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Pilot-scale submersed cultivation of *R. microsporus* var. *oligosporus* in thin stillage, a dry-grind corn-to-ethanol co-product

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Pilot-scale submersed cultivation of *R. microsporus* var.
oligosporus in thin stillage, a dry-grind corn-to-ethanol co-
product

by
Daniel Thomas Erickson

A thesis submitted to the graduate faculty in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Major: Biorenewable Resources and Technology

Program of Study Committee:
Hans van Leeuwen, Major Professor
Thomas Harrington
John Robyt

Iowa State University
Ames, Iowa
2012

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Dedication

I would like to dedicate this thesis to my grandmother Verna Erickson for always reminding me that academics are extremely important. Without her guidance I would never have taken my academic adventure. I would also like to dedicate this to the future generations of the world, by helping contribute to renewable energy science, a discipline that will inevitable flourish as humans populations continue to do the same.

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Abbreviations

TS= Thin Stillage

AA = Amino Acid

Lys = lysine

CIP = Clean in Place

DO = Dissolved Oxygen

DDG = Dried Distillers Grain

MIC = Minimum Inhibitory Concentration

VS = Volatile Solid

ppm = parts per million

MGY = Million Gallons per Year

SV = Storage Vessel

DDGS= Dried Distillers Grain w/Solubles

LWE= Lincolnway Energy

R.m.o= *Rhizopus microsporus var. oligosporus*

YM = Yeast Mold

EPA = Eicosapentaenoic Acid

SS = Stainless Steel

CFU = Colony Forming Units

COD = Chemical Oxygen Demand

TCOD = Total Chemical Oxygen Demand

TME= Total Metabolizable Energy



1600L bioreactor and author

Preface

Introduction

For the last century human transportation has been powered by petroleum driven fuels and driven by their refiners. Problems exist with these traditional fuels fueling the worlds transportation industry. Impediments include environmental complications realized with crude extraction Fig. 1, complications with transportation, combustion releases previously sequestered C into the atmosphere Fig. 2, national security issues (70s oil crisis, 9/11), additionally fossil fuels are in finite supply. The 2011 US's domestic production lead to, lead to 90,200 direct jobs, and 311,400 indirect and induced jobs, adding \$42.4 billion to the national Gross Domestic Product [1]. .



Fig 1. 205.8 million gallons of oil spilled into the Gulf of Mexico contaminating 665 miles of coastline [2]

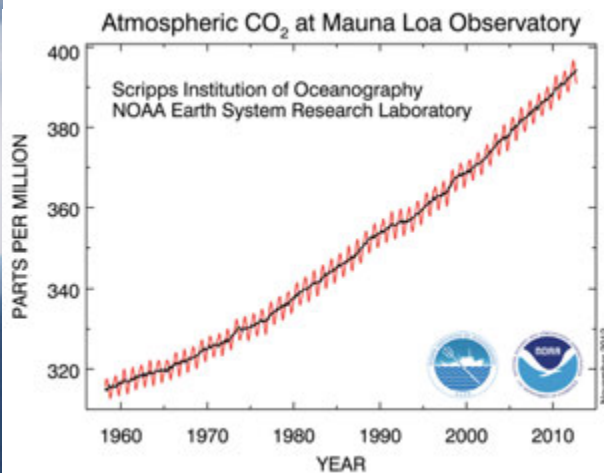


Fig 2. CO₂, the major by-product of combustion, atmospheric concentrations over the last 42 years [3]

This is why biofuels implementation into the transportation sector has seen incredible growth in the 21st century. The principal biofuel produced globally is

ethanol. In 2000 1.65 billion gallons of ethanol were produced in the in the US; in 2011 13.95 billion gallons of ethanol were produced [4]. 80% of this ethanol comes from corn dry-grind ethanol plants fed by corn grown in the Midwestern Corn Belt.

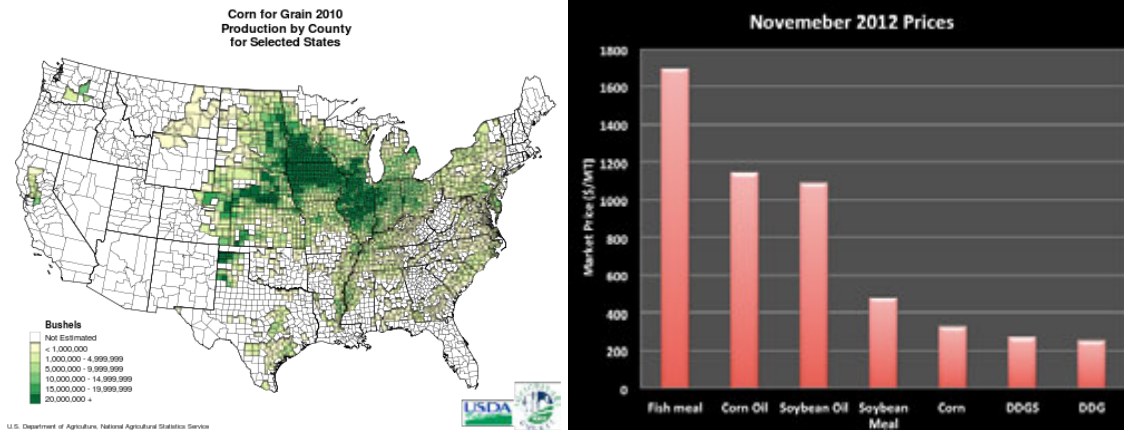


Fig 3. Cornbelt of United States of America [5]. **Fig 4.** Current prices of feed commodities [6]

Volatility in corn prices, energy prices, and fuel prices seem to be constantly threatening the economic viability of the ethanol industry. Thus there is a desire and an opportunity to develop innovative technologies that can increase the economics of industrial practices. One area that has great potential for innovation exists in co-products. The petroleum based fuel industry has done an excellent job developing co-products into profit generating commodities. The ethanol industry needs to

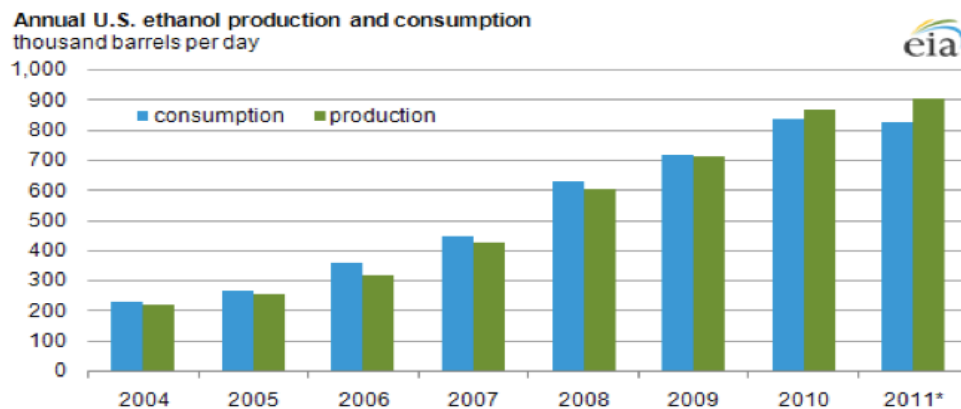


Fig 5. Production and consumption of ethanol in the US [7]

follow suit. Production and consumption of ethanol has risen considerably throughout the 21st century, thanks to the Renewable Fuel Standard. Recently E15 has been approved by EPA and will begin to replace E10; this is coupled with proliferations in production of E85 capable vehicles. So it appears that the ethanol is gaining a strong hold in the market.

The major co-product produced in concert with ethanol is DDGS, which comes from a combination of thick and thin stillage. The thick stillage is processed into distiller's dried grain, and thin stillage is evaporated into syrup and added to the distiller's dried grain to produce dried distiller's grain with solubles, DDGS. The latter is an energy intensive process that adds little value to an already low value co-product. Additionally research questions the nutritional value gained from the addition of syrup to the DDG. It appears that the evaporation of the thin stillage into syrup is more of a disposal method than anything else.

Our research aims to develop a process that not only reduces the amount of energy needed to process thin stillage, but also produce a higher value product from the ethanol production left overs in dry-grind ethanol facilities. This is done by cultivation and collection of *Rhizopus microspores var. oligosporus* biomass produced in thin stillage. To accomplish this a 1500L bioreactor was designed and developed to test the viability of the system.

Facilities

The research was conducted at 3 locations. The 1600L bioreactor was located at BECON, Biomass Energy Conversion Facility, in Nevada IA. The 50L operations, plating, and biomass research was done in 1635 Food Science and Human Nutrition. All genomic and microscopic tasks were completed in Professor Harrington's lab in 223 Bessey Hall.

Thesis Organization

The thesis is organized into 4 chapters, a literature review, the evolution of our process, a research paper, and an independent study. The literature review outlines research pertaining to thin stillage, *Rhizopus microsporus* var. *oligosporus* characteristics, and characteristics of related and contaminating organisms. The thin stillage section outlines current practices, potential usages, and chemical composition associated with thin stillage. The *Rhizopus microsporus* var. *oligosporus* section outline anthropocentric history, nutrient composition, antimicrobial properties, and enzyme production. The final section explores contaminating organisms that were found while operating the pilot scale bioreactor, as well as organisms related to *R.m.o.* that have shown industrial promise.

The evolution of our process chapter presents methodologies, operating procedures, and their justifications. It also presents information obtained that should be considered for industrial applications. Chapter 3 expresses what we learned and results of our pilot-scale operation. Chapter 4 is dedicated to the

microbial contaminants that we found molesting our process including identification and quantification.

References

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CHAPTER 1. LITERATURE REVIEW
Literature Review of Thin Stillage, *Rhizopus microsporus* var. *oligosporus*, and Co-product Fermentation

1. Thin Stillage

Thin Stillage, TS, is a co-product of dry-grind corn to ethanol production. TS generation and modification in a dry-grind facility is outlined in Fig. 1. After the corn has been processed to convert starches to sugars, resulting sugars are populated with yeasts and fungal fermentation converts sugars into an ethanol containing beer. The beer is sent to distillation columns for ethanol recovery. The bottom product is called whole stillage. The whole stillage is centrifuged and separated into 2 streams thick stillage and thin stillage. The thick stillage contains most of the

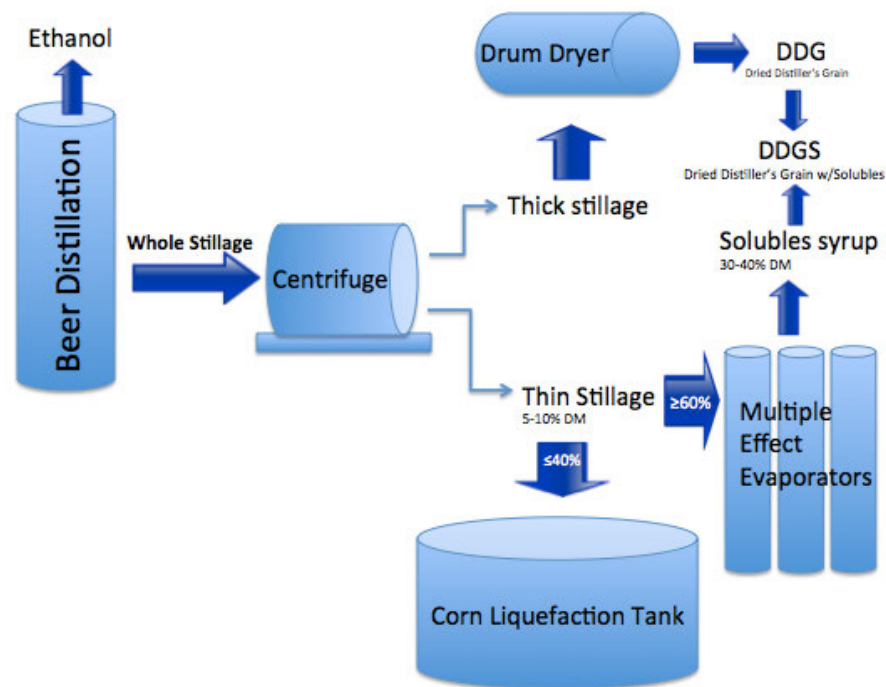


Fig. 1.1: Ethanol process after fermentation showing current co-product recovery

suspended solid fraction of the whole stillage, while the thin stillage contains most of the dissolved solids, with a total solids content of 6-9% solids. Table 1.1 shows characteristics and nutrient contents of TS. Thick stillage is dried in drum driers to distiller's dried grain, DDG. Traditionally TS is partly recycled to the front of the system as a backset for liquefaction of ground corn, and the majority of the TS is condensed by evaporation into condensed solubles known as "syrup". There is 2.5-3gal of TS produced/gal of ethanol produced in dry-grind plants.

Table 1.1. The composition of thin stillage from maize (Dusanka et al. 2009).

Parameter	Literature data	Used thin stillage
Dry matter, %	7.7	6.5-8.8
Residue starch, w/v	0.1	0.07-0.20
Total sugar, w/v	0.26	0.19-0.31
Reducing sugar, w/v	0.11	0.05-0.17
Total phosphates, mg/L	128.65	129.41-140.27
Total acids, g/100 mL	0.334	0.252-0.487
Total amino acids, g/100 mL	0.076	0.051-0.096

Recycling TS

Recycling has benefits based in waster reclamation and on residual carbohydrates, amino acids, and yeast cell degradations products, some of which can be utilized by yeast cells for ethanol production. As the amount of recirculated TS increases, bioethanol yield and starch utilization efficiencies increase (Dusanka et al. 2009). However, the amount of non-degradable residual solids in the fermentation tank also accumulates with increased recirculation, which eventually decreases yields. Another factor inhibiting yeast fermentation is the buildup of lactic and acetic acid in the fermentation tanks. These acids are predominately produced

by contaminating bacteria during the fermentation process (Skinner & Leathers 2004). These acids will inhibit optimal bioethanol production by the yeast and thus turn out costly for industrial operation. This limits TS recycling as a backset in the liquefaction of ground corn (Sankaran 2010).

Evaporation of TS

During evaporation TS is condensed in multiple effect evaporators into syrup with 30-40% dry matter. Temperature in the evaporators ranges from 210°F in first evaporator to 180° F in final evaporator (LWE control room 2012). Once condensed the syrup is added to DDG between two stages of drying to form Distillers Dried Grain with Solubles (DDGS) , while the evaporated water is condensed and returned to the front of process (Kim et al. 2008). It is debatable how beneficial the addition of the syrup is to the nutrient value available in DDG. DDGS have low and variable amino acid (AA) digestibility in animals (Stein & Shurson 2009) and is sold at low margin as an animal feed (Moreau et al. 2011). Click here for [current prices](#). AA digestibility was found to be higher in DDG when compared to DDGS thus it is hypothesized that the soluble portion added via the TS derived syrup is to blame (Pahm et al. 2008). This is due to severe and variable heating, increased fiber concentration, decreased levels of AA, and others measures associated with processing of DDG to DDGS (Stein & Shurson 2009). The severe heat discussed here must be the 210°F temperature experience in the initial evaporator.

Syrup balls form during DDGS creation and have been shown to decrease lysine available to chicks (Pahm et al. 2009). However more recent research shows that it is heating opposed to syrup balls that is responsible for the decreased AA digestibility (Soares et al. 2012). In addition to the problems with evaporation are heating temperature, GHG emissions, equipment costs, and energy costs. Typically in dry-grind corn-to-ethanol plants the production of one gallon of ethanol requires 37.98MJ of thermal energy and 1.09 kW energy (Shapouri et al. 2003). Of this 35% (13.3MJ) of the electricity and 30% (.33kW) of the natural gas/coal derived thermal energy are consumed by processing stillage into co-products/ gal ethanol(Meredith 2003).

Oil extraction from TS

Oil can be obtained from the “back end” of a dry grind ethanol plant using a proprietary process developed by GreenShift Corporation. The process uses centrifugation/heating/condensation of the thin stillage for extraction. This oil is rich in lutein and zeaxanthin (valuable for feed applications), which are partially oxidized β -carotene. GreenShift (<http://www.greenshift.com/>) claims 6 gal oil can be reclaimed for every 100 gal ethanol produced. Many ethanol plants currently have this technology implemented into their processes.

As more plants choose to extract the oil from TS, two tiers of DDGS have developed. DDGS are traded based on protein+fat=35%, and brokers are now requiring a more detailed breakdown of exact percentages of the two nutrients. Low

fat DDGS are considered a second tier product that trades for \$10-25 less/ton than DDGS form with oil left in TS. Low fat DDGS are more suitable for monogastrics than ruminants as a protein substitute. When using low fat DDGS more soybean meal can be replaced in the rations. The reduction in soybean meal can be made up with larger percentages of corn and DDGS. Amino acid profiles must be reanalyzed, but overall cost of feed should be reduced. (John Augspurger, Gavilon Agriculture and Energy Commodities 2012). So it appears that a strong market for DDG with out the solubles would be available.

Research Utilizing TS

R.m.o. has been successfully cultivated on thin stillage from corn-ethanol plants under aseptic conditions (Rasmussen et al., unpublished). The fungal biomass cultivated on the settled thin stillage supernatant had a relatively high protein content of 43% (dry weight) with various essential amino acids, including 1.8% lysine, 1.8% methionine, 1.5% threonine, and 0.3% tryptophan. Additionally a significant reduction in organic matter (up to 46%) and organic acids (71% lactic acid and 100% acetic acid) was achieved within 5 days of cultivation.

A mucoraceae family member of *R. microsporus*, *Mucor circinelloides*, has demonstrated a robust ability to growth on TS as well. In fact, as this oleaginous fungus adsorbs and assimilates the TS's oils and nutrients it produces 20g/L of high-protein fungal biomass with a lipid content of 46% oil in 48 h (Mitra et al. 2012). Also determined was that the fatty acid composition was influenced by the nature of

the growth media. Table 1.2 compares the fungal biomass grown on TS to DDGS and Table 1.3 shows variable fatty acid content when fungus is grown on different media.

Table 1.2 Proximate analysis showing the chemical characteristics of *M. circinelloides* biomass grown on thin stillage (TS) for 48 h in a 6-L airlift bioreactor, harvested by screening and oven-dried at 80 °C for 24 h, as compared to the properties of DDGS. Data are means \pm SD, n = 3 (Mitra et al. 2012).

Chemical component (%) (dry basis)	<i>M. circinelloides</i> biomass	DDGS ^a
Moisture	2.1 \pm 0.3	NA
Ash	4.0 \pm 0.5	4.6
Total carbohydrates	23.1 \pm 1.3	44
Starch	0.8 \pm 0.1	5.1
Crude fiber	19.6 \pm 1.5	10.2
Soluble sugars	2.2 \pm 0.2	NA
Total fat	39.4 \pm 2.1	11.9
Total protein	30.4 \pm 2.5	31.3

^a Belyea et al. (2004); NA: Data not provided in referred article (Belyea et al., 2004).

Table 1.3 Total lipid fatty acid composition of oil extracted from fungal cells grown on YM broth (Mucor-YM_{oil}) and thin stillage (Mucor-TSoil), as well as oil from thin stillage alone (TSoil). Compositional analysis was done by GC with prior transesterification to FAME's. Data are means \pm SD, n = 2 (Mitra et al. 2012).

Lipid class	Total fatty acid composition (wt.%)		
	Mucor-YM _{oil}	Mucor-TSoil	TSoil
C 13:0	12.5 \pm 1.4	NA	NA
C 14:0	2.3 \pm 0.1	NA	NA
C15:0	0.6 \pm 0.0	NA	NA
C16:0	15.8 \pm 0.4	15.7 \pm 1.0	15.0 \pm 0.2
C16:1	6.3 \pm 0.0	NA	NA
C18:0	4.0 \pm 0.2	2.3 \pm 0.1	2.2 \pm 0.0
C18:1	24.4 \pm 0.7	29.6 \pm 0.5	28.7 \pm 0.2
C18:2	15.7 \pm 0.2	50.0 \pm 1.6	52.5 \pm 0.2
C18:3	17.5 \pm 0.1	1.4 \pm 0.1	NA
C20:0	0.6 \pm 0.0	1.2 \pm 0.1	1.5 \pm 0.1
C22:0	0.4 \pm 0.0	NA	0.1 \pm 0.1

There has been interest in utilizing TS as a feedstock for *Clostridium pasteurianum* DSM 525 to produce biobutanol. In a preliminary study, TS was supplanted with glycerol for a carbon source, while maintaining a pH between 5-7. Results reveal that 6.2-7.2g butanol/L TS can be achieved from TS containing supplemented 20g/L glycerol (Ahn et al. 2011). Best results, 7.2g/L were obtained from TS with an initial pH of 5.8, and a growth time of 48h.

Another study analyzed the oomycete *Pythium irregulare*, and its ability to produce EPA while utilizing TS as a growth substrate. The greatest EPA production came from TS diluted to 40%; when glycerol was added to the media the fungus yielded ~16% increase in total fatty acids, but not more EPA (Liang et al. 2011). Run time on these experiments was 9 days. Table 1.4 displays the nutrient content in TS before and after run:

Table 1.4. Characterization of raw thin stillage and *P. irregulare* culture medium (Liang et al. 2011)

Characteristics	Thin stillage	Spent medium
Total solids	6.53±0.19	1.06±0.08
Total COD, g/L	112.0 ± 2.8	4.0 ±2.5
pH	4.5 ± 0.2	6.8 ± 0.4
Total carbohydrates, g/L as glucose	20.5 ± 0.2	2.5 ± 0.3
Reducing sugar, g/L as glucose	2.1 ± 0.1	0.23 ± 0.04
Glycerol, g/L	17.5 ± 0.8	0.02 ± 0.01
Total Kjeldahl nitrogen (TKN), g/L	2.5 ± 0.1	0.12 ± 0.06
Nitrate, mg/L as N	44.0 ± 0.6	8.3 ± 1.8
Ammonia, mg/L as N	35.0 ± 3.0	7.5 ± 3.2
Total phosphorus, g/L as P	1.6 ± 0.1	0.1 ± 0.03
Ortho-phosphate, g/L as P	0.99 ±0.10	0.30 ± 0.06
Lactic acid, g/L	2.23 ± 0.03	Not detected
Acetic acid, g/L	0.65 ± 0.04	Not detected
C/N ratio	5-7	-

Anaerobic digestion, AD, has been a popular topic of research on TS. The interest stems from bioconversions of co-products to useful derivatives while minimizing energy expenditures. The high organic matter and nutrient content of corn-ethanol thin stillage make AD a favorable choice over evaporator condensate. Thin stillage has high total chemical oxygen demand (TCOD), 122 g/L, and total volatile fatty acids (TVFAs) of 12 g/L. A maximum methane yield of 0.33 L CH₄/g

COD added (STP) was achieved in the two-stage process (Nasr et al. 2012).

Biogas production of up to 0.6 m³ biogas/kg volatile solids (VS) added has been reported (Schaefer & Sung, 2008). However optimal anaerobic digestion requires chemical modification and associated equipment, as well as long retention times to treat the TS (up to 65 days in batch). During AD, phosphorus is not removed in large quantities and presents potential environmental issues when present in animal feeds. Treatment with lime significantly lowers the P in centrifuged TS. The digestate from treated TS has been shown to be suitable for recycling as process water and eliminates energy intensive evaporation, yields a usable renewable energy resource, and off-sets water needs (Alkan-Ozkaynak & Karthikeyan 2011).

The problem

Since fuel ethanol is an energy source designed to replace fossil fuel (CAST, 2006), it is counter intuitive to use large amounts of fossil fuels to form bioethanol. Reducing the amount of natural gas or coal needed to treat TS would have significant effects on the amount of fossil fuels needed for the process. Overall DDGS are a low value animal feed and the condensed syrup is debatably beneficial to the precursor DDG. Considering processing of TS is particularly energy intensive while only marginally contributing to a low-value co-product; it appears to be a prime place for process improvement. Additionally there is an imminent concern considering ethanol production and water usage. Water reclamation is a pressing desire of the industry. Fungal biomass has proven to be an excellent biosorbent (Ozsoy et. al

2008) especially by removing lipids from water lipid emulsions (Srinivasan and Viraraghavan 2010). Koza et al. 2013 found that *R.microsporus* can reduce the COD from 100g/L to 60g/L in 24-40h. This will hopefully lead to increased water efficiencies in the industry. For these reasons, research has been aimed at identify potential alternative to evaporating the TS.



Picture 1.1. Concentrate tank for TS recycle



Picture 1.2. A single evaporator

2. *Rhizopus microsporus* var. *oligosporus* Characteristics

R.m.o. is a filamentous fungus that belongs to the phylum Zygomycota and is in the Mucoraceae family. First referenced in 1815, tempeh is fungal fermented food originating in Indonesia, which has long been used medicinally to increase gastrointestinal health of humans. It is most often prepared by the fermentation of soybeans by *R.m.o.* Interestingly *R.m.o.* cultures have been passed down in Indonesian culture as a heirloom to produce the protein rich tempeh. For preparation soybeans (or legume of choice) are soaked, dehulled, and partially

cooked. Then an acid, typically acetic acid, is added to create an advantageous environment for *R.m.o.* Next the treated beans are inoculated with *R.m.o.* and left for 24-36hr at ~30C. The fungal hyphae bind the soybeans together creating a vegetarian loaf. Sporulation is common on ripe tempeh. Currently Tempe is used worldwide to prepare vegetarian dishes.

Antimicrobial properties

Recently studies have been conducted monitoring GI health in animals when given *R. microsporius* biomass. One such study, analyzed the effect of Tempe on enterotoxigenic *Escherichia coli* (ETEC) adhesion to swine intestinal brush border cells. Fig 1.2 outlines adhesion of different strains of ETEC to the intestinal brush boarder cells with and without tempe supplementation. As you can see the results

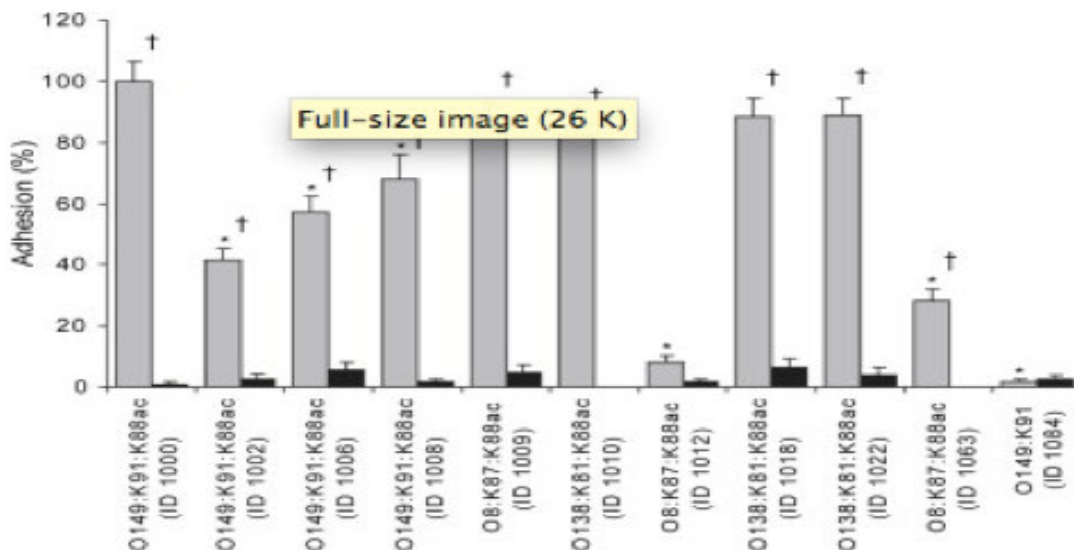


Fig 1.2. Adhesion of different ETEC strains to piglet brush border cells. Gray bars represent adhesion without addition and black bars with addition of tempe extract (2.5 g l⁻¹). Bars represent mean values, expressed as % adhesion compared to the positive control (strain ID 1000) of 12 individual brush borders. Error bars represent SEM. Bars with asterisk (*) differ significantly from strain ID 1000. Bars with † represent a significant effect of addition of tempe extract (Roubos-van den Hil 2010).

are extremely significant. Of all tempe's examined cowpea tempe had the greatest reduction in adhesion (Roubos-van den Hil 2010). Another study examined ETEC-challenged weaned piglets, and concluded that piglets fed soybean fermented by *R.m.o.* increased feed efficiency by 3%, feed intake by 13% and average daily gain by 18% (Kiers et al. 2003). This provides strong evidence that *R.m.o.* can provide health benefits to livestock animals.

Hyphal growth

Many important industrial processes utilize filamentous fungi as biofactories to produce proteins, enzymes, and/or biomass itself (Paul & Thomas 1996). Filamentous fungi chiefly grow by extending their hyphae, which are chains of elongated cells that expand at the apex of the tip cells. The cell walls are extracellular matrices primarily composed of a mixture of polysaccharides and manno-proteins that shelter the cell and resists turgor pressures (Sietsma & Wessels 2007). Arguably the most popular model for hyphal tip growth, the steady state model, begins by exocytosis of wall forming enzymes such as chitin synthases and glucanases. Then fibers of the cell wall, ex. chitin glucan chains, are synthesized at the apex. In the apex, these fibers are not yet cross-linked and the wall is still flexible. As the tip expands, sub-apical chitin crystallizes and becomes covalently bound to β -1,3-glucans, which solidifies the cell wall in the older parts of the growing hypha. It is widely thought that the hyphal cytoplasm exerts turgor pressure on the new wall, which facilitates the expansion of the flexible apex during hyphal tip growth. The Spitzenkörper is cellular component rich in F-actin and

formins and supports tip growth of fungal hyphae (Sharpless & Haris 2002). Its F-actins are essential for fungal secretions and endocytosis while formins facilitate binding F-actins together into chains. Hyphal elongation is supported by an impressive rate of intracellular transport of vesicles to the tip, through actin cytoskeleton, making filamentous fungi one of the fastest-growing cells, with rates up to 20 $\mu\text{m}/\text{min}$ (Carlile et al. 2001). Filamentous fungi contain 4 classes of myosin's, ATP-dependent motor proteins, of which Myosin-1 and Myosin-5 have essential function in hyphal growth. Fig. 1.3 shows an in-depth look at the hyphal expansion on the cellular level. Not demonstrated in this diagram is another mechanism that regulates tip growth and that is Ca^{2+} . IP_3 , inositol (1,4,5)-trisphosphate, activates Ca^{2+} channels from a subset of tiplocalized Ca^{2+} - containing vesicles that release Ca^{2+} into apical cytoplasm. The Ca^{2+} is required for fusion of wall vesicles with plasma membrane to cause tip expansion.

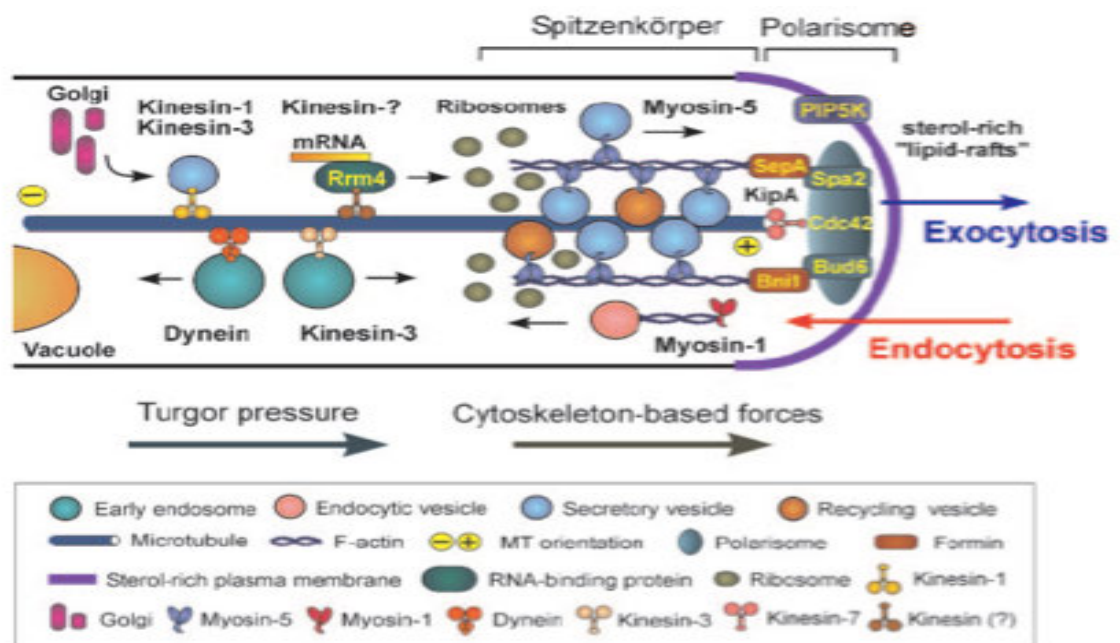


Fig. 1.3. Molecular mechanisms and machinery for hyphal growth in fungi (Steinberg 2007)

Septation, the formation of septa, which separate cells, varies greatly among fungi. In *Aspergillus nidulans*, the *myoB* gene produces myoB strings that amalgamate into contractile rings which form septa (Taheri-Talesh et. al 2012). In general Zygomycota are coenocytic, lack speta, but complete crosswalls can form to isolate old or damaged regions, or the multi-nucleated gametangia from somatic hyphae. Branching also occurs as the hyphae extend. Once biomass begins to elongate and branch it will form different morphologies based on growth conditions and density. Fig. 1.4 shows different biomass morphologies of filamentous fungi.

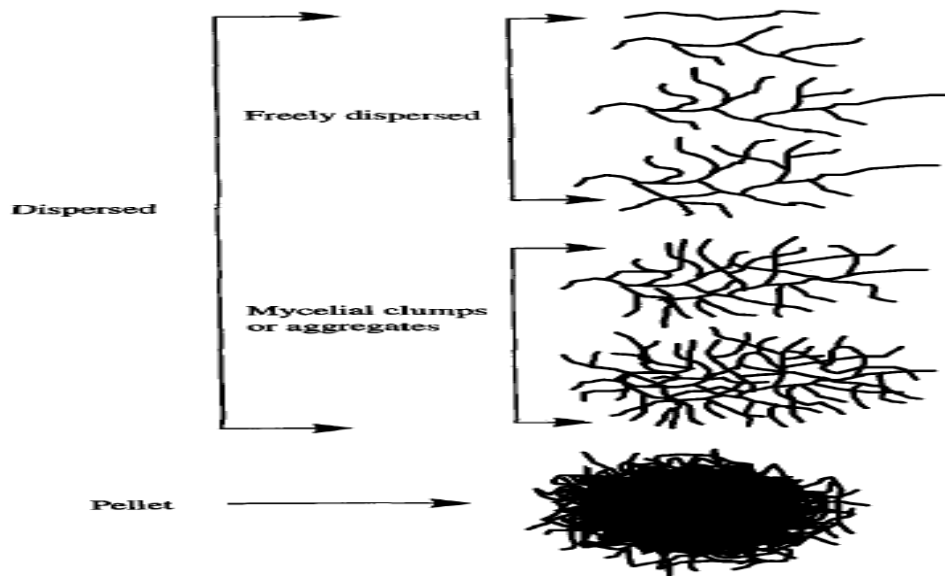


Fig 1.4. Various morphologies that occur in filamentous fungi (Paul & Thomas 1996).

Chitosan

Chitosan is a linear polysaccharide with β -(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) as the monomers which are linked at random. Its precursor, chitin, is found in the, exoskeletons of

crustaceans, insects, and mollusks, as well as cell walls of fungi. Chitosan oligosaccharides (COS) are preferred over larger polymers because the shorter chain lengths allow it to be soluble in water and free amino groups in D-glucosamine units (Jeon & Kim 2000). Currently COS primary production is done via chemical hydrolysis of shrimp and crab shells; which has a number of drawbacks including formation of toxic compounds, high risk of environmental pollution, and low production yields (Kim & Rajapakse 2005). Fungal chitin has begun to gain some market interest primarily due to the following factors: extraction of co-occurring biopolymers, no seasonal factors, and reductions in time and cost needed to perform the extractions (Cardoso et al. 2012). Research is being done utilizing a number of zygomycetes grown on industrial waste streams to produce and extract the chitin. One example of this is the usage of potato chip processing waste to feed *Rhizopus oryzae*. Figure 1.5 shows the results of the study (Kleekayai & Suntornsuk 2011).

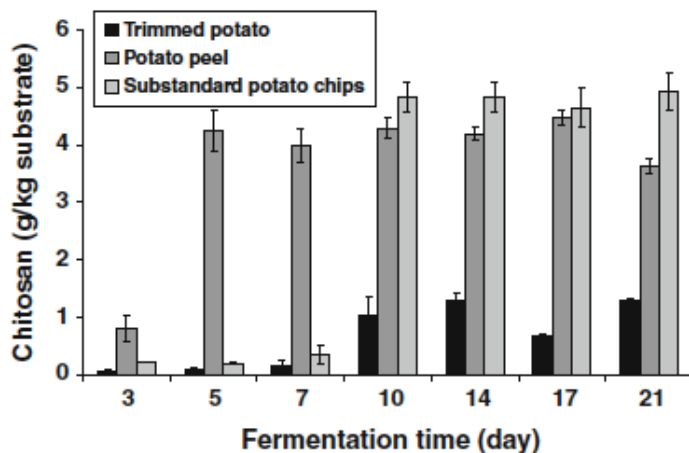


Fig. 1.5. Chitosan yields from *Rhizopus* on different potato chip production waste products.

Another research project utilized a mixture of corn steep liquor and honey to produce a media to grow *Rhizopus arrhizus* with the intent of extracting its chitin. A

maximum of 29.3mg chitin/g dried biomass was obtained while harvesting 11.71 g dried biomass/L of media (Cardoso et al. 2012). As research on family members of *R.m.o* suggests, there is promise for chitosan production from *Rhizopus spp.* Current non-fungal industrial methods of chitosan production have a multitude of problems. This is yet another promising market for *Rhizopus spp.* biomass.

Mycotoxins & Endosymbionts

Filamentous fungi have been known to cause food spoilage and produce toxic metabolites. *Fusarium verticillioides* and *Aspergillus flavus* were found to produce mycotoxins on corn (Cleveland et al. 2003). In corn silage 3 mycotoxins, citrinin, deoxynivalenol, and gliotoxin were found being produced in-vitro, including Gliotoxin at over 800ppb (Richard et al. 2007). *Rhizopus spp.* were thought to produce 2 different types of mycotoxins, rhizoxins and rhizonins. The fact is these two toxins were erroneously termed 'the first mycotoxins from Zygomycota' because it was discovered that endofungal bacteria residing in *R. microsporus* cytosol were responsible for the production of the toxic metabolites. The antimitotic polyketide macrolide rhizoxin (*Burkholderia rhizoxinica*) and the hepatotoxic cyclopeptide rhizonin (*Burkholderia endofungorum*) are toxins produced by the aforementioned bacteria. In fact, the toxicogenic *Burkholderia-Rhizopus* symbiosis was the first example of fungal bacteria symbiosis where both the fungal and bacterial partners can be cultured independently, and is believed to have evolved from a parasitism to a mutualism relationship (Lackner et al. 2009). The importance of this lies in selecting strains that are of free of *Burkholderia spp.* and not known for

endosymbiosis with the toxic bacteria. In one study after the bacteria were induced into the fungi, it was grown for 2-3 days at which point they found the toxins. In our case of a batch process where the fungi is grown for ~24h this would likely not be a problem, but *Burkholderia spp.* were detected as endosymbionts of *R. microsporus* strains. *Burkholderia spp.* can be detected with confocal laser scanning microscopy of the mycelium while the toxins can be detected by HPLC-DAD/MS (Rohm et al. 2010).

3. Enzymes

Filamentous fungi in general are famous for the diverse genetic catalogs of enzymes. This allows them to utilize a wide array of energy resources including nutrients that are not readily assimilable by genetically simpler bacteria and yeast. *R.m.o* accomplishes this primarily with the following enzymes; peptidases (protein degradation), lipases (lipid degradation), hydrolases (carbohydrate degradation), and phytase (organic to inorganic phosphorus conversion). *Rhizopus spp.* in particular, appears to be extremely well adapted to convert molecules in TS into fungal biomass. *Rhizopus spp.* have been used to produce industrial enzymes.

Peptidases

Peptidases cleave peptide bonds to liberates smaller peptides. One peptidase that has been purified and characterized from *R. microsporus* is a tripeptidyl peptidase(TPP). It cleaves tripeptides sequentially from the N terminal of the

polypeptide (Tomkinson 1999). The Rhizopus TTP characterized by Lin et al 2011, is a 136.5 kDa dimer composed of identical subunits and may play important roles in intercellular protein turnover antigen presentation, peptide hormone production, amongst other applications.

Lipases

Lipases represent a group of enzymes that have the ability to hydrolyze triglycerides. This is most commonly achieved by acting on a specific site on the glycerol backbone. *Rhizopus spp.* have been reported to produce both intracellular and extracellular lipases. Extracellular enzymes in general have benefits of simpler less expensive separation from media. Lipases are versatile enzymes that have various applications in the following industries; pharmaceutical, food, biofuel, cosmetic, detergent, leather, textile, and paper. The smallest monomeric extracellular lipase from fungal origin was isolated and purified from *Rhizopus oryzae* strain JK-1. This lipase shows remarkable thermostability when compared with other *Rhizopus spp.* lipases, and is stable in methanol and ethanol (Kantak & Prabhune 2012). This makes the enzyme a prime candidate for transesterification, an imperative step in the conversion of oil into biodiesel.

Pichia pastoris has commercial interest for its heterogeneous protein production while utilizing menthol as its sole carbon source. To increase growth and productions rates, a gene that produces lipase in *R. oryzae*, ROL, has been implemented in recombinant *P. pastoris*. Recombinant strains in combination with

co-feeding sorbitol or glycerol helps increase growth rates on methanol (Ramón et al. 2007). Mixed substrates are good strategy to increase protein production in the recombinant *P. pastoris*, however proteolytic activity was seen when glycerol was used as a co-feed substrate. (Arnau et al. 2011). The use of co-feeding has benefited other fungi used for protein production. Exploration of co-feeding sources of nutrients limiting growth, potentially could enhance production in our systems. If dry-grind ethanol plants begin to produce biodiesel from corn oils extracted from TS, glycerol would be readily available on site. Since glycerol is a major co-product of biodiesel production our system could be killing two birds with one stone, if quantity and/or quality is enhance by glycerol

Glycoside Hydrolases

Glycoside hydrolases are a group of enzymes that catalyze the breakdown of more complex sugars into simpler sugars by hydrolyzing glycosidic linkages.

Rhizopus oryzae has a large polygalacturonase gene family with 15 of 18 genes encoding unique active enzymes to break down D-galacuronic acid, a sugar acid (Mertens & Bowman 2011). *R.m.o* itself has been shown to produce glucoamylase. Fig 1.6 shows glucoamylase activity at different temperatures when grown for 24 h on starch processing wastewater. As you can see that the activity enzyme activity and biomass yield is highest at 37C, the same temperature that *R.m.o* grows efficiently in TS (Erickson et al unpublished). *R.m.o* was found to have a high conversion efficiency of the starch in the wastewater due to the aforementioned enzyme activity.

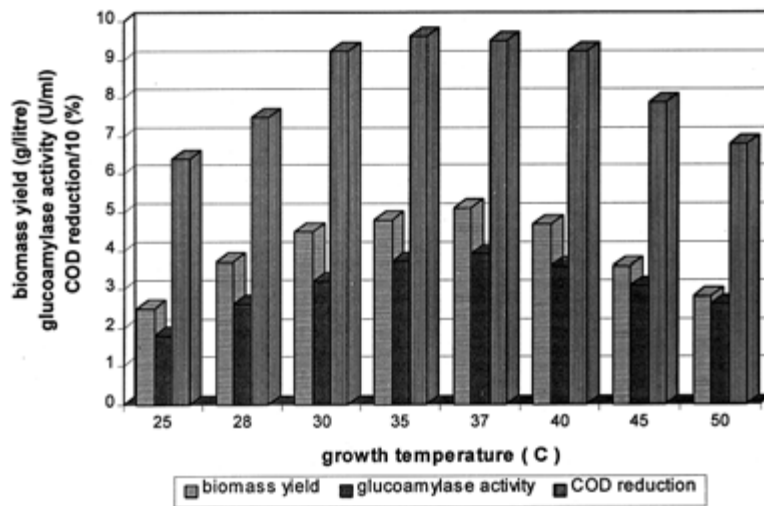


Fig. 1.6. Comparison of biomass yield, glucoamylase activity, and COD reduction of *R.m.o* grown on starch processing wastewater (Jin et al. 1999).

Phytase

Phytase is a type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid. Phytic acid is found in oil seeds and grains, in an indigestible form of organic phosphate. The phytase-catalyzed reaction yields an inorganic form a phosphorous that can be digested. This is especially important in the swine industry where phytase is often added to the hogs diets to improve adsorption. Phytase production was measured by Bogar et al. 2003, in 12 filamentous fungi on 3 different types of growth media, *Rhizopus spp.* results are displayed in Table 1.5. All of the experiments were done via solid-state fermentation, SSF, and the data clearly indicates that SSF is the superior approach to phytase production when compared to submersed fermentation. The most crucial factor affecting phytase production is the quality of C and N sources. No studies appear to have been made on phytase activity present in TS before, after, or during fermentation with *R.m.o.*

Table 1.5. Phytase production of 8 *Rhizopus* strains on 3 different media (Bogar et al. 2003)

	phytase activity (IU/g DM)		
	canola meal	coconut oil cake	wheat bran
<i>Mucor racemosus</i> NRRL 1994	4.7	14.5	7.3
<i>Mucor hiemalis</i> NRRL 13009	0.1	11.5	0.8
<i>Rhizopus microsporus</i> NRRL 3671	1.3	0.4	0.5
<i>Rhizopus oligosporus</i> NRRL 5905	5.4	2.6	0.4
<i>Rhizopus oryzae</i> NRRL 1891	4.3	5.3	0.4
<i>Rhizopus oryzae</i> NRRL 3562	3.0	5.5	0.4
<i>Rhizopus thailandensis</i> NRRL 6400	1.5	2.7	0
<i>Aspergillus ficuum</i> NRRL 3135	5.2	7.8	11.8

^a Fermentation conditions: 10 g of air-dried substrate in 500-mL cotton-plugged Erlenmeyer flask was supplemented with 23 mL of salt solution containing (g/L) NH₄NO₃ 5, MgSO₄·7H₂O 1, NaCl 1. Moisture level: 71%. Inoculum: 1 mL of spore suspension of the respective fungus. Incubation temperature: 25 ± 1 °C. Fermentation time: 3 days.

This research could be especially significant to the swine industry if Mycomeal could reduce amount of exogenous phytase needed. Considering the difference between SSF and SF it would be interesting to explore phytase production via SSF using thick stillage. Bottom line *R.m.o* produces phytase, and phytase could be valuable in animal feed commodities, especially the swine industry.

Phenolic Antioxidant activity

An antioxidant is a molecule that inhibits the transfer of electrons or hydrogen from a substance to an oxidizing agent. Antioxidants are significant because oxidation reactions can form free radicals that can damage cells. Antioxidants are commonly phenolic compounds. Solid-state fermentation on whole soybean utilizing *R.m.o*. was used to analyze the role of fungal glycoside hydrolases on phenolic antioxidant mobilization. The results revealed that carbohydrate-conjugated phenolics can be biochemically rearranged and mobilized by fungal glucosidases, and potentially by lignocellulolytic enzymes (McCue & Shetty 2003).

Additionally, fungal β -glucuronidase is thought to be involved in the biotransformation of soybean water-insoluble polymeric phenolics (lignin) into more water-soluble phenolics. Use Fig 1.7 to help understand the relationships.

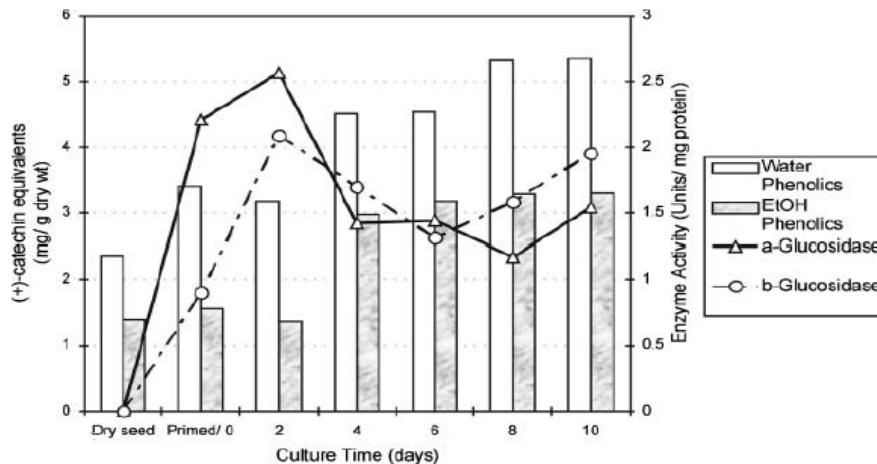


Fig. 1.7. *Rhizopus microsporus* var. *oligosporus* affect on phenolic compounds

R.m.o has also been grown on mung beans via SSF. Mung beans are known for their hypoglycemic properties and *Helicobacter pylori* inhibition. Results confirmed that SSF with *R.m.o*. can be an efficient strategy to improve the phenolic content of mung beans with associated enhancement of health-linked functionality (Randhir & Shetty 2007). Thus co-feeding a substrate containing polymeric phenols in conjunction with TS could have beneficial effects on ulceration caused by *H.pylori*.

Isoflavones

Isoflavones are organic compounds produced primarily by the Fabaceae family of plants. Isoflavones are able to act as phytoestrogens in mammals, and some are coined antioxidants. Among isoflavones, aglycones are particularly significant to humans because they are readily available for absorption. *R.m.o*.

increase the availability of aglycones by hydrolyzing isoflavone glycosides (daidzein, genistein, and glycitein) with its β -glycosidase enzymes.

	Aglycones isoflavone concentration mg/100g dry basis	
Cotyledons	0.0±0.0	6.83±0.51
10 min cooking	2.28±0.11	8.84±0.28
Soaking	3.57±0.21bB	12.27±1.38aC
30 min cooking	2.46±0.16	8.87±0.19
6 hour fermentation	3.76±5.1	12.43±0.80aB
12 hour fermentation	5.19±0.05bAB	24.19±3.61B
18 hour fermentation	9.29±1.29bAB	30.75±1.89aAB
24 hour fermentation	11.52±0.81bAB	34.78±1.97AA
6 hour refrigeration	12.71±0.55bA	35.35±2.38aA
12 hour refrigeration	11.98±1.39BA	36.32±2.28aA
18 hour refrigeration	13.16±0.38bA	36.77±2.90aA
24 hour refrigeration	12.70±0.34bA	35.44±1.48aA

Fig 1.8. Compares 2 different soybean cultivars column 1 and 2 and *R.m.o* affect on Aglycone isoflavones during Tempe process steps (Ferreira et al. 2011)

Enzyme Summary

If present in significant concentrations, harvesting enzymes produced by *R.m.o* in TS could generate profits. They could be extracted and sold directly or employed to in-house processes. Table 1.6 shows a compilation of studies that measured cellulase enzyme production on various feedstock by assorted organisms. Once again the results suggest much higher concentrations of enzymes are found in SSF than in SF experiments. Animal producers commonly provide the animals with enzymes to aid digestion efficiencies e.g. phytase. As implementation of new technologies are solidified in the biofuel industry, it is possible that economic optimization of production of certain enzymes could further increase the economic

Table 1.6. Cellulase production by assorted microbes on different substrates (Chandel et al. 2012).

Microorganism	Raw material used as carbon source	Cultivation type	Enzyme titers (U/ml) or (U/g substrate)	Reference
<i>Aspergillus oryzae</i> MTCC 1846	Saccharum spontaneum	SmF	FPase (Filter Paperase), 0.85 ± 0.07; CMCase (carboxy methyl cellulase), 1.25 ± 0.04; Xylanase, 55.56 ± 0.52	Chandel et al., 2009
<i>Bacillus subtilis</i>	Banana waste	SSF	9.6 IU/g	Tsao et al., 2000
<i>A. niger</i> NRRL3	Wheat bran	SSF	Cellobiase 215 IU/g	Weber and Agblevor, 2005
<i>Neurospora crassa</i>	Wheat straw	SmF	19.7 U/ml	Romero et al., 1999
<i>Penicillium decumbans</i>	Wheat straw	SSF	FPase 23 IU/ml	Yang et al., 2004
<i>P. janthinellum</i> NCIM 1171	Sugarcane bagasse	SmF	FPase, 0.55; CMCase, 21.58 BG, 0.58; Xylanase, 28.1 IU/ml	Adsul et al., 2004
<i>A. fumigates</i>	Wheat bran, Sugarcane bagasse	SmF/SSF	CMCase 365 U/L, FPase, 47 U/g	Grigorevski-Lima et al., 2009
<i>T. reesei</i> NRRL11460	Sugarcane bagasse	SSF	154.58 U/g	Singhanla et al., 2006
<i>Bacillus spp.</i>	Organic compost	SmF	1.333 mg glucose released ml ⁻¹ min ⁻¹	Mayende et al., 2006
<i>Humicola sp.</i> (Th10).	Paddy straw and Soybean trash	SSF	FPase, 11.43, CMCase, 15.38 and Cellobiase 90.2	Kumar et al., 2008a
<i>Fusarium chlamydosporum</i>	Sugarcane bagasse	SSF	Fpase, 95.2 IU/g; CMCase, 281.8 IU/g, Cellobiase, 182.4 IU/g, Beta-glucohydrolase, 135.2 IU/g	Qin et al., 2010
<i>P. citrinum</i>	Rice bran	SSF	Endoglucanase, 2.04 ± 0.13 FPase, 0.64 ± 0.16 IU/ml	Dutta et al., 2008
<i>Talaromyces emersonii</i>	Carob powder	SmF	Endocellulase 206 Barley beta-glucohydrolase 06.1 Beta-glucohydrolase 50.5	Gillera et al., 2010
<i>A. terreus</i> AV49	Groundnut shell	SmF	CMCase 1.147 IU/ml, FPase 0.175 U/ML	Vyas et al., 2005
<i>T. reesei</i>	Wheat bran	SmF	FPase 0.33 U/ml, CMCase 0.43 U/ml	Gomes et al., 2006

viability of *R.m.o* treating TS. The *R.m.o* system analyzed is a submersed fermentation, which may limit its ability to produce enzymes when compared to SSF. However extracellular enzymes are particularly useful because they are easier to obtain. Microwaving proved to be a good way to lyse cells. Thus if intracellular enzymes are desired, lysing of cells followed by centrifugation could be a useful way to extract intracellular enzymes from biomass. Overall enzyme production and collection are a secondary concern, as our system is based on the proteinaceous biomass, but is definitely worth exploring.

4. Fungi and co-product bioconversion

Fungi have been a popular choice in treating waste streams in various industries. The following is a collation of a few examples of this work

Rapeseed meal

Rapeseed meal, 1 of 2 major co-products in rapeseed biodiesel conversion, is low-value material that could serve as fungal growth substrate. *Aspergillus oryzae*, a fungus used in sake fermentation, has demonstrated the ability to increase free amino nitrogen, and inorganic phosphate with extracellular hydrolytic enzymes (Wang et al. 2010). This is significant because co-bioconversion of the second major co-product, crude glycerol, and the hydrolyzed rapeseed meal can add value to each product. This is an example of a fungus being used to improve the biofuel industry.

Sugarcane bagasse

Sugarcane bagasse is a cellulosic by-product of sugar cane production that is a prime candidate for second-generation cellulosic biofuel production. However high costs of enzymatic hydrolysis limits its economic viability, so a search for new cost effective bioprocesses producing cellulolytic enzymes has strong potential (Singhvi et al 2011). One such example is the use of *Aspergillus niger* to produce cellulases. Cellulases encompass endoglucanase, exoglucanase/cellobiohydrolase, and β -glucosidase and their synergistic actions that breakdown cellulosic material. The study found *A. niger* A12 to have the greatest endonuclease production potential. This was optimized when a 24h SSF was followed by a 48h submerged fermentation, which leads to a 3-fold increase in endoglucanase productivity when compared to submerged fermentation only. Germination of *A. niger* via SSF encouraged development of the filamentous form, which is superior for cell-to-

substrate interaction (Cunha et al. 2012). This presents the idea of combining SSF with SF to produce a more efficient process. This strategy may become more important to continue towards industrialization.

Sugarcane-to-ethanol vinasse

Sugarcane-to-ethanol and corn-to-ethanol plants have a similar problem; lack of value-added products coming from production by-products. For sugarcane-to-ethanol up to 13 gal of vinasse are generated for each gallon of ethanol produced. Vinasse, like thin stillage has a high chemical oxygen demand, 100-130 g/L COD, which presents a problem for disposal. Vinasse differs in that it lacks the protein content available in TS and has a higher COD/L. Fig 1.8 compares *R.m.o* biomass grown on vinasse with AA profiles of current aquaculture feed ingredients (Nitayavardhana 2010). The researchers concluded that integration of fungal treatment could improve profitability of biofuel industries. Starch processing wastewater, which is a co-product of commercial production of starch and gluten from corn and wheat, was tested as a growth media for the cultivation of *R.m.o* and *A. oryzae*. *A. oryzae* was able to grow faster and produce 10% more biomass than *R. oligosporus*, however the *R. oligosporus* biomass contains greater metabolizable energy, higher protein % (49.7 to 45.7), and higher % of essential amino acids (most importantly lysine and methionine). Additionally essentially all suspended solids were removed, 95% of COD and BOD were removed as well as 75% of N and P (Jin et al 2002).

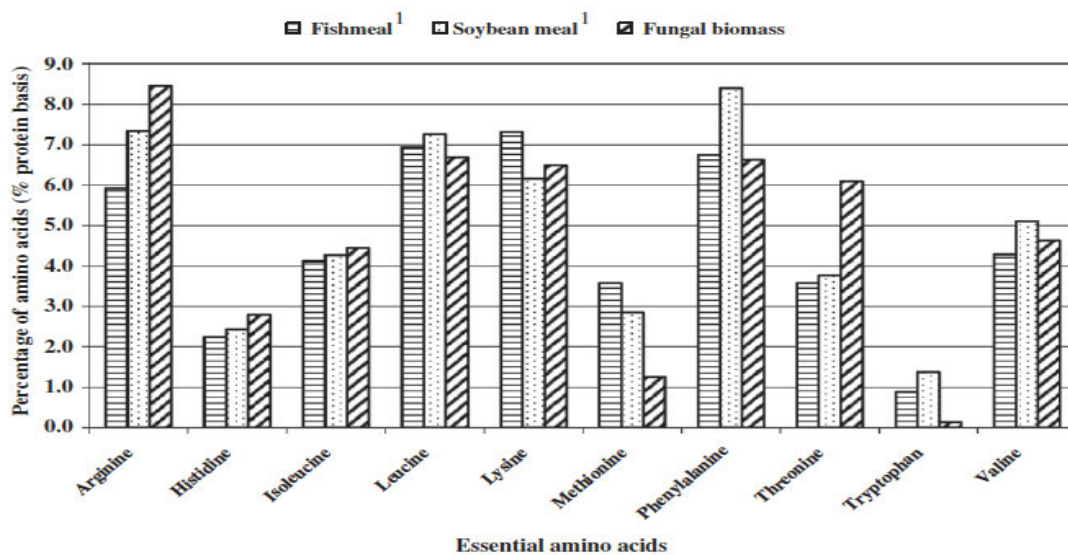


Fig. 1.8. Comparison of AA profiles of fishmeal and soybean meal to R.m.o biomass (Nitayavardhana 2010).

Spent sulfite liquor

Spent sulfite liquor, SSL, is a by-product of sulfite pulp mills. SSL is the result of delignification of wood chips in an aqueous solution of acid bisulfites in an excess of SO₂. Results express that SSL at a 50% dilution can be used successfully as a growth media for *Rhizopus spp.* with an optimal temperature of 32C. . Growth curves show vigorous growth in first 28 hrs and then stabilizes after 35hrs; with a maximum biomass yield of 7.7g/L (Ferreira et al 2012). These growth curves mimic those seen in the bioreactor work of the work of Erickson et al. 2013.

Eicosapentaenoic acid from co-products

Eicosapentaenoic acid, EPA, is an important omega-3 polyunsaturated fatty acid that has been linked to many health-promoting properties. Fungal biomass

containing EPA has solid potential as a supplement for fishmeal, an aquaculture food that we feel Mycomeal has a chance to compete with. Many studies have used *Pythium irregulare* utilized to produce EPA on agricultural products including crude soybean oil and soybean meal waste (Cheng et al. 1999), crude glycerol from biodiesel production (Athalye et al. 2009), and rendered animal proteins (Liang et al. 2011). These growth medias require further nutrient supplementation and/or dilution whereas using TS process, no supplementation is needed (Rasmussen et al. 2009, Koza et al., 2013 and Erickson et al., 2013).

5. Bioreactor Contaminates

Typically monocultures of a microbe of choice are desired in bioreactors. Corn-to-ethanol fermentation bioreactors can become contaminated lactic acid producing bacteria. Contaminates often lead to undesired results so monitoring of contaminants is necessary

Identified contaminants

Erickson et al. 2013 found four different organisms contaminating bioreactors designed to grow *R.m.o* monocultures. Two were gram-positive bacteria, *Bacillus cereus* and *Bacillus megaterium* while 2 were yeasts, *Rhodotorula mucilaginosa* and *Pichia kudriavzevii*.

B. cereus is a gram-positive, spore-forming, motile, aerobic rod that is a facultative anaerobe, and may cause two different types of food poisoning: the diarrhoeal type and the emetic type (Granum & Lund 1997). The emetic

(intoxication) is due cereulide, a 45-kDa cyclic heat-stable dodecadepsi-peptide, and diarrheal cases are caused by two toxins, a three-component heat-labile enterotoxins hemolysin and the nonhemolytic enterotoxin (NHE) (Labbé & Rahmati 2011). *B. megaterium* is an aerobic, motile, gram-positive rod that forms endospores. Endospores differ from spores in that it is not an offspring, but rather a dehydrated form of itself. It is considered non-pathogenic and is considered a saprophyte. Saprophytes are chemoheterotrophic and obtain nutrients via extra-cellular digestion of dead or decaying organic matter. *B. megaterium* often grow in chains linked to one and other by cell wall polysaccharides.

P. kudriavzevii, a yeast, has potential for bioethanol fermentation and phytase production (Chan et al. 2012). Specific strains have proven useful in the simultaneous saccharification and fermentation of rice straw (Oberoi et al. 2012) and corn stalks (Kaur et al. 2012). A toxin produced by *P. kudriavzevii* RY55 exhibited excellent antibacterial activity against several pathogens of human health significance such as *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* (Bajaj et al. 2012). *R. mucilaginosa*, a basidiomycota, a yeast that has distinctive orange/red/pink pigmented colonies and doesn't ferment sugars. *Rhodotorula spp.* are one of main carotenoid-forming microorganisms with predominant synthesis of β -carotene, torulene and torularhodin (Davoli & Buzzini, 2004).

Bacillus and mycolytic enzymes

Bacillus spp. in general are known to produce a number of mycolytic enzymes

including chitinase, chitosanase, β -1,3-glucanase, lipase, and protease, with β -1,3-glucanase and chitinase being the most significant (Helisto 2001). *B. megaterium* has been studied for its mycolytic enzymes. Its chitinase is the product of amplification of chitinase (*chiA*) gene which is 270bp. Table 1.7 compares quantities of 3 mycolytic enzymes, chitinase, β -1,3-glucanase, and protease, produced by select *Bacillus spp.* being used as a biocontrol for *Rhizoctonia solani*, basidiomycota (Solanki 2012). This strain of *B. megaterium* produces the lowest

Table 1.7. Activity of *Bacillus spp.* hydrolytic enzymes on cell wall substrate of *R. solani* (Solanki 2012)

Treatment (s)	Chitinase (μmol of GlcNAc $\text{min}^{-1} \text{mg}^{-1}$ protein)	β -1,3-glucanase (nmol of glucose $\text{min}^{-1} \text{mg}^{-1}$ protein)	Protease ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein)
<i>B. megaterium</i> MB3	14.07 \pm 1.29	5.85 \pm 0.01b	8.40 \pm 0.20
<i>B. subtilis</i> MB14	32.50 \pm 0.74	11.95 \pm 1.05	15.88 \pm 0.89
<i>B. subtilis</i> MB99	22.59 \pm 0.66	11.05 \pm 0.04	11.00 \pm 1.00
<i>B. amyloliquefaciens</i> MB101	34.09 \pm 1.07	18.16 \pm 1.05	19.84 \pm 0.19

Mean \pm SE value in the same column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to DMRT. For each experiment, values represent the means of three independent experiments

amount of chitinase, glucanase, and protease among *Bacillus spp.* examined. However in bioreactor of Erickson et al. 2013 it has shown powerful deleterious effects on fungal biomass. Taking the biomass from 7 g/L to 0 g/L of harvestable biomass within 24 h.

Another study further looked at *B. subtilis* strains and their mycolytic activities via extracellular hydrolases. Studied strains demonstrated low correlation between chitinase production and the ability of the bacteria to degrade the cell walls of *Bipolaris sorokiniana*, an ascomycete. Characterization of enzyme profiles in the studied strains revealed that β -1,3-glucanase was a more significant factor than chitinase in determining the mycolytic potential of bacteria (Aktuganov et al. 2008). This was measured on their ability to utilize the mycelium of the fungi as a growth substrate.

Aktuganov and friends also analyzed the extracellular hydrolases of *Bacillus sp.*

739 and their involvement in lysis of fungal cell walls. The key finding in this study is that only β -1,3-glucanases were able to degrade the cell walls of native fungal mycelium in the absence of other hydrolases (Aktuganov 2007). Also included were these light microscopy photos showing chronological decay.

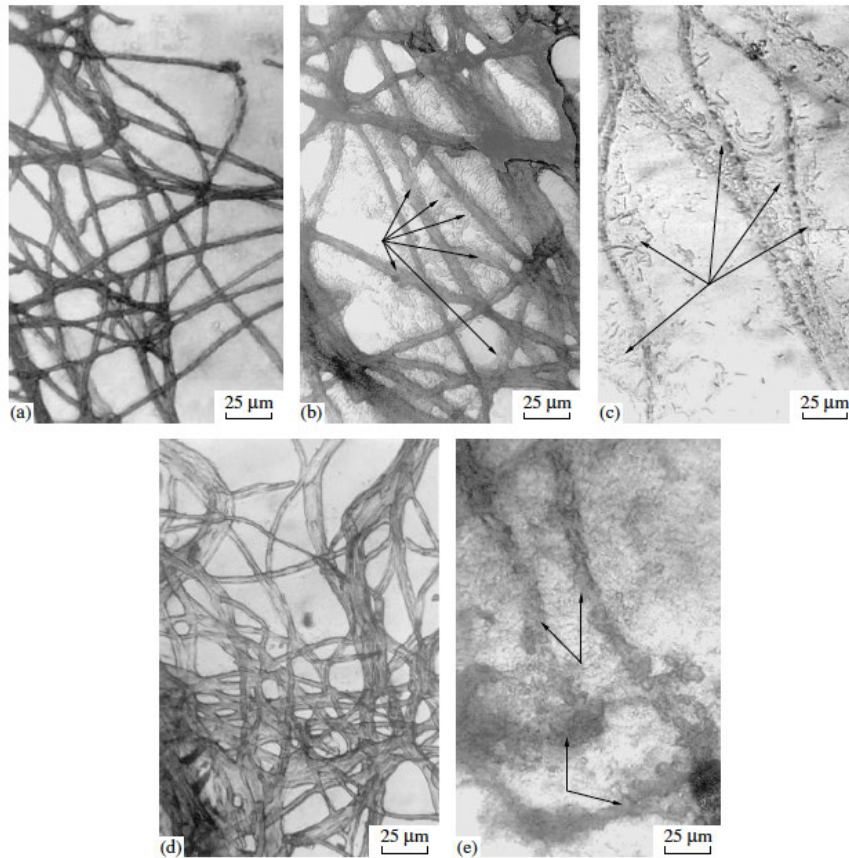


Fig 1.9. Chronological degradation of *Bipolaris sorokiniana* by *Bacillus* sp. strain 739 on potato medium a) 48 h control, b) local cell wall degradation, c) sites of bacterial mass reproduction (96 h), d) 144 h control, and e) hyphal residue after 144h

The effects of *Bacillus spp.* mycolytic enzymes on a number of Basidiomycete spp. have been analyzed. The filamentous fungi studied contain enzymes that allow them to degrade lignin, which has implications in the lumber industry. The *Bacillus spp.* were tested as potential biocontrols for these basidiomycetes. The following pictures show spheroplast, cells with cell wall almost completely removed by *B. subtilis* mycolytic

enzymes (Melent'ev 2006).

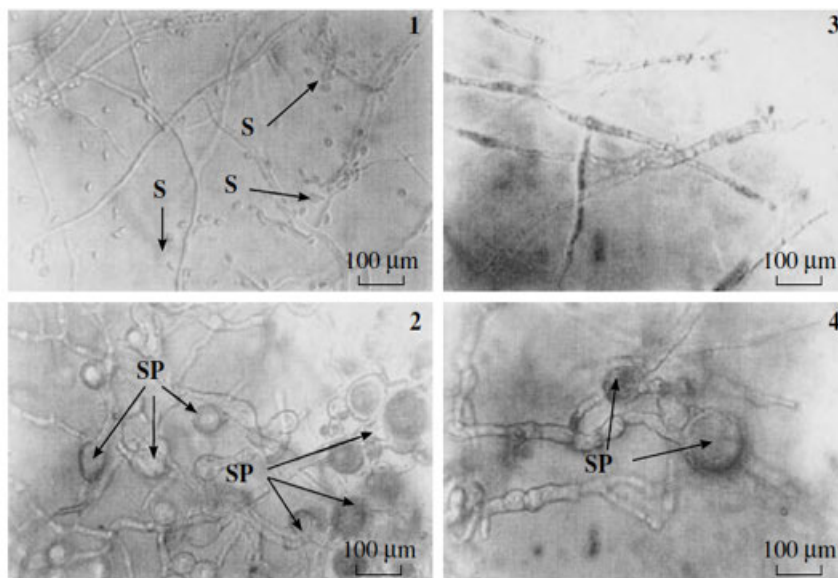


Fig. 1.10. Formation of spheroplasts on mycelium of micromycetes under exposure to bacillary metabolites detected by light microscopy (magnification, $\times 400$). Designations: 1, *P. variotii* (control); 2, mixed culture of *P. variotii* and *Bacillus* sp. X-b; 3, *P. simplicissimum* (control); 4, mixed culture of *P. simplicissimum* and *B. subtilis* IB-18; S, spores; SP, spheroplasts (Melent'ev 2006).

Bacillus spp. inhibition

Phenolic compounds are typically accepted as being more efficient against gram-positive bacteria than gram-negative bacteria. In particular thymol, carvacrol and p-Hydroxybenzoic acid are effective at inhibiting *B. cereus* at low MICs, see Fig 1.11 (Gutiérrez-Larraínzar et al. 2012).

Table 1.8 shows the MIC ($\mu\text{g/ml}$) of different compounds against bacteria and fungi. 9a inhibits DNA gyrase activity, and 9a and 9e showed unprecedented power against *B. megaterium* and *B. subtilis* while not developing any effect

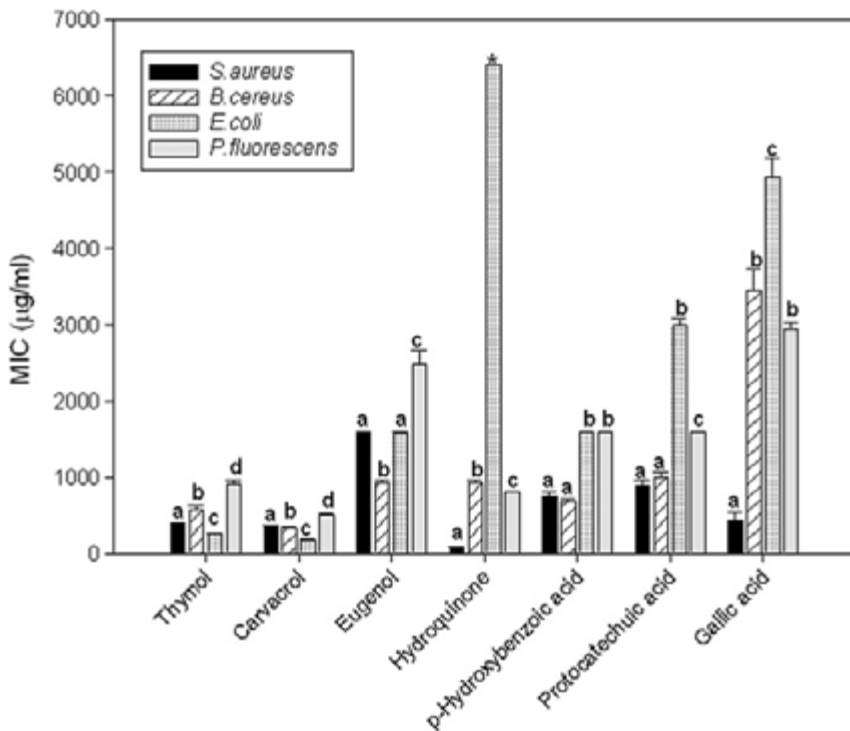


Fig. 1.11. Minimum inhibitory concentration (MIC) of phenolic compounds against the four genera/species studied. Values are the mean of those obtained for ten strains (*S. aureus*, *B. cereus* and *E. coli*) or nine strains (*P. fluorescens*). Data bearing the same letter are not significantly different amongst each other ($p < 0.05$) for one antimicrobial.

on fungi (Al-Trawneh et al 2011). Please refer to the publication for compound details.

Another substance that has shown powerful antibacterial effects on *Bacillus* spp., in particular *B. megaterium*, is mumijo. Mumijo has remarkable growth inhibition abilities towards gram-positive bacteria on complete media at neutral pH (Galgóczy et al. 2011). Mumijo is a traditional medicine of Central Asia and has been used for 1000's of years. This material is the fossil deposits of snow petrel (*Pagodroma nivea*) stomach

Table 1.8 Inhibitory activity of various compounds against bacteria and fungi, expressed as MIC ($\mu\text{g/mL}$).

Microorganism ^a	Compound ^b								
	9a	9b	9c	9d	9e	CIP	LEV	MOX	GEM
<i>Gram positive bacteria</i>									
BC	0.007	0.3	0.03	3	0.007	0.3	0.15	0.3	0.07
BM	0.000015	0.007	0.00003	3	0.000015	0.007	0.03	0.003	0.0003
BS	0.000015	0.0003	0.0003	3	0.000015	0.03	0.03	0.015	0.007
BTK	0.007	0.15	0.007	1.5	0.003	0.03	0.07	0.03	0.015
SA	0.003	0.07	0.015	6	0.003	0.3	0.07	0.03	0.015
SAR 72	0.7	12	1.5	3	0.7	100	25	12	6
SAR 4790	>100	>100	>100	>100	>100	>100	100	50	50
SE	0.03	0.3	0.15	3	0.03	0.07	0.15	0.07	0.015
SER	3	25	6	3	1.5	100	>100	50	50
<i>Gram negative bacteria</i>									
AB	0.07	0.7	3	>100	0.15	0.7	0.15	0.15	0.3
ABR	>100	>100	>100	— ^c	>100	>100	>100	50	100
EC	25	>100	>100	>100	50	0.015	0.03	0.03	0.015
ECR	>100	—	—	—	>100	100	25	50	50
HI	0.03	1.5	0.15	6	0.15	0.15	0.15	0.03	0.015
PA	>100	>100	>100	>100	>100	0.07	0.3	0.7	0.07
<i>Fungi</i>									
SC	>100	>100	>100	>100	12	>100	>100	>100	>100
CT	>100	>100	>100	>100	>100	>100	>100	>100	>100
AN	>100	>100	>100	>100	>100	>100	>100	>100	>100

^a BC, *Bacillus cereus* ATCC 11778; BM, *Bacillus megaterium* ATCC 19213; BS, *Bacillus subtilis* ATCC 6633; BTK, *Bacillus thuringiensis* var. *kurstaki* BGSC 4D1; SA, *Staphylococcus aureus* ATCC 6538; SAR 72 and SAR 4790, *Staphylococcus aureus* quinolone- and penicillin-resistant clinical isolates; SE, *Staphylococcus epidermidis* ATCC 12228; SER, *Staphylococcus epidermidis* quinolone- and penicillin-resistant clinical isolate; AB, *Acinetobacter baumannii* ATCC 19606; ABR, *Acinetobacter baumannii* quinolone- and penicillin-resistant clinical isolate; EC, *Escherichia coli* ATCC 8739; ECR, *Escherichia coli* quinolone- and penicillin-resistant clinical isolate; HI, *Haemophilus influenzae* ATCC 19418; PA, *Pseudomonas aeruginosa* ATCC 9027; SC, *Saccharomyces cerevisiae* ATCC 9763; CT, *Candida tropicalis* ATCC 1369; AN, *Aspergillus niger* ATCC 6275.

^b CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin; GEM, gemifloxacin.

^c Not tested because inactive against the corresponding quinolone-sensitive microorganism.

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CHAPTER 2. PILOT-SCALE SUBMERSED CULTIVATION OF *RHIZOPUS MICROSPORUS* VAR. *OLIGOSPORUS* IN THIN STILLAGE, A DRYGRIND CORN TO ETHANOL CO-PRODUCT

A paper prepared for submission to Applied Biochemistry and Biotechnology

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Abstract

An innovative process to add value to a corn-to-ethanol co-product, Thin stillage, was studied for pilot-scale viability. A 1500L bioreactor was designed, operated, and optimized to cultivate *Rhizopus microsporus* var. *oligosporus* via submerged fermentation in Thin Stillage. The biomass was harvested and processed into a feed suitable for storage and ultimately for animal feeding trials.

Characterization of the biomass and feed trials revealed that there is substantial potential as a nutrient dense feed supplement with 41.1% protein, 26.3% fat, and metabolizable energy on a dried basis. The amino acid profile is superior to that of DDGS, with most notably 1.7% Lys on dried basis.

This process produces a significantly more nutrient dense product than DDGS, and could increase water-reclamation in a dry-grind corn to ethanol plant. Industrially it would replace the energy intensive process of converting thin stillage into syrup that adds only \$10-25/ton to DDG, while maintaining production of DDG. Using thin stillage as used a growth media for *R. microsporus* var. *oligosporus*, should not only lead to saving in energy costs, but also generate a high-value co-product

which could lead to economic gains. Also there is still unexplored potential of enzymes, chitin, and co-culturing to further add value.

1. Introduction

As we charge into the second decade of the 21st century, world consumption of energy is increasing. There is undoubtedly a strong correlation between anthropocentric combustion of non-renewable sequestered carbon and concentration of their combustion products in our atmosphere. Biofuels eliminate or reduce the rate of proliferation at which many these compounds accumulate in our atmosphere. The United States of America's most important biofuel, in terms of volume, is ethanol derived from corn saccharides. However, volatility in the corn-to-ethanol industry seems to constantly be threatening the industry's survival. This includes corn prices, oil prices, energy prices, DDGS prices, and legislature. To help mitigate this and ensure biofuels thrive economically, it is imperative to develop innovative ways to manage waste streams or co-products. The petro-chemical industry through innovations has been able to take co-products ([other than gasoline and distillate fuel](#)) and produce profit-generating commodities. It is imperative for the biofuel industry to follow suit and maximize profits by implementing similar economic strategies that increase the value and/or decrease the expense of their co-products. Currently the United States is the world's largest ethanol producer, 13.95 billion gallons in 2011, of that 80% comes from dry-grind corn-ethanol plants ([US Department of Energy](#)). Typically in dry-grind corn-to-ethanol plants one gallon of ethanol requires 37.98MJ of thermal energy and 1.09 kW energy (Shapouri et al.

2003). Of this 35% of the electricity and 30% of the natural gas/coal are consumed by processing stillage into co-products (Meredith 2003). One such co-product is thin stillage, the leftover from distilling the fermentation mash after centrifugation to remove the distiller's grains. Thin stillage (TS) contains residual carbohydrates, amino acid and yeast cell degradations products that can be utilized. Dry-grind ethanol plants typically produce 2.5-3 gal TS/gal ethanol (2.8 gal TS/gal ethanol at LWE). This means that 27.9-33.5 billion gallons of TS are produced in the US each year. This research is focused on transitioning a low-value co-product, TS, into a higher value, more nutrient dense commodity. We utilized TS as a feedstock for *Rhizopus microsporus* var. *oligosporus*. The *R. microsporus* var. *oligosporus* biomass is the nutrient dense proteinaceous product that is generally regarded as safe by the FDA.

Previous work (Sankaran et al. 2010 and Rasmussen et al. 2010) showed that *R.m.o* grew prolifically in thin stillage. The aim of this research was to further explore the viability of this cultivation on pilot-scale systems and develop design and operational strategies, to maximize production of fungal biomass.

2. Materials and Methods

2.1 Thin Stillage (TS)

Thin stillage was obtained from Lincolnway Energy dry-grind corn-to-ethanol plant (Nevada, Iowa, USA). The TS was drawn from the plant's centrate tank and transported in a 1000L vessel to the pilot facility (3mi). Each week the TS had

slightly variable compositions due to variation in daily production. The TS was initially ~80C however to satisfy *R. microsporus* var. *oligosporus* growth conditions the TS was cooled to <40C. This is accomplished by using Havaco Corp SP600k heat exchanger with 1.55m² of heat exchange area. TS was repeatedly tested but never yielded microbial growth on YM agar. Therefore we consider the TS to be sterile.

2.2 Cultivation

Fungal strain

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 suspension was made following procedure in (Rasmussen et. al 2010). Dilution plating of spore vials revealed the suspension contained 12330 CFUs/ml spore suspension (Colony forming units, CFUs).

Inoculum preparation

Eight 2-L Erlenmeyer flasks were used to support initial spore growth. Each 2-L flask was filled with 1L of deionized water and 21g of Difco™ 271120 YM broth powder. Broth pH was adjusted to 3.95-4.10 with lactic or hydrochloric acid. The lower pH limit sustaining *supports R. microsporus* var. *oligosporus* growth in YM broth is between 2.75-2.9. Next flasks were covered with Kimberly-Clark KimGuard KC200 sterilization paper and autoclaved at 121C for 20 min. Flasks cooled until lukewarm to the touch, and then under biohood 2ml of spore suspension was added to the each flask(24660 CFUs/flask). Flasks were placed in a shaker at 37C

at 180rpm. Flasks were allowed to grow for 18-30 hrs before usage as inoculum for the 1600L bioreactor. 24h was the goal time, but due to scale of operation was not always possible. Two different morphologies were observed in the shake flask. Vials that contain spore suspension of 2330 CFUs/ml always formed fungal pellets while suspensions with 12330 CFUs/ml always-formed mycelial matrices see picture below.



Fig 1. Comparing growth of *R. microsporus* var. *oligosporus* in YM broth flasks inoculated with 5260 CFUs(l) and 24660 CFUs(r). Notice difference in morphologies and amount of growth

1600L bioreactor inoculation & growth parameters

TS was obtained from the centrate tank, transfer to 1500L bioreactor, and brought to $T < 40^{\circ}\text{C}$. The inoculation flasks (8 or 197280 CFUs) were added to 1500L bioreactor from the top through an autoclaved hose. Once inoculation has taken place, air was supplied to the fungi at static value of 7-10 ft^3/min through a manifold with 7 Cole-Parmer YO-70025-22 Ceramic Dome Fine Bubble Diffusers. A draft tube was used initially but eliminated due to equivalent results without it. The diffuser not only provides the fungi with oxygen for metabolic processes but also mixed the

growth media. This prevents settling of the fungi and helps keep the mixture homogenous. 99.9% O₂ was experimented with due to its potential to greatly reduce energy costs associated with aeration. However lack of mixing allowed fungus to settle, and reduce yields were realized. Mixing options were not explored.

2.3 Contamination and controls

Clean in place

To control contamination inside of the bioreactor a clean-in-place system was designed. Immediately after the bioreactor was drained it was rinsed with water. Prior to starting a new run, 50-150 g of KOH is added to 100L of water, steam is introduced, and CIP is started. After 1-3h at 107C the alkaline solution was drained, column rinsed, and refilled with 100L water and 2.86L of 6% NaOCl. This is allowed to circulate up until the point the TS is ready to be put in.

Controlling contamination during transportation

When transporting TS from the ethanol plant to the Pilot facility special precautions were taken to minimize contamination potential. The 1000 tank was filled with water and 2.86L of 6% NaOCl while not in use and drained only directly before use . A Gates 16GMV SAE100R4 suction hose customized with cam and groove fittings was designed to carrying TS from the centrate tank to the transportation vessel and from transportation vessel to bioreactor. A fiberglass filter was attached to the top of the vessel when draining to minimize contamination potential from air entering the transportation tank.

Air supply

For the 1600L bioreactor's air supply a Reading Technologies Inc. Eliminator 3P-090-M08-FI air filter was used to filter out anything 1 micron or larger. A King Instruments rotameter was used to control gas flow

Temperature modification

A Havaco Corp SP600k heat exchanger with 1.55m² of heat exchange area was implemented to cool the TS from up to 80C to <40C in <2 hrs.

2.4 Analytical methods

Biomass harvesting and processing

A custom harvester was fabricated to separate the fungal biomass from the liquid suspension. The harvested biomass was then placed into polypropylene bags and spun to ~75% moisture. Microwave drying takes the fungi from 75% to the desired 10%. Worth noting is that the microwave lyses the cells, which may aid in digestibility.

Fungal biomass characterization

Samples of biomass were sent to the Agricultural Utilization Research Institute (AURI) for characterization

D.O., pH, VOC, CO₂, and T

D.O. was measured with an Extech Dissolved Oxygen Meter 407510_A. The probe head was inserted 10 ft up into the column. We lacked the appropriate equipment to feed gas as a function of the fungal oxygen demands so a constant air flow was used between 7-10 ft³/min, controlled by King Instruments Company rotameter S/N 9918000001. The information was recorded by an Extech 380340 datalogger with a point taken every 3 minutes. Temperature and pH were recorded by a Madgetech pHTemp2000 data recorder, connected to Cole Palmer pH probe and SS T probe. Data points were collected every min. VOC's and CO₂ coming off the column were measured by an IAQRAE multi-gas detector. A filter was attached to the end of the collection line and held in the exhaust pipe until CO₂ and VOC measurements stabilized. Samples were periodically taken during runs.

Contamination identification and quantification

During runs where the temperature exceeded 32C (up to 55C) we typically observe little to no growth due to a contamination. Samples of contaminated media were taken from a sterilized test port attached to the 1600L bioreactor and placed in autoclaved 250ml sample bottles. The sample was then put on ice and transported to lab and dilution plated on YM agar (Difco™ 7107866). PCR was useful for fungal spp. identification while Fatty Acid Methyl Ester (FAME) analysis was performed to identify prokaryotic spp.

3. Results and Discussion

Mycomeal at 10% moisture was produced at 52g /gal TS. Optimization of fungal growth including temperature control, adequate mixing, and nutrient/enzyme supplementation could increase production and further revenue generation. However optimizing the growth does not necessarily mean optimization of profits, considering how successful a simple system is with minimal energy inputs. All results were obtained from batch process. We attempted to leave up to 20% of 24 hr growth in reactor and add additional stillage. Contamination always emerged, however if hard piped in we expect results to be different

Fig 2 shows the maximum CO₂ output, a measure of aerobic respiration, to be at 28 h. Over the next 8hrs an ~50% reduction in CO₂ output is seen. The 7-12 data on the graph represent a run where 42080 CFUs were used for inoculation opposed to 197280CFUs in the 7-18 and 8-8 runs. The difference in CFUs used for inoculation lead to a 6 h delay. To maximize growth efficiencies inoculate with at least 500CFU/gal TS. Spore germination/initial fungal growth was greatly reduced when spores were directly added to TS. Thus the spores should be germinated in YM broth.

Contamination was indicated by increased temperature due to the metabolic processes associated with growth and reproduction of contaminates (bacteria and/or yeast). Chitinase producing contaminates are especially undesirable and will reduce yields to 0g/L if not controlled. *Bacillus megaterium* and *Bacillus cereus* were both found to accomplish this when found in TS being fermented with *R.m.o.* Yeast also are not desired as they will raise temperatures above those suitable for

R.m.o and halt growth. *Pichia kudriavzevii* the primary yeast that we found to do this. However due to the nature of our project many more portals for contamination were present than would exist in an industrial setting.

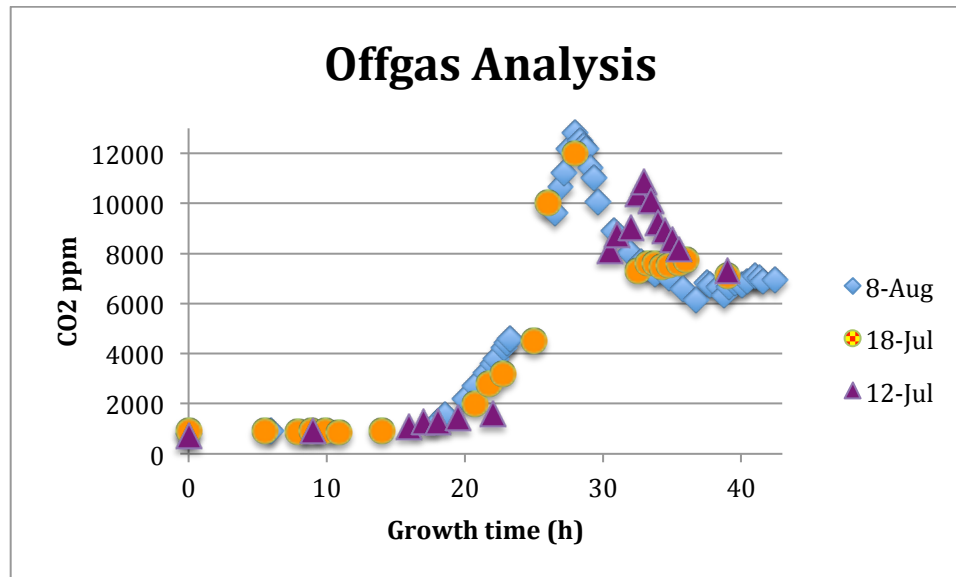


Fig. 2. CO₂ analysis of offgas coming from the reactor at various times during monoculture growth

VOCs were measured because if present in high concentrations, could produce added expenses associated with off-gas clean up. Overall VOCs are found in low concentrations. During the 6 h of growth elevated numbers, are seen potentially from residual ethanol being volatilized. Fig. 3 shows the results of VOCs analysis.

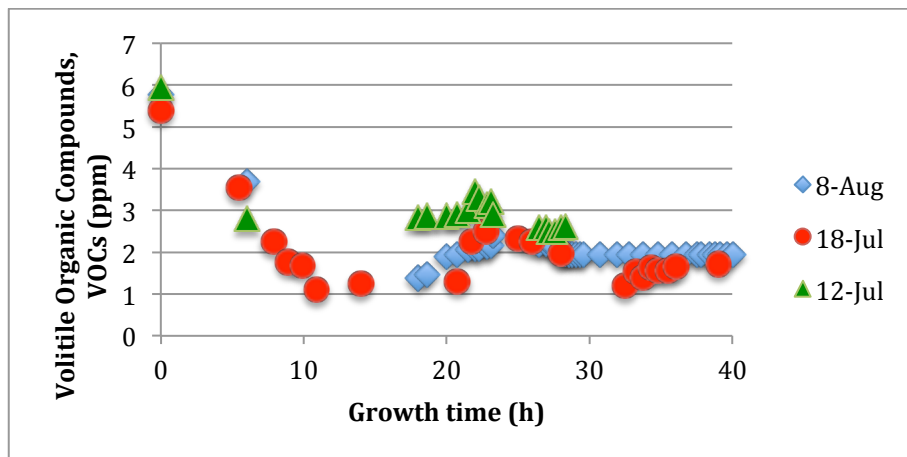


Fig. 3. VOC analysis of off-gas coming from bioreactor at various times during monoculture runs

Mycomeal, *R.m.o* grown on TS that is dried, has nutrient characteristics that can be seen in **Tables 1-3**. Mycomeal is high in protein and high in fat. Lys composition is also good when compared to corn and DDGS. Rhizopus also has been shown to significantly decrease adherence of ETEC as well as antibacterterial properties against *H. pylori*. Enzymes produced by *R.m.o* have also shown abilities to increase antioxidants available in feeds. Phytase is produced by *R.m.o* and could have profitable implications in the swine industry.

Table 1. Nutrient characteristics of mycomeal at 13.25% and 0% moisture

Moisture	AOAC 950.46b	%	13.25	-
Ash	AOAC 920.153	%	6.25	7.20
Total Fat	AOAC 991.36	%	22.80	26.28
Protein	AOAC 981.10	%	35.69	41.14
Calcium	Perkin Elmer FP-3	mg/g	0.197	0.227
Iron	Perkin Elmer FP-3	mg/g	0.245	0.282
Sodium	Perkin Elmer FP-3	mg/g	0.957	1.103
Aspartic Acid	AOAC 994.12	%	2.100	2.421
Threonine	AOAC 994.12	%	1.030	1.187
Serine	AOAC 994.12	%	0.820	0.945
Glutamic Acid	AOAC 994.12	%	2.680	3.089
Proline	AOAC 994.12	%	1.350	1.556
Glycine	AOAC 994.12	%	0.970	1.118
Alanine	AOAC 994.12	%	2.000	2.305
Valine	AOAC 994.12	%	1.400	1.614
Isoleucine	AOAC 994.12	%	1.150	1.326
Leucine	AOAC 994.12	%	2.320	2.674
Tyrosine	AOAC 994.12	%	0.820	0.945
Phenylalanine	AOAC 994.12	%	1.310	1.510
Lysine	AOAC 994.12	%	1.430	1.648
Histidine	AOAC 994.12	%	0.690	0.795
Arginine	AOAC 994.12	%	0.960	1.107
Total Amino Acids	AOAC 994.12	%	21.030	24.242
Cysteine	AOAC 994.12	%	0.33	0.38
Methionine	AOAC 994.12	%	0.52	0.60

Table 2. Micronutrient characteristics of Mycomeal at 13.25% and 0% moisture

Assay	Method	Units	LS2012142	LS2012142
alpha Carotene	AOAC 2005.07	IU/100 g	< 0.5	-
trans beta Carotene	AOAC 2005.07	IU/100 g	< 0.5	-
cis beta Carotene	AOAC 2005.07	IU/100 g	< 0.5	-
Total beta Carotene	AOAC 2005.07	IU/100 g	0.000	-
Total Carotene	AOAC 2005.07	IU/100 g	0.000	-
Vitamin A Retinol	AOAC 2001.13, AACC 86-06	IU/100 g	< 50	-
Vitamin B1 (Thiamine-HCl (US))	AOAC 942.23, 981.15, 970.65	mg/100 g	0.120	0.138
Vitamin B1 (Thiamine (EU))	AOAC 942.23, 981.15, 970.65	mg/100 g	0.0944	0.1088
Vitamin B2 (Riboflavin)	AOAC 942.23, 981.15, 970.65	mg/100 g	1.77	2.04
Vitamin B3 (Niacin)	AOAC 944.13, 960.46	mg/100 g	10.02	11.55
Vitamin B5 (Pantothenic Acid)	AOAC 945.74, 960.46, 992.07	mg/100 g	0.367	0.423
Vitamin B6 (Pyridoxine)	AOAC 961.15, 985.32, 960.46	mg/100 g	0.444	0.512
Vitamin B12 (Cyanocobalamin)	AOAC 952.20, 986.23	µg/100 g	8.338	9.612
Vitamin C	AOAC 967.22, 984.26	mg/100 g	< 0.50	-
Vitamin D	AOAC 2002.05	IU/100 g	< 40	-
Vitamin E	AACC 86-06	IU/100 g	4.19	4.83
Calcium	AOAC 975.03, 985.01, 990.08	mg/100 g	33.4	38.5
Copper	AOAC 975.03, 985.01, 990.08	mg/100 g	0.303	0.349
Iron	AOAC 975.03, 985.01, 990.08	mg/100 g	59.3	68.4
Magnesium	AOAC 975.03, 985.01, 990.08	mg/100 g	281	324
Manganese	AOAC 975.03, 985.01, 990.08	mg/100 g	2.80	3.23
Phosphorus	AOAC 975.03, 985.01, 990.08	mg/100 g	1410	1625
Potassium	AOAC 975.03, 985.01, 990.08	mg/100 g	1020	1176
Sodium	AOAC 975.03, 985.01, 990.08	mg/100 g	131	151
Zinc	AOAC 975.03, 985.01, 990.08	mg/100 g	11.3	13.0
Barium	AOAC 993.14, 2006.03	ppb	532	613
Boron	AOAC 993.14, 2006.03	ppb	3538	4078
Chromium	AOAC 993.14, 2006.03	ppb	1113	1283
Cobalt	AOAC 993.14, 2006.03	ppb	212	244
Molybdenum	AOAC 993.14, 2006.03	ppb	785	905
Nickel	AOAC 993.14, 2006.03	ppb	1753	2021
Selenium	AOAC 993.14, 2006.03	ppb	1647	1899

Table 3. Fatty Profile of Mycomeal

Assay	Method	Units	Fatty Acid	LS2012142
Fatty Acid Profile	AOCS Ce 2-66, Ce 1j-07	%	C14:0	0.1
			C16:0	11.8
			C17:0	0.1
			C18:0	2.6
			C20:0	0.3
			C22:0	0.2
			C23:0	0.2
			C24:0	0.2
			9c-C16:1	0.2
			9c-C18:1	21.2
			11c-C18:1	0.6
			9c,12c-C18:2	44.0
			10c,13c-C19:2	0.2
			11c,14c-C20:2	0.1
			6c,9c,12c,-C18:3	0.5
			9c,12c,15c,-C18:3	1.2
7c,10c,13c,16c,19c-C22:5	0.2			

Feeding trials

Two feeding trials, 1 broiler and 1 swine, were conducted using Mycomeal. Table 4 compares Mycomeal to other feed ingredients use in poultry diets. The most astounding number found is value for total metabolizable energy of the fungal biomass. Intracellular and extracellular enzymes produced by the fungi and are thought to be at least partially responsible for the extremely high TME of the biomass. Mycomeal contains 35-54% TME! **Table 4.** Proximate analysis and metabolizable energy for fungal biomass(Mycomeal), corn, soybean meal and dried distillers grains with solubles (Persia et al., 2011¹; NRC, 1994²; Waldroup et al., 2007³).

Feed ingredient	Fungal biomass	Corn	Soybean meal (48%)	DDGS
Crude protein (%)	35	8.5	47.5	26.5
<u>Methionine</u>	0.6	0.18	0.67	0.50
<u>Cystine</u>	0.50	0.18	0.72	0.54
Total sulfur amino acids	0.91	0.36	1.39	1.04
Lysine	1.48	0.26	2.96	0.71
Threonine	1.06	0.29	1.87	0.95
<u>Arginine</u>	1.02	0.71	0.94	1.09
Ether extract (%)	33.1	8.5	0.8	10.1
Total fat	23.5			10
Crude fiber (%)	9.1	3.8	3.9	7.0
Total ash (%)	5.5	---	---	4.7
Phosphorus	1.61	0.28	0.62	0.77
Starch (%)	2.67	---	---	---
<u>TME_m</u> (kcal/kg)*	5,382	3,470	2,485	2,845

Feeding of weaned piglets, also showed promise in rate of gain, but the results have yet to be published. Personal feeding trial done by author reveal that Rhode Island reds, and cochin chickens enjoy Mycomeal. Peacocks also find it suitable for eating.

4. Conclusion and Industrial Implications

R. microsporus var. *oligosporus* can be successfully cultivated in TS directly from centrate tank with no chemical modifications. Sterility is key, and monoculture is desired. The implementation of fungal bioreactors in ethanol plants would reduce non-renewable energy demands, produce a highly proteinaceous generally regarded as safe feed product, and treat wastewater stream. If current production could be scaled up Mycomeal at 10% moisture could be produced at 52g Mycomeal/gal TS. If a 50MGY generates 2.75gal TS/gal ethanol 7,865 ton Mycomeal/year. If \$500/ton mycomeal would generate \$3,932,5000 in revenue per year. This would not account for energy savings. It is important to keep in mind that the plant would still produce DDG but not DDGS. The nutrient content makes Mycomeal a very suitable food for a multitude of animals including swine, poultry, fish, and decapoda. Considering its G.I. health promoting benefits in mammals this should also help its viability.

Industrially a continuous or semi-continuous bioreactor would be necessary. Co-culturing could have substantial industrial implications that could tailor biomass composition to feed application. Give example . However yeast have proven to be detrimental to *R. microsporus* var. *oligosporus* due to their rapid metabolism causing T surges that escalate temperatures for above *R. microsporus* var. *oligosporus* comfort zone (55C).

Retro-fitting existing infrastructure would be ideal for implementation into existing plants and capturing heat exchanger energy via TS should be explored. Optimization of O₂ delivery should be obtained while maintaining adequate mixing.

Use of pure O₂ would reduce the blower energy demand by ~80%, but mixing energy demand would increase. Enzyme production and purification should also be explored for economic viability.

Acknowledgements

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CHAPTER 3. EVOLUTION OF PILOT-SCALE PROCESSES

1. Introduction

Contemporary human society is based on nonrenewable energy resources. Crude oil, coal, and natural gas located below the Earth's surface, has sequestered thousands if not millions of year's worth of carbon containing compounds. Humans found that by extracting and modifying crude oil into derivatives, they could harness its stored energy for transportation. For the last century this and coal/natural gas combustion, have been basically the only methods fueling our societies. However, concerns have risen regarding crude oil derivatives' cost, environmental effects, and extraction methods. Thus biofuels, fuels obtained from biological carbon-fixation, have been gaining interest. Biofuels are able to mitigate many of the aforementioned problems associated with petroleum-based fuels. Currently the most important of these mitigations include national security issues, environmental concerns, and renewability. Of all the biofuels produced, bioethanol is being produced in the largest quantity. Increasing economic viability of ethanol will help solidify and expand its production domestically and globally. This is where our research comes in, utilizing a co-product to generate a valuable commodity. Changing an expensive energy intensive process yielding a low value feed-supplement, into a less energy intensive process yielding more valuable product. Biofuels are cleaner, safer, and environmentally healthier alternative to petroleum based fuels, especially those that are imported.

Methodology and Equipment

50L Bioreactor

We used 2 SS 50L bioreactors. There are 5 1in ports in the bottom of each with the central port used for diffuser insertion and gas induction. One port on the side is for harvesting and sampling and 2 ports in the top are for inoculation and gas release. Casters and handles were added for ease of transport.

1600L Bioreactor

A tall slender A 20ft SS bioreactor with a 24in diameter (1600L) was used. The large height to diameter ratio helps increase oxygen transfer efficiencies in the bioreactor. The design also aids in mixing. There are 10 1in ports located on the outside of the column and one on the bottom in the center for gas induction. There are 3 additional ports found on the top, the central port was used for the spray ball. The gas is piped in by $\frac{1}{2}$ SS pipe that is goosenecked at the top to prevent any potential backflow. A polycarbonate window is located at the bottom of the reactor and attached with 30 bolts. A port 6in from the top is where the HE feeds into the reactor.

Thin Stillage, TS

All TS was collected from Lincolnway Energy LLC in Nevada IA. It was typically taken directly from the centrate tank, where it would come out between 65-80C with a pH of 3.8-4.2. The bright yellow corn-to-ethanol co-product was also

collected from storage tanks during instances where plant operations were down. Collection of TS from 3-5 day old occurred when plant cleaning and maintenance procedures were being performed. We saw no significant difference in the growth of *R.m.o.* on the older stillage. This demonstrated older TS could be successfully utilized as a growth media.

TS pH Control

Initially hydrochloric acid was pumped into the TS to control the pH. The theory behind adding the acid was the acidic environment, pH= \sim 4, would inhibit bacterial growth, while maintaining an ideal environment for the filamentous fungi. Inconsistencies in contamination prevention decreased its viability. In addition multiple equipment failures occurred and this presented safety issues. During runs with failed acid induction, we were able to successfully cultivate fungal biomass. The combination of the aforementioned, lead to the elimination of acid supplementation

Air filtration

Since our goal is to grow *R.m.o.* in pure culture on TS it is necessary to take all steps to prevent other opportunistic microbes from entering our reactors. Air filtration has been implemented into various steps of our process to help ensure that no contaminating microbes are entering the bioreactors from the air.. At the shake flask level Kimberly-Clark KimGuard KC200 sterilization paper is rubber banded to the top of 2L flasks to prevent any microbes from entering during shaking. This same technique is used on all autoclaved hoses and fitting to maintain

sterilization until directly before usage. The 50-L bioreactor utilizes a Pall Corp. Acro 50 .2 μ m PTFE filter to treat air going to diffuser. The 2 ports on top 50L facilitate gas leaving the reactor, and these are also covered with Kimberly-Clark KimGuard KC200 sterilization paper as well. However on extended runs in 50-L an oily layer is formed on bottom side of filter causing restricted airflow.

For the 1600L bioreactor a Reading Technologies Inc. Eliminer 3P-090-M08-FI air filter is used to filter out anything 1 micron or larger. This should be adequate to remove all incoming yeasts (3-4 μ m) and most bacteria (.2-2 μ m). The air comes from an air compressor system that supplies air to the entire facility.

Dissolved Oxygen, DO

An Extech 407510_A dissolved oxygen meter to measure DO was used, linked into an Extech 380340 data logger. This combination allowed generation of DO curves that in turn were used as a measurement of growth. To control the amount of gas flow to the 1600L a King Instrument Company I75302317C07 rotameter with control valve was used. Air was supplied at a static 8-10 SCFM (standard ft³/min) was used for the 1600L bioreactor. For the 50L reactor a VWR flow meter was used and a static value of 22-28 LPM was set. Technologies that monitor D.O. and adjust O₂ flow rates accordingly exist, but were not implemented. Obviously during the initial stages of growth small fungal populations require less O₂. Industrially it will be important to realize and optimize these technologies to reduce energy demand. Reduced airflow will have mixing implications.

Aeration

To optimize the amount of oxygen available to the fungi, air diffusion is imperative. The gas diffusers transform air or oxygen into small spheres with a large surface area to volume ratios. This permits greater fungal absorbance efficiencies; the O₂ is absorbed and utilized as the primary electron acceptor for aerobic energy production. The design of the 1600L column itself benefits the absorption efficiency. With a height of 20ft and a 2ft diameter the bioreactor provides a large distance for the diffused oxygen to travel before leaving the column. In the 1600L bioreactor a SS manifold was designed that holds 7 ceramic diffusers. When pure O₂ runs were performed the manifold was removed a single ceramic diffuser was placed in the 1600L.

Porous diffusers

We have tested a number of diffusers. Initially the 1600L had a single large rubber membrane diffuser. We were able to successfully cultivate biomass in the 1600L bioreactor with this diffuser. However when exposed to a high temperature ~97C, the diffuser was permanently enlarged. Concerns about the rubber's ability to withstand high chlorine and high alkali solutions, pH 11, were also discussed. We experienced the most success with the Cole Palmer ceramic dome fine bubble diffuser. Fine bubble diffusion typically implies <2mm gas bubble diameter. The ceramic diffuser is able to withstand high T (autoclavable) inside the reactors as well as the aforementioned chemical environments. The diffusers can be simply removed and disassembled for cleaning. Since autoclaving the 1600L reactor is not

an option, cleaning of diffusers is accomplished by disassembling, cleaning, reassembling, and reattaching to the manifold. Steam was forced through the diffusers while simultaneously running the CIP with KOH and then chlorine. When no gas is being forced through the ceramic diffusers liquid will penetrate and back fill your airlines. This has contamination implications since it occurs after the air filter. A valve was placed on the airline and closed in times when liquid is in the column but no air is being blown into diffusers.

However, two primary problems exist with the ceramic diffuser for usage of growing *R.m.o* in TS. First the diffusers are rigid and after repeated cleaning and handling many of them have been broken. Secondly fungal hyphae of the can penetrate into the diffuser, which is especially apparent during extended growth runs (<2 days). This is exaggerated when the flow of air is reduced or pure oxygen is utilized. When this occurs a decrease in efficiency of the oxygen transfer is realized and the problem progressively becomes worse through the fermentation period.

SOP ceramic diffuser cleaning

1. Remove viewing window of the 1600L reactor
2. Holding the outside SS portion diffuser twist CC
3. After removed, unscrew the bolt and place bolt and rubber washer in cleaning solution.
4. Carefully remove ceramic diffuser and rubber ring and place in cleaning solution
5. Reassemble don't tighten bolt down too tight and put sealant tape on threads that go to manifold



Ceramic porous diffuser and manifold



Viton diffuser

Mixing

No mixing strategies were employed in this research other than that achieved from the diffused gases. R.m.o. can reproduce vegetatively via hyphal fragments; therefore theoretically it is possible to create more CFUs by designing mixing strategies to shear hyphae to make culture-forming fragments. However the lack of developed septa in *Rhizopus* spp. limit how effective this may be. It was noticed that strata form in the bioreactor and biomass seems to grow in the bottom 1/3 but not in the rest of the column.

Volatile Organic Compounds

VOC analysis done revealed that there are 1.9-6 ppm VOC depending on time of sampling. VOCs were at highest concentration during the first hour of operation and gradually decreases as run continues. I hypothesize that residual ethanol is responsible for the at least part of the initial concentrations.



Draft tube inside of 1600L bioreactor

Methodology

Draft tube

During initial experiments both the 50L and 1600L were equipped with a draft tube. It was thought that a draft tube was necessary to enabling fungal growth via the draft tube principal. The draft tube principal is based on a cylindrical tube with slightly greater diameter than the impeller (in our case the diffuser), and is elevated slightly over the impeller. The gas travels through the tube and out the top creating continuous cyclic flow.

TS Transportation and transfer equipment

To transfer TS from LWE to the PP an empty enzyme storage vessel, SV, provided by LWE were used. To keep the 1000L SV as sterile as possible it is filled with 1000L water, 1000L and 2.83L of 6% NaClO. When weather is above freezing the tank was kept in a sunny area outside for the UV radiation sterilization and temperature increase. During the early stages we would open the 6in cap on the top of the tank and fill via a rubber hose attached to the centrate tank. This presented multiple problems when trying to maintain sterility during transfer of TS. First opening the top of the SV presented an opportunity for microbial introduction. Second the rubber hose was not sterile and actual had biofilm formation on interior surface. The outside was rubbed down with 80% ethanol. Remarkably we were successful on many occasions with the system of filling.

As the process was refined we had a custom autoclavable hose made that could tolerate autoclaving. Each end of the hose had a female cam and groove fitting attached to it. This type of attachment facilitates quick attachment and detachment. A male cam and groove piece was attached to the bottom of SV and another male piece was attached to the centrate tank at the ethanol plant. This allowed us to take the autoclaved hose and quickly attach the hose to both centrate tank and SV, minimizing the contamination potential.

A 1in ball valve was attached to the top of the SV to control the movement of air into the SV. During the filling step the pressure pushes air out through the 1 in opening, and is closed immediately after filling. However when transferring the TS from the SV to the 1600L the top would have to be opened to relieve negative

pressure that occurs during transfer. This presents an obvious opportunity for microbes and spores in the air to find their way into the TS, this also occurs when emptying out the bleach solution. To combat this problem a filter was fabricated with a 4 in plastic pipe with a 1 in MIP that can be screwed in the 1in ball valve on top of SV. Fiberglass wool is stuffed into the 4 in pipe and prevents microbes from entering the SV from air being drawn in.



SV attached to pump to fill 1600L bioreactor, utilizing a fork lift to create head pressure. Notice filter on top of SV.

SOP Filling SV with TS

1. Attached filter to top, open valve, and drain the bleach solution from SV
2. Close valve and remove filter
3. Load the SV into the truck
4. Flush centrate tank line with ~3 gallon of TS
5. Remove rubber hose from cam and groove male piece and clean inside and outside of male piece with 80% ethanol and lab towel
6. Attach the custom autoclaved hose to the centrate tank
7. Using 80% ethanol clean the male cam and groove piece on the bottom of SV.
8. Attach custom autoclaved hose to the SV
9. Open valve on top and bottom of SV
10. Fill the SV with 1000L of TS.
11. Using a rubber glove or thin foil quickly cover the hose's female cam and groove piece after squirting with 80% ethanol.

1600L Feedstock Cooling

The TS from the ethanol plant comes out of the centrate tank at 65-80C. The desired temperature for growth of *R.m.o* is 37C. Initially the column would be filled with TS and a fan would cool the outside of the 1600L reactor. However this provided a long period of residence time for the TS without being inoculated with our filamentous fungus.

To reduce the time in which the column contained uninoculated TS a column cooler was developed. The column cooler was made out of $\frac{3}{4}$ in Pex tubing with a hole drill every 6 inches and a hose attachment coming from a T that circularized the pex. A hose was connected to the column cooler and run water down the outside of the column. This was much quicker than the previous method of cooling and when combining the column cooler with airflow cooling time was reduced from 10h to 2h. Yet this method still provided a longer than necessary fungi-free residence time, while fueling any contaminating microbes with O₂. After a few failed runs it was decided not provide any air to the TS because it could provide any

contaminating aerobic organisms a head start. Cutting off the air led to the discovery of temperature striations that form inside the column in the absence of mixing. Since the Temperature probe is positioned at the bottom of the column, on one run it was thought that the T had gotten down to the appropriate level 37C. However after inoculation of the TS and air induction the Temperature spiked up to 48C, killing the fungal inoculum. Extreme T gradients are found inside the column if not properly mixed. So a more ideal method was still desired.

At this point we designed and built a heat exchanger that cross-flows water over copper pipe. We used ½ in copper tubing and 1½in schedule 80 PVC to build the exchanger. With this method, we would precisely control the temperature of TS entering the column to the nearest .25 of a degree. Typically the TS was cooled from 75C to 37C from the time it left the SV to the time it entered the column. This works amazingly with one caveat, sterilization. The heat exchanger had a series of 7 10 ft. copper sections that run parallel inches from each other, making mechanical cleaning impossible with our equipment. Additionally the copper also proved to not be the ideal material. The lines were sometimes filled with a NaClO solution when we are not running, up to 10 days.. The copper would leave a rust colored residue in the solution. Further steam cleaning the heat exchanger caused leaks in the rubber pressure seals needed to contain cross-flow water.

Our fear of contaminants entering through the heat exchanger lead us to revert back to the column cooling method. However this time we doubled it up and used 2 column coolers in combination with air supply. Increased confidence in column cleaning reduced our fear of using air to aid cooling. This doesn't have the

immediate cooling capability of the heat exchanger, but eliminates ~70ft of pipe that needs to be cleaned and sterilized. However when considering continuous or semi-continuous operations, the TS needs to be cooled before being added to *R.m.o.* containing media in the column. Industrially this would require the TS to be cooled prior to addition.



Heat exchanger attached to 1600L



Column cooler (up to 2 of them)

Preparation of 2L Shake Flasks

Initially to start fungal spores 4 x 2L Erlenmeyer flasks were filled with 1L of distilled water and 21 grams of YM broth (Difco™ 271120). For the first 2.5 years we used hydrochloric acid to reduce the pH of the broth from 6 to 4. However we also tried lactic and acetic acid to accomplish this. The justification being that lactic and acetic acids are the primary problematic acids in TS. Thus the usage of these acids could enzymatically acclimate the organisms to these acids. No differences in

2L growth were observed when using lactic acid in place of hydrochloric acid. After acid addition, cover flask opening with Kimberly-Clark KimGuard KC200 sterilization paper and place into autoclave. Once broth has cooled to the appropriate T, we would add 1ml of spores to each flask under biohood.

Different quantities of spores were added to the flasks, 1ml vs 2ml. Placing a full vial, 2ml, into flasks yields higher fungal populations in flasks. Despite 1ml containing ample amount of spores to successfully cultivate fungus on the pilot scale, more spores gave us more CFUs. So we now add 2 ml to each flask. To determine if the 2L flasks were *R.m.o.* monocultures .1ml samples were plated on Difco™ 71210 YM agar. All tests of 24h flasks revealed a monoculture of *R.m.o.* Signifying contamination was not coming from 2L flasks or the spore vials.

SOP for inoculation of 2L flasks

1. Weigh out 21g of YM broth in weigh boat
2. Place 21g of YM into 2L Erlenmeyer flask
3. Add 1L of distilled water to the shake flask
4. Place on stir plate and stir until the solution is homogenous – heat maybe added to reduce time needed but be careful not to scorch
5. Add Acetic/lactic acid to the broth to reduce pH to 4
6. Place custom rubber stopper onto the flask and cover opening with Kimberly-Clark KimGuard KC200 sterilization paper and secure with rubber band.
7. Place into autoclave and autoclave for ~15 min or until the autoclave tape shows black stripes
8. Remove from autoclave and allow growth media to cool
9. Under the biohood add 2ml of *R.m.o.* spore stock to the flask and recover Kimberly-Clark KimGuard KC200 sterilization paper
10. Place inoculated flasks into the shaker at 175 rpms and 37C
11. After 24 hours or when flocs have reached the size of a pea they are ready for TS inoculation

Incubation

Incubation of the flask occurred in a shaker @ T=37C and 175rpm. Decreased T leads to increases in time needed for maturation. The best indicator of to readiness of spores for inoculation was size. It is important to get the *R.m.o.* into the TS at the appropriate maturity. This is when the flocs are the size of a pea. If the flocks are too small extra time is needed and sometimes only minimal growth is seen (most likely due to contamination). If the flocs are too large then we also witness the same phenomena. Once the shaker was left for 5 days and one large floc was form in one of the 2L flasks. It is important to catch the spores while they are in the active growing stage.

50-L bioreactor design evolution

Initially the 50-L had a stainless steel lid that used 4 clamps to attach the lid to the bioreactor. As time went on the lid began to warp. The warping caused the lid to no longer fit tightly on top of the 50-L. Without a tight seal we obviously had a portal for unwanted microbial induction. At first 50-L of TS in an autoclaved 50L carboy was transported from Lincolnway Energy to the cold room on campus. After TS reached T≤37C the lid was removed and the TS into the 50-L bioreactor. This has obvious disadvantages when trying to maintain sterility.

Next the 50L bioreactor was modified so that we could use an autoclaved hose to directly pump the TS into the autoclaved 50L bioreactor. This was much more sanitary than the previous method, but created a problem knowing how much TS was in the bioreactor. To combat this a hole was drilled in the 50L bioreactor and

a fabricated test tube provided a peep sight into the 50-L reactor at the 46L mark. Then the entire reactor was placed in the cold-room, which eliminated taking the top off the autoclaved reactor. However the lid was still problematic.

A new lid design that featured a SS flange, an autoclavable rubber seal, a polycarbonate lid, and a SS ring was implemented. The 2 SS pieces sandwiched the seal and lid with 12 bolts creating a great seal. The lid also contains 3 ports to allow ample air to exit the reactor without creating pressure. The ports were covered with Kimberly-Clark KimGuard KC200 sterilization paper. As growth time went over 24h bottom side of the paper covering these ports began to get covered with an oily film. This begins to obstruct airflow from the reactor and could cause holes to form in paper.



50L prior to modifications with cooler



Modified 50L

The 50-L bioreactor preparation

To promote a microbe free environment in the 50L we take a number of steps. First the assembled reactor, with the diffuser inside, is autoclaved. All ports on the top of the reactor are covered with the Kimberly-Clark KimGuard KC200 sterilization paper. The air induction line and the filling and draining/filling port are also covered with the Kimberly-Clark KimGuard KC200 sterilization paper. After everything has been autoclaved we need to put in instruments (see SOP).

To maintain a steady temperature a SS aquarium-heating element was placed into one of the 4 1in ports at the bottom of the 50L. This facilitated a constant T, optimizing growth inside the 50L. *R.m.o.* will attach itself to the heater and during longer runs and a burnt popcorn smell will arise from the biomass adhering then burning to the heater.

SOP for putting instruments into the 50L/1600L

1. With firm tooth brush and Simple Green all purpose cleaner clean all port threading and remove tape from all plugs. Retape, and replace plugs in ports that are not going to be used instruments. In instrument ports no retaping of plugs in necessary
2. Autoclaved 50L or run CIP in 1600L.
3. Partial unscrew plug
4. Put plumbers tape on threads of instrument
5. Sanitize instrument of choice with 80% ETOH- in case of SS heater apply ETOH and then ignite
6. Quickly remove the plug and place in instrument

50L heat exchanger

A heat exchanger was developed specifically for the 50L to reduce the time needed to cool the TS inside of the 50L. It was made with a 20ft piece of ½ copper pipe.

SOP for 50L Heat Exchanger

1. Attach heat exchanger to the black filling hose on one end and an autoclavable braided hose at the other end
2. Using Kimberly-Clark KimGuard KC200 sterilization paper cover the ends of each attached hose and autoclave
3. Fill plastic tote with ice and water and submerge HE
4. At ethanol plant always run ~3 gal through the plants hose. Detach hose and clean the nipple with ethanol inside and out.
5. Remove the Kimberly-Clark KimGuard KC200 sterilization paper paper and attach black hose to nipple and repeat for attachment to 50L.
6. Using a slow flow fill the 50L ~10 min to achieve appropriate temperature

Inoculation of 50L bioreactor

Once the 50L has been filled with TS and the desired temperature has been attained, we can inoculate the 50-L with the 2L flasks. This is accomplished by using a 2½ft autoclavable hose with a ½in ID. The hose has a 1/2in copper pipe piece attached to one end and a modified rubber stopper at the other end.

SOP for 50L inoculation

1. Attach air line with in-line filter to air induction line
2. Turn on air to mix and then sample T to make sure it is appropriate ~37C
3. Turn off air
4. Remove Kimberly-Clark KimGuard KC200 sterilization paper and quickly attach the copper pipe end of the hose into the copper coupler that is integrated into the polycarbonate lid.
5. Remove filter paper from 2L shake flask and link flask to modified rubber stopper
6. Elevate the flask to transfer contents (if a circular motion is use while transferring a cyclone will form helping transfer all of the flocs)
7. Repeat for each flask
8. Recover the copper coupler with Kimberly-Clark KimGuard KC200 sterilization paper and band
9. Turn on air to 25 cm³/min and set T to 37C
10. Start applicable devices
11. Take zero hour sample

1600L reactor clean in place (CIP)

At first our method for cleaning, combating contamination, was rinsing the interior of the column with water and then filling it with ~900ppm bleach water solution and let it sit for 6-14 days proved to be ample time to facilitate successful runs with no other cleaning method. However, the prolonged time was not desirable and an oily film was noticed sticking to the inside wall of the column. This was potentially providing a protective environment for microbes to live.

Next a steam line was installed which allowed us to raise the T in the column to 97C. Originally we thought that this would lead to a phase change in the oily film and this would remove the film. Conversely this was not the case, and the film would still coat the inside of the column after extended periods at ~97C. The next progression was to fill the column with water and then add NaOH until the pH reached 10.5-11. After letting the solution sit for a few days you would see a layer of oil accumulated at the bottom on the 1600L reactor. The basic bath was followed up by filling the column with the 900-ppm bleach solution and let sit. After draining the bleach solution a soapy residue would still remain. The NaOH would cause saponification to occur causing the inside surface of the reactor to have a soapy residue. When KOH was used in the same manner we saw minimal saponification form when compared the NaOH method. Unsatisfied with the quality of cleanliness as well as the time needed to clean the column, we decided to venture to Olde Main brewery and study their CIP system. This led to our development of our CIP system, which includes a ??? spray ball connected to a 3/4hp Dayton LTAC21SA pump. We designed the system so that we could run the CIP thru the heat exchanger

and the 1600L. This allows us to clean the entire system internally, and minimize the amount of water needed for cleaning from 1600L x 2 (NaOCL and KOH/NaOH) to only 75-100L for each. A surfactant provided by LWE made by Mist Chemical, Excelon 210, was added at recommended quantities to the alkali solution. It is claimed to improve the cleaning efficiency of the caustic solution while providing antifoaming properties. Excelon 210 has been approved by the FDA center for veterinary medicine to be used in animal feed. This system proves to be very effective and inside of column looks new after running.



Oil film inside of 1600L



Settled oil film from inside of the 1600L when treated with KOH

SOP 1600L CIP

1. Immediately after harvesting the 1600L rinse with water until effluent stream is a transparent stream. Also rinse out the HE.
2. Remove all probes placed in column and place in plugs (destruction of probes may occur at high T)
3. Remove cover and remove the porous diffuser from the manifold and disassemble and clean each diffuser (see SOP)
4. Replace the porous diffusers onto the manifold and reattach ppolycarbonate window.
5. Insert T probe into port with plumbers tape

6. Fill the column with 75-100L H₂O and add ~100g of KOH/NaOH. Then add corresponding volume of mist chemical surfactant to column through sample port
7. Attach steam hose to 1600L port and turn on pump
8. Let run for at least 3 hr and then switch valve to HE for 3 hr. If very dirty can clean for an hour, empty, and repeat step 6.
9. Shut off steam, shut off pump, and drain caustic solution.
10. Rinse the column with ~20 gal water through test port and again fill with 75-100L water. Add 2.86L of NaOCl 6% and run for at up until inoculation. No steam
11. Replace the probes into column as described earlier in SOP
 - *periodically remove top and clean, also pay special attention to overflow/exhaust pipe. If TS bubbled over during the run it creates a film inside the pipe, and may need cleaned to help maintain sterility

1600L Inoculation

Everything discussed in the paper so far is leading up to the point in which we fill the 1600L reactor with TS and inoculate it with the contents of the 50L. When I first took over the project This involved priming the pump with water followed by removing the top of the SV and placing an unsterilized rubber hose into the TS. Once filled, allowed it to cool, and then would remove the top of the 50L put the hose in into the 50L inoculum. Once near the bottom I would start to fill with water to flush all inoculum out of the lines and into the column. Amazingly this worked on the occasion! However time has lead us to the refined process described below

1600L Inoculation SOP

1. Turn on all the heat exchangers
2. Remove the SV with forktruck from the back of F150
3. Place fiber filter on top of the SV and open valve
4. Use ethanol to dose MIP attached to pump and use lighter to ignite
5. Remove Kimberly-Clark KimGuard KC200 sterilization paper paper from end of black hose soak with ethanol and swiftly attach to pump
6. Elevate the forklift to generate head pressure
7. Begin filling the column with TS and monitor the T, and adjust HE for target T of 37C
8. Turn on the air to provide mixing (T striations occur inside the column and T gage is at bottom)

9. After filling with ~500L stop pump, shut valves and dose 50L MIP with ethanol ignite then attach black hose
 10. Lift 50L reactor onto scissor lift and elevate the lift
 11. Shut off HE*
 12. Turn on pump and empty contents of the 50L
 13. Dose the SV MIP with ethanol and ignite, then reattach the black hose to the SV
 14. Reengage HE
 15. Open valves, start pump and continue filling until the SV is empty
 16. Return to ethanol plant and fill SV with 500L of TS
 17. Repeat step 13 and finish filling the 1600L reactor
 18. Set up the air flow rate 10 ft³/min
 19. Start recording all data
 20. Close valve to column and wash out heat exchanger with water and the fill with 900ppm NaClO solution
- *The HE is shutoff when inoculating the 1600L with the 50-L. This is to minimize the shock that the *R.m.o.* has to experience during the transfer period. However many successful runs have been executed without shutting them down; however I feel that it is good microbiological practice.

1600L sampling

Sampling from the 1600L is needed primarily for 2 reasons, biomass yield calculations and contaminate identification. The samples are taken from a 1" port ~4ft from the bottom of the column. Coming from the port is a street 90° elbow attached to a 250ml SS pipe with a 1" valve on each side. Attached to the distal valve is a nossal that is compatible with the 250ml sample bottles. Both of the aforementioned reasons for sampling have problems associated with sampling. First there is potential for contamination inside 250ml tube. Opening the valve completely and quickly is necessary to optimize the biomass yield sample. Reduce in flow volume can lead to skewed biomass readings.

1600L Sampling SOP

1. Empty out the bleach solution
2. Open proximal valve and allow the sampling pipe to fill, close proximal valve, open distal valve, and drain pipe 2x. Use strainer on 2nd time to see biomass

3. Quickly and completely open the valve proximal to the column while keeping distal valve closed. Partial opening may restrict biomass giving skewed result
4. Shut proximal valve after 1-2s
5. Dose the nipple with ethanol
6. Open distal valve and quickly empty sample into autoclaved 250ml sample
7. Quickly cap the container, label, and place in the fridge
8. Fill the sample port with a concentrated bleach solution (recipe 200ml 6% NaOCl and 1000ml water)

T, pH, D.O., and off-gas

To measure the pH and temperature we used a Madgetech pHTemp2000 data logger with a stainless steel temp probe and Cole-Palmer 27003-00 pH probe. All pH calibrations are performed with Fischer scientific SB101-500 buffer solutions. D.O. was measured with an Extech Dissolved Oxygen Meter 407510_A and collected with an Extech 380340 data logger every 3 minutes. The probe head was inserted 10 ft up into the column. Calibrations were performed prior to each run. VOC's and CO₂ coming off the column were measured by an IAQRAE multi-gas detector. A filter was attached to the end of the collection line and held in the exhaust pipe until CO₂ and VOC measurements stabilized. Samples were collected randomly when available.

Harvesting

After the fungi has matured, ~24 hours, in the TS, it is time to harvest the fungal biomass. This is accomplished by running the suspended biomass over a 3mm pitched screen in a custom made harvester. After using the screen once it was observed that a significant amount of biomass was escaping through the screen. So an additional vinyl coated screen with .4mm hole size was laid on top of the 3mm screen. With the additional screen the fungi would conveniently coagulate and roll

down in cylindrical globs. The effluent stream is derived from all remaining growth media that is not retained by screen. The effluent drains into a temporary storage tank and is disposed of.



Harvester

Di-screen configuration

Harvester in action

Dewatering

Initially we took the biomass directly from the screen and put it into 2 types of bags burlap and polypropylene. Polypropylene bags are far superior due to their durability, cleanability, and biomass retention. When hung up BECON personnel gave the bags the nickname teabags. Fellow tenants began to comment on the teabags odor after 6 days. During these extended hanging times the outer biomass dewateres well, but an oil layer covers the inside of the bag. This significantly restricts any further dewatering after 3 days. After 24h an average 39% of initial weight was lost. Hyphae and then spores would appear on biomass that was in direct contact with air with 2 days. The spores were removed prior to storing, before drying would take place. To reduce dewatering time we designed a dewatering trough. We used recycled stainless steel screen provided by LWE, to produce a rigid surface for dewatering. Scraping and pressing the biomass helped

remove additional water but lead to increased biomass losses. Once again a 4mm screen was laid over top of this SS screen to retain more biomass. After the secondary dewatering screen we would place the biomass into polypropylene bags. Industrially Teabags are infeasible so we created the Fungifuge. The Fungifuge is a 110V washer modified to facilitate centrifugation of the teabags. After 10 min of centrifugation the fungal biomass has a playdo consistency (~75% moisture). Once dewatered, the biomass is transferred from the bags into 5 gal buckets and stored at 10C.



Infamous Teabag



Secondary dewatering trough



Fungifuge

Drying

For drying the fungal biomass we explored many options including a modified clothes dryer, oven drying, and microwave drying. A pilot scale microwave provided by Cellencor Inc. was used to dry most biomass. After the 1st pass through the microwave the biomass appeared wetter. This was due to cell lysing caused by the microwaves, which exposed intercellular contents. For intracellular contents analysis the biomass could be placed in bags, and then spun in Fungifuge. The effluent stream could be collected and tested. Power settings were optimized to dry

the fungi without burning the outer layer. This created a laborious situation with >10 passes needed to get the biomass $\leq 15\%$ moisture. However the end product was superior when compared to other methods. The biomass would start off yellow and change to a golden brown at 15-18% moisture. The dried biomass has a Grapenut cereal appearance and texture. This is Mycomeal.



Colleagues feeding the microwave(L) and fellow scientists enjoying summer drying (R)



Microwave used for biomass drying provided by Cellencor

Results

TS pH control

We were able to successfully harvest fungi with the acid supplementation, with partial acid supplementation (equipment failure runs), and without acid.

Industrially, lactic and acetic acids cause growth disruption of fermentation yeasts. This is why only $\sim\frac{1}{2}$ of the TS is recycled to the fermentation tanks while the other $\sim\frac{1}{2}$ is evaporated. If the acids primary goal is to prevent bacterial growth, it would be more feasible to use an food grade antibiotic; especially if it is desired to recycle the effluent from harvesting into the fermenters.

Draft Tube

The draft tube was first removed from the 50L reactor, and we saw no change in biomass yield. Next the draft tube was removed from the PP reactor during a location change in summer of 2011. We have successfully cultivated *R.m.o.* in our 1600L many times with and without the draft tube. It is worth noting; in our 1600L pure O₂ run a draft tube was not present. Thus it may become more vital to have a draft tube as process is scaled up and O₂ flow rates are optimized.

DO

The most accurate curves were been generated from our 50L reactor because issues of maintaining a monoculture in 1600L reactor. However DO typically bottomed out at .2-.4 g/L after starting out 10 g/L.

Diffusers

In the 50L bioreactors a single ceramic diffuser was typically used to supply O₂. However when using pure oxygen we opted to a Viton diffuser to combat the fungal penetration that is associated with the ceramic diffusers at low air flows. The

Viton diffuser was unable to be autoclaved so this required the 50L top to be removed after autoclaving. This always presented us with contamination issues so simultaneous runs comparing the Viton and ceramic were unsuccessful.

For industrial application a combination of the heat and chemical tolerance found in the ceramic porous diffuser, and the hyphal penetration prevention of the Viton/rubber membranes are desired. Industrially a semi-continuous or continuous system is the finest option so growth into the ceramic diffusers will be problematic. It seems the best option would be Viton diffuser with a melting $T > 110\text{C}$.

Air vs O₂

One of the primary expenses associated with our system is gas delivery. Industrially blowers would be utilized to push air into bioreactors. By using pure O₂ ~ 1/6 of the energy would be needed to supply the bioreactor with the same amount of O₂. The reduction in flow rate also limits the amount of off-gas (exhaust) coming out of the bioreactor. The volume reduction reduces the amount of gas that potentially needs treated/scrubbed. We proved that it is possible to use pure O₂ to cultivate *R.m.o.* in both the 50L and 1600L bioreactors. On the 50L scale growth was comparable to that achieved with air. However in 1600L, a reduction in biomass yield was realized. It is believed that insufficient mixing is the primary cause of the reduction in yield. This type of system would not only have the additional expense of the cryogenic system but also costs associated with mixing technologies.

Mycomeal

Mycomeal is an energy dense food supplement composed of *R.m.o* biomass grown on TS. Mycomeal is high in protein and fat. Lysine, an essential amino acid, is found in relatively high concentration that has important implications for swine nutrition. AA and fatty acid profile are shown below. Tests were completed by Agricultural Utilization Research Institute, AURI.

Assay	Method	Units	LS2012080	LS2012081
Moisture	AOAC 950.46b	%	14.97	31.25
Ash	AOAC 920.153	%	5.71	4.22
Total Fat	AOAC 991.36	%	18.37	15.92
Protein	AOAC 981.10	%	30.24	22.47
Calcium	Perkin Elmer FP-3	mg/g	0.402	0.286
Iron	Perkin Elmer FP-3	mg/g	0.216	0.072
Sodium	Perkin Elmer FP-3	mg/g	0.960	0.668
Aspartic Acid	AOAC 994.12	%	1.76	1.26
Threonine	AOAC 994.12	%	0.81	0.64
Serine	AOAC 994.12	%	0.75	0.63
Glutamic Acid	AOAC 994.12	%	2.47	1.74
Proline	AOAC 994.12	%	1.20	0.77
Glycine	AOAC 994.12	%	0.85	0.61
Alanine	AOAC 994.12	%	1.68	1.32
Valine	AOAC 994.12	%	1.16	0.82
Isoleucine	AOAC 994.12	%	0.93	0.66
Leucine	AOAC 994.12	%	1.99	1.34
Tyrosine	AOAC 994.12	%	0.71	0.57
Phenylalanine	AOAC 994.12	%	1.11	0.79
Lysine	AOAC 994.12	%	1.16	0.83
Histidine	AOAC 994.12	%	0.60	0.45
Arginine	AOAC 994.12	%	0.82	0.69
Total Amino Acids	AOAC 994.12	%	18.00	13.12

Assay	Method	Units	Fatty Acid	LS2012080
Fatty Acid Profile	AOCS Ce 2-66, Ce 1-62	%	C14:0	0.2
			C16:0	17.1
			C18:0	3.3
			C20:0	0.6
			C22:0	0.3
			C24:0	0.4
			C16:1	0.2
			C18:1	25.4
			C18:2	49.7
			C19:2	0.2
			C20:2	-
			C18:3	1.6
			C20:4	0.3
			(EPA)	C20:5
(DPA)	C22:5	none detected		
(DHA)	C22:6	none detected		

Assay	Method	Units	LS2012080	LS2012081
Cysteine	AOAC 994.12	%	0.38	0.27
Methionine	AOAC 994.12	%	0.49	0.30

Discussion

R.m.o can be easily grown on TS on the pilot scale as long as a monoculture can be maintained. Industrially there should be no problem achieving this, considering the sterility advantages of a directly piped-in system. The Mycomeal

product is highly nutritious, containing over 30% protein and 18% fat at 15% moisture. This in combination with high very TME, 5382 Kcal/Kg, and GI health benefits, gives Mycomeal tremendous potential as a feed supplement. At least 130 CFUs/L of TS should be used optimal bioreactor efficiencies. Lower CFUs will eventually yield similar biomass results, but more time will be needed to reach equivalent yields. It appears that this system could significantly enhance the economic viability of dry-grind corn to ethanol production.



A Gallus gallus domesticus rooster enjoying 100% Mycomeal

CHAPTER 4. CONTAMINATION IDENTIFICATION AND QUANTIFICATION

1. Problem

During runs where the temperature exceeds 32C we observe little to no growth. In fact the T would continue to rise up to 55C if the bioreactor was not emptied. The increased temperature is almost certainly due to the metabolic processes associated with growth of unwanted bacteria and/or yeast in our growth media. Initial plating test done on contaminated samples revealed at least 2 morphologically distinct organisms that were present in high populations in comparison to the desired *R.m.o.* This is not saying that there are only 2 present, but rather there are at least two different organisms that maybe responsible. Another phenomena that can be observed when batches are left in the bioreactor for extended periods of time with elevated temperatures is biomass reduction. Good growth at low temperatures for the first 24-36h followed by temperature elevation leads to reduction and then disappearance of *R.m.o.* biomass.

For industrial implementation our process needs to be semi-continuous or continuous. This prevents the need to go through the aforementioned process each time we want to grow a reactor full of fungus. Unfortunately, to date, have only had 1 semi-successful semi-continuous run. Contamination is to blame for the failure in all-unsuccessful cases. We need to identify what is

contaminating our bioreactor, where it is getting in, and how we can control it.

Improvements

We detected contamination in the 50-L on multiple occasions, thus we decided to remove this step and directly inoculate the 1600L bioreactor with the 2-L shake flasks. This resulted in successful fungal growth on every attempt. However, with this method we witnessed a delay in growth inside the 1600L, and quantifiable growth did not occur until the 30+ hr mark.

The delay in growth subsided when we switched over to a new box of spores that produced mycelial matrices instead of the fungal pellets (see Pic. 1). These flasks presented us with good growth after 20 hrs. and quantifiable growth after only 13hrs.

Importance

Considering that our research has made it to the pilot scale and presents auspicious results, the next progression would be industrial implementation. There are several investors that have very strong interests in commercializing this process. However when asked about contamination I could only give them vague guesses onto what organism/organisms are responsible for the contamination that results in failure/disappearance of fungal biomass.

2. Methods

Collection of sample from the 1600L bioreactor

9. Empty out the bleach solution
10. Open proximal valve and allow the sampling pipe to fill, close proximal valve, open distal valve, and drain pipe 2x. Use strainer on 2nd time to see biomass
11. Quickly and completely open the valve proximal to the column while keeping distal valve closed. Partial opening may restrict biomass giving skewed result
12. Shut proximal valve after 1-2s
13. Dose the nipple with ethanol
14. Open distal valve and quickly empty sample into autoclaved 250ml sample
15. Quickly cap the container, label, and place in the fridge
16. Fill the sample port with a concentrated bleach solution (recipe 200ml 6% NaClO and 1000ml water)

Plating

Immediately after collecting a sample, it was put in a cooler full of ice, and driven to the lab. All plating was done in a biohood on YM agar plates. An hour typically elapsed from time sample was taken until time the plates were placed in the incubator.

SOP for dilution plating

1. Turn on hood and clean with 80% ethanol
2. Shake or vortex sample bottle after removing it from cooler
3. Fill each of the dilution tubes with 9.9ml of .85% NaCl solution.
4. Take .1ml from the sample. In events of thick growth it will be hard to pipette out this small amount due to the fungal filaments. I found that pouring some sample into the inside of the lid would help .1ml attainment.
5. Unscrew 15ml test-tube lid and place .1ml sample inside. Rescrew cap
6. Vortex the test-tube and take a .1ml sample from this tube in put into next test-tube.
7. Repeat 5 and 6 until all desired dilutions have been formed
8. Using 3 plates/dilution tube, vortex desired dilution test-tube, and take .1 ml and ejaculate onto YM agar plate located on plate spinner.
9. Using a sterile a hockey stick, spread the .1ml evenly over the YM media. Repeat for each dilution plate.
10. Place plates in incubator at T=35C

SOP for species isolation

1. Remove plates from the incubator
2. Under biohood open plate with sterile loop obtain isolated colony
3. In 4 quadrants streak the isolated colony over the YM agar plate
4. Place back in incubator at T=35C
5. Repeat process to ensure isolation.

Entry Point

Plating results revealed that no contaminants are present in the spore stock vials, the 2L autoclaved shake flasks after >24h spore growth, or the thin stillage itself. This tells us that the contamination is coming from transfer of the TS into the 1600L, inside the 1600L bioreactor, the air supply, or the 1600L inoculation process.

Rhizopus microsporus var. oligosporus spores

Freeze-dried culture of the fungus *Rhizopus microsporus var. oligosporus* was obtained from the American Type Culture Collection (ATCC 22959, Rockville, MD, USA). Stock cultures were maintained on yeast malt (YM) agar (Teknova, Hollister, CA, USA).

SOP for spore collection and storage

1. Inoculated plates with .1 ml spore stock and incubated at 25°C for 3-4 d
2. Add dilution water containing 0.85% (w/v) NaCl and 0.05 % (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA) to the plates and scrape growth from plates and collect in beaker.
3. Take spore suspension and filter through glass wool in aseptic conditions and collect filtered spore suspension
4. Mix spore suspension with yeast malt (YM) broth (Himedia Laboratories, India) in 1:1 ratio (v/v).
5. Add glycerin (20% [v/v]) to the spore suspension
6. Place in sterile 2-ml cryovials and store in ultra-low freezer @ -80°C

Half way through the research we had a change in personnel who prepared the spore vials. Oddly when using vials prepared by the new personnel, we witnessed different fungal morphologies in 2L shake flasks. Traditionally spores would grow into spheres, pellets, with small hyphal projections coming out of all sides. In contrast, the new spore vials produced a web of

fungal biomass, a fungal matrix, which is visually distinct from the fungal pellets seen previously. Also we saw much more robust growth in the flasks containing the fungal matrices. It was observed that if the 1600L bioreactor was inoculated with the spherical spores it would take ~24h to see growth. Contrariwise inoculation with the fungal matrix containing flasks produced similar results within 13h.

In attempt to understand why this had occurred experiments were conducted to look at colony forming units (CFUs) in both spore vials. Triplicates were performed on 3 individual vials of each of the 2 types tested. Following the dilution plating procedure described about, results showed that the pellet forming vials contained 2553 CFU/ml while the matrix forming vials contained 12330 CFUs/ml. The difference in spore density is thought to cause the morphology shift.



Pic. 1. Difference between 2 spore stock growths at 15 h

Pic. 2 24h spore growth (pellets morphology)

Isolation

Contaminates were isolated from contaminated contents of the 1600L bioreactor by methods described above. The above-mentioned species isolation method was engaged once morphological monocultures had been isolated on YM plates. After mature colonies were formed (24-48h) a loop was used to scrape individual colonies to obtain DNA template for PCR. The biomass was placed in 100 μ L of PrepMan Ultra (Applied Biosystems Forest City, CA, USA) and vortex for 30s. The vials were then placed in the Thermocycler (MJ Research Inc. HBA-1600 Waltham, MA, USA) with the following cycling conditions: 56C for 30 min, 100C for 10min, followed by a 12 h hold at 4C. The vials are then spun for 2 min at 12000rpm, and 50 μ L of template was transferred to new tube. This formed the template needed for PCR.

PCR

The ITS region of the 18s ribosomal DNA was amplified by the primer pair of ITS-1F and ITS-4, prepared by Iowa State University DNA sequencing synthesis facility. The amplification reaction consisted of 25 μ L DI water, 10 μ L 5x buffer, 1 μ L of 10mM dNTPs, 8 μ L of 25mM MgCl₂, 2.5 μ L DMSO, 1 μ L of 50 μ M ITS-1F, 50 μ M of ITS-4, .5 μ L of 5U/ μ LTaq, and 1 μ L DNA template. After aliquoting out and adding the template, vortex vials for 30s. Vials were

placed in the Thermocycler (MJ Research Inc. HBA-1600 Waltham, MA, USA) with the following cyclers conditions: 1. 55C for 2 min, 2. 94C for 95s for initial denaturing, 3. 58C for 60s for annealing, 4. 72C for 80s for extension, 5. 95C for 70s for denaturing, and steps 3-5 were repeated 35 times. This is followed by 52C for 60s for final annealing, 72C for 15min for final extension, and a 4C hold. The PCR product was purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Buckinghamshire, United Kingdom) and quantified with Spectrophotometer (NanoDrop ND-100 Wilmington, DE, USA). Automated sequencing was performed at the Iowa State university Sequencing and Synthesis Facility. Then a blast search was performed at the National Center for Biotechnology Information, and contaminants were identified.

Fungal Culprits

After sequencing, the results showed that 2 different yeasts were present in samples taken. One was identified as *Pichia kudriavzevii* strain RCEF4907 (basionym *Issatchenkia orientalis*) with 384/385bp matching. Identification used the 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. *P. kudriavzevii* grown on YM agar for ~24hrs at 35C. Morphologically on the

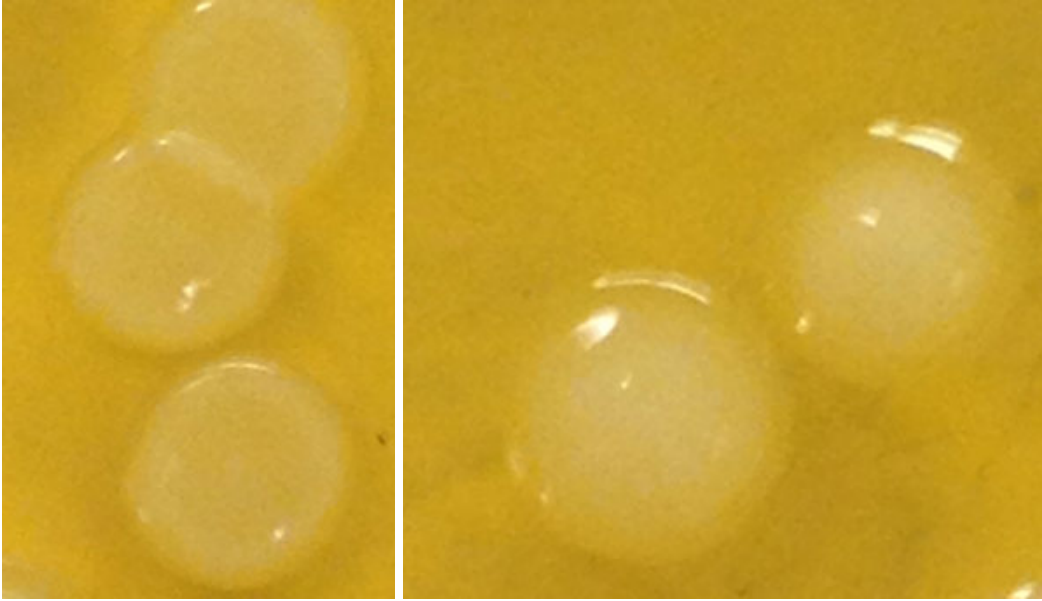
agar the yeast produces a circular colony with a raised white dot in the center of the circle.

From the 50L bioreactor, plating of samples revealed another eukaryotic organisms. This was morphologically distinct in that colonies appeared as a salmon orange and bright pink color. Populations of this organism were seen in very high numbers however this might have been inflated by untimely plating to of samples (2 days at 10C). PCR results identified this to be *Rhodotorula mucilaginosa* strain AUMC 7248 via 18s ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. 571/575 base pairs compared matched. As mentioned earlier the 50L was eliminated from the process, and this organism has not resurfaced itself since.

Bacterial culprits

For prokaryotic organisms universal bacterial primers were used for PCR reactions. After unsuccessfully producing PCR product on numerous attempts, Fatty Acid Methyl Ester (FAME) identification method was used. Based on results, 2 *Bacillus* species were identified with confidence. *Bacillus megaterium*-GC subgroup A was identified with a Sim Index of .803. Morphology of *B. megaterium* on YM agar is an off white circular colonies that are slimy when touched with a consistency that mimics semen. The

second, *Bacillus cereus*-GC subgroup A was identified with a Sim Index of .567. Morphology of *B. cereus* on YM agar is off-white circular colonies, with a depression in the middle making the colonies resemble a doughnut.



B. cereus (l) and *B. megaterium* (r) grown for 24h @ T=35C on YM agar

Contaminate quantification

Here are results from our last contaminated run @ 24hr saw a mixture of *Bacillus megaterium*-GC subgroup A and *Bacillus cereus*-GC subgroup A in ~1:1 ratio. Dilution plating revealed 1.56×10^7 CFU/ml @45.33h growth and 3.13×10^8 @69.16h growth.

Rhizopus oligosporus

Isolation was accomplished by taking organisms directly from the spore vial, plating them onto YM agar plates, and then isolating individual colonies and transferring to new plate (2X minimum). There were concerns about whether the 2 morphologies produced were the exact same organism. The

DNA sequences revealed that both morphologies were the same organism, *Rhizopus microsporus* isolate F6-03. This was determined by the 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence, and 28S ribosomal RNA gene, partial sequence. 438/454 base pairs matched and all of the 16 unmatched pairs were at positions where an N had been placed.

Discussion

Typically the spike in temperature not only means an inhibition of growth, but also reduction or elimination of fungal biomass. However in one run the temperature spike up to 55C but we still has a significant amount of biomass in the reactor. We had trouble explaining this until we identified the contaminates. As it turns out *Bacillus spp.* produce a chitinase that degrades the chitin in the hyphal biomass. This leads to the degradation of hyphae and thus reduction in biomass. If the bioreactor is only contaminated with yeast the biomass quits accumulating when $T > 40$ but is not destroyed.

It would seem that as long as the contamination can be controlled that a semi-continuous or continuous system is feasible. Maintaining the *R.m.o.* monoculture in the bioreactor is of the utmost importance when considering these systems. However, I hypothesize that co-culturing could be very useful if successful co-cultivation can be achieved by growing organisms that have varying enzyme catalogs, and biomass properties. This could be developed

into a system that produces fungal biomass tailored to needs of the animals that the biomass is being fed to. One example such example would be the use co-culture to produce a fishmeal for salmon. Astaxanthin has been increasingly used as a feed and food pigment in the aquaculture industry, and also regarded as a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity [1-3]. Astaxanthin can be produced by *Mucor circinelloides* [4]. *M.circinelloides* can be successfully cultivated on TS with high levels of lipids in its biomass [5]. Both the Astaxanthin and the extra lipid content would add to the proteinaceous *R.m.o.* biomass value and viability as salmon fishmeal. Many other examples exist.

Sources

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Addendum

Rhodoturula 'O'

TAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTaGTGAATATAGGAC
GTCCAACCTTAACCTGGAGTCCGAACCTCTACTTTCTAACCTGTGCAYTTGTTTGGGATAGTAACTCTCGCA

AGAGRGC GAAC TCTATTCACTTATAAACACAAAGTCTATGAATGTATTAATTTTATAACAAAATAAAAC
 TTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
 AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCCTCCATGGTATTCCGTGGAGCATGCCTGTTG
 AGTGT CATGAATACTTCAACCCTCCTCTTTCTTAATGATTGAAGAGGTGTTTGGTTTCTGAGCGCTGCTGGC
 CTTTASGGTCTAGCTCGTTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGCGTAATAGAC
 TATTGCTGAGGAATTCTAGTCTTCGGACTAGAGCCGGGTTGGGTTAAAGGAAGCTTCTAATCAGAATGTC
 TACATTTTAAGATTAGA

RHIZOPUS 'RO'

ATATAGGCTATAATGGGTNNGCCTGTTCTGGGGTTTGATCNATGCCAATCANGATTACCTTTCTTCTTTG
 GGAAGGAAGGTGCCTGGTACCCTTTACCATATACCATGAATTCAGAATTGAAAGTATAATATAATAACAAC
 TTTTAAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTANCAAAGTGCATAACTAGTGTGAATTG
 CATATTCGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGGATCTTCTATAGAGTACGCTTGCTTC
 NNTATCATAACCAACCCACACATAAAATTTATTTTATGTGGTGATGGACAAGCTCGGTTAAATTTAATTAT
 TATACCGATTGTCTAAAATACAGCCTCTTTGTAATTTTCATTAATTACNAACTACCTAGCCATCGTGCTTT
 TTTGGTCCAACCAAAAAACNT

White Dot 'WD'

ATTACTGTGATTTAGTActaCAcTGcGTGAGCGGAACGAAAaCAAAAaCaCcTAAAATGTGGAATATAgCATAT
 AGTSGaCAAGAGAAaTcTAcGAAAAACAAACAAAACCTTCAACAACGGATCTCTTGGTTCTCGCATCGATGA
 AGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT
 GCGCCCTCGGCATTCGGGGGGCATGCCTGTTTGAGCGTCGTTTCATCTTGCGCGTGCGCAGAGTTGGGG
 GAGCGGAGCGGACGACGTGTAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGcGAAgCTGGCCGAGCG
 AAcTAGACTtTTTTtCAGGGACGcTTGgcggcc

CHAPTER 5 GENERAL CONCLUSIONS

Mimicking the industrial revolution, an automotive revolution has begun to take hold. A shift is occurring where automotive companies are focused on developing cars that have a lower environmental impact, in the form of increasing energy efficiencies available in fuels. Hybrids are being developed combining internal combustion engines with electricity to maximize energy efficiencies. Vehicles are developing versatility in the types of fuels they can use; almost all new vehicles are E85 capable. Currently infrastructure is beginning to transition from an one that relies solely machines powered by polluting non-renewably fueled vehicles to one powered by biofuel. This is not limited to cars and trucks but all types of vehicles, motorcycles, ATVs, military, boats, planes, and construction equipment. Ethanol is rooting itself into the fuel sector.

Considering the need, importance, and viability of the research aforementioned, industrial potential is strong. One of greatest assets of this process is its simplicity. No chemical modification is necessary for growth media and air is relatively simply filtered and diffused. The fungus is generally regarded as safe by the FDA and has been medicinally used by humans for centuries. *R.m.o* demonstrates rapid growth in low retention time, which is impressive. There is still huge potential for optimization in the form of co-feeding and/or co-culturing. Co-feeding could lead to increased biomass yields, customizable biomass composition and/or directed enzymes production. Co-culturing could be accomplished by growing organisms simultaneously whose biomass can be combined to be more complete feed supplement. This could lead to customized feeds for specific target markets that

contain desired AAs, FAs, pigments, and antimicrobial substances. The chitosan contained within the cell walls also has economic potential. Continuous or semi-continuous systems will have to be developed. Pure oxygen or directed gas diffusion should also be examined for economic viability. Overall this has huge implications to the biofuel industry and could seriously benefit the process. Don't forget that R.m.o biomass is generally regarded as safe for human consumption and could provide protein to humans, in protein starved regions of the globe.



Myself and Professor Hans van Leeuwen eating mycomeal

Renewable Energy and Hawaii

Renewable energy makes the most economic sense in places with high-energy costs, because the energy produced is more valuable. Couple that with an abundant natural resource (sun, wind, waste), then it can actual become a profit generating system (ex solar panel over its lifetime). Add into the mix that the location is extremely isolated, renewable energy should be obligatory. On Kauai HI the electricity prices are highest in the country at .48/kWh, with 4.5-7 kWh/m²/day (nrel.gov). Hawaii is extremely isolated so not only are non-renewable energy sources expensive to get here, but are also more environmentally taxing. Considering the gas prices and sugarcane potential in HI; ethanol should rule to fuel pumps. This would develop a sustainable, renewable fuel supply which would be convenient if times arise where shipping fossil-fuel out to the islands doesnt rank high on the totem pole. In other words produce energy security, in particularly susceptible location. Ethanol production would not only create quality jobs, but also keep plenty more money in the state. I want to help make Kauai a prototype for completely sustainable energy system. This will be achieved by ethanol production from sugarcane, biodiesel from WVO and optimized seed oil, and solar and wind for electricity generations. Methane via anaerobic digestion should also be implemented.

Autobiography

I was born in Harlan, Iowa on May 8th 1985. I grew up with my mother in 2 sisters and lived in Harlan until I graduated from Harlan Community High School in 2004. Here I was captain of the 2003 state champion football team and achieved a 3.5 gpa while accumulating 20 college credits through Iowa Western Community College. For the summer of 2004, I moved to Des Moines, IA and attended Des Moines Area Community College summer semester. In Fall 2004 I enrolled at Iowa State University in the mechanical engineering program. After a year of unsuccessful academics, I moved to Okoboji IA and worked as a bartender and waiter in the fine dining industry for the summer. In November of 2005, I decided to move to Hawaii. Despite making a substantial amount of money over the summer, I left for HI with my backpack, \$350, and my diamondback mountain bike. I didn't know one person in HI, I didn't have a place to live, I didn't have a job, but most importantly I didn't have fear. After 3 weeks of island hopping, mountain biking, and camping I ended up on the island of Kauai, with only \$20 left in my pocket. I realized a job was imperative and found 2 good one, routinely riding my mountain bike 25 miles for one work place to the other. Homeless, after work I would pop up my tent on the day's beach of choice. After a few weeks I had saved up enough money to buy a 1987 Jeep (\$600). I lived in the Jeep for couple more weeks before I was able to get money in-line for housing. After a year I realized that serving rich tourist food and beverages was not my life calling, and I decided to a return to school was obligatory. So for summer of 2006 I returned to Okoboji to work and then returned to Kauai enrolling at Kauai Community College in fall 2006. Here I discovered that I had an interest in biology. Realizing that the KCC could no longer satisfy my educational requirements, I enrolled at the University of Hawaii at Manoa for Spring 2007. Here my interest in biology became a passion. I work 35 hrs/wk at a 5 star restaurant where I was required to wear a full tuxedo. Despite having my computer stolen a week before finals and having surgery that required 22 staples to hold my abdominal cavity together, I still managed a 3.5 GPA. Realizing that cost of living required me to work too much I decided to once again migrate to Okoboji for the summer and worked all summer, including 50 straight nights, in order to stockpile some cash. In the fall 2007 I enrolled at the University of Oregon. At UO I completed my BS in biology with a minor in chemistry and spent a summer at Oregon Institute of Marine Biology. Realizing that renewable energy has an imminent place in the future of humans and well being of the earth, I came full circle and returned Iowa State University to obtain Master's degree in Biorenewable Resources and Technologies. I was a research assistant, and worked on the project you have just read about. While in grad school I bought and restored a house in Des Moines. I then accumulated 2 more properties a 3 acre farm in Exira and a commercial storage shed in Mingo. I moved back to Hawaii in the fall of 2012 in hopes to lead HI to energy independence via renewable energy. The first mission is to do this in the form of a restaurant/bar on Kauai by changing how restaurants think about energy. Hopefully contributing to the renewable energy revolution on the island.