia. Thin stillage particles attached to the surface of the fungal biomass and within it, particularly inside the mycelia on the agitator blades. The final biomass weights provided in Table 3.1 include the freeze-dried fungal biomass and attached thin stillage solids, which were collected at the end of the experiments. The harvested biomass tended to increase with increasing aeration rates.

The experiment performed on the soluble-fraction of thin stillage had lower biomass production (13 g/L), in part since no thin stillage suspended solids were present to attach to the mycelia. The resulting yield was 0.43 g dry fungal biomass/g SCOD removed. Assuming a similar yield for the thin stillage with particles, the fungal biomass production in 6 d with 0.8-vvm aeration would be approximately 14 g/L based on SCOD reductions. The initial thin stillage suspended solids was 20

g/L, and the final biomass production was 30 g/L (an increase of 10 g/L). The lower actual biomass increase of 10 g/L compared to the expected fungal growth of 14 g/L, based on SCOD removal, confirms that suspended solids were both removed by attachment to the fungal biomass and by biodegradation to carbon dioxide and water. In the 50-L cultivation with 1.0-vvm aeration, more of the fungal mycelia and thin stillage solids remained in suspension. The production of 28 g dry biomass/L thin stillage was lower than obtained in the bench-top bioreactors, in part because centrifugation did not recover as much of the thin stillage solids as attachment to fungal mycelia.

Table 3.1. Comparison of biomass production during fungal cultivation of thin stillage in stirred bioreactors (5 L) at increasing aeration rates. (n=3)

Air flow (vvm)	Cultivation length (d)	Final biomass (g dry wt/L)^a
0.4	7	27 ± 3
0.8	6	30 ± 3
1.0	6	36 ± 4

^a Weight of freeze-dried biomass, including fungal mycelia and attached thin stillage solids.

Fungal protein and amino acids

The average crude protein content of *R. microsporus* mycelia grown in YM broth for inoculation of the 50-L cultivations was 39% (w/w). The fungal biomass cultivated on the settled thin-stillage supernatant (no suspended solids) had up to 43% (w/w) crude protein. This fungal protein could be fed to the nonruminants, swine and poultry.

Lysine, methionine, tryptophan, and threonine are the main amino acids of concern for nonruminants (Cheeke, 2005). Lysine and methionine are usually the first-limiting amino acids in corn-soybean meal diets for swine and poultry, respectively. Based on the amino acid data in Table 3.2, the dried fungal biomass had almost two-thirds the lysine and over 2.5 times the methionine contents of soybean meal (% as-fed basis). The fungal lysine, threonine, and tryptophan contents, however, were all comparable to soybean meal on a percent of protein basis (Figure 3.13). Biomass protein contents and digestibility depend on harvesting, dewatering, and drying methods, as well as amounts of enmeshed stillage solids. Improved dewatering should increase the protein fraction (%) of the fungal biomass and, consequently, the lysine content (% as-fed). Water reclamation and fungal protein content may be enhanced by supplementing thin stillage with readily-bioavailable inorganic nitrogen sources.

Table 3.2. Amino acid composition of biomass on as-fed basis (10% moisture).

Amino acid	Fungal biomass^a (%)	Soybean meal^b (%)	Corn^b (%)	DDGS^b (%)
Lysine	1.8	3.0	0.3	0.6
Methionine	1.8	0.7	0.2	0.5
Threonine	1.5	1.9	0.3	1.0
Tryptophan	0.3	0.7	0.1	0.3

^a Freeze-dried fungal biomass product includes enmeshed thin stillage solids (n=1).

^b Data from NRC (1998).

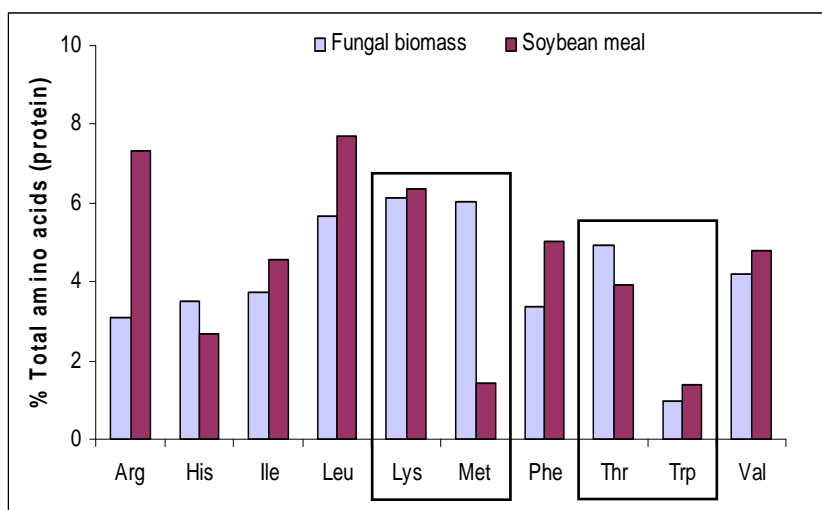


Figure 3.13. Essential amino acid composition of fungal biomass (n=1), cultivated in thin stillage, and soybean meal (NRC, 1998) as a percent of protein.

Conclusions

Fungal treatment of thin stillage in corn ethanol plants is an innovative technology to reclaim water, save energy and potentially enzymes, and produce an additional valuable coproduct. Recycling fungal-treated water directly could provide substantial energy savings by avoiding the current practice of evaporating and condensing water from thin stillage. The high-protein fungal biomass produced could be fed to nonruminants. The fungal biomass could also be used as raw material for the extraction of valuable biochemicals, such as chitosan. Fungal cultivation of thin stillage has the potential to make ethanol production more energy efficient and more sustainable, to reduce costs, and to produce an additional value-added coproduct. Future research on fungal cultivation of thin stillage will focus on improving the mode of aeration in order to reduce the treatment time, such as employing an airlift design and producing biomass on a pre-commercial scale.

Acknowledgements

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CHAPTER 4. PRELIMINARY INVESTIGATION OF AIRLIFT DESIGNS FOR FUNGAL TREATMENT OF CORN ETHANOL STILLAGE

A paper prepared for submission to *Biotechnology and Bioengineering*

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Abstract

This research investigated cultivation of the food-grade fungus *Rhizopus microsporus* var. *oligosporus* in airlift bioreactors on thin stillage from a local dry-grind corn ethanol plant. Non-sterile, batch experiments in 4.5- and 24-L airlift bioreactors showed prolific fungal growth, and fungal mycelia filled the bioreactors within 2–3 d. In 4.5-L experiments, the rapid growth of mycelia impeded the flow of liquid around the internal draft tube, limiting the cultivation length of time. Removing fungal biomass by coarse screens enabled the experiments to be extended, but was difficult to implement continuously on bench-scale and tended to result in fungal growth transitioning from suspended to attached mode. Soluble COD, lactic acid, and acetic acid reductions, critical for in-plant water reuse, reached 54, 100, and 100%, respectively, within 5 d of fungal inoculation. A SCOD removal of up to 74% was achieved in 7 d. Glycerol was removed by up to 99% in 6 d. Removal

efficiencies in airlift bioreactors on these smaller scales were not as high as achieved in 50-L stirred bioreactor experiments. Future research will employ a larger, pilot-scale airlift bioreactor with continuous screening and removal of fungal biomass. This scale of fungal cultivation will also enable production of sufficient biomass to assess the comprehensive feed value using animal feed trials.

Keywords: Thin stillage; fungus; animal feed; water reuse; ethanol plant; distillers dried grains with solubles (DDGS); airlift bioreactor

Introduction

Dry-grind corn ethanol facilities, the mainstay of fuel ethanol production, typically grind, cook, enzyme-treat, and ferment corn to produce ethanol from starch. The ethanol is separated by distillation, which leaves behind copious amounts of stillage containing the leftover corn protein, oil, fiber, and residual carbohydrates as well as yeast cells. Most of the solids, distillers grains, are removed from stillage with centrifuges. The centrate, thin stillage, is partially recycled to the corn fermentation process, but the recycle is limited to 50% to prevent the build-up of solids and of the fermentation byproducts lactic and acetic acids. The remaining thin stillage is currently concentrated by flash evaporation, an energy-intensive process, blended with distillers grains, and dried to produce distillers dried grains with solubles (DDGS). Although the water vapor could be condensed, the volatile compounds are then also recovered and still limit recycling; more treatment is required, which is a serious deterrent to recycling water. The coproduct DDGS is used for livestock feed, but is low in essential amino acids, e.g., lysine (<1%) (Bregendahl, 2008). Co-feeding DDGS to hogs and chickens is restricted based on

low content and digestibility of lysine and methionine, the first-limiting amino acids in corn-soybean meal diets (Cheeke, 2005).

Public opinion of the biofuel industry, in particular corn ethanol, has been poor recently, igniting debates on the perceived environmental impacts (e.g., indirect land use changes), high ratio of energy inputs to outputs, and competition with food production (RFA, 2009). The industry has diligently worked on negating these arguments and on investigating process improvements to reduce energy inputs and to produce more nutritious feed coproducts. Dry-grind corn ethanol plants, for instance, generate 5–6 gal stillage per gal ethanol after distillation (Dunn, 2008; van Leeuwen, 2009). Creating value-added coproducts from stillage and recycling water is important as the annual U.S. fuel ethanol production exceeds 10 billion gallons (RFA, 2009); profitability hinges on the sale of both the ethanol and the coproducts.

Our research aimed to find a better and more cost-effective way to water recycling, in addition to producing a valuable high-protein animal feed. Wheat milling and corn wet-milling streams have been used as substrate for cultivation of the food-grade fungus *R. oligosporus*, achieving significant reductions in COD of up to 80–90% (Jasti et al., 2006, 2008, 2009; Jin et al., 1998, 1999 a–f, 2001a,b, 2002, 2009; Rasmussen et al., 2007; Sankaran et al., 2008, 2010). Our previous lab-scale batch studies in stirred bioreactors achieved prolific fungal growth under non-sterile conditions (Chapter 3; Rasmussen et al., 2010). Organic matter (as measured by total chemical oxygen demand, COD), glycerol, and organic acids removals, critical for in-plant water reuse, reached 80, 100, and 100%, respectively, within 5 d of fungal inoculation. The fungus was found to contain 2% lysine, 2% methionine (corn

contains 0.3% and 0.2%, respectively), and 43% crude protein, enhancing the nutritional value as a nonruminant feed.

The goal of this study was to investigate an airlift design, in place of stirring, for fungal treatment of thin stillage. Airlift designs are appropriate for filamentous fungi, which have a high oxygen demand and sensitivity to shear (Jin et al., 2001). Airlift bioreactors also have the potential advantages of simple construction, low energy consumption (at the same aeration rates), and high mass, momentum, and heat transfer rates compared to stirred bioreactors.

Materials and Methods

Thin stillage

Thin stillage samples were collected in sterile 20-L carboys from a local dry-grind corn ethanol plant, Lincolnway Energy (Nevada, IA). Samples were stored at 4°C prior to use. Suspended solids in the thin stillage settled during storage within one day (Figure 4.1). Supernatant from settled thin stillage was used as substrate to prepare fungal mycelia inoculum, in order to acclimate the fungus, and to determine the effect of thin stillage particles on fungal growth/morphology. As reported in our stirred-bioreactor study (Rasmussen et al., 2010), fresh thin stillage had a pH of 3.8–4.7. The total and soluble chemical oxygen demand (COD) of fresh thin stillage ranged from 81–98 g/L and 49–60 g/L, respectively. The total and reducing sugar contents averaged 17 and 6 g/L, respectively. The suspended solids contents of thin stillage was 20–30 g/L and settled thin-stillage supernatant was 0.2 g/L.



Figure 4.1. Thin stillage suspended solids settled during storage for 1 d in a sterile 20-L carboy at 4°C.

Fungal culture

Freeze-dried culture of the fungus *Rhizopus microsporus* var. *oligosporus* was purchased from the American Type Culture Collection (ATCC 22959, Rockville, MD, USA). Fungal spore suspensions were prepared and stored ultra-low frozen (-75°C) in sterile 2-ml cryovials, as discussed in our stirred-bioreactor study (Rasmussen et al., 2010). The harvested spore count of 2.4×10^6 spores/ml was determined by haemocytometer counts. The contents of one vial (4.8×10^6 spores) were used as spore inoculum in draft-tube cultivations. Mycelia inoculum was prepared by thermal sterilization of settled thin-stillage supernatant (1 L adjusted to pH 4.0) in a 2-L Erlenmeyer flask, inoculation with 1 vial of spore suspension, and incubation (37°C) at 150 rpm shaking for 3 d.

Bioreactor setup and operation

Batch fungal cultivations on thin stillage and settled thin-stillage supernatant were performed in a bench-top, draft-tube bioreactor (5 L) with a 4.5-L working volume (Figure 4.2). The bioreactor was sterilized by autoclaving with water for 45 min at 121°C and filled aseptically using a peristaltic pump. The thin stillage

substrate was inoculated with one vial of fungal spore suspension (2.4×10^6 spores). In experiments started from fungal mycelia, the substrate was inoculated with 2% (v/v), or 0.3% (dry wt/v), of mycelia inoculum prepared previously using settled thin stillage supernatant. The culture conditions for draft-tube experiments were maintained at pH 4, 37°C, and 1.0 or 1.5 L/L/min (v vm) aeration. The temperature and pH were chosen based on successful fungal research treating corn ethanol wet-milling wastewater (Jasti et al., 2006; Jasti et al., 2009) and on our previous work using thin stillage (Rasmussen et al., 2010). The pH was checked daily and adjusted using sterile 4 N HCl and 4 N NaOH. The bioreactor was submerged in a water bath to maintain the temperature at 37°C. Sterile water was added on a daily basis to replace evaporative losses.

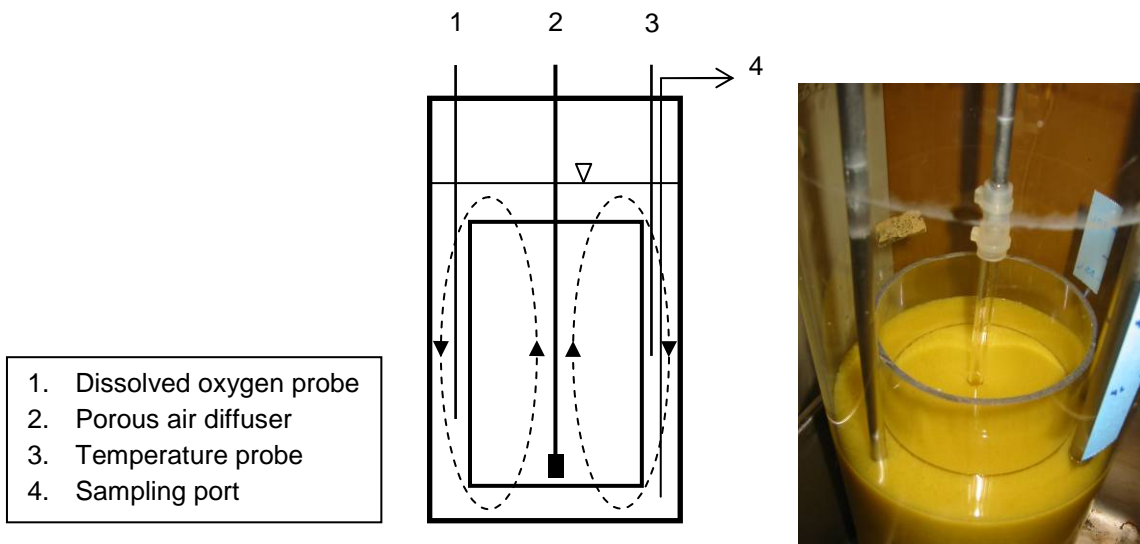


Figure 4.2. Schematic of 5-L, draft-tube bioreactor – aeration created liquid movement shown with arrows (left); photo of draft-tube setup and aseptic addition of thin stillage (right).

Airlift experiments were scaled up to a 24-L working volume, with an external recycle design (Figure 4.3) replacing the internal draft tube used in bench-top experiments (4.5 L). Preliminary scaled-up airlift experiments were performed under similar culture conditions with pH 4, 37°C, and 0.4 – 0.8 L air/L thin stillage/min (vvm); the soluble-thin stillage fraction was used as substrate to assess reactor performance before fungal cultivation with thin-stillage solids present. The airlift reactor was disinfected by filling with deionized water and bubbling ozone for 1 h, drained, and inoculated with fungal mycelia (2% [v/v]) cultured previously in heat-sterilized thin-stillage supernatant.

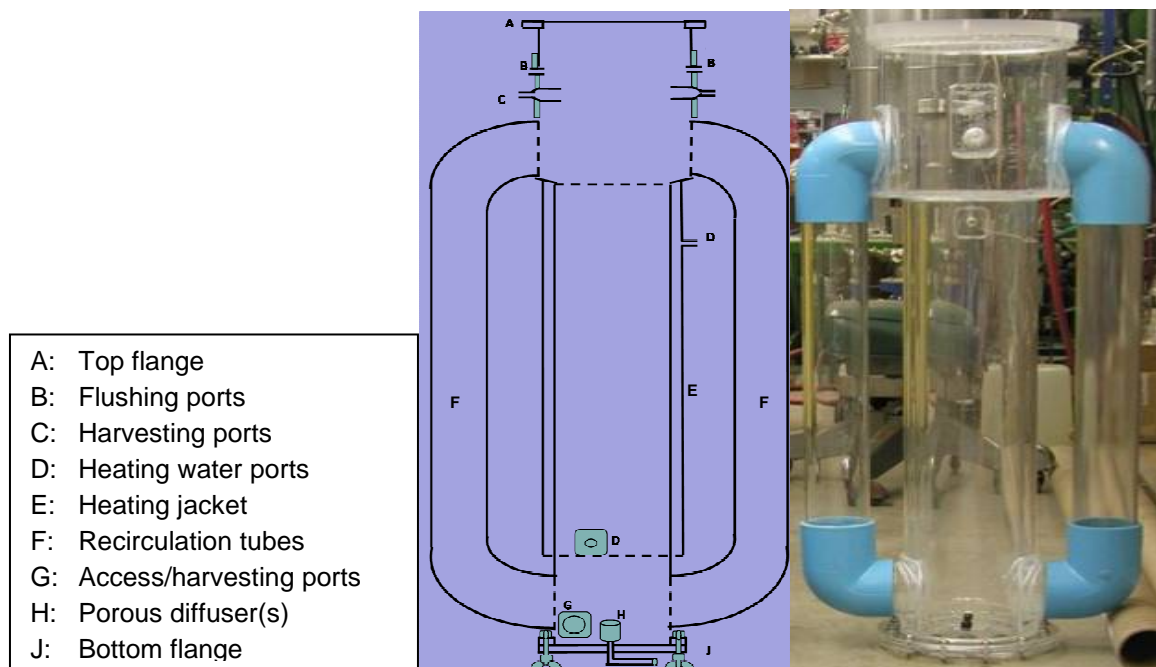


Figure 4.3. Schematic of airlift external-recycle bioreactor (24 L) – liquid recycle in external tubes designed to promote oxygen transfer and fungal pellet formation and recovery (left); photo of airlift external-recycle bioreactor (right).

In preliminary airlift experiments using the 4.5-L, draft-tube bioreactor, the substrate (thin stillage or settled thin-stillage supernatant), aeration rate (1.0 or 1.5 vvm), and inoculum (spore or mycelia) were varied to determine the impact on fungal growth (Table 4.1). The two sets of experiments are divided by substrate type for discussion purposes. Experiments using the settled TS supernatant were performed to demonstrate the differences in fungal growth with and without suspended solids, to provide estimates on fungal yields (g biomass/g SCOD removed), and to clearly show the liquid/biomass movement in the airlift bioreactors.

Table 4.1. Airlift experiments performed using a 4.5-L draft-tube and a 24-L external-recycle bioreactors.

Exp. set	Substrate^a	Bioreactor	Inoculum	Aeration (vvm)
1	Thin stillage	Draft tube (4.5 L)	Spores	1.0
1	Thin stillage	Draft tube (4.5 L)	Spores	1.5
2	Settled TS supernatant	Draft tube (4.5 L)	Spores	1.0
2	Settled TS supernatant	Draft tube (4.5 L)	Spores	1.5
2	Settled TS supernatant	Draft tube (4.5 L)	Mycelia	1.5
2	Settled TS supernatant	External recycle (24 L)	Mycelia	0.8

^a TS stands for thin stillage. Thin stillage suspended solids settled during storage (4°C) within 1 d. The supernatant was pumped aseptically from the carboys for use as substrate in Exp. set 2.

Sample analyses

Feed thin stillage and bioreactor samples were analyzed for lactic acid, acetic acid, and glycerol contents using a Waters high pressure liquid chromatograph (HPLC) (Millipore Corporation, Milford, MA, USA). The HPLC system included a Waters Model 401 refractive index detector, Bio-Rad Aminex HPX-8711 column – dimensions 300 mm x 7.8 mm (Bio-Rad Chemical Division, Richmond, CA, USA),

column heater, autosampler, and computer controller (Kunduru and Pometto, 1996). Sample constituents were isolated using a mobile phase of 0.012 N sulfuric acid, flow rate of 0.8 ml/min, injection volume of 20 μ l, and column temperature of 65°C. The COD analysis of fresh thin stillage and daily bioreactor samples was performed as described in Standard Methods (APHA/AWWA/WEF, 2005).

Effluent and biomass collection

The bioreactor head plate, with attached draft tube, was unscrewed and lifted from the vessel to recover the process effluent and attached biomass. Fungal biomass was collected from the vessel wall, draft tube, probes, and ports. Suspended biomass was recovered from the effluent by centrifuging for 20 min at 7,277 x g, decanting the supernatant, and rinsing with 0.85% NaCl solution in water. Attached and suspended biomass was freeze-dried to quantify fungal biomass production and to avoid heat-drying effects on the feed quality for research purposes. Compositional analyses were performed on this fungal biomass product.

Results and discussion

Visual observations

As observed in stirred experiments, fungal growth and morphology during draft-tube cultivations depended on the initial suspended-solids concentration and the aeration rate. Experiment set 1 investigated fungal cultivation on thin stillage, whereas Experiment set 2 used settled thin-stillage supernatant. Table 4.2 provides a summary of the experiments and visual observations.

Experiment set 1 (thin stillage): suspended fungal growth was visible within 2 d of spore inoculation using 1.0-vvm aeration (Figure 4.4). Accumulation of mycelia

was observed on the vessel wall above the liquid level and between the submerged draft tube, probes/ports, and vessel wall. The fungus continued to agglomerate and grow. By day 3, the fungal biomass obstructed the flow of liquid around the draft tube, and the liquid was well-clarified. More time (4–5 d) was required to achieve liquid clarification in stirred bioreactor experiments (Rasmussen et al., 2010). The air flow was increased to 1.5 vvm in order to facilitate the flow of liquid and the growing biomass around the draft tube. Increasing the air flow favored biomass attachment to the vessel wall above the liquid level, with no visible accumulation between the submerged draft tube, probes/ports, and vessel wall (Figure 4.5). Unlike the effluent with 1.0-vvm aeration, the liquid remained turbid with thin-stillage suspended solids despite extending the fermentation to 7 d.

Table 4.2. Summary of visual observations and fungal cultivation length for experiments performed using a 4.5-L draft-tube bioreactor, except as noted.

Exp. set	Substrate ^a	Inoculum	Aeration (vvm)	Fungal growth & morphology	Cultivation length (visible mycelia)
1	Thin stillage	Spores	1.0	Filamentous; obstructed flow	3 d (2 d)
1	Thin stillage	Spores	1.5	Filamentous; attached above liquid level	7 d (3 d)
2	TS super.	Spores	1.0	Large spherical pellets; obstructed flow	7 d ^b (2 d)
2	TS super.	Spores	1.5	Smaller, filamentous (fluffy) pellets	5 d (3 d)
2	TS super.	Mycelia	1.5	Smaller, fluffy pellets	2
2 ^c	TS super.	Mycelia	0.8	Small spherical pellets	7 ^b

^a “TS super.” stands for settled thin-stillage supernatant. Thin stillage suspended solids settled during storage (4°C) within 1 d. The supernatant used as substrate in Exp. set 2.

^b Fungal pellets filled the bioreactor in 3 d; experiment extended to 7 d by screening out biomass.

^c Scaled-up, external-recycle airlift bioreactor (24 L) was used.

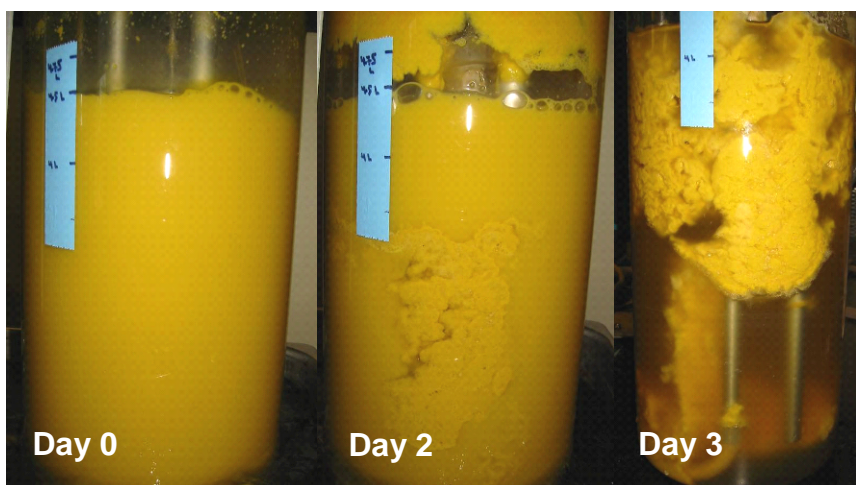


Figure 4.4. Exp. set 1: fungal cultivation of thin stillage with 1.0-vvm aeration on the day of spore inoculation (day 0), day 2, and day 3 – experiment ended.

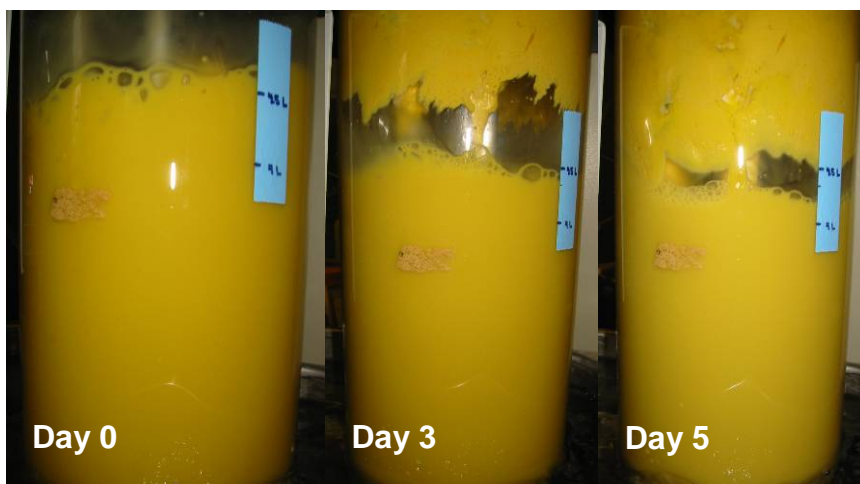


Figure 4.5. Exp. set 1: fungal cultivation of thin stillage with 1.5-vvm aeration on the day of spore inoculation (day 0), day 3, and day 5. Experiment ran 7 d.

Experiment set 2 (settled thin-stillage supernatant): small fungal pellets were observed within 2 d of spore inoculation during draft-tube cultivation of settled thin-stillage supernatant using 1.0-vvm aeration (Figure 4.6), similar to stirred bioreactor results (Rasmussen et al., 2010). The spherical pellets grew in size and filled the draft-tube bioreactor by day 3, with mycelia accumulating between the draft tube,

probes/ports, and vessel wall. Suspended fungal pellets floated when aeration was turned off (Figure 4.6, Day 3*). The 1.0-vvm aeration experiment was extended to 7 d by screening out most of the fungal biomass from the bioreactor on day 3. The fungus continued to grow, but only in attached mode on the probes/ports and draft tube and not in suspension. Increasing the aeration to 1.5 vvm during subsequent experiments, again to facilitate liquid and biomass movement, resulted in more turbulence and foam (Figure 4.7); small pellets were observed on day 3 only, a day later than with 1.0-vvm aeration. The pellets filling the bioreactor by day 5 were smaller and more filamentous compared to the spherical pellets formed using 1.0-vvm aeration.

Mycelia inoculation of settled thin-stillage supernatant, instead of spores, and the higher aeration rate of 1.5 vvm resulted in rapid fungal growth (Figure 4.8). Mycelia filled the bioreactor within 2 d of inoculation. The fungal growth was filamentous; flocs were smaller and appeared fluffy, similar to the 5-d pellets starting from spores (Figure 4.7). In the scaled-up airlift experiment (24 L, 0.8-vvm aeration), fungal pellets filled the bioreactor within 3 d of mycelia inoculation (Figure 4.9). The pellets were harvested by coarse screens with 1/4" openings; the fungal biomass product was readily dewatered to 20–30% solids by mild pressure, e.g. hand squeezing, simulating a pressure filter. The liquid following fungal harvesting was returned to the bioreactor for further fungal degradation of the residual organic materials. Experiments using thin stillage (including suspended solids) in the 24-L airlift bioreactor were attempted subsequently, but were not successful, because of

issues with leaking at the recirculation-tube connections. Leaking and build-up of oil on the walls, in particular, was a problem using thin stillage in this acrylic bioreactor.

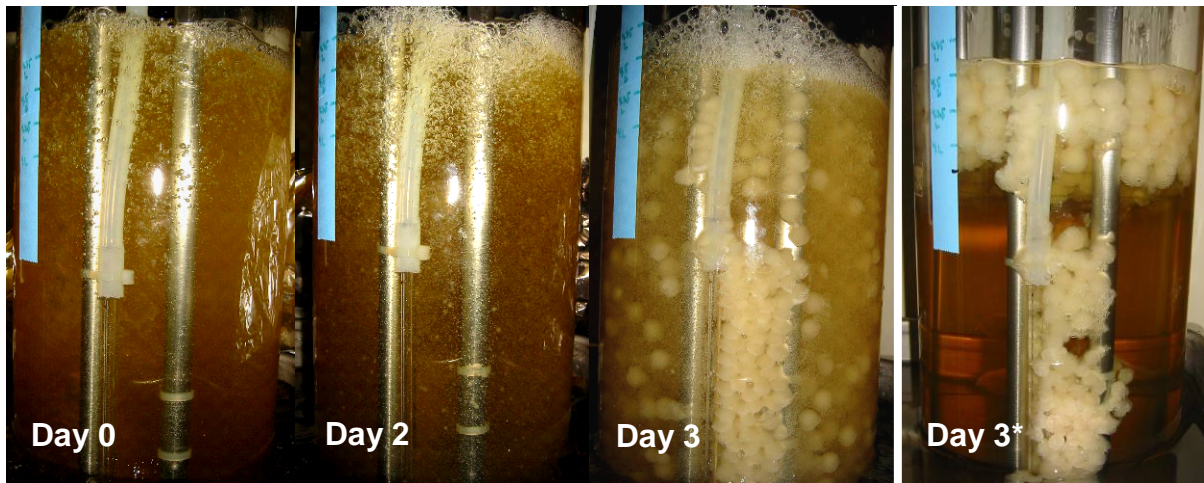


Figure 4.6. Exp. set 2: fungal cultivation of settled thin-stillage supernatant with 1.0-vvm aeration on the day of spore inoculation (day 0), day 2, and day 3. *Mycelia floated on day 3 with aeration turned off. Experiment ran 7 d; most fungal biomass removed on day 3 to continue running the cultivation.

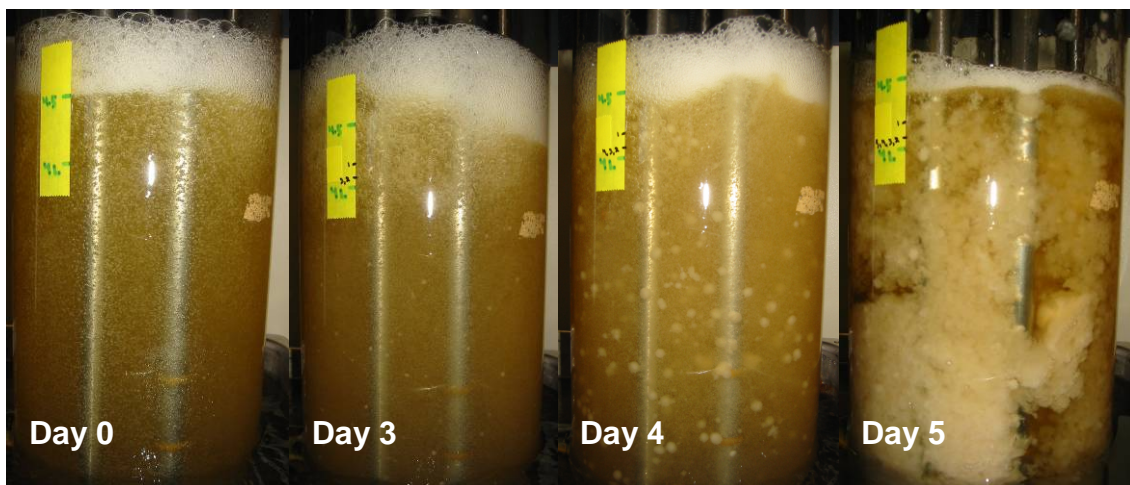


Figure 4.7. Exp. set 2: fungal cultivation of settled thin-stillage supernatant with 1.5-vvm aeration on the day of spore inoculation (day 0), day 3, day 4, and day 5 – experiment ended.

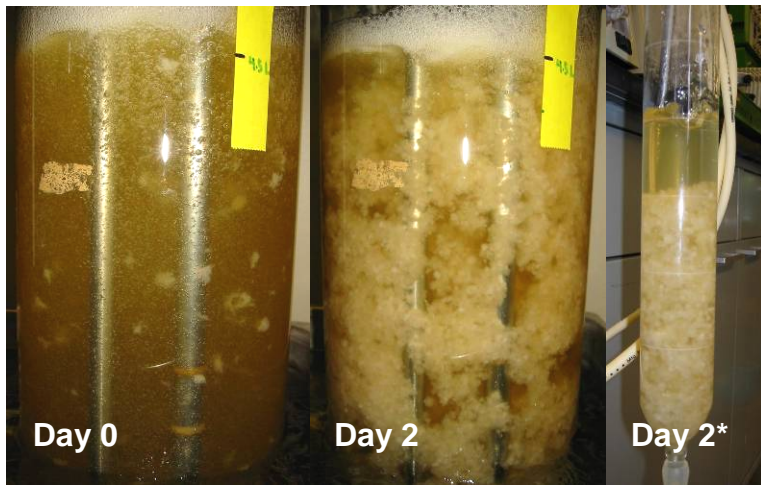


Figure 4.8. Exp. set 2: fungal cultivation of settled thin-stillage supernatant with 1.5-vvm aeration on the day of mycelia inoculation (day 0) and day 2 – experiment ended. *Mycelia settled on day 2 when pumped into a 500-ml burette.



Figure 4.9. Exp. set 2: scaled-up airlift fungal bioreactor cultivating settled thin-stillage supernatant (24 L) with 0.8-vvm aeration on the day of mycelia inoculation (day 0) and filled with mycelia on day 3. The fungal pellets collected on day 3 were separated by coarse screens and dewatered to 20–30% solids by hand squeezing.

Organics (COD) removal

Experiment set 1 (thin stillage): aeration rate, 1.0 or 1.5 vvm, did not affect the daily trends in SCOD reductions during 4.5-L, draft-tube fungal cultivations of thin stillage using spore inoculum (Figure 4.10). Slightly higher biodegradation was achieved by day 3 with aeration of 1.5 vvm (29% reduction) than with 1.0 vvm (25% reduction); this difference may be attributed to the obstructed liquid flow around the draft tube by day 3 with 1.0-vvm aeration. By comparison, in stirred experiments, SCOD removal was similar with 24% reduction in 3 d with 1.0-vvm aeration (Rasmussen et al., 2010). High aeration rates resulted in more rapid biomass accumulation (fungal and thin stillage solids) above the liquid level and reductions in liquid volume. The SCOD reductions in the 1.5-vvm draft-tube experiment steadily increased to 54 and 74% by days 5 and 7, respectively (Figure 4.10). The final SCOD reduction for 1.0-vvm stirred experiments was lower at 42% (day 5).

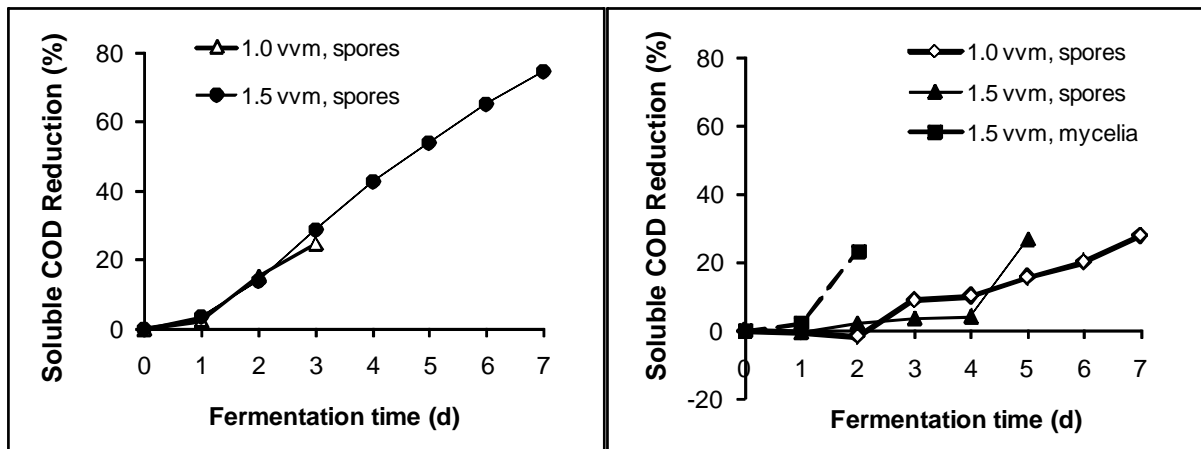


Figure 4.10. Soluble COD reductions during fungal cultivation of thin stillage (Exp. set 1, left) and settled thin-stillage supernatant (Exp. set 2, right) in a 4.5-L, draft-tube bioreactor using spore or mycelia inoculum and aeration rates of 1.0 or 1.5 vvm.

Exp. set 2 (settled thin-stillage supernatant): the initial SCOD of settled thin-stillage supernatant (50–55 g/L) was within the SCOD range of values for thin stillage as expected. Using 1.0-vvm aeration and spore inoculum, the SCOD in the settled thin-stillage supernatant only started to decrease by day 3, with 9% removal (Figure 4.10). The higher reduction (25%) with suspended solids may indicate that the solid particles provide a nutrient that enhances growth. It may also be a result of the different fungal morphologies, filamentous versus spherical pellets with and without suspended solids, respectively. Increasing the aeration to 1.5 vvm resulted in a lower SCOD reduction of only 4% in 3 d. The slower SCOD removal correlates with the delayed fungal growth with aeration of 1.5 vvm compared to 1.0 vvm (Figures 4.7 and 4.6, respectively); increased turbulence and foaming may have affected growth. By comparison, stirred bioreactor experiments (0.8-vvm aeration) using settled thin-stillage supernatant had higher SCOD removals of 19 and 41% in 3 and 5 d, respectively (Rasmussen et al., 2010). This improved SCOD removal may be a result of better mixing and more numerous, smaller fungal pellets. The flow of liquid around the draft tube at this 4.5-L scale was obstructed by the growing fungal biomass.

Mycelia inoculation of settled thin-stillage supernatant resulted in fungal biomass filling the draft-tube bioreactor in 2 d with 1.5-vvm aeration (Figure 4.8). The SCOD was reduced by 23% in 2 d, compared to almost no SCOD reduction in 2 d starting from spores (Figure 4.10); this emphasizes the lag time for growth from spore inoculum. The dissolved oxygen (DO) level using mycelia inoculum dropped from 100% to 80% of saturation in 1 d and to 20% in 2 d. The DO was calibrated to

100% saturation based on 1.5-vvm aeration of fresh settled thin-stillage supernatant at 37°C. The DO in stirred experiments was less than 5% within 1–2 d using 1.0-vvm aeration. Thus, the draft-tube design resulted in higher DO levels in the liquid during fungal cultivations.

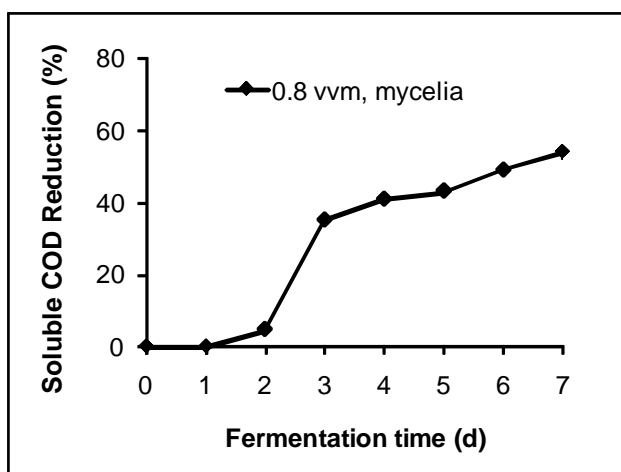


Figure 4.11. Exp. set 2: soluble COD reductions during fungal cultivation of settled thin-stillage supernatant (without suspended solids) in a 24-L airlift bioreactor using mycelia inoculum and an aeration rate of 0.8 vvm.

Scaling up to a 24-L, external-recycle airlift design, fungal removal of thin stillage organic materials was minimal until a substantial jump in SCOD reduction by day 3 (Figure 4.11); this coincided with the dramatic fungal growth observed between days 2 and 3 (Figure 4.9). The fungal growth on day 3 indicates a lag time in starting up a batch experiment with mycelia inoculum of 2% (v/v). Operation in continuous mode would eliminate the 2- to 3-d lag time delays observed in batch experiments. Fungal biomass was harvested on day 3 from the airlift bioreactor, and the liquid recovered was returned to the bioreactor. The aeration was reduced, from 0.8 to 0.4 vvm, to determine bioreactor performance at lower aeration rates. The

resulting reduction in mixing and oxygen transfer, or a nutrient deficiency, may explain the slower fungal growth and organics removal after day 3. The SCOD jumped to a 41% reduction in 3 d and continued to decrease steadily to a 58% reduction in 7 d, the end of the experiment (Figure 4.11).

Organic acids and glycerol removal

Initial lactic and acetic acids concentrations in fresh thin stillage, mainly resulting from bacterial contamination during ethanol fermentation, ranged from 2.3–3.1 g/L and 1.0–1.3 g/L, respectively. Thin stillage contained 13.9–14.7 g/L of glycerol, a byproduct of yeast fermentation under stressed conditions (Walker, 1998). Lactic acid contents in all but one experiment remained steady throughout the fungal cultivations on thin stillage and settled thin-stillage supernatant (Figure 4.12). The exception was cultivation on thin stillage using spore inoculum and 1.5-vvm aeration; lactic acid removal began by day 2 (54%) and was completely removed by day 5 (Figure 4.12). By comparison, during stirred 5-L experiments, lactic acid reductions were observed on days 3–4 only and did not reach 100%.

The acetic acid contents of draft-tube experiment samples was reduced by 100% after 2 d of fungal cultivation on thin stillage (Exp. set 1) using spore inoculum (1.0- and 1.5-vvm aeration) (Figure 4.13). Complete acetic acid removal was also achieved in 2 d with settled thin-stillage supernatant (Exp. set 2) using mycelia inoculum in draft-tube cultivations. Likewise, acetic acid was completely removed in 2 d during stirred experiments with 1.0-vvm aeration (Rasmussen et al., 2010). More time (3–5 d) was required to remove all the acetic acid in draft-tube cultivations of settled thin-stillage supernatant from spores due to the fungal-growth lag time.

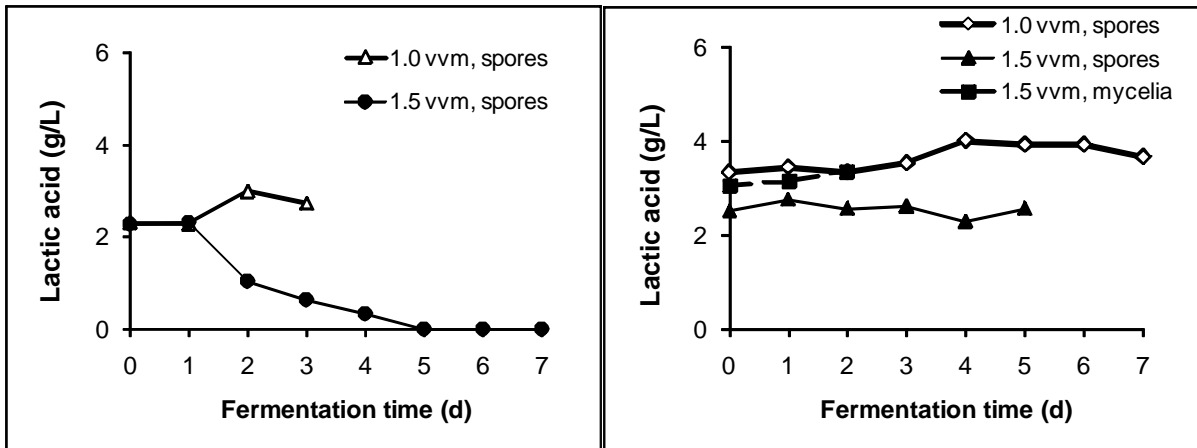


Figure 4.12. Lactic acid sample contents during fungal cultivation of thin stillage (Exp. set 1, left) and settled thin-stillage supernatant (Exp. set 2, right) in a 4.5-L, draft-tube bioreactor using spore or mycelia inoculum and aeration rates of 1.0 or 1.5 vvm.

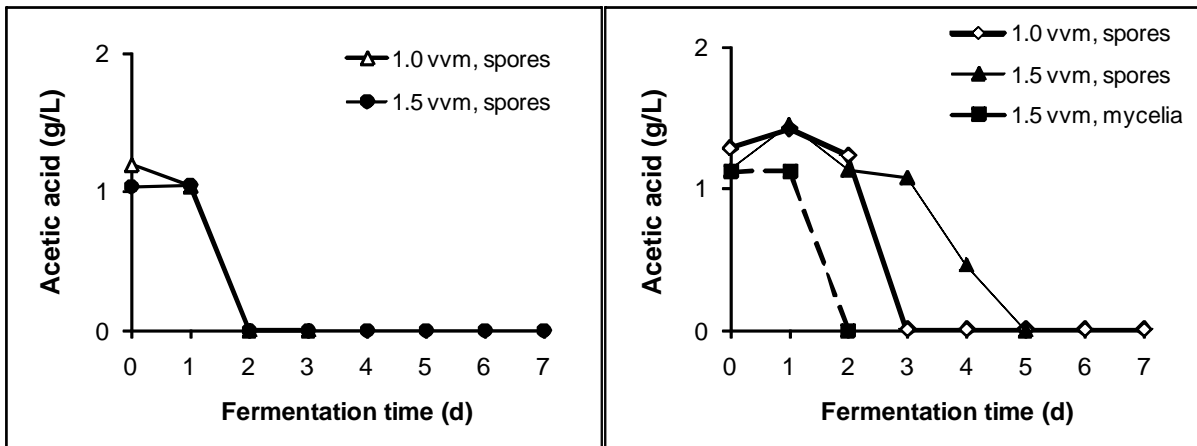


Figure 4.13. Acetic acid sample contents during fungal cultivation of thin stillage (Exp. set 1, left) and settled thin-stillage supernatant (Exp. set 2, right) in a 5-L, draft-tube bioreactor using spore or mycelia inoculum and aeration rates of 1.0 or 1.5 vvm.

Glycerol levels remained steady, similar to lactic acid, in almost all draft-tube experiments (Figure 4.14). The exception was the fungal cultivation of thin stillage using spores and 1.5-vvm aeration; the glycerol began to decrease by day 3 and was reduced to less than 0.2 g/L by day 6 (99% reduction). The glycerol contents of

stirred experiment samples began to decrease by days 3–4; final glycerol reductions was 35% (day 6) (Rasmussen et al., 2010).

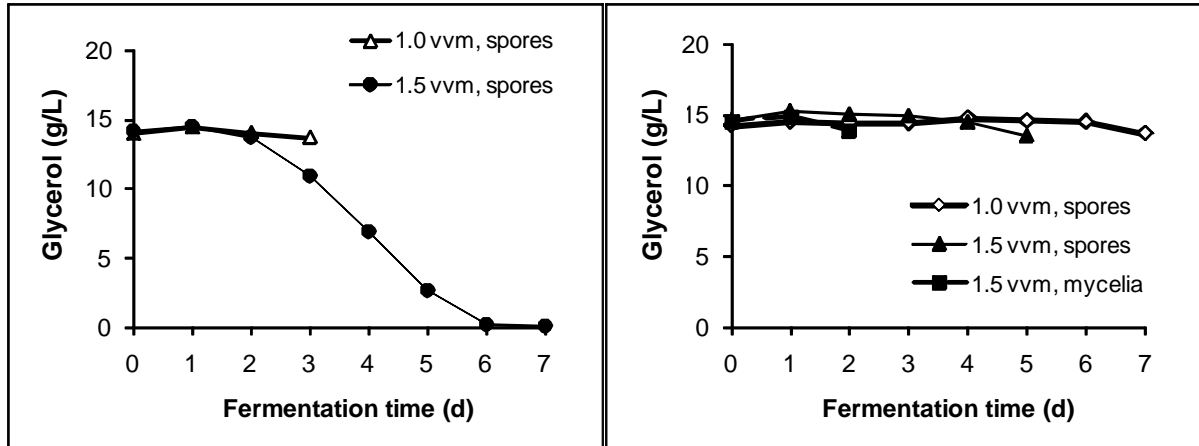


Figure 4.14. Glycerol sample contents during fungal cultivation of thin stillage (Exp. set 1, left) and settled thin-stillage supernatant (Exp. set 2, right) in a 4.5-L, draft-tube bioreactor using spore or mycelia inoculum and aeration rates of 1.0 or 1.5 vvm.

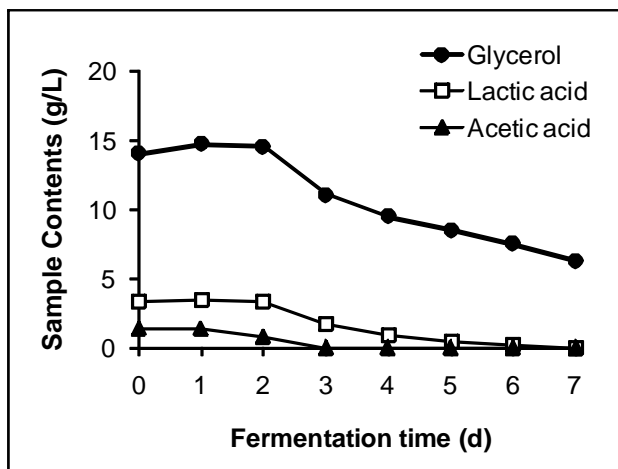


Figure 4.15. Exp. set 2: glycerol, lactic acid, and acetic acid sample contents during fungal cultivation of settled thin-stillage supernatant in a 24-L external-recycle airlift bioreactor using mycelia inoculum and 0.8-vvm aeration rate.

The scaled-up airlift bioreactor (24 L) experiment on settled thin-stillage supernatant (Exp. set 2: mycelia inoculum and 0.8-vvm aeration) had reductions in

glycerol, lactic acid, and acetic acid of 25, 51, and 100%, respectively, by day 3 (Figure 4.15). Glycerol removal of 57% and lactic and acetic acids removals of 100% were achieved by the end of the experiment on day 7.

Fungal biomass production

Fungal biomass production on settled thin-stillage supernatant (Exp. set 2), without thin stillage suspended solids, was found to be in the range of 5.6–7.0 g dry wt/L (Table 4.3). Despite the longer cultivation times (5–7 d) starting from spore inoculum, the final biomass production was similar to experiments with mycelia inoculum (2 d). The ratios of biomass production to SCOD removal were also similar for all experiments, between 0.45 and 0.54 g dry biomass/g SCOD removed. The stirred experiment on settled thin-stillage supernatant produced a higher yield of 13.2 g dry fungal biomass/L in 6 d, but with a slightly lower ratio of 0.43 g dry fungal biomass/g SCOD removed (Rasmussen et al., 2010).

Table 4.3. Biomass production for draft-tube fungal cultivations of settled thin-stillage supernatant (Exp. set 2).

Inoculum	Air flow (vvm)	Fermentation length (d)	Final biomass (g dry wt/L)	Yield (g biomass produced/g COD removed)
Spore	1.0	7	6.3	0.49
Spore	1.5	5	6.7	0.45
Mycelia	1.5	2	5.6	0.47
Mycelia	1.5	2	7.0	0.54

Fungal biomass composition

Compositional analyses were performed on the fungal biomass product grown on thin stillage. Comparisons with the traditional feed ingredients, corn and soybean meal, and with dry-grind corn ethanol coproducts are provided in Table 4.4 (on a dry basis). The freeze-dried biomass consisted of fungal mycelia in addition to enmeshed thin stillage solids. The crude protein content of the fungal biomass (28% w/w) was lower than expected based on previous work using *R. oligosporus*. Jin et al. (2002) reported *R. oligosporus* grown on starch processing wastewater to consist of approximately 50% crude protein and 1% fat (w/w). The lower crude protein and much higher crude fat (33% w/w) composition of our fungal biomass product may be attributed to the thin stillage substrate and the contributions of enmeshed thin stillage particles. Crude protein measurements on our fungal biomass cultivated in settled thin-stillage supernatant were also much higher (43% w/w), but did not include thin stillage suspended solids (Rasmussen et al., 2010).

The crude protein content of our fungal biomass sample grown in thin stillage was comparable to conventional DDGS and to the no-cook process DDGS (POET's Dakota Gold BPXTM) (Table 4.4). The crude fat content of the fungal biomass, however, was three times higher, and linoleic acid represented nearly half of the fat fraction. High linoleic contents are advantageous for poultry (Bregendahl, 2008), but may be detrimental for swine by increasing the softness of pork fat (high carcass iodine values) (Stein and Shurson, 2009). If extracted, the fat content of the fungal biomass may serve as a good substrate for biodiesel production. The fungal biomass product was lower in fiber (NDF and ADF) compared to the DDGS

products, which is a benefit to nonruminants by enhancing the nutritional value and reducing nonruminant gut fill.

In feed protein, lysine, methionine, tryptophan, and threonine are the main limiting amino acids for nonruminant diets (Cheeke, 2005). Lysine and methionine are the first-limiting amino acids in corn-soybean meal diets for swine and poultry, respectively. Based on the amino acid data in Table 4.4, the dried fungal biomass had 40% of the lysine and almost 60% of the methionine contents of soybean meal, the typical protein supplement for animal diets. Relative to conventional DDGS, the lysine content of the fungal biomass product was 60% higher. Biomass protein contents and digestibility depend on harvesting, dewatering, and drying methods, as well as the proportion of enmeshed stillage solids. Improved dewatering should increase the protein fraction (%) of the fungal biomass and, consequently, the amino acid contents. Further research is needed on the feed quality of the fungal biomass product, as it would be produced on commercial scale.

The minerals phosphorus and sulfur are important to consider for feed ingredients. The phosphorus composition (1.65%) of the fungal biomass product was substantially higher than that of the other feed ingredients, for instance, almost 6 times higher than corn, which is a potential benefit for nonruminant feed. The fungal biomass may, thus, displace the inorganic phosphorus supplement typically added, dicalcium phosphate, which would reduce ration costs (Bregendahl, 2008). The sulfur content of the fungal biomass was roughly half that of the DDGS products. Low-sulfur composition is advantageous for livestock diets. Sulfur contents in conventional DDGS and Dakota Gold BPXTM can vary substantially from

0.3% to well over 1% (Batal and Dale, 2003; Spiels et al., 2002; POET Nutrition, 2009; University of Minnesota, 2008). The sources of sulfur in DDGS, in addition to the corn grain, are the sulfur in yeast, well water, and sulfuric acid added during ethanol production (Bregendahl, 2008). Sulfur concentrations of 0.4% of the diet can cause the toxic disorder polioencephalomalacia in cattle (NRC, 1980).

Table 4.4. Compositional comparison of fungal biomass, corn, soybean meal, and dry-grind corn ethanol coproducts.

Composition (% dry basis)	Fungal biomass product ^a	Corn ^b	Soybean meal ^b	Conventional DDGS ^c	Dakota Gold BPX ^d
Crude protein	28.2	9.3	52.8	29.8	28.5
Crude fat	33.1	4.4	3.3	11.3	11.1
NDF	21.1	10.8	9.9	36.2 ^e	26.0
ADF	8.3	9.3	6.0	13.4 ^e	9.5
Ash	5.7	–	–	6.4 ^f	5.1
Lysine	1.42	0.29	3.36	0.85 ^g	1.04
Methionine	0.42	0.19	0.74	0.61 ^g	0.63
Tryptophan	0.27	0.07	0.72	0.24 ^g	0.27
Threonine	1.09	0.33	2.06	1.17 ^g	1.14
Linoleic acid	15.9	2.16	0.67	4.89 ^h	–
Phosphorus	1.65	0.31	0.77	0.87	1.00
Sulfur	0.55	0.15	0.49	0.94 ^g	0.99

^a Freeze-dried fungal biomass product includes enmeshed thin stillage solids.

^b Data from NRC (1998).

^c Data from Waldroup et al. (2007) except as noted.

^d Data from Poet Nutrition (2009).

^e Mean of 5 samples reported by Fastinger et al. (2006) (Bregendahl, 2008).

^f Data from Spiels et al. (2002).

^g Mean of 39 samples reported by Stein et al. (2005, 2006), Fastinger and Mahan (2006), Urriola et al. (2007), and Pahn et al. (2008); data compiled by Stein and Shurson (2009).

^h Data from NRC (1994).

Fungal process add-on and economics

Based on lab-scale stirred experiments (Rasmussen et al., 2010) and preliminary airlift results, the next research step is to employ a larger, pre-commercial-scale airlift bioreactor for fungal cultivation of thin stillage. A scaled-up, draft-tube bioreactor should reduce liquid-flow obstruction, which was problematic in 4.5-L experiments; stainless steel construction and an internal draft tube should result in less issue with oil build-up and leaking, as occurred in the 24-L external-recycle (acrylic) bioreactor. Large quantities of fungal biomass are needed to evaluate the comprehensive nutritional value of the biomass product using animal feed trials. The methods for dewatering and drying the biomass could greatly impact its feed quality and should be as close as possible to future commercial-scale processing. The fractions of fungal biomass and enmeshed thin stillage particles in the product, in particular, affect the biomass composition, as observed by crude protein measurements. The fungal process add-on envisioned for biomass production (i.e., cultivating fungal biomass, separating by screens, dewatering by centrifuge/belt filter, and drying) at a full-scale ethanol plant is shown in Figure 4.16.

A fungal process add-on has the potential for substantial economic benefits to a commercial dry-grind corn ethanol plant. For a 110 MGY corn ethanol plant, 330 MGY of thin stillage is available for fungal cultivation assuming 6 gal thin stillage per gal ethanol and 50% backset. The potential estimated profitability for the fungal process add-on based on laboratory findings to date is as follows:

Income and savings:	\$23.5 million/y	(21.4 ¢/gal EtOH)
Capital costs for add-on:	\$12.0 million	(\$2.4 million/y; 2.2 ¢/gal EtOH)
Operational costs:	\$8.2 million/y	(7.5 ¢/gal EtOH)

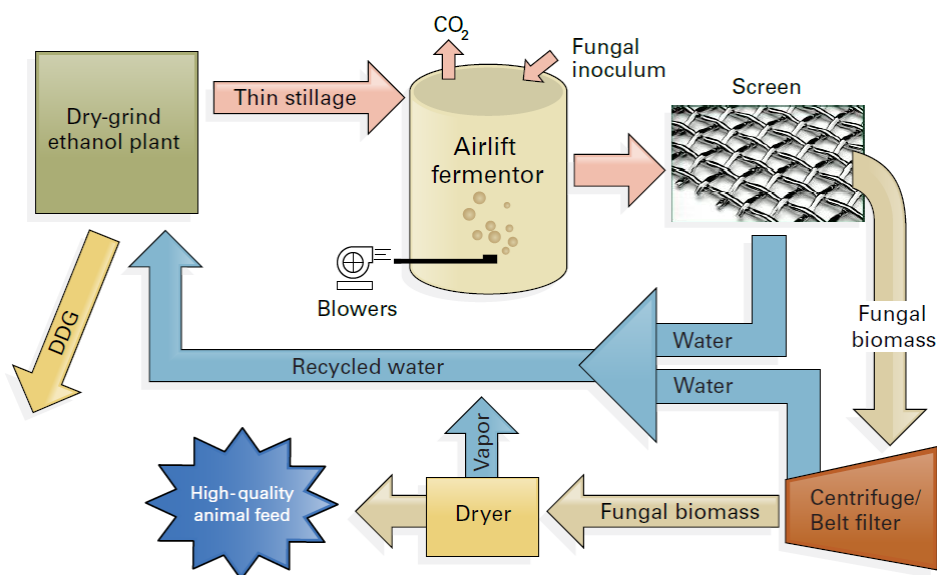


Figure 4.16. The fungal process add-on envisioned for a full-scale dry-grind corn ethanol plant.

A 110 MGY ethanol plant could potentially profit by 11.7 ¢/gal ethanol (\$12.9 million/y) at natural gas-fired plants, with a payback period of approximately 6 months. The potential income is generated by fungal biomass sales, based on 80% COD removal and 0.4 g fungal biomass/g COD removed and a dried product value of \$225/ton. This value for the fungal biomass product is conservative, given our collaborating feed experts' estimate of \$300/ton. The potential savings are a benefit of lower energy inputs by avoiding water evaporation from thin stillage and from water/enzyme recycling. The energy-cost savings are based on the average natural gas price in 2008 of \$9.5/MBTU (EIA, 2009). The amortization cost for the capital expenditures (\$2.4 million/y) was calculated for 15% interest/y over 10 years. The proposed fungal treatment requires fermentation tanks, aeration equipment, and biomass separation (screens), dewatering, and drying equipment, as well as piping and storage tanks. Estimated operational costs of \$8.2 million/y will be required for

aeration, pumping, centrifugation/pressing/drying fungal biomass and for operational personnel.

The energy savings will vary from plant to plant depending on the equipment efficiencies and whether the plant is currently recovering and reusing some of the heat from evaporation. The cost of natural gas is another important factor to energy-cost savings. Natural gas has fluctuated substantially over the last few years, from \$13.1 in July 2008 to \$4.3 in August 2009 per MBTU (EIA, 2009). Nonetheless, the fungal process has the potential to significantly improve commercial dry-grind corn ethanol profitability. A more in-depth analysis of the process economics will be performed as part of the pre-commercial-scale airlift investigation, which is the next research phase.

Conclusions

A novel fungal process for thin stillage treatment in an airlift bioreactor has been developed to reclaim water at ethanol plants, to save energy and enzymes, and to produce an additional valuable coproduct – fungal protein – for swine and poultry feeds. This process could make ethanol production more sustainable and profitable (roughly 12 ¢/gal ethanol), as well as create new jobs and enhance rural prosperity. It has potential for adaptation to other industrial wastewater applications and could reduce the complexity and energy expenditure of current approaches, particularly in the food industry. Aeration is a critical factor to fungal growth, and an airlift reactor seems to be most suitable to ensure high rates of aeration. Future research will employ a larger, pre-commercial-scale, stainless steel bioreactor for airlift fungal cultivation on thin stillage. This bioreactor will help address issues with

obstructed liquid and biomass flows by scaling up in size and should be less problematic with oil build-up (and leaking as occurred in the 24-L acrylic bioreactor). It will also enable production of large quantities of fungal biomass for research on downstream processing – dewatering and drying of biomass – and for animal feed trials.

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CHAPTER 5. REDUCING BACTERIAL CONTAMINATION IN FUEL ETHANOL FERMENTATIONS BY OZONE TREATMENT OF UNCOOKED CORN MASH

A paper prepared for submission to *Biotechnology and Bioengineering*

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Abstract

Ozonation of uncooked corn mash from the POET BPXTM process was investigated as a potential disinfection method for reducing bacterial contamination prior to ethanol fermentation. Corn mash (200 g) was prepared using POET ground corn and POET corn slurry and was ozonated in 250-ml polypropylene bottles. Lactic and acetic acid levels were monitored daily during the fermentation of ozonated, aerated, and non-treated corn mash samples to evaluate bacterial activity. Glycerol and ethanol contents of fermentation samples were checked daily to assess yeast activity. No yeast supplementation, no addition of other antimicrobial agents (such as antibiotics), and spiking with a common lactic acid bacterium (LAB) found in corn ethanol plants, *Lactobacillus plantarum*, amplified the treatment effects.

The laboratory-scale ozone dosages ranged from 26–188 mg/L, with very low estimated costs of \$0.0008 to 0.006 per gallon ethanol. Ozonation was found to

decrease the initial pH of ground corn mash samples, which could reduce the sulfuric acid required to adjust the pH prior to ethanol fermentation. Lactic and acetic acid levels tended to be lower for samples subjected to increasing ozone dosages, indicating less bacterial activity. The lower ozone dosages in the range applied achieved higher ethanol yields. Preliminary experiments on ozonating POET corn slurry at low ozone dosages were not as effective as using POET ground corn; however, corn slurry samples contained recycled antimicrobials from the backset. The data suggest additional dissolved and suspended organic materials from the backset consumed the ozone or shielded the bacteria.

Keywords: ozonation; no-cook; BPXTM process; corn mash; fuel ethanol; bacteria; dry-grind ethanol; lactic acid

Introduction

POET leads the fuel ethanol industry in streamlining corn dry milling to improve ethanol production efficiency, as well as their feed coproduct, Dakota Gold BPXTM distiller grains. The efficient BPXTM, or no-cook, process involves raw starch hydrolysis that, like the conventional process, adds enzymes to convert starch to sugars and adds yeast to ferment sugars to ethanol, but without the cooking step. POET invested years of development to take the patent-pending BPXTM process to commercial scale in 2004 (POET, 2009). The BPXTM process is now used by 24 of the 26 POET biorefineries. POET produces over 1.5 billion gallons of ethanol annually in a network spanning seven Midwestern states.

The POET BPXTM process for dry-grind corn ethanol production has important advantages over the conventional process. The energy-intensive liquefaction and

cooking steps are omitted, thus reducing plant energy requirements by 8–15% (POET, 2009). The BPX™ process achieves higher ethanol yields by improving starch accessibility, lowers volatile organic carbon (VOC) emissions, cuts cooling water needs, and enhances the nutrient quality, flowability and anti-caking properties of the distillers dried grains with solubles (DDGS) coproduct. Amino acid digestibility in DDGS samples varies significantly among conventional ethanol facilities, attributable to heat damage from corn mash cooking and DDGS drying (Batal and Dale, 2006; Fastinger et al., 2006; Fontaine et al., 2007; Bregendahl, 2008). Heat damage is responsible for the lower amino acid digestibility in DDGS than in corn grain; the essential amino acid lysine, which is low in corn, is especially susceptible (Fontaine et al., 2007; Stein et al., 2006). Removing the cooking step, therefore, improves the feed quality of Dakota Gold BPX™ DDGS over conventional DDGS by reducing heat damage of the protein (POET Nutrition, 2009).

Fuel ethanol fermentations are not conducted under pure culture conditions (Skinner and Leathers, 2004). High-temperature jet cooking (90°–165°C) in the conventional process not only gelatinizes the corn starch, it helps reduce contamination and yield losses caused by unwanted lactic acid bacteria (LAB) (U.S. Grains Council, 2007). Contamination in fuel ethanol fermentations originates from bacteria present on the corn from the fields (Bryan, 2006). Bacteria compete with yeast for nutrients – glucose, trace minerals, vitamins, and free amino nitrogen – and lower ethanol yields by diverting glucose to lactic and acetic acids, which are inhibitory compounds to yeast (Barbour and Priest, 1988; Maiorella et al., 1983; Makanjuola et al., 1992). In addition to yield losses by chronic LAB contamination,

acute infections arise unpredictably and can result in plant shut-downs for cleaning – an expensive loss in productivity (Makanjuola et al., 1992; Narendranath et al., 1997; Skinner and Leathers, 2004).

Gram-positive and gram-negative bacterial isolates from fuel ethanol production include species of *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, *Clostridium*, and *Lactobacillus* (Lushia and Heist, 2005; Skinner and Leathers, 2004). *Lactobacilli* sp. are the most prevalent in the industry and are ubiquitous in nature. In a study of two commercial dry grind ethanol plants by Skinner and Leathers (2004), the genus *Lactobacillus* was isolated most frequently, representing 38 and 77% of total bacterial isolates from the first and second plants, respectively. Gram-positive LAB are ethanol-tolerant, grow faster than yeast, and produce organic acids which inhibit yeast.

Bacterial growth is currently controlled at POET biorefineries by dosing with the antibiotic virginiamycin (Lactrol[®]) and an antimicrobial agent (IsoStab[™]) and by operation at low pH levels (pH 4.2). Conventional ethanol plants, despite jet cooking, also add antibiotics to fight chronic or acute bacterial infections (Bayrock and Ingledew, 2003; Narendranath and Power, 2005; Skinner and Leathers, 2004; Stroppa et al., 2000). If no antibiotics are used, it is common for a facility to lose 1–5% of its potential ethanol yield (Bryan, 2006). Rising concerns about the use of antibiotics and the emergence of antibiotic-resistant bacteria is creating a demand for alternative methods of controlling bacteria.

Cleaning and sanitation are important microbial control measures in the corn ethanol industry. Infections in process tanks and continuous yeast propagators, as

well as resistant biofilms within the process, can continually reintroduce bacterial contaminants into the fermentors (Bischoff et al., 2009; Skinner and Leathers, 2004). Large yeast inoculums ($\geq 2\%$ [v/v]) help control contamination during fermentations; however, yeast growth and fermentation rates are still reduced by high lactobacilli concentrations (Thomas et al., 2001).

Penicillin and virginiamycin are the antibiotics available commercially for fuel ethanol production (Connolly, 1997; Lushia and Heist, 2005). Virginiamycin, produced by *Streptomyces virginiae*, has had limited application in human medicine but extensive use as a growth-promoting additive in animal feed (Cocito, 1979). The recommended dose of virginiamycin in fuel ethanol fermentations is 0.25–2.0 ppm (Hynes et al., 1997). The use of antibiotics has limitations. Antibiotic applications are essentially selection experiments for resistant microorganisms. Hynes et al. (1997) observed reductions in the effectiveness of virginiamycin after extended fermentations, possibly resulting from its breakdown by lactobacilli (Dutta and Devriese, 1981). *Lactobacillus* sp. with higher tolerance to virginiamycin have been isolated from fuel ethanol plants that dose with virginiamycin. Moreover, bacterial isolates have emerged with resistance to both virginiamycin and penicillin (Bischoff et al., 2007; Lushia and Heist, 2005).

Hop acids offer a natural alternative to antibiotics for fuel ethanol production (Bryan, 2006). Many of the organic acids found in hops exhibit antimicrobial activities. Hop-compounds inhibit growth by disrupting the transmembrane pH gradient of microbes (Haakensen et al., 2009; Simpson, 1993). Hops have long been used in the brewing industry to contribute to the flavor of beer, by adding

bitterness and aroma, and to improve product shelf-life. IsoStab™, a liquid hop extract, is a commercially-available product for fuel ethanol applications. U.S. producers using IsoStab™ have obtained higher ethanol yields with less fluctuation.

A variety of disinfectants have been investigated on lab scale for fuel ethanol fermentations, including hydrogen peroxide, chlorine dioxide, potassium metabisulfite, 3,4,4'-trichlorocarbanilide, and lactate with a lactate-tolerate yeast strain (Chang et al., 1997; Gibbons and Westby, 1986; Meneghin et al., 2008; Narendranath et al., 2000; Oliva-Neto and Yokoya, 1998; Skinner and Leathers, 2004; Watanabe et al., 2008). Bacteria were inhibited over yeast by these agents, but the effectiveness depended on the bacterial strain (Skinner and Leathers, 2004). Chlorine dioxide and ozonation are common alternatives to chlorination for disinfection of drinking water. Meneghin et al. (2008) evaluated the effect of chlorine dioxide against bacteria prevalent in ethanol fermentations (e.g., *Lactobacillus plantarum* and *L. fermentum*). The minimum inhibitory concentration (MIC) for chlorine dioxide on the bacteria and on the yeast inoculum were determined using nutrient media; the effectiveness in nutrient media is likely different than in the high suspended-solids corn mash at fuel ethanol plants. The MICs ranged from 10–125 ppm for the bacterial strains tested; *L. plantarum* had the highest MIC of 125 ppm. Yeast growth was also affected at chlorine dioxide concentrations over 50 ppm.

The objective of this research was to investigate ozonation as a potential method for minimizing bacterial contamination during ethanol fermentation in the POET BPX™ process. Ozone is a powerful disinfectant, with an oxidation potential ($E_0=2.08$) higher than that of chlorine dioxide ($E_0=0.95$) and hydrogen peroxide

($E_o=1.78$) (AWWA, 1999). Ozone has a short half-life and leaves no residual, reverting back to oxygen. This research seeks to replace antibiotics with the disinfection method – corn mash ozonation – and to enable operation at a higher pH more conducive to ethanol fermentation.

Currently, sulfuric acid is added to lower the corn mash pH prior to fermentation. Operating at a higher fermentation pH would reduce sulfuric acid requirements, if any, and the resulting sulfur content in the distillers grains. The coproduct could have an advantage in marketing as antibiotic-free and reduced-sulfur distillers grains. Corn grain is fairly low in sulfur (0.13%; NRC, 1998). The sulfur contents in conventional DDGS and Dakota Gold BPX™ are greater (0.84 and 0.91%, respectively) and can vary substantially from 0.3% to more than 1% (Batal and Dale, 2003; Spiels et al., 2002; POET Nutrition, 2009; University of Minnesota, 2008). The additional sources of sulfur are contributed by the yeast, well water, and sulfuric acid added during ethanol production (Bregendahl, 2008). Sulfur concentrations of 0.4% of the complete diet can be toxic to cattle by causing polioencephalomalacia (NRC, 1980). The sulfur content of DDGS can limit its inclusion rate as a feed ingredient. Ozonation, in place of antibiotic addition and lower fermentation pH, could therefore save money by reducing operational expenses (i.e., antibiotic and chemical costs) and improve the feed quality of the DDGS.

Research was conducted to establish an appropriate laboratory-scale ozonation setup for corn mash, to determine the impact of a range of low ozone dosages on bacterial activity during fermentation, and to estimate ozonation costs.

Fermentation experiments were conducted following ozonation and aeration to assess whether lower lactic acid contents in corn mash resulted from ozonation as a disinfect or from possible stimulatory effects of aeration on the yeast. No other antimicrobial agents (e.g., Lactrol[®] and IsoStab[™]) were added. Select samples were spiked with *L. plantarum* prior to ozone treatment and were not yeast-supplemented to amplify differences in lactic acid production. Preliminary experiments were subsequently performed using corn slurry to evaluate the effectiveness of ozonation for reducing bacterial contamination under conditions more similar to plant operation, including the contributions of recycled water sources.

Materials and Methods

Corn mash preparation

Ground corn and corn slurry were obtained from POET Biorefining in Jewell, IA. The corn slurry obtained from POET contains the ground corn in a slurry with recycled water streams, such as backset. Figure 5.1 illustrates the corn mash preparation, ozone treatment, and simultaneous saccharification and fermentation (SSF) procedures. Corn mash was prepared in 250-ml polypropylene (PP) bottles with 35% (w/w) ground corn (200 g mash per bottle) and deionized water or with 200 g corn slurry per bottle. Urea was added (0.03% [w/w]) as a nitrogen source. The final corn mash volume was 186 ml per bottle. Bottles were shaken for 30 min at 250 rpm and room temperature to slurry contents. The pH was adjusted to 5.0 in select experiments. In preliminary work (pH 4.2), all controls with and without Lactrol[®] and/or IsoStab[™] added had similar values for lactic and acetic acids, glycerol, and ethanol, which indicated minimal bacterial contamination even without

ozonation (results not shown). Therefore, no antibiotics or other antimicrobials, i.e., Lactrol® or IsoStab™, were added in experiments reported in this publication. The POET corn slurry experiments, however, contained recycled antimicrobials contributed in the backset from previous fermentations at the plant. Select samples were also LAB-spiked and/or not yeast-supplemented to amplify the differences in treatment effects.

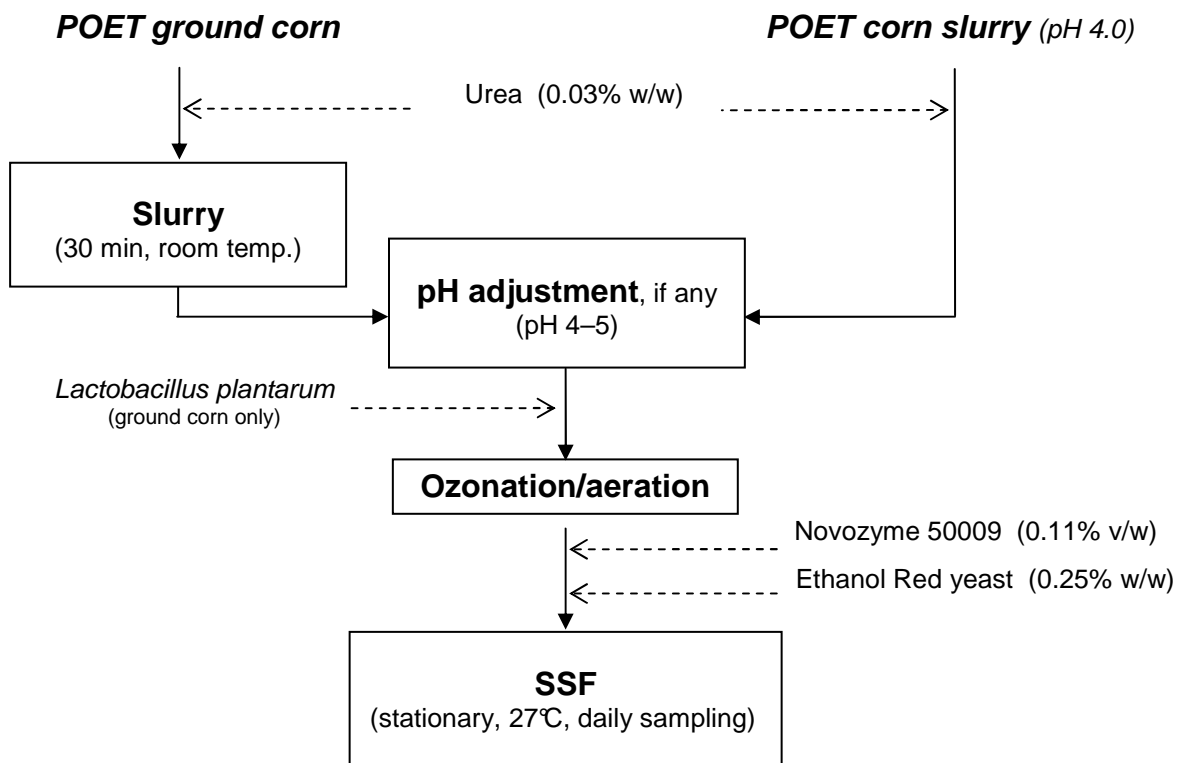


Figure 5.1. Corn mash preparation, ozonation/aeration, and fermentation using POET ground corn and POET corn slurry.

Microorganisms

Select corn mash samples were spiked with *Lactobacillus plantarum*, a lactic acid bacterium, obtained from the American Type Culture Collection (ATCC 14917,

Rockville, MD) prior to ozone treatment. Stock cultures of *L. plantarum* were freeze-dried and stored previously in sterile skim milk at 4°C. Fermentis Ethanol Red (Lesaffre Yeast Corporation, Milwaukee, WI), containing the yeast *Saccharomyces cerevisiae*, was added (0.5 g) after ozone treatment for ethanol fermentation.

Ozonation

The laboratory-scale installation used to apply the ozone treatment is shown in Figure 5.2. Ozone gas was generated from oxygen supplied by a compressed-air gas cylinder using a corona-discharge ozone generator (TOG C2B, Trigon, Glasgow, Scotland). Moisture and hydrocarbon traps (Restek, State College, PA) were placed between the ozone generator and compressed-air cylinder to remove impurities in the feed gas. A mass flow controller (GLC17, Aalborg, Orangeburg, NY) was used to control the gas flow rate.

Ozone gas was bubbled into the corn mash samples (200 g) in 250-ml PP bottles with a flow rate of 500 ml/min. The ozone concentration in the gas phase was measured by the potassium iodide titration method. The ozone dose, or consumption, was calculated based on ozone concentrations in the influent and effluent gas from the bottle over time. Corn mash samples were ozonated and residual ozone in off-gas measured by titration after varying ozonation times. This setup was used to determine the effects of different ozone dosages on ethanol fermentation of corn mash samples prepared from ground corn or corn slurry. Aeration treatment, as a control, was also performed using this equipment. The aeration and ozonation procedures were the same (e.g., similar gas flow rate), but with the ozone generator turned off.

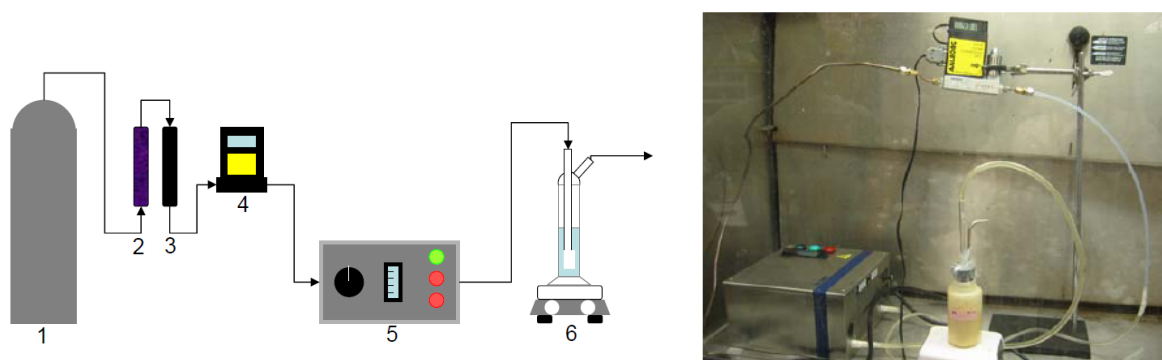


Figure 5.2. Setup and photo of ozonation of corn mash: (1) compressed air tank, (2) moisture trap, (3) hydrocarbon trap, (4) mass flow controller, (5) corona-discharge ozone generator, and (6) corn mash sample (200 g) in 250-ml PP bottle.

Ethanol fermentation

Following ozonation, raw starch hydrolyzing enzyme (Novozyme 50009, 0.11% [v/w]) and Ethanol Red (0.25% [w/w]) were added to ozonated, aerated, and non-treated corn mash (Figure 5.1). Bottles were shaken for 2 min at 250 rpm to mix contents and incubated without shaking at 27°C until ethanol levels reached a plateau – up to 168 h. SSF samples were HPLC-analyzed on a daily basis.

Sample analyses

The ethanol, glycerol, lactic acid, and acetic acid contents of corn mash and SSF samples were quantified using a Waters high pressure liquid chromatograph (HPLC) (Millipore Corporation, Milford, MA). The HPLC system was equipped with a Waters Model 401 refractive index detector, column heater, automatic sampler, and computer controller. The Aminex HPX-8711 column (300x7.8 mm, Bio-Rad Chemical Division, Richmond, CA) separated sample constituents with a mobile phase of 0.012 N sulfuric acid, flow rate of 0.8 ml/min, injection volume of 20 μ l, and column temperature of 65°C (Kunduru and Pometto, 1996).

Experiment sets

A pH experiment was conducted prior to fermentation. Aeration treatment was included to confirm that ozonation, rather than aeration and mixing, lowered the initial corn mash pH prior to fermentation (0 h).

The ethanol fermentation experiments reported in this publication are divided into three sets – two with POET ground corn and one with POET corn slurry – as outlined in Table 5.1.

Ground corn (Experiment sets 1 & 2)

Two sets of fermentation experiments with ozone-treated corn mash prepared from ground corn were performed. The first set included spiking with *L. plantarum* before ozone treatment (0–188 mg/L) of corn mash; no yeast and no other antimicrobial agents were added to select bottles to further amplify the treatment effects. The second set included ozonation and aeration treatments of corn mash prior to ethanol fermentation. Select bottles were spiked with *L. plantarum*. Fermentation experiments were conducted with aeration treatment as a control to determine whether lower lactic acid contents in daily samples resulted from ozonation as a disinfect/oxidant or from possible stimulatory effects of aeration on the yeast.

Corn slurry (Experiment set 3)

A preliminary set of fermentation experiments was conducted with POET corn slurry to assess the effectiveness of ozonation for reducing bacterial contamination under conditions more similar to plant operation, including contributions of recycled water streams. The recycled backset contributed other antimicrobials (Lactrol[®] and

IsoStab™) in these experiments. Corn mash samples were prepared with/without the addition of yeast, with the slurry initial pH of 4.0 and adjusted to pH 5.0, and with/without ozone treatment, as shown in Table 5.1.

Table 5.1. Experiments performed using corn mash prepared with POET ground corn and POET corn slurry^a.

Exp. set	Corn mash	Treatment	Initial pH ^b	LAB spiked	Yeast added
1	Ground corn	Ozone (0–188 mg/L)	5.0	Yes	No
1	Ground corn	Ozone (0–152 mg/L)	5.0	Yes	Yes
2	Ground corn	None	4.7	No	Yes
2	Ground corn	Air (60 min)	4.7	No	Yes
2	Ground corn	Ozone (60 min)	4.7	No	Yes
2	Ground corn	None	4.7	Yes	Yes
2	Ground corn	Air (60 min)	4.7	Yes	Yes
2	Ground corn	Ozone (60 min)	4.7	Yes	Yes
3	Corn slurry	None	4.0	No	Yes
3	Corn slurry	None	4.0	No	No
3	Corn slurry	None	5.0	No	Yes
3	Corn slurry	Ozone (60 min)	5.0	No	Yes

^a No antimicrobials were added in these experiments; however, corn slurry obtained from POET contained backset, which has Lactrol® and IsoStab™ from previous fermentations.

^b Initial pH refers to the pH after adjustment and before treatment. Without adjustment, the pH of corn mash prepared using ground corn was 4.7 and using corn slurry was 4.0.

Results and Discussion

Ozone dosages

The results of ozonating corn mash samples for various treatment times in order to determine ozone consumption, i.e., ozone dosage, are illustrated in Figure 5.3. Ozone consumption is the difference between the ozone applied to the corn

mash, $f(x)$, and the unused ozone exiting the headspace of the treated corn mash over time (off-gas), $g(x)$; it equals the green area in Figure 5.3. The following fitted equations were used to calculate ozone consumption/dosage:

$$\begin{aligned} \text{Applied ozone (mg/min)} &= f(x) = 0.996 \\ \text{Off-gas ozone (mg/min)} &= g(x) = 0.00319x + 0.000538x^2 & 0 \leq t \leq 37.5 \text{ min} \\ \text{Off-gas ozone (mg/min)} &= g(x) = 0.884 & 37.5 \text{ min} \leq t \end{aligned}$$

$$\int_0^t f(x)dx - \int_0^t g(x)dx = 0.996t - 0.00159t^2 - 0.000179t^3 \quad 0 \leq t \leq 37.5 \text{ min}$$

$$\int_0^t f(x)dx - \int_0^t g(x)dx = 0.112*(t-37.5) + 25.7 \quad 37.5 \text{ min} \leq t$$

The ozone dosages computed for each treatment time are provided in Table 5.2. In our preliminary work, the additional dosages of 26 and 75 mg O₃/L were tested. The higher dosages (127, 152, and 188 mg/L) were chosen for replicate experiments, as reported in this publication.

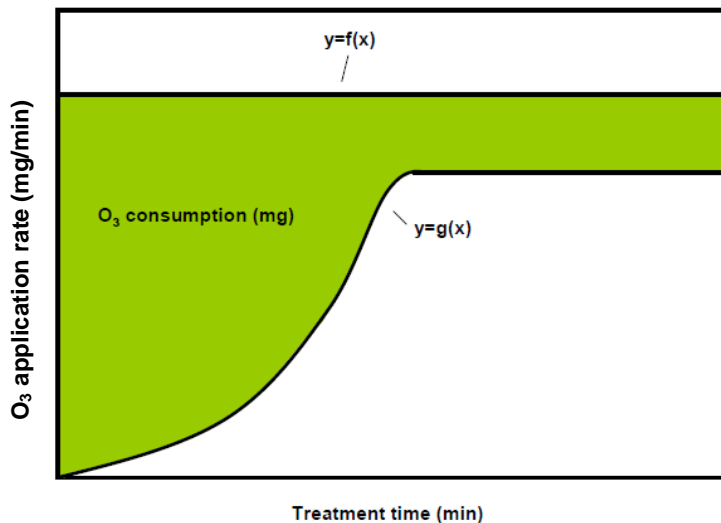


Figure 5.3. Ozone consumption/dose (the green area) as a function of ozonation treatment time for corn mash samples. The curve was fit based on the effluent ozone discharge rates, $g(x)$, observed in corn mash over time. The influent ozone application rate was constant, $f(x)$. (n=4)

Ozone cost estimates

The ozone cost estimates include operating costs to purchase electricity for the ozone generator and to prepare air for the ozonation. The costs of electricity and air are calculated as follows:

Electricity (typical ozone generators): 10 kWh/kg ozone (Rakness, 2005)
 Electricity: \$0.07/kWh, 2009 average for industrial consumers (EIA, 2009)
 Typical electricity cost for air feed drying: approximately 2 kWh/kg ozone

Capital costs: since the ozone dosage has not yet been optimized in relation to corn mash formulations in industry, including recycled water sources, the required ozone generator size for a typical plant cannot be determined. As a rough guide, the amortization costs will equal the energy costs. The cost of ozone, therefore, can be expected to amount to roughly \$1.68/kg. This cost still needs to be determined to a greater degree of accuracy. Table 5.2 presents the estimated ozonation costs based on the ozone dosages applied in experiments to date.

Table 5.2. Ozone dosages applied to corn mash and estimated costs in our study using different ozonation times.

Ozonation time (min)	Ozone dose ^a (mg O ₃)	Ozone dose ^b (mg O ₃ /L corn mash)	Estimated cost ^c (\$/1000 gal ethanol)
5	5	26	0.84
15	14	75	2.39
30	24	127	4.04
60	28	152	4.82
120	35	188	5.97

^a Ozone dose/consumption was determined by ozonating corn mash samples. The resulting equations used for ozone calculations (mg) are: $0.996t - 0.00159t^2 - 0.000179t^3$ for $0 \leq t \leq 37.5$ min, and $0.112*(t-37.5) + 25.7$ for $t \geq 37.5$ min.

^b The volume of ozonated corn mash was 0.186 L; ozone dose (mg) was divided by this volume.

^c Estimated cost was calculated from ozone dose (mg/L) based on the conversion factor of 3.79 L/gal, an ozone cost of $\$1.68 \times 10^{-6}$ per mg, and assuming 20% v/v final ethanol content (or 1 gal ethanol to 5 gal corn mash).

Initial pH experiment

The initial pH of corn mash prepared using ground corn, prior to fermentation (0 h), was adjusted to 4.2 or 5.0. The pH of corn mash samples tended to decrease with increasing ozone treatment times/dosages (Figure 5.4). The pH of ozonated samples (initial pH 5.0) started to decrease after 15 min of ozonation (75 mg O₃/L), reaching an average pH of 4.8 in 60 min (152 mg O₃/L). With an initial pH of 4.2, the corn mash pH was also reduced by 0.2 pH units to 4.0 after 60 min of ozonation. Aeration using the same procedure, but no ozone generated, did not affect the corn mash pH. This finding shows that the ozone or ozone byproducts, rather than aeration and additional mixing, were responsible for the corn mash pH reductions observed in all experiments using ground corn.

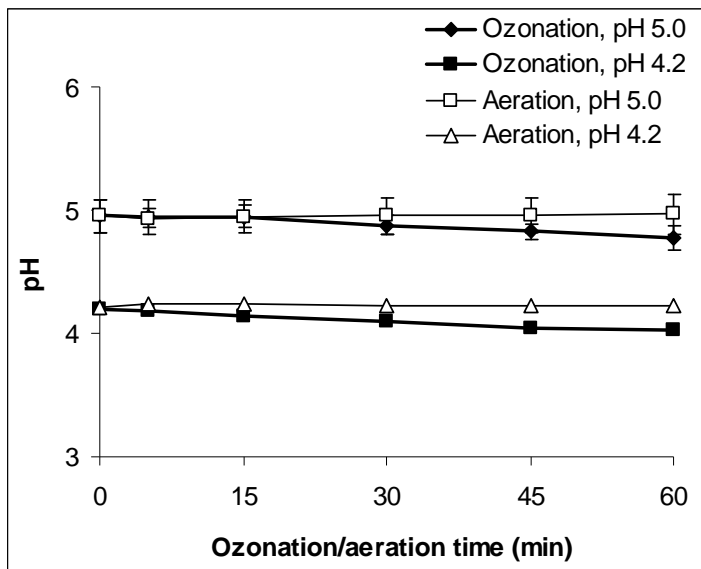


Figure 5.4. Initial pH experiment: changes in pH observed during ozonation and aeration of corn mash samples prepared using ground corn and adjusted to different initial pH values, 5.0 (n=3) and 4.2 (n=1).

Corn mash samples were analyzed on the HPLC before and after treatment to detect any changes in peaks, in particular for organic acids. No consistent changes were observed for ozonated and aerated samples. A slight increase in acetic acid of 0.35 g/L occurred in one ozonated sample, but not in replicate samples. There were no changes in acetic acid in aerated samples. No lactic acid was detected in any samples before or after ozonation/aeration.

Ground corn (Experiment sets 1 & 2)

Daily pH & lactic acid contents

Experiment set 1 (Figure 5.5): the daily pH values of ozonated and non-ozonated corn mash samples decreased the first 24–48 h of fermentation only. The exceptions were samples with higher ozone dosages (152 and 188 mg/L) and no yeast added; the pH of these samples started decreasing after 24–48 h of fermentation and stabilizing by 96–120 h. All pH trends corresponded with increases in lactic acid contents. Higher daily pH values, and less lactic acid, were observed in samples subjected to higher ozone dosages.

Statistical analysis was conducted to compare the significance ($p < 0.05$) of 96-h fermentation samples. As expected, lactic acid production in samples without yeast added was significantly higher than in samples with yeast added. Yeast inoculum size affects the ability to compete with bacteria for the same substrate – fermentable sugars (Thomas et al., 2001). Increasing the ozone dosages from 0 to 127 mg/L and from 127 to 152 mg/L, without yeast added, resulted in significantly lower ($p < 0.05$) lactic acid contents; no significant difference was observed between 152 and 188 mg ozone/L. These differences were amplified by not supplementing

with yeast. With yeast added, the 152 mg/L ozone dosage resulted in significantly lower lactic acid contents than the 127 mg/L dosage, indicating less bacterial activity; there was no significant difference, however, between 0 and 127 mg/L dosages.

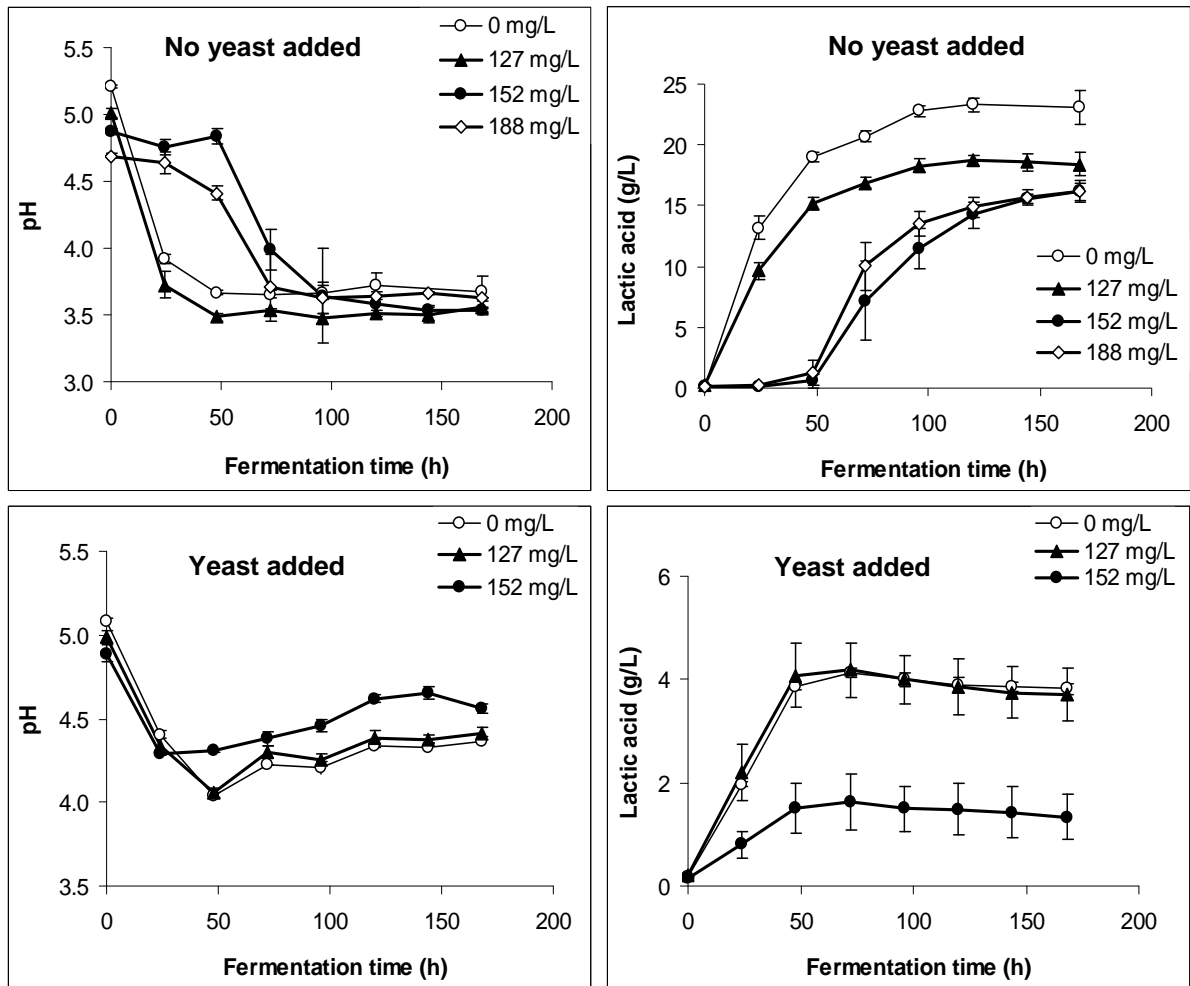


Figure 5.5. Exp. set 1: samples were spiked with *L. plantarum* and ozonated. Select samples had yeast added. Corn mash pH was adjusted to 5.0 prior to ozonation. No other antimicrobial agents (Lactrol® or IsoStab™) were added. The daily pH (left) and lactic acid contents (right) of fermentation samples from corn mash prepared using ground corn. (n=3)

Experiment set 2 (Figure 5.6): the daily pH values of ozonated, aerated, and non-treated corn mash samples decreased the first 24–48 h of fermentation only, without exception. These results agree with Experiment set 1 since yeast was added to all samples in this set. Likewise, the daily pH values for all samples corresponded with increases in lactic acid contents.

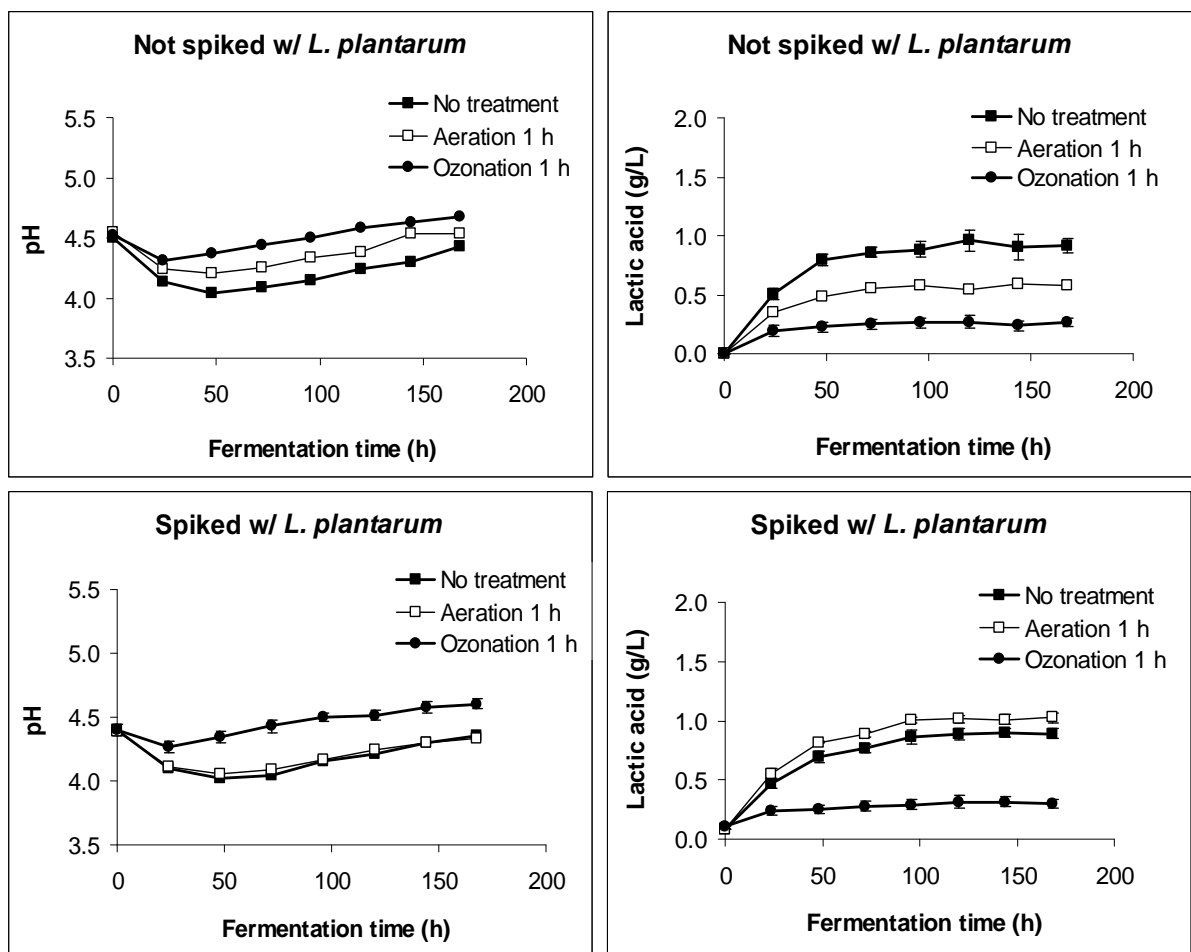


Figure 5.6. Exp. set 2: select samples were spiked with *L. plantarum* prior to treatment. Samples were ozonated or aerated for 60 min, except for controls with no treatment, and yeast was added. No other antimicrobial agents (Lactrol® or IsoStab™) were added. The daily pH (left) and lactic acid contents (right) of fermentation samples from corn mash prepared using ground corn. (n=3)

Non-treated samples tended to have lower daily pH values and higher lactic acid concentrations. Ozonated samples had the highest pH values and least lactic acid throughout the fermentation period, indicating less bacterial and more yeast activity. LAB-spiked, aerated samples had similar trends to non-treated samples. Non-LAB-spiked, aerated samples had lower lactic acid values than non-treated samples, which suggest the indigenous LAB populations prefer a reduced oxygen environment. Thus, aeration was only effective for reducing lactic acid production in samples without LAB-spiking, but ozonation was still more effective. Statistical comparisons of 96-h fermentation samples showed that all differences in lactic acid contents among ozonated, aerated, and non-treated samples were significant ($p < 0.05$).

Glycerol & ethanol contents

Experiment set 1: glycerol production was the lowest in corn mash treated with 127 mg/L of ozone prior to fermentation, compared to non-treated and 152-mg/L ozone-treated samples (Table 5.3). The 127-mg O_3 /L samples had the highest acetic acid and ethanol contents, which create osmotic stress on the yeast triggering glycerol production (Walker, 1998); lactic acid contents, however, were comparable to non-treated samples (Figure 5.5, yeast added). Reduced glycerol contents were also observed in preliminary experiments with increasing ozone dosages, indicating the yeast were less stressed compared to non-ozonated samples.

The daily ethanol contents of ozonated and non-ozonated corn mash samples began to plateau by 96 h of fermentation (Figure 5.7). With yeast added, the 96-h ethanol concentration ranged from 150 to 154 g/L. The ethanol concentrations in

96-h samples treated with 127-mg/L ozone dosage were significantly higher ($p < 0.05$) than in samples with no ozone treatment (Table 5.3). Samples subjected to 152 mg/L ozone dosage had more ethanol than non-ozonated samples, but the difference was not significant. Ethanol production was observed in corn mash samples even without yeast added (up to 61 g/L in 96-h, non-ozonated samples); the ground corn used to prepare the corn mash was obtained from POET and presumably contained yeast from exposure during storage at the ethanol biorefinery. Without supplemental yeast, ozone dosage had no significant effect on ethanol contents in 96-h samples (Figure 5.7).

Table 5.3. Comparison of glycerol and ethanol contents of 96-h fermentation samples from corn mash prepared using ground corn with yeast addition after ozone treatment^a. (n=3)

Exp. set ^a	LAB spiked	Treatment ^b	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (g/L)
1	Yes	None	0.7	10.2	150
1	Yes	Ozone (127 mg/L)	1.0	9.3	154
1	Yes	Ozone (152 mg/L = 60 min)	0.7	9.8	152
2	No	None	0.2	10.4	147
2	No	Aerated (60 min)	0.2	10.7	147
2	No	Ozone (60 min)	0.4	8.6	143
2	Yes	None	0.2	10.4	145
2	Yes	Aerated (60 min)	0.3	10.5	145
2	Yes	Ozone (60 min)	0.4	8.8	143

^a No other antimicrobial agents (Lactrol[®] or IsoStab[™]) were added.

^b Initial pH values, prior to ozonation, of Exp. sets 1 and 2 were 5.0 and 4.7, respectively. The initial fermentation pH values were 4.8 and 4.5, respectively.

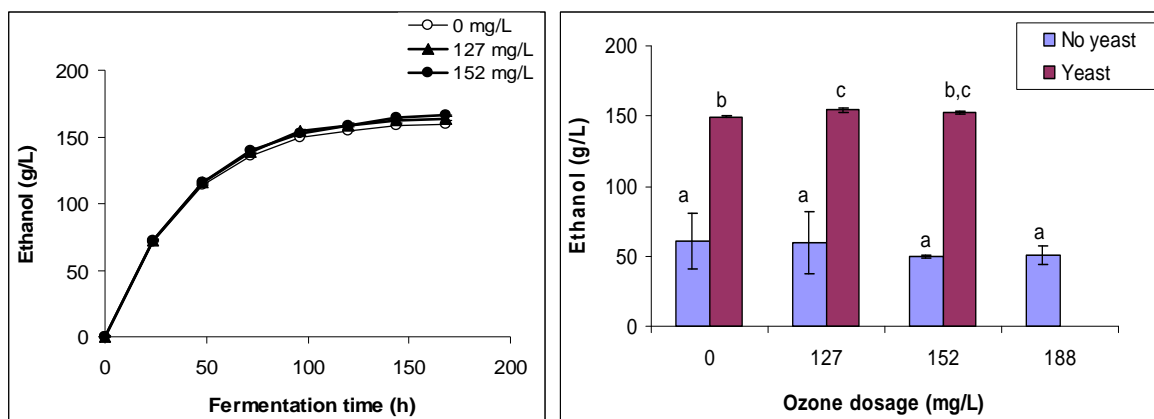


Figure 5.7. Exp. set 1: ethanol contents of daily fermentation samples from corn mash prepared using ground corn, spiked with *L. plantarum*, ozonated, and yeast added (left). No other antimicrobial agents (Lactrol[®] or IsoStab[™]) were added. Comparison of ethanol contents of 96-h samples (right). (n=3)

Experiment set 2 (Table 5.3): the daily glycerol trends in corn mash samples were not affected by spiking with *L. plantarum*. The aerated and non-treated samples had similar glycerol production throughout the fermentation period, with slightly more glycerol in the aerated samples. Ozonated samples had the least glycerol after 24 h of fermentation, which coincided with lower daily ethanol production.

The ethanol concentrations in all samples, LAB-spiked and non-spiked, began to plateau by 96 h of fermentation, as observed in Experiment set 1. The 96-h ethanol concentration ranged from 143 to 147 g/L. Spiking with *L. plantarum* resulted in somewhat less ethanol than in non-spiked samples (Table 5.3). The ozonated samples had less ethanol and less lactic acid (Figure 5.6), than aerated and non-treated samples in this set of experiments, which accounts for less glycerol production. The ethanol findings suggest that higher ozone dosages (152 mg/L) may negatively impact ethanol yields, despite lowering lactic acid contents,

depending on the initial pH. The starting pH (post-treatment) for fermentations in the first and second sets of experiments was 4.8 and 4.5, respectively.

Corn slurry (Experiment set 3)

Yeast addition

Corn mash samples were prepared using POET corn slurry (pH 4) by adding urea, enzymes, and yeast. No yeast was added to select samples. As expected, samples without yeast added had higher levels of lactic acid within 24 h and acetic acid within 48 h of fermentation (Figures 5.8B and 5.8C). Higher yeast inoculum size enables the yeast to be more competitive with the bacteria. The addition of yeast resulted in significantly ($p < 0.05$) higher ethanol contents within 24 h of fermentation (Figure 5.8E); 96-h samples had an average ethanol yield of 146 g/L, with yeast added, as compared to 130 g/L without yeast added.

Initial pH

The initial pH of corn mash prepared with corn slurry from POET was 4.0. This experiment was performed to investigate the effect of increasing the initial corn mash pH to 5.0 on ethanol fermentation with yeast added. Corn mash samples were prepared at slurry pH 4.0 and with pH adjusted to 5.0. The pH of corn mash samples dropped the first 24 h of fermentation (Figure 5.8A); corn mash with initial pH 4.0 and 5.0 decreased to pH 3.6 and 4.0 in 24 h, respectively. The pH increased gradually to pH 3.8 and 4.2 by the end of the experiment (168 h). The lactic and acetic acid contents increased the first 24 to 48 h of fermentation, coinciding with decreasing pH values (Figures 5.8B and 5.8C). Production of lactic/acetic acids and glycerol (Figure 5.8D) was higher in samples at adjusted pH 5.0 than at slurry pH

4.0, which illustrates an active LAB population. Growth of lactic acid bacteria reduces at pH less than 4.0. Ethanol production was affected by the increased levels of organic acids; this was demonstrated by higher ethanol concentrations at the slurry pH 4, indicating higher yeast activity.

Ozonation

Corn mash (adjusted pH 5) prepared with POET corn slurry was ozonated to investigate potential reductions in bacterial activity. Lactic acid production in ozonated samples was somewhat lower than in non-ozonated samples – e.g., 12.0 versus 12.5 g/L in 48 h, respectively – up to 120 h (Figure 5.8B). The acetic acid contents of ozonated samples, however, were higher throughout the fermentation – e.g., 2.6 versus 2.2 g/L in 48 h (Figure 5.8C). The initial acetic acid contents, after ozonation, were 0.3 g/L higher in the ozonated samples, which helps explain this finding. These results suggest that ozonation is not as effective in samples prepared using corn slurry in place of ground corn. Additional dissolved and suspended organic materials contributed from the backset may have consumed most of the ozone or shielded the bacteria. The recycled antimicrobial agents, Lactrol[®] and IsoStab[™], and the antioxidants in hops (IsoStab[™]) in the corn slurry from the backset may have reduced the effects of ozonation compared to non-ozonated controls.

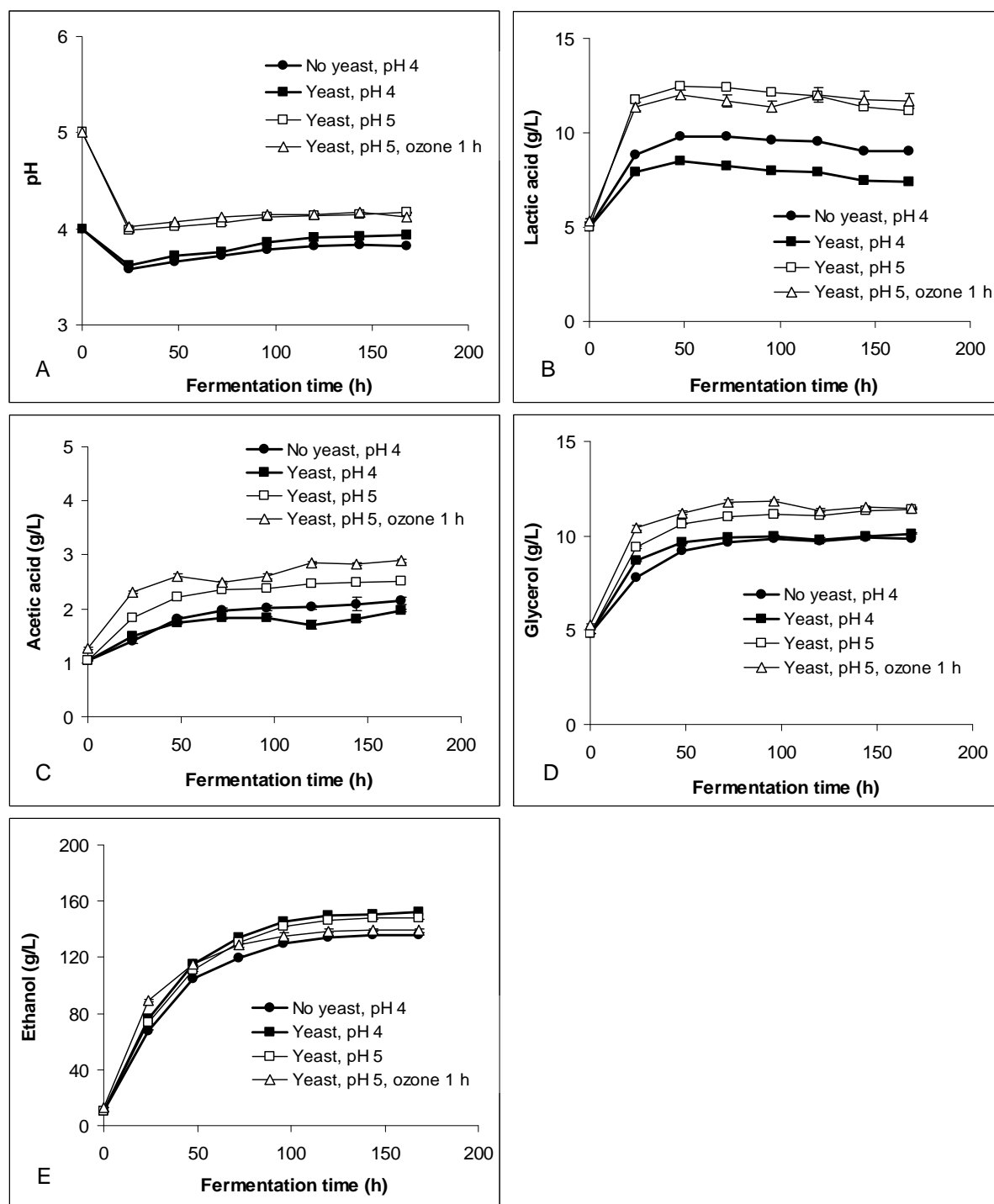


Figure 5.8. Corn mash prepared using POET corn slurry, which contains recycled antimicrobials, Lactrol[®] and IsoStab[™], in the backset from previous fermentations. (A) pH, (B) lactic acid, (C) acetic acid, (D) glycerol, and (E) ethanol contents of daily fermentation samples from non-ozonated/ozonated corn mash samples, with or without yeast added, and initial pH of 4 or 5 (n=3).

Conclusions

Fermentation experiments were conducted with ozone treatment and without the addition of other antimicrobial agents, such as antibiotics. The experiments performed using POET corn slurry, however, contained the recycled antimicrobial agents Lactrol[®] and IsoStab[™]. Based on the findings of this research using POET ground corn and POET corn slurry, the following may be concluded:

Ground corn (Experiment sets 1 & 2)

- Ozonation could be used to lower corn mash pH and, therefore, reduce sulfuric acid requirements to adjust the pH prior to fermentation. Reducing sulfuric acid addition would result in chemical cost savings, as well as less sulfur in the DDGS, which is beneficial for use as livestock feed.
- Lower lactic acid levels were observed in experiments with ground corn; on this basis, ozonation may reduce bacterial contamination, and thus antibiotic dosages, during fuel ethanol fermentations.
- The impact of ozonation on ethanol yields, regardless of lowering lactic acid contents, depends on the ozone dosage and initial fermentation pH.

Corn slurry (Experiment set 3)

- *Yeast addition:* the yeast inoculum size affects the ethanol yields and enables the yeast to be more competitive than the bacteria, as expected.
- *Initial pH:* ethanol production was higher at initial pH 4.0 than pH 5.0, indicating more yeast activity. Thus, without another method of controlling bacteria, a lower pH – less conducive to yeast growth – is required to achieve

higher ethanol yields with longer fermentation times, as observed in the corn ethanol industry.

- *Ozonation*: ozone treatment, as applied to ground corn mash, is not as effective in samples prepared using corn slurry. Dissolved and suspended organic materials contributed from the recycled water sources likely consumed the ozone or shielded the bacteria. In addition, recycled antimicrobials were present in the corn slurry contributed from the backset, which may reduce the impact of ozonation relative to non-ozonated controls.

Additional experiments using POET corn slurry, and the various recycled water streams at a POET ethanol biorefinery, may help clarify the effectiveness of ozonation under fermentation conditions closer to those in plant operation. Higher ozone dosages and only using recycled water sources that do not contain antimicrobials could be investigated.

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CHAPTER 6. GENERAL SUMMARY AND CONCLUSIONS

The objective of the research presented in this thesis was to evaluate two novel approaches – fungal cultivation and ozone treatment – to enhance dry-grind corn ethanol process efficiency, sustainability, and coproducts. Corn ethanol production generates copious amounts of stillage (5–6 gal per gal ethanol), which consists of the leftover protein, fat, fiber, and residual starch and sugar from the corn grain and yeast cells. Most thin stillage is concentrated by flash evaporation, blended with distillers grains, and dried to produce DDGS – requiring substantial energy inputs. Thin stillage is an ideal feedstock for fungal cultivation; it is pasteurized, rich in nutrients (COD up to 100 g/L), and low in pH, favoring fungal growth over bacteria.

The first research project cultivated the food-grade fungus *R. oligosporus* on thin stillage from a local dry-grind corn ethanol plant. Batch experiments in bench-top and small pilot-scale, stirred and airlift bioreactors showed prolific fungal growth under non-sterile conditions within 2–3 d. Suspended solids, COD, glycerol, and organic acids removals, critical for effluent recycle as process water, reached 100, 80, 100, and 100%, respectively, within 5 d of fungal inoculation. Additional airlift experiments on a larger pilot-plant scale is needed to improve oxygen transfer, while reducing the liquid flow issues encountered on the smaller scales used in this research.

Fungal cultivation in place of water evaporation from thin stillage could save substantial energy inputs in corn ethanol production, as well as reduce fresh water requirements and generate fungal biomass. The fungus produced in these studies

contained 2% lysine, 2% methionine (corn contains 0.3% and 0.2%, respectively), and 43% crude protein, enhancing the nutritional value as a poultry and livestock feed. The overall benefits of water and enzyme recycling, process energy reductions, and value-added feed production address the industry goals of improving ethanol production efficiency, sustainability, and coproducts.

Rising concerns about the use of antibiotics and the emergence of antibiotic-resistant bacteria is creating a demand for alternative methods of controlling bacteria during fuel ethanol production. Bacterial competition reduces ethanol yields by diverting glucose to the end-products lactic and acetic acids, which inhibit yeast growth. Contamination by lactic acid bacteria, in particular, is currently controlled by cleaning, addition of antibiotics, and operation at low pH levels. The objective of this research project on the POET BPX™ (no-cook) process was to investigate ozonation as a potential method for minimizing bacterial contamination and fermenting at a higher pH more conducive to yeast growth. Operating at a higher fermentation pH could reduce sulfuric acid requirements and the distiller grains' sulfur content. The coproduct could have an advantage in marketing as antibiotic-free and reduced-sulfur distillers grains.

A laboratory-scale ozonation setup successfully ozonated corn mash prepared from POET ground corn and POET corn slurry with subsequent ethanol fermentation. For experiments conducted using ground corn, corn mash pH after ozonation decreased with increasing ozone dosages/times. Aeration alone did not reduce the corn mash pH, indicating that the ozone treatment was responsible. Ozonation, with and without bacterial spiking, lowered lactic acid levels relative to

the aerated and control samples. Ethanol yields were reduced or improved depending on the initial fermentation pH and ozone dosage applied. Ozonation could, thus, be used to help control bacterial contamination during ethanol fermentations. Ozonation could also reduce sulfuric acid addition required to lower pH prior to fermentation by lowering the mash pH and by enabling operation at a higher pH. Reducing sulfuric acid requirements would result in chemical cost savings, as well as less sulfur in the DDGS, which is important for use as livestock feed.

Preliminary experiments were subsequently performed to assess the effectiveness of similar ozone dosages under conditions closer to plant operation, including the recycled water streams; corn slurry was obtained from a local POET ethanol plant. In these experiments, higher organic acid and lower ethanol contents were observed in corn mash prepared without yeast supplementation. This finding demonstrates that the yeast inoculum size affects the ethanol yields and enables the yeast to be more competitive than the bacteria. Lactic and acetic acid production was higher in control corn mash samples at initial pH 5.0 than at pH 4.0, suggesting more bacterial activity. Ethanol production was higher at initial pH 4.0, indicating more yeast activity. Thus, without a method of controlling bacteria, a lower pH is employed which is less conducive to yeast growth and requires longer fermentation times are required to achieve higher ethanol yields, as observed by the corn ethanol industry. Ozone treatment of corn mash prepared using corn slurry, at the dosage applied to ground corn mash, was not as effective compared to non-ozonated controls. Dissolved and suspended organic materials contributed from the backset

may have consumed most of the ozone or shielded the bacteria. In addition, antimicrobials contributed from the backset in the corn slurry may have reduced the impact of ozonation. Additional experiments using POET corn slurry, and the various recycled water streams at a POET ethanol plant, as well as higher ozone dosages would help clarify the effectiveness of ozonation under fermentation conditions closer to those in plant operation.

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APPENDIX. PRELIMINARY BENCHTOP (1.75 L) STIRRED FUNGAL BIOREACTOR RESULTS

The following findings were taken and condensed from our 2007 American Society of Agricultural and Biological Engineers (ASABE) Meeting Paper entitled “Bioconversion of Thin Stillage from Corn Dry-Grind Ethanol Plants into High-Value Fungal Biomass.”

Abstract

Conventional dry-grind corn ethanol plants generate considerable amounts of low-value thin stillage. Thin stillage is currently concentrated by flash evaporation, an energy-intensive process, to produce syrup, which is blended with distillers dried grains (DDG) to produce DDGS. The condensate is recycled as process water. Thin stillage is generated in pasteurized condition and is rich in organic compounds and other nutrients, with a chemical oxygen demand (COD) of 90 g/L. The low initial pH of 4 and high nutrient levels make it an ideal medium for fungal cultivation. In this research, the fungus *Rhizopus oligosporus* was cultivated on thin stillage obtained from a local dry-grind ethanol plant. Preliminary batch tests in a 1.75-L reactor showed prolific fungal growth under non-sterile conditions and COD removal of up to 86%. Suspended solids decreased from an initial 2 to 3% to as low as 0.03%, with partial removal by attachment to the fungal biomass. The effluent was well-clarified with a light yellow tint. Solids separation is very important, and the effluent from the fungal process could potentially be recycled as process water with minimal further treatment. The fungal biomass is high in lysine (corn protein is low) making it a nutritionally beneficial livestock feed; if fungal cell walls are disrupted, it

can be co-fed with DDG to monogastrics. The ability to feed more of the byproducts to hogs and chickens would help resolve the anticipated corn grain shortfall created by the booming ethanol industry. The fungal biomass could also be used to extract chitin/chitosan.

Introduction

The objective of this research was to conduct preliminary bench-scale experiments to evaluate the efficacy of a fungal system for organic removal and protein-rich biomass production on thin stillage from a dry-grind corn ethanol plant.

Materials and Methods

Substrate

Thin stillage samples were obtained from Lincolnway Energy (Nevada, IA), a dry-grind corn ethanol plant. Samples were collected in sterile 10- and 20-L carboys and were stored at 4°C. The pH of the fresh thin stillage was acidic in the range of 3.8 to 4.5, and the chemical oxygen demand (COD) averaged 90 g/L, of which 50 g/L was soluble. The total and reducing sugar contents were 18 and 3 g/L, respectively. Suspended solids varied from 2.2 to 2.6%, and the mean nitrogen content was 0.57%.

Fungal culture

Freeze-dried culture of the fungus *Rhizopus oligosporus* was obtained from the American Type Culture Collection (ATCC 22959, Rockville, MD, USA). The culture was rehydrated and revived in yeast mold (YM) broth (Difco Laboratories, Sparks, MD, USA) at 25°C. Plates of potato dextrose agar (PDA) (Difco Lab) were inoculated with the revived culture and incubated at room temperature for 5 to 7

days. Fungal spores were harvested from the surface of the plates using sterile deionized water containing 0.1% (w/v) peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). Glycerin (20% v/v) was added to the spore suspension prior to ultra-low freezer storage in sterile 2-ml cryovials at -75°C . The harvested spore count of 2.4×10^6 spores/ml was determined by haemocytometer counts.

Reactor setup and operation

Fungal cultivation on the thin stillage was performed in batch mode in 1.75-L fermentors with 1.25-L working volume (Figure 1).

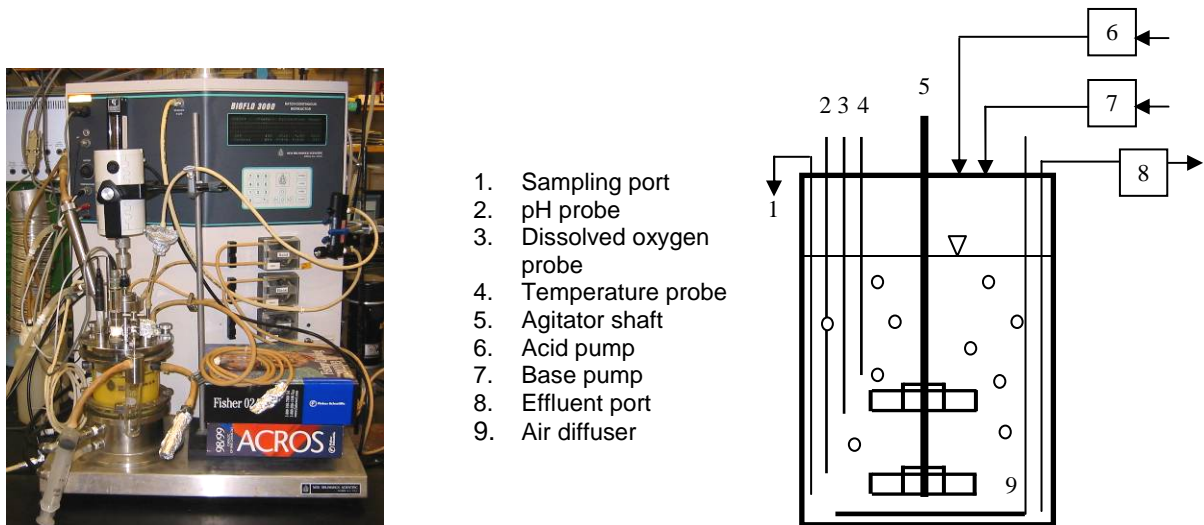


Figure 1. Reactor setup and schematic of a 1.75-L fermentor.

Mycelial inoculation

The reactor was filled with 1.25 L of potato dextrose broth (PDB) (Difco Lab) and sterilized by autoclaving for 40 min at 121°C . The sterile PDB was inoculated with two vials of fungal spore suspension (4.8×10^6 spores) to cultivate mycelia prior

to the addition of thin stillage. Mycelia were grown in PDB for 6 d with the culture conditions of pH 4.0, 37°C, 150 rpm agitation, and 0.8 vvm air flow. The PDB was then drained from the reactor, with the mycelia remaining in the vessel, and 1.25 L of thin stillage was added aseptically using a peristaltic pump. The fermentation proceeded with thin stillage under similar culture conditions. Within four days, fungal mycelia filled the vessel. Most of the fungal biomass was removed from the vessel walls and agitator shaft on day 4; the liquid collected was returned to the vessel for further biodegradation.

Repeat-batch mycelial inoculation

Mycelia from the previous batch experiment were used as inoculum for this experiment. The vessel was filled aseptically with 1.25 L of thin stillage using a peristaltic pump. The fungal cultivation ran for 8 d with the culture conditions of pH 4.0, 37°C, 250 rpm agitation, and 0.8 vvm air flow.

Results and Discussion

Mycelial Inoculation

Thin stillage was added to the 1.75-L reactor containing fungal mycelia grown previously on PDB (Day 0). After 4 d of cultivation, fungal mycelia filled the vessel (Figure 2). Most of the fungal mycelia were removed on day 4, and the liquid was returned to the vessel to convert residual organics into fungal biomass. The biomass was harvested again on day 7 (Figure 3).



Figure 2. Thin stillage in 1.75-L vessel (1.25-L working volume) started from mycelia on day 0 with biomass removed on day 4. Liquid was returned to the reactor for additional bioconversion of the organic materials to fungal biomass.



Figure 3. Fungal biomass harvested from the reactor on day 7. Views of fungal mycelia, from left to right, as the head plate was lifted from the reactor, inside the vessel, and on the agitator shaft.

The initial total and soluble COD of the thin stillage (day 0) were 88 and 50 g/L, respectively (Figure 4a). By day 7, the total COD was reduced by 75% to 22 g/L, and the soluble COD had decreased by 50%. The total and volatile suspended solids were close in value throughout the experiment (Figure 4b). The overall reduction in suspended solids was from 22 to 0.2 g/L in 4 d. The increase in suspended solids on day 4 resulted from solids attached to the fungal biomass becoming re-suspended in the liquid as biomass was removed from the vessel and the liquid returned. The lactic acid and ethanol concentrations decreased from 1.4

and 1.7 g/L on day 0 to 0.6 and 0.0 g/L by day 7, respectively (Figure 4c). No acetic acid was detected in the samples.

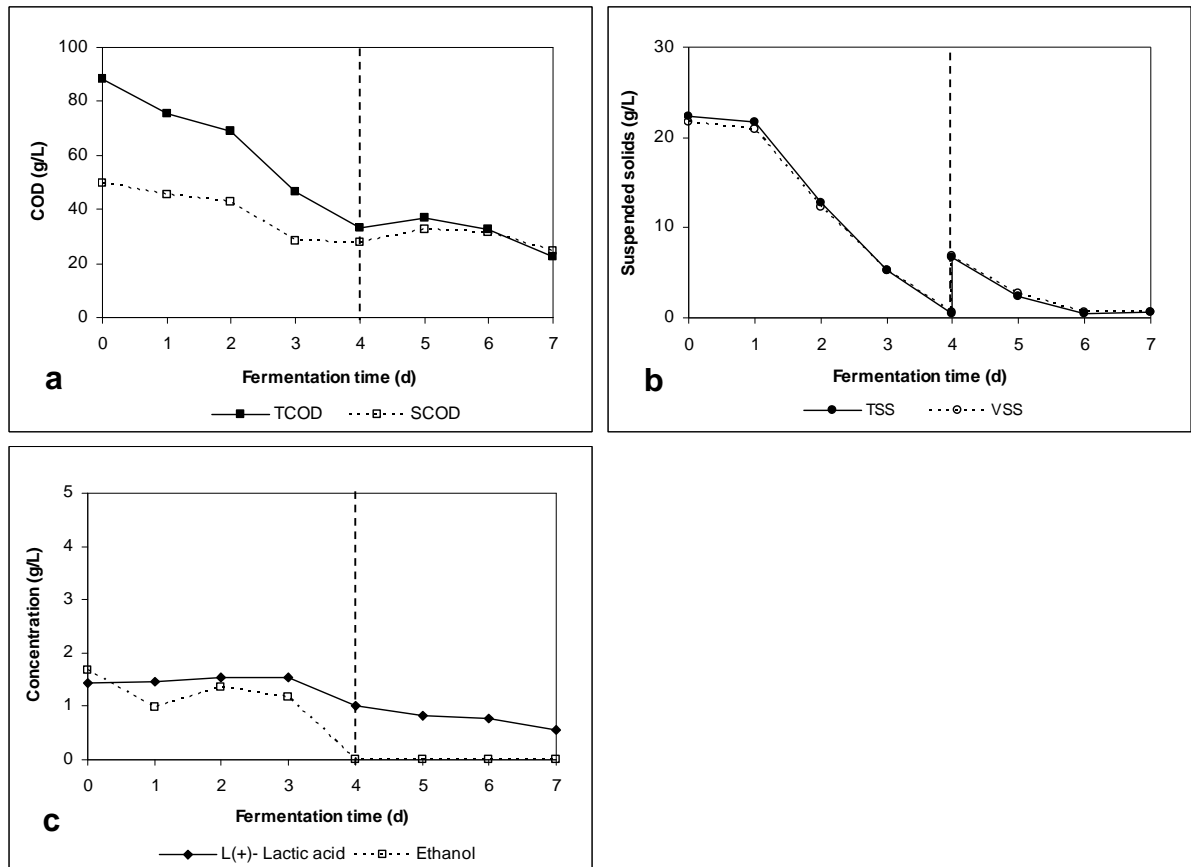


Figure 4. Results for the reduction in (a) total and soluble COD (TCOD, SCOD), (b) total and volatile suspended solids (TSS, VSS), (c) lactic acid and ethanol in samples from the 1.25-L experiment with mycelial inoculum. The dotted line on day 4 indicates the removal of fungal biomass from the vessel, the return of the liquid fraction, and continued fermentation. (n=1)

Repeat-batch mycelial inoculation

Fungal mycelia on the agitator shaft from the previous batch experiment were used as inoculum for this experiment. The vessel was filled with 1.25 L of thin stillage, and the fungus was cultivated for 8 d (Figure 5). Solids from the thin stillage

and fungal mycelia collected initially along the reactor walls, particularly above the liquid level. Mycelia also accumulated on the agitator shaft and between the probes and walls. The liquid became well-clarified with a yellow tint between days 4 and 5 (Figures 5 and 6). Fungal mycelia and sporangiospores were observed under the microscope on days 2 and 3 (Figure 7).

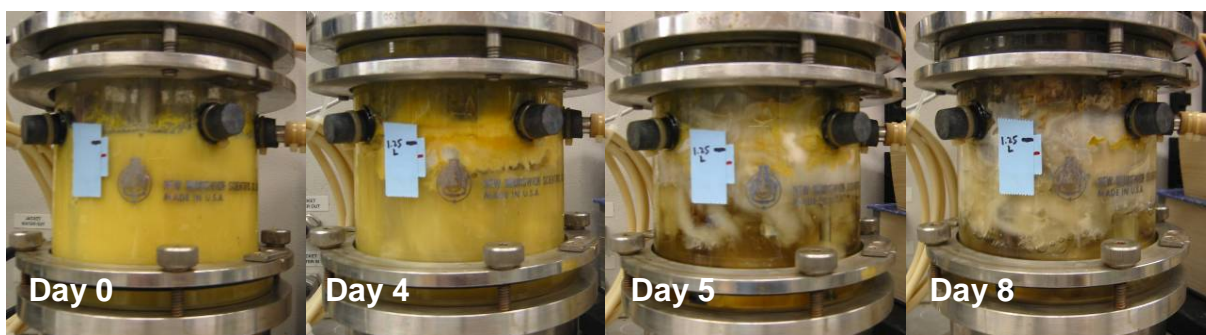


Figure 5. Thin stillage in 1.75-L vessel (1.25-L working volume) inoculated with mycelia from previous batch experiment (day 0) and fermented until day 8.

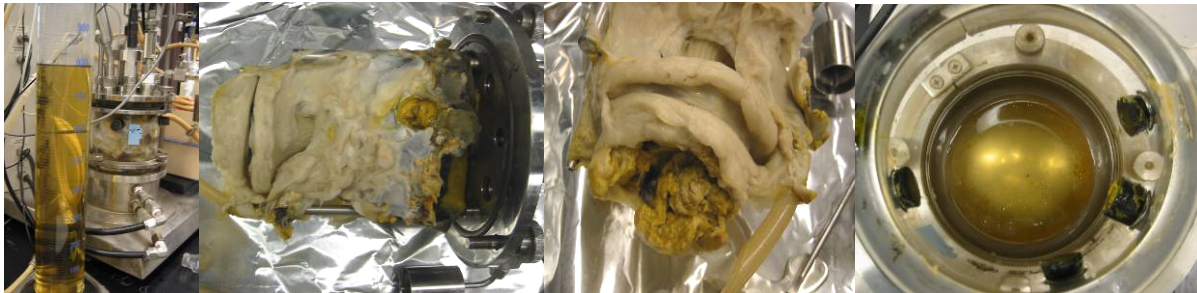


Figure 6. From left to right, thin stillage fermented with *R. oligosporus* and collected through the effluent port; fungal biomass attached to the agitator shaft and reactor head plate; solids within the fungal mycelia; and view inside the vessel after removing the head plate.

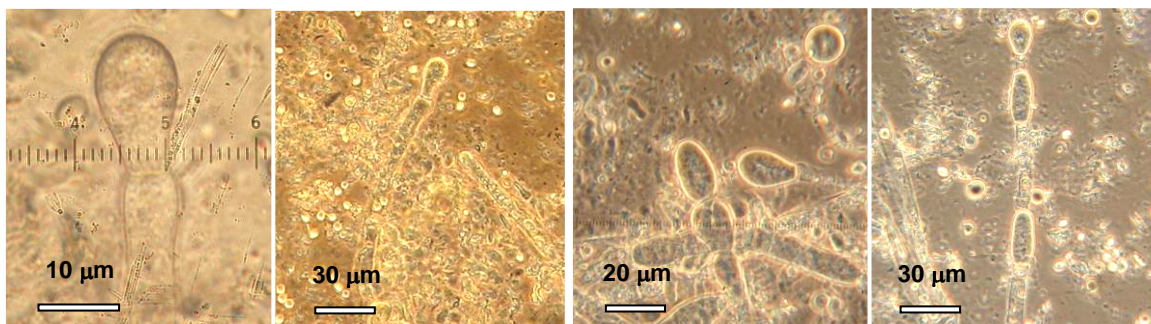


Figure 7. Microscopic observation of reactor samples from day 2 (left) and day 3 (right).

The total and soluble COD of the thin stillage on day 0 were 92 and 49 g/L, respectively (Figure 8a). The reduction in total COD was close to linear with 13 g/L remaining on day 8, a removal of 86%. The SCOD decreased by 76% to 12 g/L by day 8. The volatile and total suspended solids in the feed thin stillage, 26 g/L, were reduced to 3 g/L by day 5 and 0.3 g/L by day 8 (Figure 8b). The lactic acid and ethanol concentrations also decreased, from 1.3 and 1.8 g/L on day 0, respectively, to 0 g/L by day 7 (Figure 8c). No acetic acid was detected in the samples. The total and reducing sugar content of effluent samples (Day 8) were 3.8 and 0.8 g/L, as compared to an initial 18.1 and 3.4 g/L in the feed thin stillage, respectively.

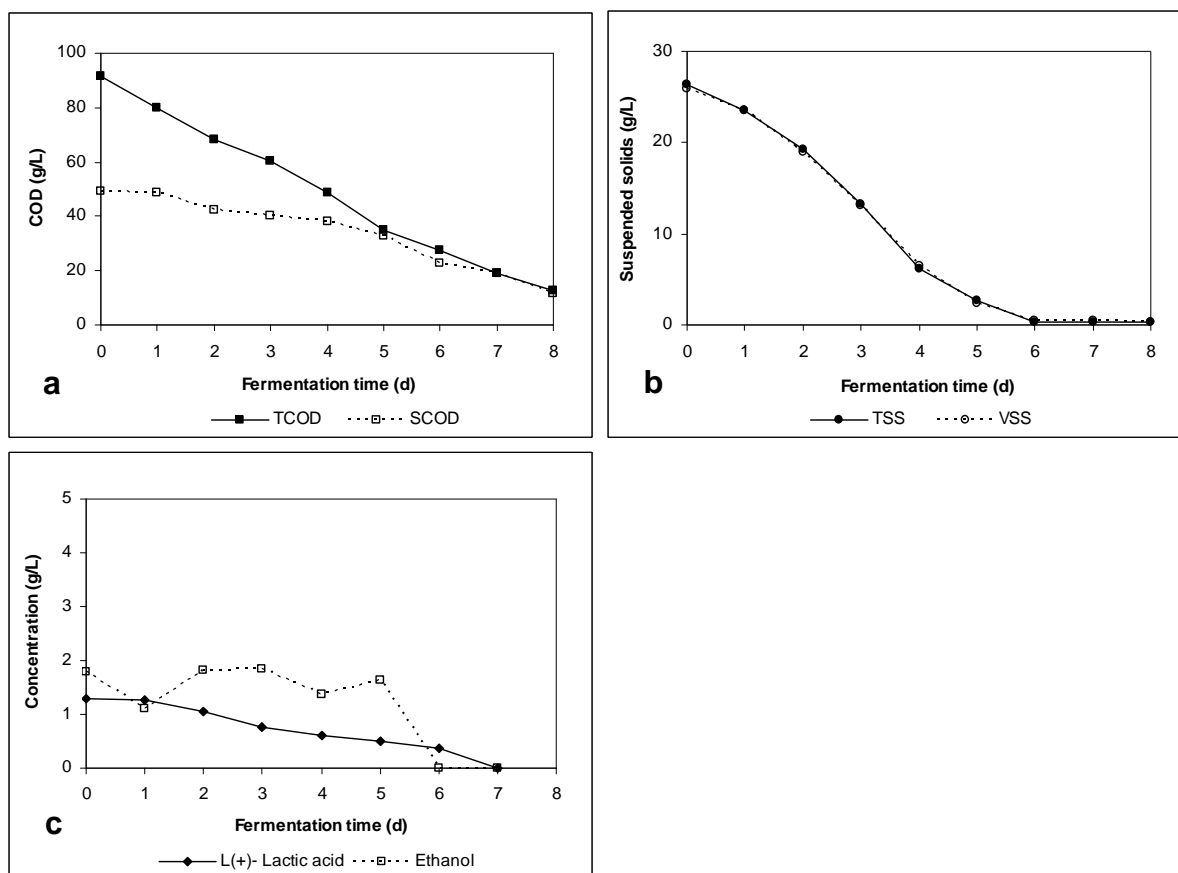


Figure 8. Results for the reduction in (a) total and soluble COD (TCOD, SCOD), (b) total and volatile suspended solids (TSS, VSS), (c) lactic acid and ethanol in samples from the 1.25-L experiment inoculated with mycelia from the previous batch experiment. (n=1)

The typical total and soluble COD of the feed thin stillage for the two batch experiments were about 90 and 50 g/L, respectively (Table 1). The total organic matter (TCOD) decreased up to 79% in 7 d and 86% in 8 d. The higher COD reduction on day 7 by mycelia cultivated on thin stillage as compared to mycelia grown initially on potato dextrose broth (79% as compared to 72%) may be due to acclimation of the fungus to the thin stillage or increased oxygen transfer and mixing at the higher agitation speed.

Table 1. Comparison of thin stillage COD reduction by *R. oligosporus* in two batch experiments.

Inoculum type	Agitation (rpm)	TCOD (g/L) (Day 0)	SCOD (g/L) (Day 0)	COD reduction (%)
Mycelia grown on PDB	150	88	50	72 (Day 7)
Mycelia from previous batch exp.	250	92	49	79 (Day 7) 86 (Day 8)

Conclusions

Fungal growth in the thin stillage was visible within a couple days of mycelial inoculation, and mycelia filled the reactor by day 4. The total organic matter (TCOD) was reduced by as much as 79% in 7 d and up to 86% to 13 g/L in 8 d. The suspended organics in the reactor samples decreased from 2 to 3% initially to as low as 0.03%. Solids from the thin stillage were removed in part through incorporation within the fungal biomass. Liquid collected from the effluent port was well-clarified with a light yellow tint. Solids separation before returning the water to the corn fermentation tanks is very important to avoid build-up of non-degradable substances, and the effluent from the fungal process could potentially be recycled with minimal further treatment. This process could result in substantial energy savings by avoiding the current practice of evaporating and condensing water from thin stillage. The fungal biomass could serve as an important dietary supplement because of the high lysine content and be co-fed with DDG to monogastrics, which may require fungal cell wall disruption prior to feeding. The fungal biomass could also be used as a raw material for the extraction of valuable biochemicals such as chitosan.

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