PROTEIN-RICH FUNGAL BIOMASS CULTIVATION ON AGRO-INDUSTRIAL WASTES/RESIDUES FOR AQUACULTURE FEED PRODUCTION WITH SIMULTANEOUS ORGANIC REMOVAL

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ABSTRACT

Global aquaculture industry faces an ever increasing challenges of acquiring feed that is cost efficient yet environmentally sustainable. Single cell protein (SCP) from the edible fungus *R.oligosporus* has high potential as aquaculture feed as it is nonpathogenic to humans and has high essential amino acid and fatty acid content for fish feed. Additionally, to minimize cost and promote sustainable development, fungal protein can be cultivated on low-cost wastes/residues, preferably from agricultural industries which are high in organics and nutrients. In this research, fungal biomass was investigated for its ability to grow on variety of agro-industrial wastes/residues. Sugarcane molasses, unmarketable papaya juice, and sugarcane vinasse were examined for their potential as substrates. Efficiency of organics removal, quantified as soluble chemical oxygen demand (sCOD) was also examined to determine feasibility of the process as a bioremediation technology. Small scale optimization studies showed that the fungus can successfully be cultivated on all three of the agro-industrial wastes/residues. Molasses, however, yielded the highest specific fungal biomass of 0.41 ± 0.02 (g biomass/g sCOD removed) at COD concentration of 50 g/L, and pH of 5.0. Both molasses and vinasse achieved fungal pellet growth, while papaya juice only supported free mycelial growth.

Sugarcane molasses was selected for the bench-scale studies to further demonstrate the feasibility of the bioremediation process. Fungal fermentation was conducted in two 2.5-L working volume bubble column reactors. Maximum fungal biomass yield of 4 grams of dry biomass per liter of molasses was achieved after 48 hours of cultivation. Organics removal of 56 ± 4.23 % (quantified as % sCOD removed) as well as significant solids and nutrients removal were also obtained. However, bacterial contamination was detected beginning at 16 hours post

spore inoculation, and may have assisted the organics and nutrient removal. Molasses-derived fungal biomass had a protein content (38%), essential fatty acid profile and *in vitro* protein digestibility (~80%) comparable to that of commercial fish feed. Importantly, lysine, a limiting amino acid in fish feed, was in high amount (8.6%).

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CHAPTER 1: INTRODUCTION

1.1 Background

During 2017-2020, the global aquaculture feed industry is predicted to experience a steady compound annual growth rate (CAGR) of 8-8.5% due to the growing aquaculture industry (1). Global demand for fish and seafood is increasing rapidly, owing largely to the rising middle-class income levels in emerging countries such as China, India, and Brazil. To meet this demand, within the next five years, around 52% of the global fish supplies are predicted to be from farmed fish. Moreover, the global aqua-feed market, which accounted for USD 54.41 billion in 2016 is expected to reach USD 98.15 billion by 2022 (1). However, the expanding aquaculture industry faces an increasing challenge of feeding farmed seafood and fish with nutritious yet economically and environmentally sustainable alternative diets (2). In aquaculture, 50% of the production cost comes from feed and fishmeal is the single most important source of protein in aquaculture feed (3). Unfortunately, over the past two decades, fishmeal price has quadrupled to more than USD 2000 per metric ton (4) while worldwide depletion of marine fisheries has been resulting in the global shortage of fishmeal (5). For instance, the Peruvian anchovy fishery, the world's largest fishery for fish feed, is constantly in threat in El Niño years and therefore, during such years, the price of fishmeal in the marketplace can fluctuate drastically (6).

As a consequence of the rising fishmeal prices, aquaculture farmers have been turning into more affordable protein from plant sources. Soybean meal is one such protein source that contains around 47-50% protein, which is comparable to that of fish meal (7). In fact, soybean has become a key ingredient in aquaculture feed in recent years. However, although soybean

meal has a high lysine content, it is deficient in other essential amino acids for fish feed, including sulfur-containing amino acids and tryptophan, (7). Soybean meal also has high levels of anti-nutritional components such as protease inhibitors, allergens, oligosaccharides, lectins, and saponin (8). Amino acid deficiencies coupled with anti-nutritional components can have adverse effects on digestibility of nutrients and performance of fish (8). Additionally, negative ecological footprint of crop feed arises from a large amount of land conversion and the destruction of ecosystems. In South America, for instance, almost 4 million hectares of forest are razed yearly and converted to agricultural land for soybean cultivation for aquaculture feed alone (9).

As traditional feed components - fishmeal and soybean meal - are scarce yet costly, and nutritionally deficient yet environmentally unsustainable, respectively, current developments in aquaculture feed production are looking into alternative sources of proteins. Materials from krill, seafood by-products, protist, and fungi (10), and land animal proteins such as feather meal and meat and bone meal have been suggested as feasible replacements (11). Krill is, however, a costly limited resource while land animal proteins in aquaculture feed are restricted in some parts of the world, particularly in the European Union due to potential bovine spongiform encephalopathy (BSE) outbreak (12).

Amongst the suggested alternative protein sources, single cell protein (SCP) from fungal biomass has high potential as aquaculture feed. SCP is defined as “the dried cells of microorganisms such as bacteria, yeast, molds, algae, actinomycetes and higher fungi grown in large-scale culture systems for use as protein sources in human foods or animal feeds” (13). The SCP fungal biomass has essential amino acid content comparable to that of commercial

aquaculture feed (fishmeal and soybean meal), with exception of methionine and phenylalanine. Furthermore, the biomass is rich in lysine and tryptophan, the most important amino acids in aquaculture feed, as well as essential fatty acids (14)(15). Fungi also have the advantage of yielding high amounts of protein over short periods of time while not competing with human food sources (16). A feasible SCP is an edible filamentous fungal species *Rhizopus microsporus* (*var. oligosporus*) which is given Generally Recognized as Safe (GRAS) status by the U.S Food and Drug Administration (FDA) (17). The fungus is traditionally used as a starter culture for the traditional Indonesian delicacy tempeh (18), yet has recently been explored for fungal bioremediation and protein production (19).

Additionally, to minimize cost and promote sustainable development, fungal protein can be cultivated on low-cost wastes/residues, preferably from agricultural industries which are high in organics and nutrients (20). It is especially of importance in the case of tropical regions such as Hawaii that generate tremendous amount of biomass from food, agriculture, and forestry industry, compared to other parts of the world (21). The state of Hawaii has made a resolution aimed at achieving zero-waste by 2045 through waste/residue bioconversion, including conversion into protein-rich animal feed (22). Hawaiian Islands produce a wide range of agricultural feedstock that include food crops such as sweet potato, papaya, and sugar cane (23). Sugarcane distillery vinasse, cane molasses, as well as unmarketable waste papaya fruits are all abundant yet low-cost feedstock that can be utilized as substrates for fungal biomass production in the Islands.

1.2 Objectives of the Study

Based on these rationales, the overall objective of this research is to investigate the feasibility of protein-rich fungal (*R.oligosporus*) biomass production on low-value agro-industrial /wastes/residues for aquaculture feed application and bioremediation. Specific objectives of this research are to:

1. Determine the feasibility of the agro-industrial wastes/residues – sugarcane molasses, papaya juice, and sugarcane vinasse – as substrates for fungal biomass cultivation through batch optimization studies.
2. Evaluate the fungal fermentation in a bioreactor system for a process scale-up and assess organic removal efficiency
3. Determine the chemical composition, nutritional value, and digestibility of the fungal biomass for aquaculture feed applications

CHAPTER 2: LITERATURE REVIEW

2.1 Value-added Processing of Agro-industrial Wastes/Residues

2.1.1 Background

Agro-industrial wastes/residues include negative to low-value waste and residual materials from manufacturers of food, beverage, tobacco, textile, clothing, wood products, paper, paper products and printing, and rubber and rubber products. These wastes/residues are generally nutrient-rich, yet low strength in terms of toxicity (24). Agro-industrial wastes/residues are attracting ever-expanding interest as readily available yet cheap renewable substrates for production of chemicals, materials and biofuels. It has been estimated by the US Department of Energy that up to 500 million metric tons of such raw materials can be readily available each year in the United States for a price tag of 20-50$/metric ton (25). In the United States alone, more than 15 million metric ton of solid wastes are generated yearly from fruit and vegetable processing industries (26), while 80 billion gallons of wastewater are produced from the same industry each year (27).

Unfortunately, a great portion of the food processing wastes/residues is treated as waste and pose disposal problems for the associated industries (28). Often, these waste and residues are either dumped into municipal bins, or are left to rot due to lack of proper infrastructure to handle such quantity of biomass and established commercial utilization. Aside from adding extra cost to the processors, disposal of such wastes/residues directly into the landfill and soil may cause serious environmental issues (29).

On the other hand, as wastes/residues from the fruits and vegetable industries usually contain soluble sugars, organic acids, vitamins, and various nutrients, these can be bioprocessed into production of variety of valuables such as fuels, chemicals and animal feed (25)(30). However, such resources need to be treated with methods that have minimal impact on the environment as well as human health and enable recycling of organic waste material. Food production continues to consume excessive amounts of energy, water, and nutrients; thus, recycling of organic matter should be explored beyond the scope of using food waste as an energy source (31). Therefore, bioconversion of fruit and vegetable processing waste/residues into value-added products can be a sustainable solution for reducing environmental pollution and improving food security (29).

2.1.2 Sugarcane Molasses

Sugarcane molasses, a by-product of the sugar crystallization process, is a viscous dark liquid rich in sucrose (50% to 60-63%), suspended colloids, heavy metals, vitamins and nitrogenous compounds (32)(33). It is a relatively inexpensive yet abundant raw material. Each metric ton of cane yields approximately 3.0 percent of molasses, with a global yearly total production of 16 million metric tons (34). Today, Brazil, India, and China are the major producers of sugar cane molasses, with Brazil alone accounting for more than half of all cane sugar exports (35). Additionally, molasses is the main substrate for ethanol production in India (36).

Aside from ethanol, molasses has been utilized as a substrate for the production of a number of industrial important chemicals, such as sorbitol (37), lactic acid (33)(38)(39), citric acid (40)(41) polysaccharide (32), welan gum (42), succinic acid (43), trehalose (44),

biosurfactants (45), astaxanthin (46), poly e-L-lysine and poly L-diaminopropionic acid (47), lactic acid (48), L-ornithine (49), erythritol (50), poly-L-malic acid (51), 2,3-butanediol (52) and butanol (53). Enzymes including invertase (54), and alkaline protease (55) have also been produced on molasses. It is also the principal carbon and energy source for the production of a SCP *Saccharomyces cerevisiae* (baker's yeast) (13).

2.1.3 Sugarcane Vinasse

Distillery industry, on average, generates 12-16 L of effluent spent wash (also commonly named vinasse) per liter of alcohol produced (56). It is a negative value residual generated during alcohol production characterized by its dark color, high temperature, low pH, high organics content, and potassium. Due to its high organics and nutrient profile, disposal of the liquid to the environment would pose severe environmental problems (57). As such, fertirrigation (fertilization and irrigation) has been the most commonly utilized disposal method. The considerable amount of potassium in the effluent not only acts as a fertilizer, but also reduces water input for plant growth (58). However, fertirrigation has harmful effects on soil and groundwater in the long run (59). Continues disposal of the effluent on crops increases the risk of soil salinization, metal leaching, phototoxicity, odor nuisance and alterations in soil quality, including nutrient imbalance and reduced alkalinity (60)(61)(62).

As an alternative, a few large-scale management have been operating adequately, such as vinasse recycling to fermentation streams (64), energy (65)(53), and animal feed production (64). Cristiano E. Rodrigues and Bo Hu have reported that a recent development of vinasse utilization have been focusing on production of large variety of valuables including bioemulsifiers (65), biopolymers (66), biofuel (67), enzymes (68), microbial biomass (69),

fungal biomass (62)(70), algae (71), laccase (72), and much more (73).

2.1.4 Waste from Unmarketable Papaya Fruits

The state of Hawaii commercial papaya production is a USD 8.2 million industry which produces in excess of 25 million pounds (~11'300 metric tons) fruits annually (74). Such unmarketable papaya account for 35-50% of the total fruit production, which translates to a huge revenue loss for the growers (74). As reported by Matthew K. Loke and Pingsun Leung, the average post-harvest waste of fresh fruits is around 9.7% (75), owing largely to the global obsession with cosmetically perfect looking fruits and vegetables.

The state of Hawaii has an ambitious plan to eliminate all waste by the year 2045 which aims to decrease dependency of Hawaii on fossil fuel and animal feeds import while improving the profitability of Hawaii's agriculture by creating value from its waste streams (22). Currently, a number of research have been focusing on utilizing papaya waste for extracting valuable chemicals such as derivation of protease enzyme (76) and pectin (77) from the peels, and various oils from the seeds (78). Additionally, as the fruit wastes are rich in soluble sugars, organic acids and various nutrients, unmarketable papaya can be utilized for the production of protein enrichment by microbes (79), ethanol (80)(78)(81), or other value-added products, such as polyhydroxyalkanoate PHA (82). Production of single cell protein, particularly yeast production for aquaculture feed supplementation was achieved by Kang, Hsu-Ya, 2007 (82). In accordance with the waste elimination plan mentioned above, the state of Hawaii has invested in the production of biofuels from papaya waste, with the use of heterotrophic algae that would potentially convert waste from the crop into biodiesel (83).

2.2 Single Cell Protein

Professor Carroll Wilson first coined the term "single cell protein" at Massachusetts Institute of Technology in May 1966. The term refers to the dried cells of bacteria, yeast, molds, algae, actinomycetes, and higher fungi produced for use in human and animal diets as protein sources. Single cells protein (SCP) has historical roots in Germany where, during the First World War, approximately 50% of the imported protein sources came from yeast (84). However, SCP as an aquaculture feed is a recent innovation (13). SCP is regarded as a highly promising alternative protein source for inclusion in fish feed (85)(86)(87). It has been found by Hardy (1996) that the well-balanced amino acid profiles of SCP make it a comparable alternative to fish meal when being added to the diet of trout and salmon (88). Aside from the desirable amino acids content, SCP has many other advantages such as fast growth, ability to be produced on industrial waste products, and proficient nutrient content that include B-vitamins, pigments, and β-glucans (89). Anupama et al., 2000 have suggested that the organisms suitable for SCP generally should be non-pathogenic to plants, humans, and animals, feasible as food and feedstock, have good nutritional values, do not contain toxic compounds, and have low production costs (90). Although SCP has high nucleic acid content that may lead to uric acid precipitation, nucleic acid is not a toxic component and it causes problems only when taken in excessive amounts (91).

The most common microorganisms utilized in the production of SCP are bacteria *Cellulomonas* and *Alcaligenes,* algae *Spirulina* and *Chlorella*, fungi *Trichoderma*, *Fusarium*, and *Rhizopus*, and yeasts *Candida* and *Saccharomyces*. One of the advantages of cultivating microorganisms is that they can be grown on variety of substrates such as agricultural wastes and

effluents, industrial wastes, and natural gas (92).

Table 2.1 Comparison of typical values of single cell protein (SCP), fishmeal, and soybean meal contents

Single cell protein[1]	**Protein (% dry weight)**	**Fat (% dry weight)**	**Ash (% dry weight)**	**Nucleic acid (% dry weight)**
Yeast	45-55	2-6	5-10	6-12
Fungi	30-45	2-8	9-14	7-10
Algae	40-60	7-20	8-10	3-8
Bacteria	50-65	1-3	3-7	8-12
Traditional feed[2]	**Protein (% dry weight)**	**Fat (% dry weight)**	**Ash (% dry weight)**	**Nucleic acid (% dry weight)**
Fishmeal	60-72	8 -12	10-17	<1
Soybean meal	47-50	1-3	5 -9	<1

[1] Miller and Litsky (1976) (93)

[2] Rasmussen et al. (2007)(94); Swick (95)

Raw materials containing mono and disaccharides are excellent substrate choices for the production of SCP as most microorganism digests glucose, other pentose and hexose sugars, as well as disaccharides. Potential substrates for SCP include bagasse, citrus wastes, sulfite waste liquor, molasses, animal manure, whey, starch, sewage and many others (92).

2.3 Microorganism

2.3.1 The Kingdom of Fungi

The Kingdom of fungi includes eukaryotic unicellular organisms such as yeasts and molds, as well as more complex multicellular organisms known as mushrooms. Fungi are heterotrophs, much like animals, absorbing dissolved nutrients by secreting enzymes. They have high diversity in preference for growth substrate, and thus, fungi are essential decomposers in nature and play a major role in nutrient cycling. Most fungi are either obligate aerobic or facultative anaerobic, that is, prefer oxygen but will undergo fermentation with oxygen limitation (96). Fungi can be classified into three different sub groups: filamentous fungi (molds), yeasts, and mushrooms.

2.3.2 Filamentous Fungi

The diverse and ubiquitous nature of the fungi makes it near-impossible to circumscribe the filamentous fungi briefly, but it can be described as eukaryotic microorganisms which, for at least some part of their life cycle, display a mycelial growth habit and absorb nutrition from the extracellularly digested material (96). Despite diverse array of habitats ranging from air, water, plants, and animals to organic and inorganic debris, many filamentous fungi find their way into water system (97). Most require comparatively high levels of water to enable vegetative growth (98) and the majority of fungi prefer wet aerobic conditions (99). Therefore, filamentous fungi are suitable for submerged state fermentation (SSF) such as cultivation in wastewater. Additionally, in SSF fungi not only can produce valuables such as protein-rich biomass, biochemicals and enzymes, but also the filamentous nature of the fungi facilitates separation and recovery from the liquid phase (100).

2.3.2.1 Optimal Growth Conditions

Generally, carbohydrates are the major source of carbon. Utilization of simple sugars as carbon sources are adapted by virtually all fungi. Many fungi are also habituated to metabolize complex carbon polymers of plant and animal origin, especially cellulose-based materials (101). However, growth conditions and nutrient preferences vary drastically between species. Fungi can, therefore, grow in a variety of substrates such as wastewater from various agricultural industries. In addition to carbon, another major element needed for fungal growth is nitrogen source from nitrates, nitrites, ammonium or other organic sources. Typically, C to N ratio should be kept at 10:1 although there are some exceptions. Certain fungi are adapted to high N levels, as in the case of *Coprophilus* fungi, while some prefer very low levels as in wood-decay fungi (98).

Aside from nutrients, temperature, pH, and oxygen are other important factors that play crucial roles in fungal growth. Majority of fungi employed by the wastewater facilities are mesophiles that thrive at around 20-40 °C, while preferred pH for most fungi is less than 5.0. In protein production, pH range of 4.0 to 6.0 seems to give the best yields of mycelium and protein. Additionally, since most fungi are obligate aerobes, oxygen levels are critical for fungal growth (102)(103). However, a wide range of genera show the capacity to grow under low oxygen conditions (98). Studies on tempeh molds have shown that optimal growth conditions are at pH values of less than 6.0 (103), temperatures around 40°C, and oxygen concentrations above 1% (v/v) (104).

2.3.2.2 Fungal Growth Kinetics

In liquid culture, single-celled fungi such as yeast follows a typical bacterial growth curve, including the stages of - lag, acceleration, exponential, deceleration, stationary, and decline phases. In contrast, since filamentous fungi lack cell division from each individual cells,

it relies on the hyphal tip for growth, which grows at a constant linear rate and generally follows three stages - lag phase, linear phase, and decline phase (105). Maximum growth rate can only be attained when all the nutrients, including gaseous oxygen is supplied in excess. Growth rate typically declines when any of the nutrients become depleted. Although in most cases, the limiting nutrient is usually the carbon source (such as glucose), in industrial scale fermenters where high biomass densities are desirable, oxygen can be the limiting nutrient. The onset of stationary phase of a fungal culture can be determined by either nutrient exhaustion, lack of oxygen, accumulation of toxic metabolites, or any combination of any or all of these deciding factors (106).

2.3.2.3 Fungal Pellets

Filamentous fungal morphology ranges from freely dispersed mycelia to distinct pellets. Pellets are largely considered a desirable morphology in laboratory studies due to low broth viscosity resulting in Newtonian flow behavior, ease of harvest, low viscosity of the fermentation broth, high yield of some proteins, and elimination of fungal growth around impellers (107). Fungal growth in the form of pellets provides substantial nutrient as well as oxygen transfer and reduces adverse effect on bioreactor performance. Because active growth zone where the growth occurs is around the surface of the pellets, fungal inoculation with a small pellet size is preferable due to the increased surface area (108). The phenomenon of pellet formation is one of the most studied areas in filamentous fungal research due to its' advantages (108). It has been suggested that several factors such as pH, oxygen level, temperature, medium composition, nutrients, and presence of ions are associated with pellet formation (109).

2.3.3 *Rhizopus Microsporus var. Oligosporus*

The filamentous fungus, *R. oligosporus,* belongs to the phylum *Zygomycota*, order *Mucorales*, and family *Mucoraceae* (110). *R. oligosporus* spores are heterogeneous, which have a large size and shape variance. The species has only been isolated from tempeh and other fermented products and never from nature, and therefore have been suggested to be a domesticated form of *R. microsporus* (111). They range in shape from sub-globose to globose and have a size variance of greater than 3 μm (111). The fungus was first described by Dr. Kendo Saito from Tokyo Imperial University in 1905 as being the microorganism most responsible for the fermentation of Indonesia delicacy, tempeh (112). *R. oligosporus* has been widely used in Indonesia in soybean tempeh production since the ancient times (113). During the tempeh production, soybeans are bound by the mycelium, forming a cake, and enzymes from the fungi make the product more digestible by humans (114). It is also used for fermentation of '*ontjom*' from pressed peanuts, and '*bonkrek*' from pressed copra alone or mixed with soybeans (115). Industrially, the fungus has been used in the production of industrial enzymes (116)(117)(118), and treatment of waste and wastewater (119)(118)(119).

The fungus is not associated with the production of any metabolites harmful to humans (110). Additionally, U.S Food and Drug Administration (FDA) recognizes the fungus as safe (GRAS status) (120) and has been classified as a biosafety level 1 (BSL-1) organism.

2.3.3.1 Growth Conditions

It is a *saprobic* microorganism, one that prefers environment rich in organic matter but relatively free from oxygen (121). *R. oligosporus* is capable of utilizing wide range of protein (122) and sugars, including glucose, fructose, galactose, xylose, and maltose. However, Sorenson and Hesseltine (1996) reported that the fungus did not use sucrose and raffinose as growth

substrates (63). Moreover, sugar alcohols such as sorbitol, glycerol, and mannitol may be utilized by the fungus, but are less preferable than the sugars (102). It is also capable of growing on both organic (yeast extract, peptone, and amino acids) and inorganic (ammonium, nitrate, and nitrite) nitrogen sources (63). Graham et al. (1976) have found that maximum mycelial growth for the fungus occurs after 48 hours at 37 °C, and at pH of either 3.0, 4.0, or 5.0. However, a significant growth difference was reported between pH of 5.0 and 6.0 (123).

In conclusion, a large demand exists for economically viable yet environmentally sustainable aquaculture feed. Although plant based feeds such as soybean meal have been an economically competitive option, such feed do not resolve the need for more environmentally conscious alternatives that have minimal carbon foot print. As the agro-industrial sector generates massive amount of nutrient rich residues and waste with high treatment cost, adaptation of biorefinery concept coupled with development of value-added product production is a feasible solution that would also address the global aquaculture feed demand.

Single cell protein from microorganisms such as bacteria, yeasts, algae, and fungi are protein-rich nutrients that can be produced on low to negative cost substrates, including agro-industrial wastes/residues. Commonly used treatment of such residues/wastes add undesirable cost to the associated industry. Therefore, it is a plausible solution to utilize such materials for the production of value-added products. An edible fungal biomass is a viable option as it is rich in protein and safe in terms of toxicity to humans and animals. Protein rich fungal biomass cultivated on agro-industrial residues/wastes have high potential to become sustainable yet economically advantageous alternatives in aquaculture feed.

However, it is crucial to put emphasis on fungal biomass yield and product recovery.

Yield and composition of fungal biomass varies with cultivating conditions, including put not limited to, substrate concentration, pH, temperature, oxygen availability and nutrients. Bacterial contamination is another factor that may adversely affect the nutritional composition of the fungal biomass. To maximize yield and protein content, screening for desirable fermentation condition is of outmost importance.

CHAPTER 3: MATERIALS AND METHODS

3.1 Substrate Preparation

Three different agro-industrial byproducts/residues – sugarcane molasses, sugarcane vinasse, and papaya waste – were examined in the fungal fermentation as potential substrates. Crude molasses was collected from Hawaiian Commercial and Sugar (HC&S) Co. in Maui, Hawaii and kept at 4°C until use. Pre-treatment was conducted with 0.1-0.4% H_2SO_4 (sulfuric acid) hydrolysis at 120 °C for 20 minutes. Vinasse was collected from Manulele Distiller, LLC in Oahu, Hawaii and kept at 4°C until use. Papaya was harvested from Waimanalo Research Station in Oahu, Hawaii. Papaya fruits were then seeded, peeled and blended with food grade blender for homogeneity. Blended slurry was then centrifuged at 15,557 x g for 10 min to extract the juice out. Extracted papaya juice was kept at -20°C until further use.

3.2 Fungal Spore Inoculum Preparation

The food-grade, freeze-dried fungal species, *Rhizopus oligosporus* (*var. microsporus*) was obtained from the American Type Culture Collection (ATCC # 22959, Rockville, MD, USA). Fungal spores were reactivated in sterilized deionized water and cultivated on Potato Dextrose Agar (PDA) (Difco Laboratories, Sparks, MD, USA) plates for 5 d at 30°C. The spores were then harvested with spore suspension solution of 0.1% (w/v) peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). Harvested spores were centrifuged at 6300 × g for 20 minutes, adjusted to concentrations of 4×10^6 spores/mL (based on hemacytometer count (Hausser Scientific, Horsham, PA)) and kept in 20% (v/v) glycerol at -29°C (R 404 freezer, Summit commercial freezer, Bronx, NY, USA) until further use.

Table 3.1 Characteristics of the three agro-industrial wastes/residues

Parameters	Molasses (hydrolyzed, 20 times diluted)	Papaya juice	Sugarcane vinasse
pH	2.50 ± 0.33	5.08 ± 0.12	3.78 ± 0.08
Total solids (TS) (%)	5.19 ± 0.0047	10.45 ± 0.031	2.93 ± 0.10
Volatile solids (VS) (% of TS)	83.89 ± 0040	94.89 ± 0.083	73.81 ± 0.15
Total suspended solids (TSS) (%)	0.48 ± 0.12	0.73 ± 0.0040	0.23 ± 0.026
Volatile suspended solids (VSS) (% of TSS)	85.04 ± 2.39	95.56 ± 1.19	83.66 ± 4.16
Chemical oxygen demand (COD) (g/L)	56.02 ± 2.76	132.96 ± 2.85	55.72 ± 3.24
Soluble chemical oxygen demand (sCOD) (g/L)	52.725 ± 5.01	112.46 ± 2.30	50.05 ± 2.26
Total Kjeldahl Nitrogen (TKN) (mg/L)	375.0 ± 37.5	431 ± 26.52	187.5 ± 25.8
Sucrose (g/L)	1.93 ± 0.96	N/D	N/D
Glucose (g/L)	7.81 ± 0.86	44.5 ± 0.25	N/D
Fructose (g/L)	9.13 ± 1.23	42.81 ± 0.45	N/D
Sulfate (g/L)	3.87 ± 0.21	0.357 ± 0.087	1.85 ± 0.086
P (mg/L)	25.46 ± 1.53	118.65 ± 0.07	56.76 ± 0.47
K (g/L)	2.49 ± 0.081	2.54 ± 0.0092	4.07 ± 0.019
Ca (mg/L)	368.3 ± 10.47	303.25 ± 1.77	277.9 ± 2.83
Mg (mg/L)	221.25 ± 8.27	286.85 ± 0.07	112.4 ± 0.99
Na (mg/L)	328.05 ± 3.84	87.87 ± 0.07	11.07 ± 0.04
Fe (mg/L)	34.80 ± 0.66	0.47 ± 0.014	21.25 ± 0.16
Mn (mg/L)	6.72 ± 0.15	0.07 ± 0.014	3.07 ± 0.00
Zn (mg/L)	0.13 ± 0.014	0.42 ± 0.014	2.86 0.00
Cu (mg/L)	0.18 ± 0.014	0.23 ± 0.00	7.18 ± 0.035
B (mg/L)	0.95 ± 0.41	2.00 ± 0.021	0.07 ± 0.00

N/D – not detected

Mean value ± standard deviation

Sample size (n) = 3

3.3 Experimental Design and Optimization Studies

Full factorial experimental design with substrate concentrations (25 and 50 g/L COD) and pH (4.0, 5.0, and 6.0) as factors were examined for batch optimization studies. All three substrates were diluted to desired concentrations with distilled water. 250-ml Erlenmeyer flasks containing 100 ml substrate were sterilized at 120°C for 20 min (HICLAVE^{TM} HVE-50, Hirayama, Amerec Instruments Inc., Lafayette, CA, USA). Molasses was hydrolyzed with 0.1-0.4% sulfuric acid (H_2SO_4). Substrates were supplemented with ammonium sulfate ($(NH4)_2SO_4$) and potassium di-hydrogen phosphate (KH_2PO_4) as sources of nitrogen (N) and phosphorus (P), respectively, to a sCOD:N:P ratio of 100:5:1. Media were then inoculated with 0.5% (v/v) fungal

spores and incubated in the incubator shaker (New Brunswick Scientific Innova®42) at 37 °C and 150 rpm for 3 days. pH was adjusted daily with 1 N sodium hydroxide (NaOH) and 1 N sulfuric acid (H_2SO_4). After the 3-day run, fungal biomass was collected with USA Standard Test Sieve with 250-µm nominal opening size, thoroughly washed with tap water and dried at 70°C (Lindberg Blue M MO1450A-1) for 24 h until constant weights were measured. Specific fungal biomass yields measured in grams of dry biomass per grams of sCOD removed were reported.

3.4 Bench Scale Fermenter Experiments

Fungal cultivations were conducted in two 2.5-L working volume bubble column bioreactors. Fermentation broth containing 2.5 L of sterile, hydrolyzed molasses, and nutrient ratio of 100 sCOD: 5 N (supplied as $(NH4)_2SO4$): P (supplied as KH_2PO_4) was inoculated with a 5 mL (4 x 10^6 spores/mL) spore suspension. Fungal cultivation was conducted at the optimal conditions found in small scale experiments (pH 5.0, 37°C, molasses COD concentration of 50 g/L) and a 1.5 vvm air flow. pH was adjusted daily with 0.2% sodium hydroxide and 0.2% sulfuric acid. After the 3-day fermentation, wet fungal biomass was collected with USA Standard Test Sieve with 250-nm nominal opening size. Dry biomass weight was taken following a 24-hour incubation at 70°C. Effluent was collected for analysis in a time-series. Biomass yield was determined as g dry biomass/ L of media.

3.4.1 Bioreactor Configuration

Two identical, 2.5 L working volume, bubble column reactors made of clear acrylic plastic with a thickness of 0.5 cm were used in this research. The cylindrical reactors had inner

diameters of 14cm and heights of 40 cm. Porous air diffusers at the bottom of the riser section supplied air, which were passed through 0.1 µm pore size polytetrafluoroethylene (PTFE) membrane filters (Whatman, FlorhamPark, NJ, USA) to minimize contamination.

3.5 Analytical Methods

3.5.1 Mineral Analysis

Mineral analyses were conducted by the Agricultural Diagnostic Service Center (ADSC) at the University of Hawai'i at Mānoa (Honolulu, HI, USA). Samples were analyzed for boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), and zinc (Zn).

3.5.2 pH

The pH of the samples were measured with a pH meter (accumet* AB15+, Fisher Scientific, Fair Lawn, NJ, USA) equipped with pH probe (accuTupH* # 13- 620-183A, Fisher Scientific, Fair Lawn, NJ, USA).

3.5.3 Solids

Solids contents of the substrates were measured following the procedures outlined in Standard Methods (APHA/AWWA/WEF, 2005): total solids (TS) – method 2540 B, total suspended solids (TSS) – method 2540 D, and volatile solids (VS) and volatile suspended solids (VSS) – method 2540 G.

3.5.4 Total Chemical Oxygen Demand (TCOD) and Soluble Chemical Oxygen Demand

(sCOD)

Total chemical oxygen demand (COD) and soluble chemical oxygen demand (SCOD) were measured according to Standard Methods (APHA/AWWA/WEF, 2005) and the US Environmental Protection Agency (USEPA) reactor digestion method (# 10212; HACH Company, Loveland, CO, USA). sCOD was determined by filtering the samples through a 0.45 µm pore size Whatman cellulose membrane filter (Whatman, Florham Park, NJ, USA) prior to analysis. COD measurement was taken by a spectrophotometer (HACH DR5000, HACH Company, Loveland, CO, USA).

3.5.5 Total Kjeldahl Nitrogen (TKN) and Sulfate

Total Kjeldahl Nitrogen (TKN) was determined though the Nessler Method (# 8075; HACH Company, Loveland, CO, USA). Sulfate, measured as SO_4^{2-} was analyzed through HACH method #8051 (HACH Company, Loveland, CO, USA). All readings were obtained by the HACH spectrophotometer.

3.5.6 Dissolved Oxygen

Dissolved oxygen reading was taken every 60 s with an InPro® 6800 Series polarographic 0_2 sensor 12/25 mm (Mettler Toledo, Mettler Toledo Inc., Columbus, OH, USA) attached to Eppendorf BioFlo® 120 reactor (Brinkmann Instruments Inc., Westbury, NY, USA).

3.5.7 Bacterial Contamination Determination

Broth sample was taken in time-series and analyzed for bacterial contamination. Bacterial contamination was measured by incubation on actidione agar (HiMedia Laboratories Pvt. Ltd.,

Mumbai-400086, India) with 0.1 mg/mL cycloheximide (VWR International, LLC, Solon, OH) at 37°C for 24 hours.

3.5.8 Sugar Concentrations

Sugar concentrations were determined with Waters High Pressure Liquid Chromatography (HPLC) (Waters, Milford, MA). An Aminex® HPX-87N, 300 mm x 7.8 mm column (Bio-Rad Laboratories Inc., Hercules, Ca) with 0.01 M disodium phosphate (Na_2HPO_4) mobile phase was used. The peaks were compared to three standard sugars: glucose, fructose, and sucrose.

3.5.9 Biomass Yield and Characterization

For weight analysis, fungal biomass was weighed following a 24-hour incubation at 70°C, until constant weight was read. Yield was reported as g dry biomass per liter of substrate. For biomass characterization, biomass cultivated on crude molasses was freeze-dried, and sent to the Department of Aquatic Feeds and Nutrition at the Oceanic Institute (Waimanalo, HI) for analysis.

3.5.10 *In vitro* Protein Digestibility

The *in vitro* protein digestibility was measured through the pepsin-pancreatin enzyme method (Akesan and Stahman, 1964). Pepsin (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and pancreatin (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) method was followed by the Kjeldahl method (Section 3.5.5) for crude protein determination, using the multiplying factor of 6.25.

3.5.11 Statistical Analysis

The sample analysis of various parameters as well as fungal biomass yield and organics removal tests were conducted in triplicates. Results were interpreted by JMP® Data Analysis Software from SAS Institute Inc. (100 SAS Campus Drive Cary, NC 27513). The statistical significances were determined with a one-way analysis of variance (ANOVA) followed by a Tukey's test. Superscript letters denote significantly different/same results.

CHAPTER 4: RESULTS AND DISCUSSIONS: FUNGAL GROWTH OPTIMIZATION STUDIES

4.1 Rationale

Chemical composition of the three different substrates - sugarcane molasses, papaya juice, and sugarcane vinasse – may have adverse effect on fungal growth. The high organic constituents and viscosity of the fermentation broths could constrain yield as well. Additionally, of the three types of fungal morphology – suspended mycelia, clumps, and pellets – pellet formation is most desirable as it provides the best mass transfer (124). As for the optimal pH, Moore-Landecker (1990) reported that fungi may have two optimal pH ranges as lower pH increases iron availability whereas higher pH supports enzyme activity (102). Therefore, optimization of fungal growth conditions is crucial in establishing favorable conditions for maximum fungal biomass yield. Investigation for the various substrate conditions should be set in a full factorial design to account for possible interactions between the factors of interest.

4.1.1 Full Factorial Optimization Study

Full factorial experimental design with substrate concentrations (25 and 50 g/L COD) and pH (4.0, 5.0, and 6.0) as factors were examined for batch optimization studies for each of the three substrates – sugarcane molasses, papaya juice, and sugarcane vinasse. Prolific fungal growth was observed in all three of the substrates, suggesting that various agro-industrial wastes/residues can be used for fungal cultivation. Overall, organic reduction quantified as sCOD removal (%) was between 24-37%. Pellet formation, however, was only observed in molasses and vinasse samples.

4.1.2 Specific Fungal Biomass Yield

The highest fungal biomass yields of 0.41 ± 0.02, 0.39 ± 0.03, and 0.37 ± 0.02 (g biomass/g sCOD removed) were achieved for 50 g/L COD molasses with pH of 5.0, 25 g/L COD papaya juice with pH of 5.0, and 25 g/L sugarcane vinasse with pH of 5.0, respectively. The least amount of fungal biomass yield was reported from 25g/L COD molasses with pH of 6.0 (0.20 ± 0.03 g biomass/ g sCOD removed), 50 g/L papaya juice with pH of 6.0 (0.21 ± 0.03 g biomass/ g sCOD removed), and 25 g/L COD sugarcane vinasse with pH of 4.0 (0.20 ± 0.03 g biomass/ g sCOD removed) (Table 4.1).

Table 4.1 Specific fungal biomass yield for sugarcane molasses, papaya juice and sugarcane vinasse at various concentrations and pH

	Specific yield (g biomass/ g sCOD removed)		
Factors \ Substrate	Sugarcane molasses	Papaya juice	Sugarcane vinasse
COD: 25g/L, pH 4.0	0.21 ± 0.04	0.25 ± 0.04	0.20 ± 0.03
COD: 25g/L, pH 5.0	0.24 ± 0.02	0.38 ± 0.02	0.37 ± 0.02
COD: 25g/L, pH 6.0	0.20 ± 0.03	0.27 ± 0.05	0.26 ± 0.02
COD: 50g/L, pH 4.0	0.38 ± 0.06	0.22 ± 0.03	0.16 ± 0.05
COD: 50g/L, pH 5.0	0.41 ± 0.08	0.23 ± 0.02	0.31 ± 0.01
COD: 50g/L, pH 6.0	0.30 ± 0.02	0.21 ± 0.03	0.17 ± 0.03

Mean value ± standard deviation
Sample size (n)= 3

The highest fungal biomass yield for all three samples was in the range of 0.37 – 0.41 (g biomass/ g sCOD removed), while the least amount of the yields was in the range of 0.20 – 0.21 (g biomass/ g sCOD removed), suggesting consistency between the three substrates.

For all three substrates, pH of 5.0 was the most suitable pH, while pH of 6.0 resulted in the lowest specific biomass yield. It is in agreement with the fact that *R.oligosporus* prefers low

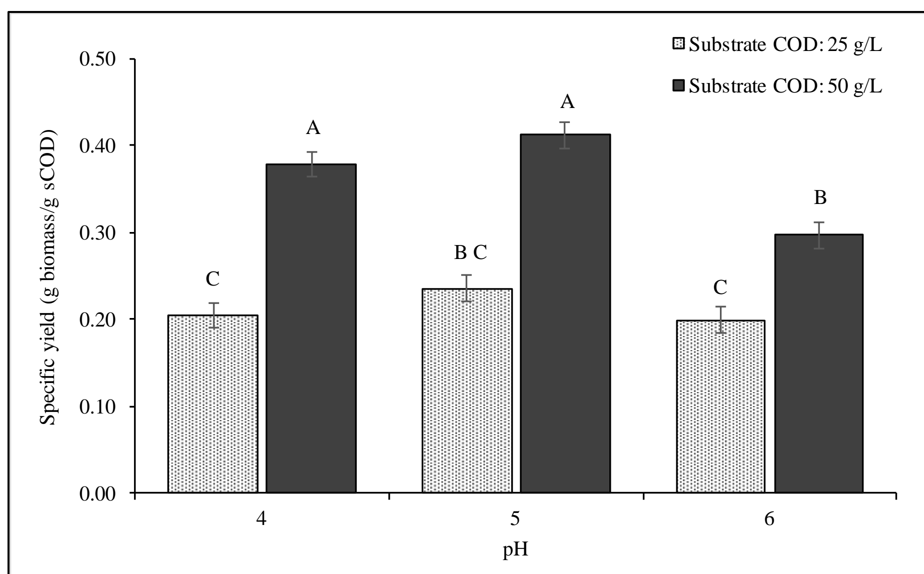

Figure 4.1 Specific fungal biomass yield for sugarcane molasses with various concentrations and pH

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

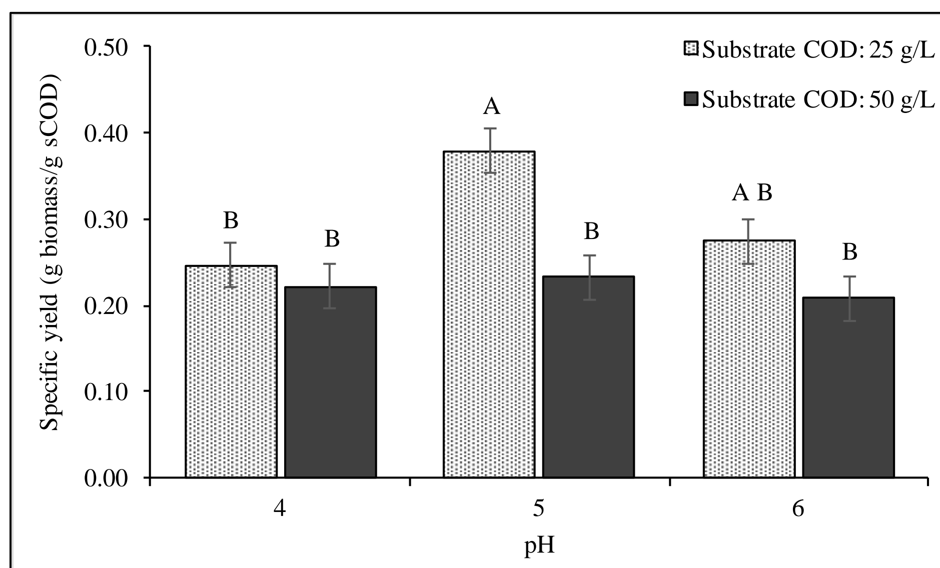

Figure 4.2 Specific fungal biomass yield for papaya juice with various concentrations and pH

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

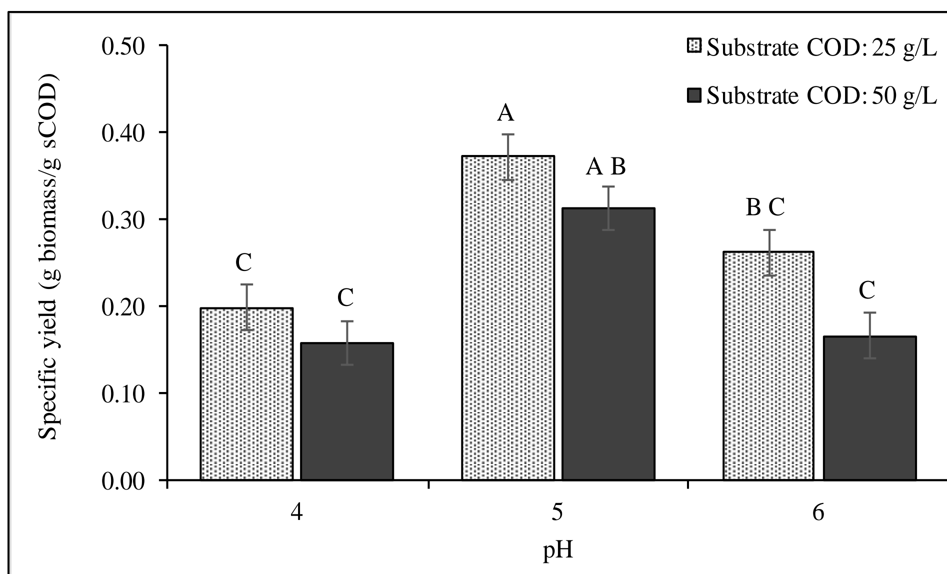

Figure 4.3 Specific fungal biomass yield for sugarcane vinasse with various concentrations and pH

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

pH for fermentation, particularly pH of 5.0. pH of 6.0 could be adversely affecting the biomass yield due to possible bacterial contamination, resulting in nutrient competition and therefore, suppression of fungal growth. It is important to mention that, for the small-scale optimization studies, it was impossible to precisely maintain the pH at desired points. When adjusting pH on a daily basis, it was observed that pH tended to drop slightly overnight possibly due to production of various primary and/or secondary metabolites.

Note that for the investigation of pH effects, midpoints of 4.5 and 5.5 were not chosen. The study involving 5 different pH levels – 4.0, 4.5, 5.0, 5.5, and 6.0 with molasses as substrate showed no detectable differences between the pH of 4.0 and 4.5, and 5.0 and 5.5. Therefore, to conserve resources, only the pH of 4.0, 5.0, and 6.0 were investigates.

In terms of COD concentrations, molasses COD of 50 g/L yielded the highest biomass,

while for both papaya juice and vinasse, 25 g/L was the most suitable for optimal fungal yield. Highly concentrated papaya juice is very viscous and low biomass yield at high papaya juice concentration could be due to oxygen limitation resulting from thick viscosity. As for the vinasse samples, Santos et al., (2008) have determined that vinasse has a fungicidal activity and suppressed the growth of variety of fungi. This could explain the growth inhibition at higher vinasse concentration as the fungicidal activity would be elevated at higher concentration. However, it was noted by Santos et al. 2008) that due to limited bibliography, suppression of fungal growth by vinasse was a difficult matter to explain (125).

As for pellet formation, molasses and vinasse samples yielded pellets, while papaya juice supported only free mycelial growth. For bioreactor fermentations, formation of free filamentous mycelia can potentially cause several undesirable issues such as growth around impellers, resulting in oxygen limitation and increase in medium viscosity (126). However, it was unclear as to why papaya juice only generated free mycelial biomass.

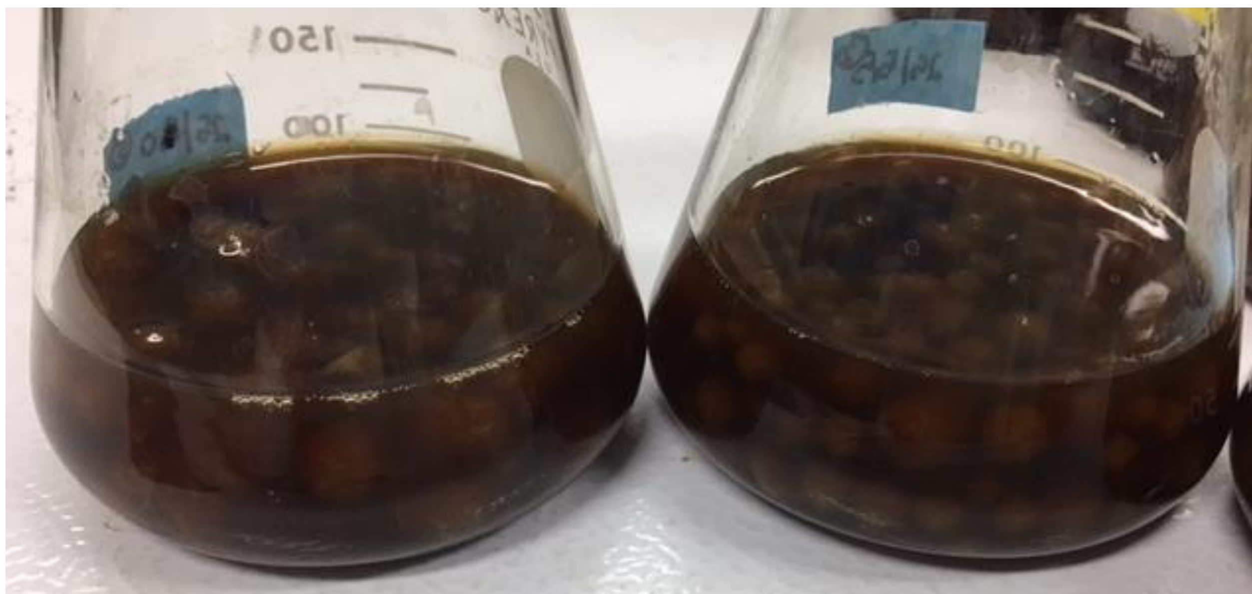

Figure 4.4 Fungal pellet formation in sugarcane molasses

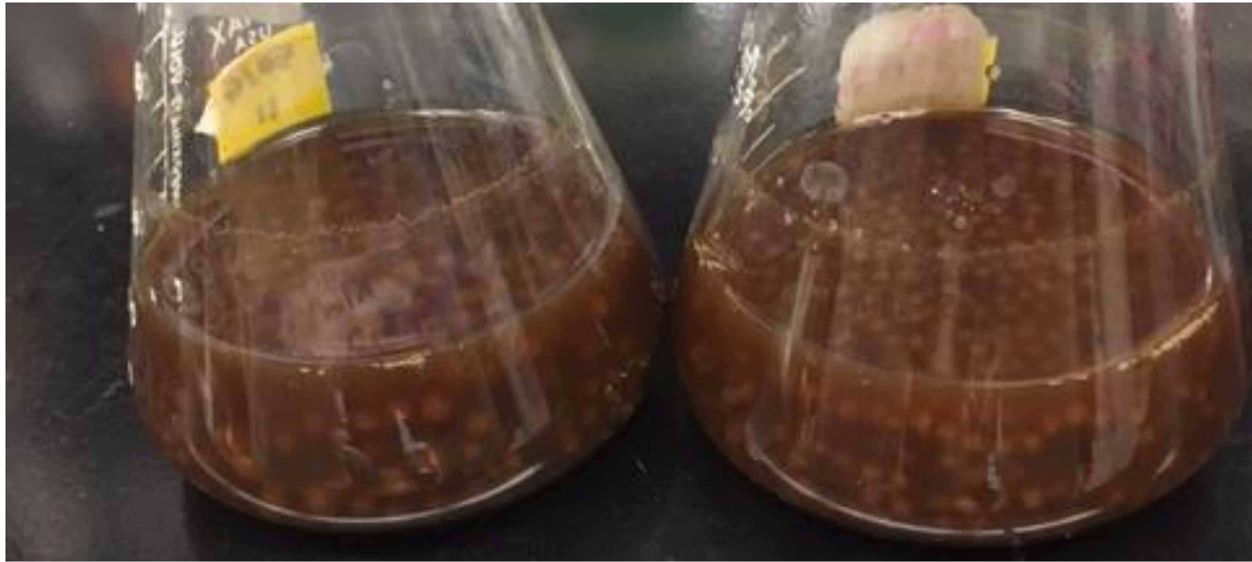

Figure 4.5 Fungal pellet formation in sugarcane vinasse

Since operating parameters - inoculum level, incubator shaker rotation speed, aeration, temperature, and pH – were kept the same for all three of the substrates, characteristics of the fermentation broth could be the most likely factor affecting the fungal morphology. Molasses and vinasse have higher salt and ion concentrations compared to papaya juice. These salt particles may be serving us physical support for the mycelia to attach and grow around them, forming pellets. As it has been found that formation of compact pellets favors higher fungal protein yield (127), fungal morphology is an important factor to consider when choosing fermentation conditions.

Overall, amongst the three substrates, sugarcane molasses yielded the greatest amount of fungal biomass. Both the 25 g/L and the 50 g/L COD molasses concentrations at pH 5.0 yielded statistically same results in term of specific fungal biomass yield. Therefore, it can be deduced that sugarcane molasses could support fungal growth even at higher concentrations than the investigated ones. However, when determining the optimal molasses concentration, it is

important to take into account the cost that comes with the use of clean water. Additionally, in term of fungal pellet formation, molasses and vinasse were proven to be advantageous compared to the papaya juice.

4.1.3 Organic Removal

The overall removal of organics, quantified as sCOD reduction (%) was 24-37%, depending on the cultivating conditions. Interestingly, for each of the substrates, not much difference was observed across different fermentation conditions. For molasses, the range of sCOD removal was 32.5-37.5%, with no statistical difference, while cultivation on papaya juice and sugarcane vinasse achieved organics removal of 26.9%-35.8% and 24.2-30.3%, respectively.

Table 4.2 sCOD removal of sugarcane molasses, papaya juice and sugarcane vinasse at various concentrations and pH

	sCOD removal (%)		
Factors \ Substrate	Sugarcane molasses	Papaya juice	Sugarcane vinasse
COD: 25g/L, pH 4.0	32.50 ± 2.34	33.80 ± 4.59	30.31 ± 4.37
COD: 25g/L, pH 5.0	34.05 ± 1.56	30.50 ± 2.87	30.22 ± 3.53
COD: 25g/L, pH 6.0	36.49 ± 4.09	27.38 ± 3.40	24.16 ± 2.26
COD: 50g/L, pH 4.0	37.53 ± 3.90	34.18 ± 3.00	25.79 ± 3.65
COD: 50g/L, pH 5.0	35.97 ± 2.05	35.80 ± 4.06	25.22 ± 2.83
COD: 50g/L, pH 6.0	34.40 ± 3.01	26.94 ± 2.35	25.10 ± 1.45

Mean value ± standard deviation

Sample size (n)= 3

The only substrate showing statistical difference at the 95% confidence interval in regards to sCOD removal was papaya. Even then, the difference was minimal compared to the specific fungal biomass yield (Table 4.2).

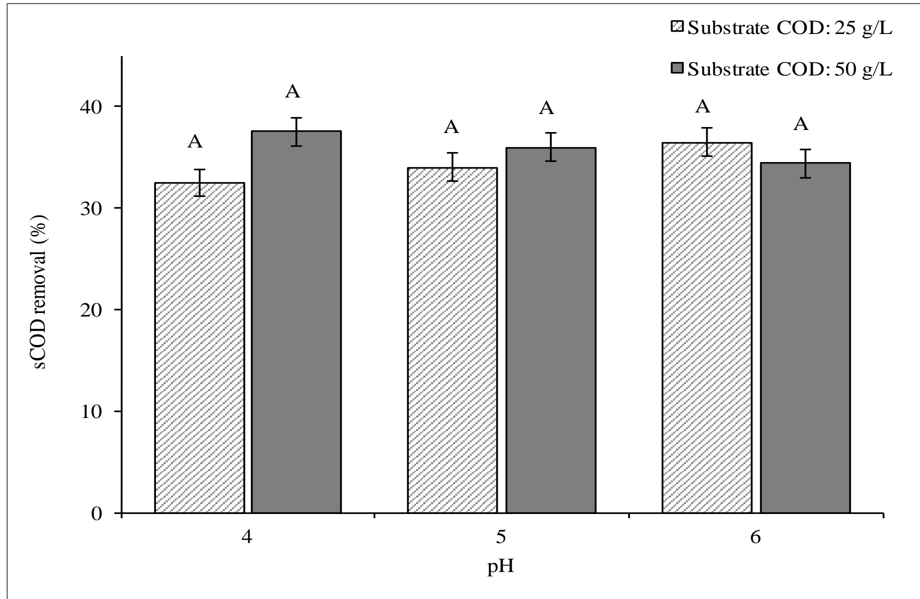

Figure 4.6 sCOD removal of fungal fermentation on sugarcane molasses at various cultivation conditions

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

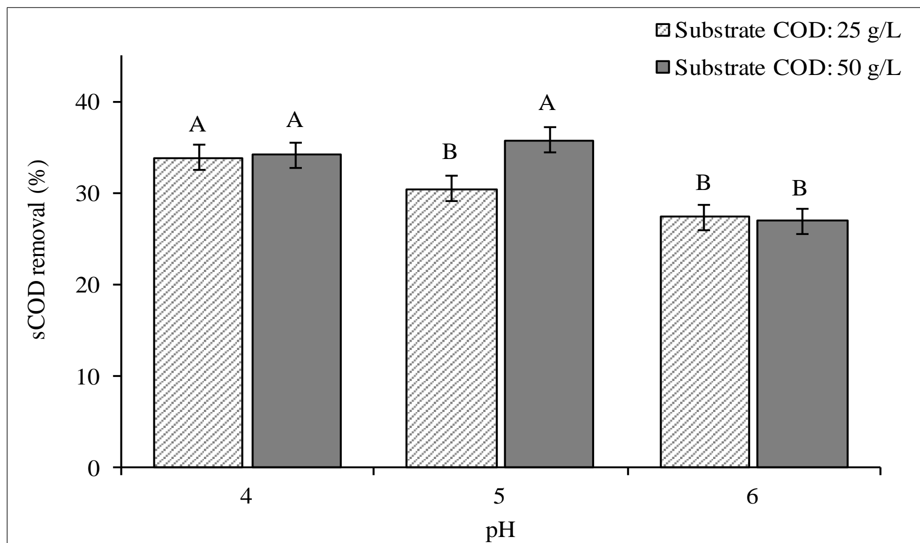

Figure 4.7 sCOD removal of fungal fermentation on papaya juice at various cultivation conditions

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

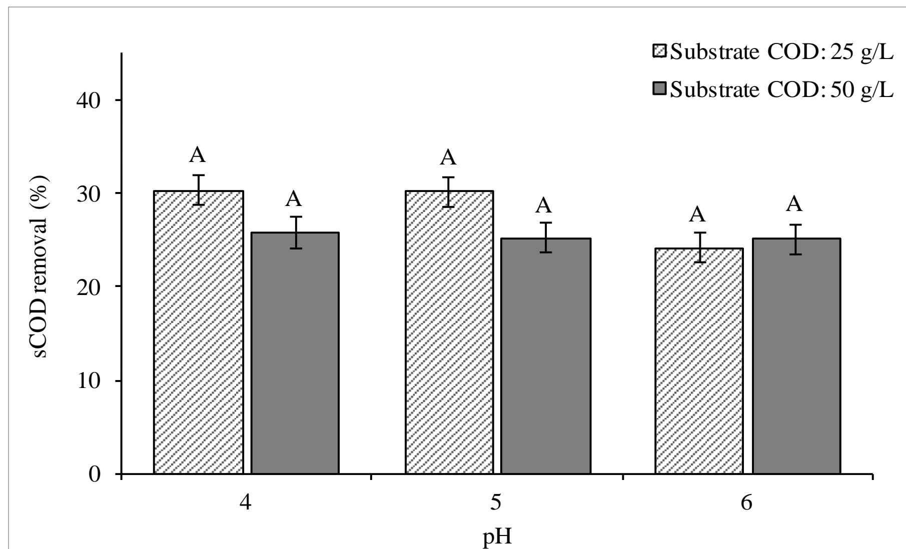

Figure 4.8 sCOD removal of fungal fermentation on sugarcane vinasse at various cultivation conditions

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

Moreover, removal of sCOD did not have a linear relationship with fungal yield, meaning reduction of organics did not directly attribute to fungal growth. This finding is agreement with the findings by Nitayavardhana S., (2012) that studied *R. oligosporus* growth on three different vinasse samples (63). Microorganism degrade organic matter for not only growth, but for various other purposes such as respiration and secondary metabolite production. The observation therefore implies that not all degraded organics are being converted to production of the fungal biomass. Since minimal statistical difference is observed as far as organics removal is concerned, specific fungal biomass yield can be the sole factor when determining fungal growth conditions.

In conclusion, all three of the agro-industrial wastes/residues successfully supported the growth of the fungus *R.oligosporus,* suggesting that variety of such low to negative-value substrates can be utilized in fungal bioremediation. However, this optimization study investigated the fungal fermentation only in a laboratory-scale. Fungal cultivation in a bioreactor

would provide a better control of operating parameters such as pH, air supply, mixing, and temperature.

CHAPTER 5: RESULTS AND DISCUSSIONS: BIOREACTOR SCALE-UP STUDY AND EVALUATION OF ORGANIC REMOVAL

5.1 Rationale

Bioreactor scale-up is a crucial step in investigating feasibility of the system as an industrial scale technology. However, scale-up is one of the biggest challenges in bioprocess technology. Certain parameters, such as pH and temperature are scale-independent, while others such as oxygen transfer rate and agitation speed are scale-dependent and need to be regulated carefully. A bubble column bioreactor provides high mass transfer and low energy consumption as mixing is facilitated by rising air bubbles. Particularly for pellet formation, eliminating extensive mechanical shear while promoting good mixing and adequate air supply is of paramount importance.

The small-scale optimization studies showed that out of the three agro-industrial wastes/residues studied, sugarcane molasses is the most feasible substrate for fungal cultivation and therefore was chosen for the large scale bioreactor studies. Molasses fermentation produced the highest amount of specific fungal biomass while also achieving uniform fungal pellet growth. Additionally, molasses is an abundant yet low cost substrate with long shelf life and high nutrient concentration.

5.2 Bioreactor Scale-up Study

5.2.1 Biomass Yield

72-hour fungal fermentation in bubble column bioreactor yielded roughly 4 grams of dry

fungal biomass per liter of molasses (with COD of 50 g/L). Rapid growth occurred between 12 and 48 hours post spore inoculation. Fungal yield reached a maximum at around 48 hours and stayed unchanged for the next 24 hours. A slight decline of biomass yield was observed after 48 hours, although this was not statistically significant.

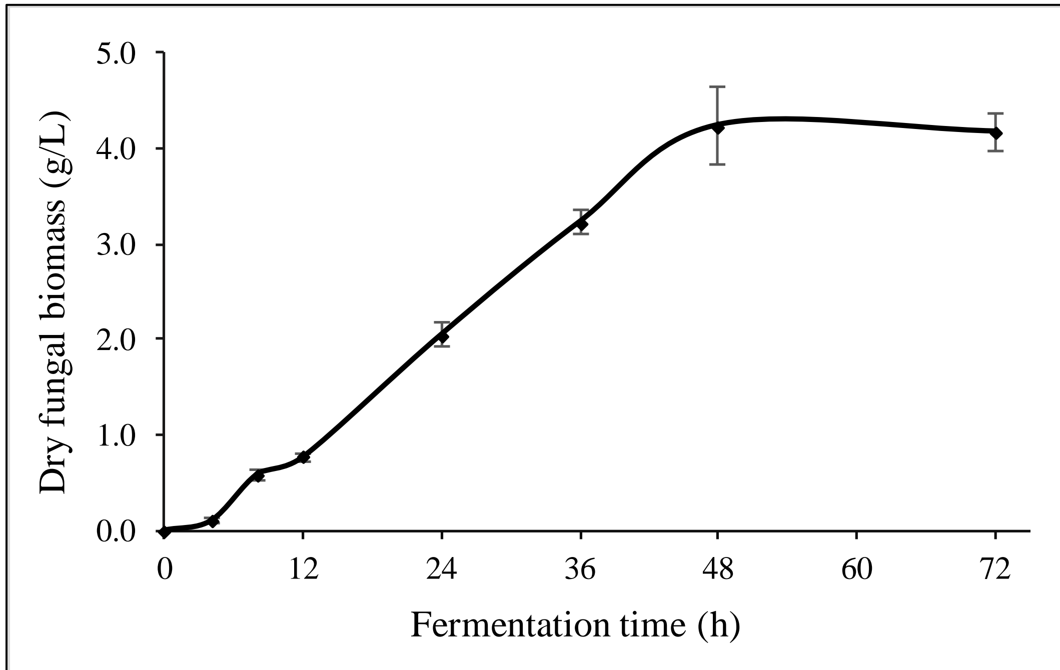

Figure 5.1 Growth kinetic of *R.oligosporus* cultivated on sugarcane molasses

The results suggest that since maximum fungal yield can be achieved after only 48 hours, fungal cultivation can be terminated at 48 hours, saving time and resources. It is however, important to take into account the sCOD removal data in order to determine the best cultivation time for both fungal biomass yield, as well as organics removal. Additional research to compare composition of the biomass at certain time intervals may be of benefit when determining the most suitable fermentation time.

5.2.2 Consumption of Organics and Sugars

Unlike the biomass growth kinetics, rapid sCOD removal was achieved between 12 and 60 hours. sCOD remained unchanged until the 12th hour. After 60 hours of fermentation, no additional sCOD removal was observed. The overall organics removal was 56 ± 4.23 %, which is a better removal efficiency compared to the small scale studies, which only resulted in maximum removal of 37.5%. This could be due to number of factors, including more efficient mixing, better pH control and better oxygen transfer rate.

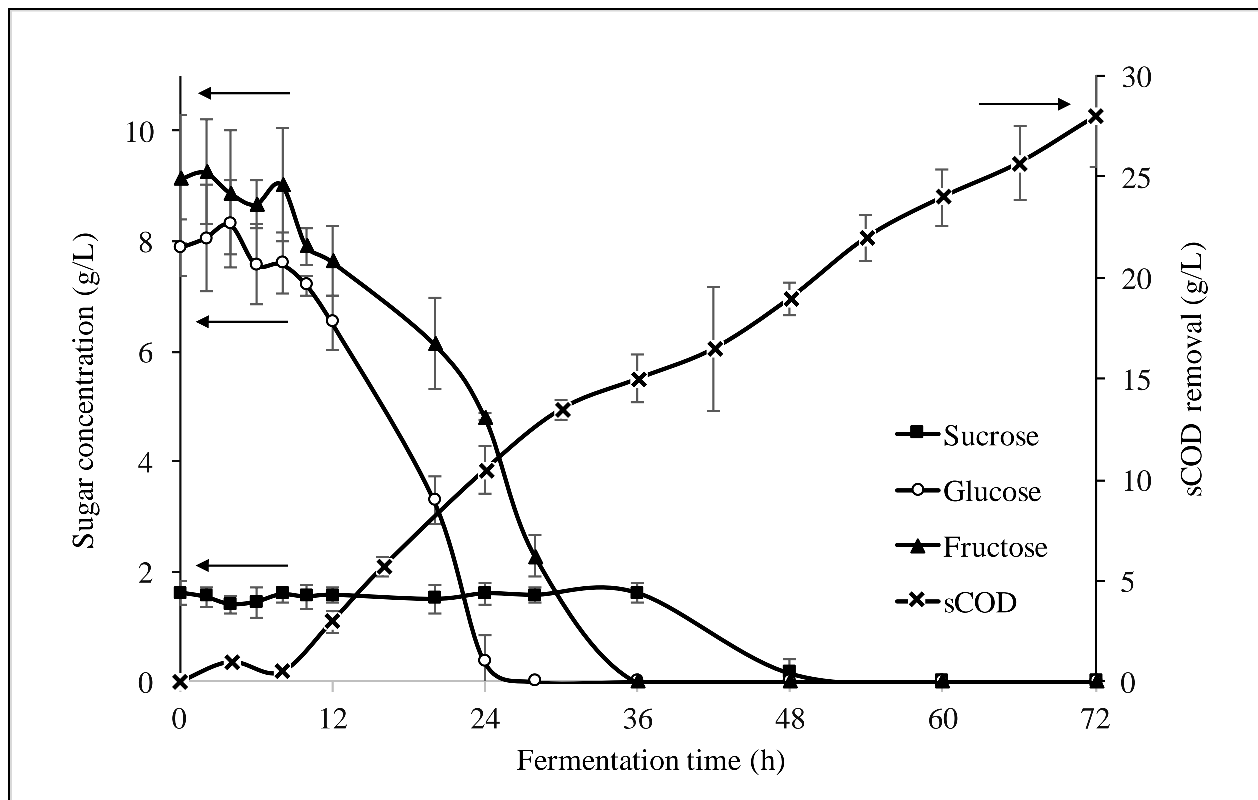

Figure 5.2 Consumption of sugars and organics overtime

If more efficient organics removal is desired, fermentation should be allowed to proceed for at least for 60 hours. However, it is important to note that after the 48th hour, bacterial contamination may be a reason for continued sCOD reduction. Although molasses is sterilized, and fermentation occurs in aseptic conditions, contamination could still be an aiding factor to the

continued organics removal.

On the other hand, most rapid consumption of reducing sugars occurred between 12 and 24 hours. A steady decline in fructose and glucose concentration is observed until the 36th hour, when concentrations of the reducing sugars reach a negligible amount. Up until the 12th hour of cultivation, almost no noticeable sugars reduction is observed, which is consistent with the organics removal data, as well as the biomass kinetics data. Exponential growth of fungal biomass was observed to begin at around 12 hours of fermentation, suggesting that the microorganism is successfully converting reducing sugars into fungal biomass.

However, it is important to note that at around 36 hours, sucrose starts to get consumed as well. *R. oligosporus* is reported to not utilize sucrose as a carbon source. Glucose and fructose are the most preferred carbon sources of the fungi (63). As sCOD (which includes sucrose) continued to decline even after maximum fungal biomass is achieved at 48 hours of cultivation, a possible bacterial contamination is suspected. If indeed the system is contaminated, early termination of the process may lower the risk of undesirable contamination effect on the valuable protein product.

5.2.4 Dissolved Oxygen Consumption and Bacterial Contamination

According to the dissolved oxygen time series data, rapid drop in oxygen concentration begins at around 10 hours of fermentation time, which is 2 hours earlier than the rapid organics consumption and exponential fungal biomass growth. Between 10 to 12 hours, a sharp decline in dissolved oxygen concentration is observed, which is followed by a steady decline until the 36th hour. At 36 hours of cultivation, another sharp decline is observed. A steady depletion of oxygen

then follows. The first sharp drop in oxygen concentration can be attributed to the rapid consumption by the fungus, which coincides with sugar depletion data.

However, the second drop is most likely due to bacterial contamination as this coincides with the sucrose consumption data as well. In fact, the system was contaminated with bacteria at around 16 hours of fermentation. Exponential growth of bacteria was observed between 36 and 60 hours, although a steady increase in colony forming units (CFU) was observed after 16 hours of initial spore inoculation. A rapid increase in CFU is observed at around 36 hours, which is

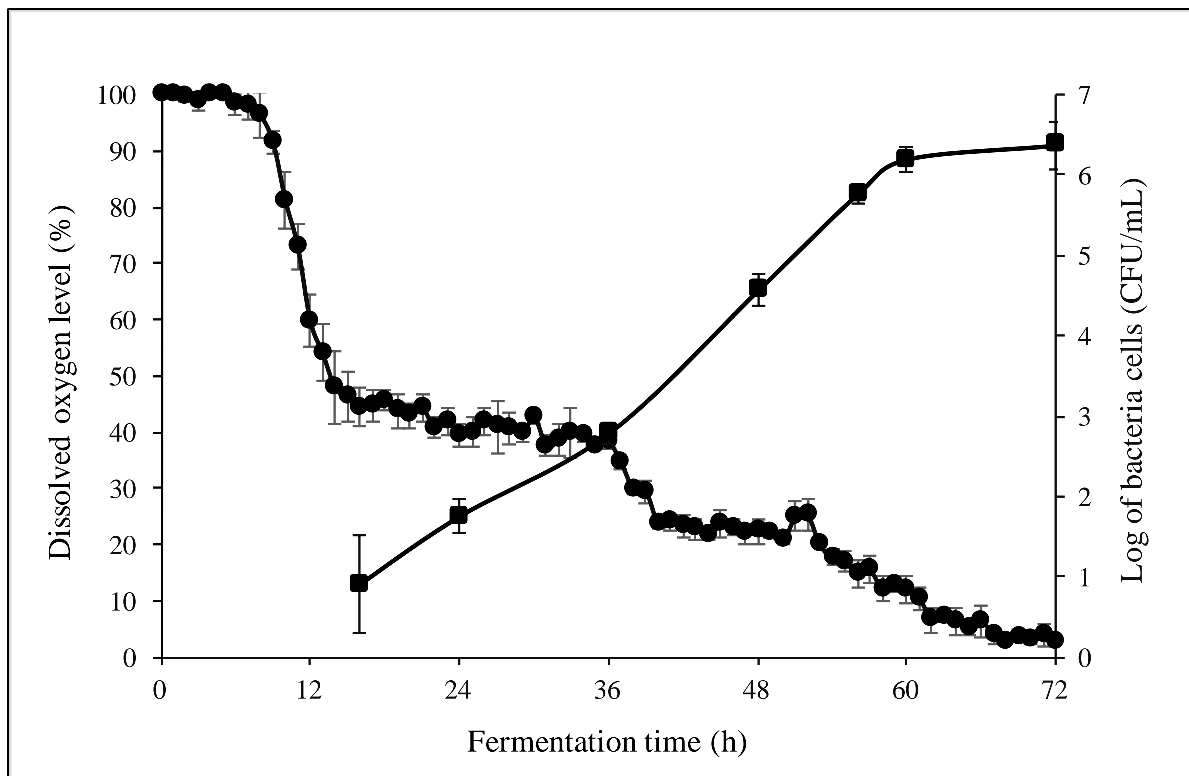

Figure 5.3 Dissolved oxygen consumption and bacterial contamination

consistent with the sucrose consumption as well as dissolved oxygen depletion data. The bubble column bioreactor was disinfected with freshly prepared 10 % bleach and all connecting tubes were sterilized by autoclaving at 121°C for 20 minutes, a standard practice for decontamination.

Additionally, because molasses samples from 0 to 12 hours showed no contamination, contamination likely occurred post inoculation, possibly due to foaming of the broth. Although a sterile foam trap was utilized, foaming was difficult to contain at all times. A pressure built up inside the bioreactor (due to aeration) coupled with excessive foaming put a strain on system closures, making them more susceptible to contamination. Studies to eliminate bacterial contamination is, therefore, crucial.

Bacterial contamination has a few adverse effects on the system, including competition for resources, thereby minimizing fungal biomass yield, and possible change of the nutritional composition of the final product. It is therefore important to adapt a process that would eliminate contamination. One possible solution is to change the operating parameters to favor fungal growth over bacterial proliferation. Operating the system at a lower pH, possible at pH of 4.0 in this case, may minimize the growth of opportunistic microorganisms. Another possibility is to terminate the fermentation earlier than 72 hours to minimize growth of other microorganisms. As noted earlier, bacteria started to grow rapidly at around 36 hours of cultivation while fungal biomass reached a maximum yield at 48 hours post inoculation. Therefore, terminating the operation of the system between 36-48 hours would prevent further growth of undesirable microorganism.

Another possible parameter to control bacterial contamination in this particular case could be oxygen limitation. In this study, between 36 and 72 hours, dissolved oxygen concentration dropped from about 40% of saturation level to about 3% of saturation level. 3% of saturation level is much lower than the critical level of 10-50% required for the growth of obligatory aerobic microorganisms. However, *R.oligosporus* is a saprobic microorganism, one

that prefers environment rich in organics yet relatively low in oxygen. Therefore, a further study of cultivating *R.oligosporus* in oxygen limited conditions would demonstrate the effectiveness of oxygen limitation as a contamination control parameter.

Figure 5.4 Fungal cultivation in a bubble column bioreactor

Figure 5.5 Bubble column bioreactor

5.3 Effluent Characterization

Effluent was collected and analyzed for organics, solids, and minerals composition following fungal fermentation. TCOD and sCOD were reduced by roughly 56%. No glucose, fructose or sucrose were detected in the effluent. Total solids, volatile solids, total suspended solids, and volatile suspended solids were reduced by 31.4%, 29.6%, 43.7%, and 38.2%, respectively. Total Kjeldahl Nitrogen (TKN) was reduced by 69.9%, while phosphorus was reduced by 57.2%. As organics, solids, and nutrient removal was significant, it can be concluded

that fungal bioremediation is a plausible solution for treatment of agro-industrial wastes/residues. Since nitrogen and phosphorus was still present following the cultivation, reduction of the added nutrients accordingly would not only be economical but also environmentally sustainable as well.

Table 5.1 Composition of the fermentation broth following fungal fermentation

Parameters	(hydrolyzed, 20 times diluted)	Parameters	(hydrolyzed, 20 times diluted)
pH	5.45 ± 0.33	Sulfate (g/L)	4.21 ± 0.34
Total Solids TS (%)	3.56 ± 0.011	P (mg/L)	10.98 ± 6.99
Volatile Solids VS (% of TS)	54.09 ± 0087	K (g/L)	2.23 ± 0.45
Total Suspended Solids (TSS) (%)	0.27 ± 0.08	Ca (mg/L)	304.3 ± 30.98
Volatile Suspended Solids VSS (% of TSS)	52.56 ± 4.87	Mg (mg/L)	220.00 ± 16.55
Chemical Oxygen Demand (COD) (g/L)	26.02 ± 1.98	Na (g/L)	3.07 ± 0.23
Soluble Chemical Oxygen Demand (sCOD) (g/L)	23.14 ± 3.30	Fe (mg/L)	8.35 ± 0.71
Total Kjeldahl Nitrogen (TKN) (mg/L)	113.0 ± 15.06	Mn (mg/L)	1.53 ± 0.15
Sucrose (g/L)	N/D	Zn (mg/L)	0.8 ± 0.004
Glucose (g/L)	N/D	Cu (mg/L)	0.11 ± 0.031
Fructose (g/L)	N/D	B (mg/L)	0.82 ± 0.11

N/D – not detected

Mean value ± standard deviation

Sample size (n) = 3

Concentration of sulfate, unfortunately, was detected in excessive amount. Sulfate is added to the molasses as sulfuric acid (H_2SO_4) for hydrolysis and as ammonium sulfate ($(NH4)_2SO_4$) for nutrient supply. High concentration of sulfate ion can cause mineralization of water, corrosion of reinforced steel, negative effects to mammals and generation of generation of highly corrosive hydrogen sulfide in the sewer system (128). Concentration of sulfate ion can be minimized in the system by using a different acid for hydrolysis and replacing ammonium sulfate with an alternative nitrogen source, such as urea.

In summary, a successful cultivation of the fungal biomass was achieved in a bench-top bioreactor system using sugarcane molasses as the substrate. The fungus was able to attain a

maximum concentration of 4 g/L in dry weight basis. However, contamination was present as indicated by the bacterial contamination as well as dissolved oxygen and sugar depletion studies. Overall, the system was able to achieve a significant amount of organics and nutrient removal. The produced fungal biomass had essential amino acid and fatty acid composition comparable to the traditional fish feed.

CHAPTER 6: RESULTS AND DISCUSSIONS: PROTEIN-RICH FUNGAL BIOMASS CHARACTERIZATION

6.1 Fungal Biomass Characterization

Fungal biomass was found to contain around 38.1% crude protein, 6% crude lipids, and 15.5% ash. Both fishmeal and soybean meal, contained higher protein than *R.oligosporus* (Table 6.1). However, typical dietary protein requirements for fish and shrimp are 32-45% and 25-42% on dry weight basis, respectively (129). Crude lipid content was significantly higher than the lipid content of soybean meal, which roughly has about 1.43% (130)(Table 6.1). Since lipids are energy-rich nutrients that can substitute protein in aquaculture feed, a high lipid concentration is desired.

Table 6.1 Composition of molasses derived fungal biomass vs fishmeal and soybean meal

Feed	Protein (% dry weight)	Fat (% dry weight)	Ash (% dry weight)
Fishmeal[1]	60 - 72	8 - 12	10 - 17
Soybean meal[1]	47 - 50	1 - 3	5 - 9
R.oligosporus	38.1	6	15.5

[1]Rasmussen et al. (2007)(94); Swick (2001)(131)

It is also important to note that molasses derived fungal biomass had an *in vitro* protein digestibility of ~80%, which is comparable to fishmeal and soybean meal (both roughly 85%) (131). Protein alone is not an adequate parameter of the feed as not all protein can readily be digested by animals.

6.1.1 Essential Amino Acid Composition

Further analysis was conducted to determine amino acid profile of the fungal biomass. Overall, as indicated on Table 6.2, the essential amino acid content of fungal biomass was comparable to the standards set by FAO and WHO (World Health Organization) (132). Although fungal biomass generally tends to be deficient in methionine and phenylalaline, *R.oligosporus* was found to contain adequate amounts of both of these amino acids. Furthermore, lysine content was relatively high at 8.6%. Lysine is the most critical amino acid in aquaculture feed as it is often the limiting ingredient in aquatic feed and is critical for optimal fish growth (133).

Table 6.2 Essential amino acid composition of the molasses derived fungal biomass

		Percent composition (protein basis)	
Amino Acids		***Rhizopus oligosporus***	**FAO Standard[1]**
Non-essential AA	Alanine	8	Not available
	Aspartic acid + Asparagine	9.7	Not available
	Cysteine	1.2	Not available
	Glutamic acid + Glutamine	10.5	Not available
	Glycine	6.5	Not available
	Proline	4	Not available
	Serine	4.5	Not available
	Tyrosine	4.5	2.8
Essential AA	Tryptophan	1.8	1.4
	Arginine	6.2	2
	Histidine	1.1	2.4
	Isoleusine	5.7	4.2
	Leucine	8	4.8
	Lysine	8.6	4.2
	Methionine	2.5	2.2
	Phenylalaline	5.6	2.8
	Threonine	6.4	2.6
	Valine	7	4.2
Subtotal of Non-Essential AA		**50.7**	Not available
Subtotal of Essential AA		**49.3**	Not available
Total		**100**	Not available

[1]FAO/WHO (1991)(132)

6.1.2 Essential Fatty Acid Composition

Fatty acid profile of the molasses derived fungal biomass showed that the fungal biomass is a feasible alternative for aquaculture feed. Fatty acid composition is one of the most crucial

Table 6.3 Fatty acid composition of the molasses derived fungal biomass

Fatty Acid	**Lipid #**	**%**
Octanoic acid	C8:0	0.020
Decanoic acid	C10:0	0.010
Dodecanoic acid	C12:0	0.290
Tetradecanoic (Myristic) acid	C14:0	3.860
Pentadecanoic acid	C15:0	0.350
Palmitic acid	C16:0	22.99
Palmitoleic acid	C16:1n-7	1.830
Hexadecenoic acid	C16:1n-9	1.930
Heptadecanoic acid	C17:0	0.060
Hexadecadienoic acid	C16:2n-4	0.080
Hexadecatrienoic acid	C16:3n-4	0.010
Stearic acid	C18:0	14.83
Oleic acid	C18:1n-9	18.53
Octadecenoic acid	C18:1n-7	0.180
Linoleic acid	C18:2n-6	17.12
Gamma Linolenic acid	C18:3n-6	2.790
Octadecatetraenoic acid	C18:4n-3	0.020
alpha-Linolenic acid (ALA)	C18:3n-3	1.760
Eicosanoic (Arachidic)	C20:0	0.010
Eicosenoic acid	C20:1n-9	N/D
Eicosatrienoic acid	C20:3n-3	0.260
Eicosatetraenoic acid	C20:4n-3	0.140
Arachidonic acid	C20:4n-6	N/D
Eicosapentaenoic acid	C20:5n-3	N/D
Docosapentaenoic acid	C22:5n-3	N/D
Docosapentaenoic acid	C22:5n-6	N/D
Docosahexaenoic acid	C22:6n-3	0.560
Nervonic acid	C24:1	0.000
Identified		**87.63**
Unidentified		**12.37**
Total Fatty Acids		**100.0**

parameters in aquaculture feed as it can have significant influence on the tissue fatty acid composition, particularly in fish (133). Essential fatty acids were found to be in satisfactory amounts in the biomass; linoleic acid (17.12%), linolenic acid (1.76%), eicosapentaenoic acid (EPA) (0.146%), and docosahexaenoic acid (DHA) (0.56%), (Table 5.3). Linoleic acid, C18:2n-6, and linolenic acid, C18:3n-3 can not be synthesized by fish, thus they must be supplied in a diet. However, it it important to emphasize that the requirement of fatty acid in feed diet varies significantly depending on the metabolic pathway of the target animals (133).

CHAPTER 7: CONCLUSIONS

Global aquaculture industry is in an ever increasing demand for cost effective yet environmentally sustainable feed. This research investigated the feasibility of agro-industrial waste/residue-derived fungal biomass as alternative aquaculture feed.

The following conclusion are drawn based on this research:

1. The small scale optimization studies showed that all three of the investigated agro-industrial wastes/residues - sugarcane molasses, papaya juice, and sugarcane vinasse - were feasible substrates for fungal cultivation. The best fermentation pH was found to be 5.0, while optimum concentration varied from substrate to substrate. Overall, molasses produced the highest fungal biomass yield of 0.41 ± 0.02 (g biomass/g sCOD removed).
2. In scale-up bioreactor studies, highest yield of 4 grams of fungal dry biomass per liter of molasses was achieved at 48 hours post spore inoculation. Reducing sugars were consumed altogether at 36 hours, although organics content continued to decrease. Dissolved oxygen level dropped significantly after 36 hours, reaching around 3% of saturation level. Bacterial contamination was observed at 16 hours of fermentation and continued to increase exponentially until settling at around 60 hours of cultivation.
3. Fungal biomass cultivated on agro-industrial wastes/residues has a potential to serve as protein-rich alternative ingredient for aquaculture feed. The biomass has essential amino acid and fatty acid profile as well as digestibility (~80%) comparable to that of commercial protein sources. Lysine, a rate-limiting amino acid was detected in high percentage in the protein. Supplementing the biomass with other commercial protein sources may address the low methionine and phenylalaline content of the fungal protein.
4. Agro-industrial wastes/residues need to be treated with methods that have minimal

impact on the environment, human health, and enable recycling of organic waste material. Production of protein-rich fungal biomass on agro-industrial wastes/residues has dual merits as waste bioremediation and as production of high-value fungal protein with potential use as aquaculture feed.

CHAPTER 8: FUTURE RESEARCH

Further research on production of protein-rich fungal biomass on agro-industrial wastes/residues should investigative the following aspects:

- Pilot scale study to investigate design criteria for industrial scale applications is crucial. Pilot scale study should address depletion of dissolved oxygen
- Investigation of the fungal biomass as animal feed ingredient is pivotal to determine its potential in commercial applications. Aquaculture feeding trials would provide insights on this matter. Techno-economical analysis should be conducted to justify the commercialization of the process
- Studies of eliminating bacterial contamination are crucial in maximizing yield and quality of the fungal biomass

APENDIX A: STATISTICAL ANALYSIS OF FUNGAL GROWTH OPTIMIZATION STUDIES

Fungal biomass yield and organics removal were statistically analyzed with one-way analysis of variance (ANOVA) and Tukey's mean comparison test at 95% confidence level. Sample size of 3 was used for each test

Sugarcane Molasses

ANOVA

	Source	DF	Sum of Squares	F Ratio	Prob > F
Specific biomass yield	Substrate COD (g/L)	1	0.100	155	**<.0001**
	pH	2	0.017	13.4	**0.0009**
	Substrate COD (g/L)*pH	2	0.006	4.62	**0.0326**
sCOD removal	Substrate COD (g/L)	1	11.82	2.05	0.1777
	pH	2	0.745	0.06	0.9377
	Substrate COD (g/L)*pH	2	38.25	3.32	0.0713

Papaya Juice

ANOVA

	Source	DF	Sum of Squares	F Ratio	Prob > F
Specific biomass yield	Substrate COD (g/L)	1	0.028	14.1	**0.0028**
	pH	2	0.019	4.73	**0.0305**
	Substrate COD (g/L)*pH	2	0.012	2.89	0.0946
sCOD removal	Substrate COD (g/L)	1	13.70	2.57	0.1347
	pH	2	166.5	15.6	**0.0005**
	Substrate COD (g/L)*pH	2	28.97	2.72	0.1061

Sugarcane Vinasse

ANOVA

	Source	DF	Sum of Squares	F Ratio	Prob > F
Specific biomass yield	Substrate COD (g/L)	1	0.019	12.9	**0.0037**
	pH	2	0.089	30.1	**<.0001**
	Substrate COD (g/L)*pH	2	0.002	0.79	0.4764
sCOD removal	Substrate COD (g/L)	1	36.80	4.76	**0.0498**
	pH	2	42.70	2.76	0.1033
	Substrate COD (g/L)*pH	2	32.69	2.11	0.1637

APENDIX B: SUGARCANE MOLASSES STUDY WITH 5 PH LEVELS

Sugarcane molasses study with 5 pH levels – 4.0, 4.5, 5.0, 5.5, 6.0 – showed that there was no statistically significant difference on fungal biomass yield between runs with pH 4.0 and 4.5 and 5.0 and 5.5.

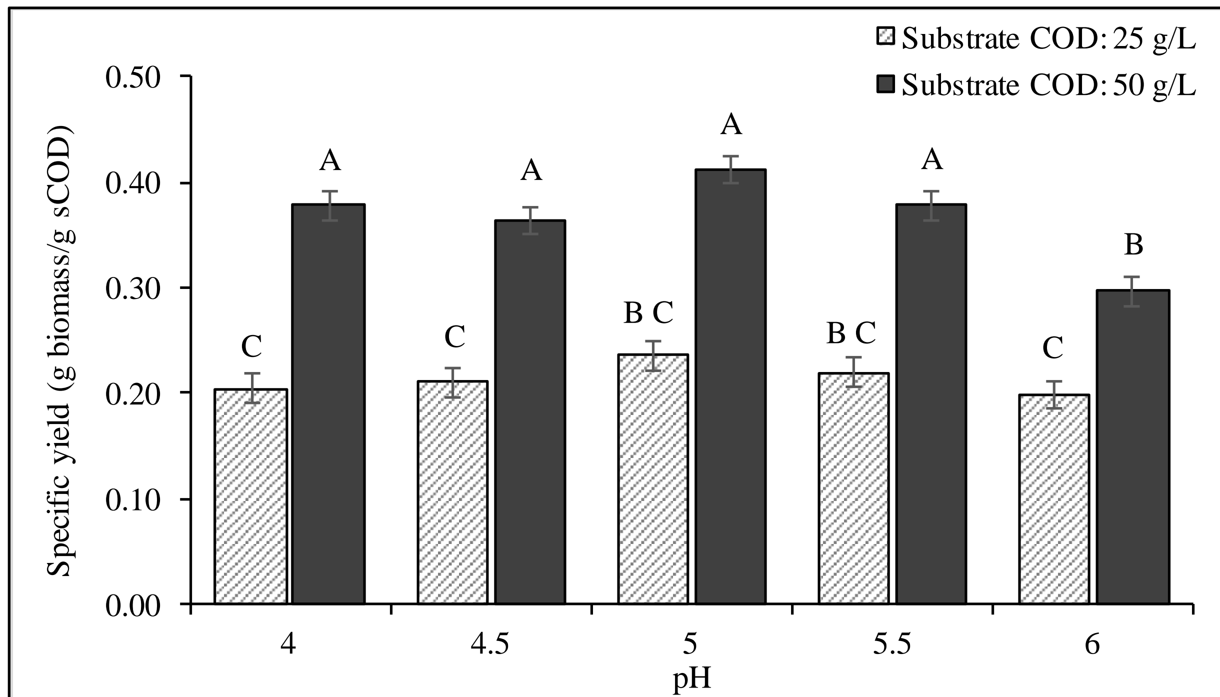

Figure B.1 Specific fungal biomass yield for sugarcane molasses with various concentrations and 5 pH levels

Superscript letters denote statistical difference/similarity

Sample size (n)= 3

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