PROTEIN-RICH FUNGAL BIOMASS PRODUCTION ON SUGARCANE VINASSE FOR ANIMAL FEED APPLICATIONS WITH CONCOMITANT WATER RECLAMATION

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ABSTRACT

The sugar-to-ethanol production process generates a significant amount of low-value residue known as vinasse. Vinasse has a high organic content measured as chemical oxygen demand (COD) of over 100 g/L, and its direct disposal has a negative impact on the environment. This research investigated the feasibility of fungal technology as a cost effective and environment-friendly approach in utilizing the vinasse as a sole substrate for producing a protein-rich edible fungus, *Rhizopus microsporus* (var. *oligosporus*) with concomitant wastewater reclamation. Optimization studies showed prolific fungal growth at pH 5.0 on vinasse with nutrient (nitrogen and phosphorus) supplementation. The fungus grew exponentially during the first 24 hours using reducing sugar as a major constituent to support its growth. Ethanol at concentrations greater than 5.0% (v/v) adversely affected the fungal growth through change in mycelial morphology.

Fungal fermentation in an airlift bioreactor (2.5-L working volume) under various aeration rates (0.5, 1.0, 1.5, and 2.0 $volume_{air}/volume_{liquid}$/min (vvm)) was investigated for the potential large-scale fungal biomass production. The fungal biomass yield was found to depend on the aeration rate, and the aeration rate of 1.5 vvm resulted in the highest fungal biomass yield of 8.04 ± 0.80 ($g_{biomass\ increase}/g_{initial\ biomass}$) with significant reduction in organic content ~ 80% (or 26 g/L) measured as soluble chemical oxygen demand (SCOD). Vinasse-derived fungal biomass contained approximately 50% crude protein with 84% *in vitro* protein digestibility. Essential amino acids contents of the fungal biomass were comparable to commercial protein sources (fishmeal and soybean meal) for animal feeds, with the exception of methionine and phenylalanine. Importantly, the fungal biomass contained high lysine (~ 8% on protein basis), which is the most critical amino acid required in animal feed. Moreover, fungal biomass had essential fatty acids including linoleic, linolenic, eicosapentaenoic (EPA), and docosahexaenoic (DHA), which could further benefit fungal biomass as an aquatic feed. The integration of innovative fungal technology with sugar-based ethanol production could provide an opportunity for producing food-grade fungal protein for animal feed application with simultaneous wastewater reclamation for in-plant use or for land applications.

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CHAPTER 1: INTRODUCTION

1.1 Background

Rapid growth in biofuel production has been driven by the policy mandates and the global renewable energy goals. Particularly in the United States, regarding the Energy Policy Act of 2005 (EPAct 2005, P.L. 110-58), established a Renewable Fuel Standard (RFS), coupled with the Energy Independence and Security Act (EISA) of 2007 (P.L. 110-140, H.R. 6), it is required that the use of renewable fuel be increased to 36 billion gallons (136 billion liters) annually by 2022 (Brown et al., 2007). Moreover, the Agricultural Outlook report projects that global ethanol production is expected to reach 48 billion gallons (182 billion liters) by 2021 (OECD, 2012) from the current 29 billion gallons (110 billion liters) (RFA, 2012). Presently, first generation feedstocks, such as sugarcane/sugar beet and starch-based grains (corn and sorghum) and tubers (cassava), are mainly used for ethanol production (Joshi et al., 2011; Shrestha et al., 2012). Specifically, sugarcane is largely used for ethanol production in Brazil, the second largest ethanol producer following the United States. Brazilian ethanol derived from sugarcane is expected to represent ~ 28% of global ethanol production in 2021 (OECD, 2012). While domestic ethanol production and consumption in the U.S. is primarily derived from corn, sugarcane-based ethanol is likely to become more competitive as the world raw sugar prices are projected to decrease.

However, biofuel production is not without concerns. A considerable increase in biofuel production would simultaneously generate a large amount of low-value residues that may have detrimental effect on the environment if disposed without proper treatment, and may also raise serious concern on the long-term sustainability of biofuel industries if not converted into revenue generating co-products. Sugar-to-ethanol industries, for example, generate up to 13 gallons of an organic-rich liquid stream following ethanol recovery, know as *vinasse*, for each gallon of ethanol produced

(Pereira, 2008). Depending on substrate for ethanol fermentation, the vinasse is brownish to dark color with low pH of 4-6 and extremely high organic content of 50-150 g chemical oxygen demand (COD)/L (Nandy et al., 2002; Goldemberg et al., 2008; España-Gamboa et al., 2011). Consequently, a direct vinasse disposal causes severe environmental concerns. Although, fertirrigation (fertilization + irrigation) is widely applied as a vinasse disposal method (application of less than 32,073 gallons of vinasse/acre (30,000 m^3 of vinasse/km^2) has been found to have no negative impact on groundwater contamination (Goldemberg et al., 2008)), this can cause the increasing risk of soil salification, metal leaching, alterations in soil quality (such as nutrients imbalance and reduction of alkalinity), phytotoxicity, and odor nuisance (Navarro et al., 2000; Smeets et al., 2008). To reduce the vinasse volume, recirculation and concentration of vinasse have been suggested for sugar-based ethanol facilities, but they are not cost effective and the vinasse recirculation has an adverse effect on ethanol yield (Moura and Medeiros, 2008). Therefore, sugar-based ethanol plants urgently need an environment-friendly and sustainable option for vinasse disposal.

Conventional biological wastewater treatment using mixed bacterial communities results in no other benefits than an environmental protection. For example, an aerobic wastewater treatment, such as the activated sludge process, which mineralizes organic compounds into carbon dioxide and water, generates considerable amount of bacterial biomass known as sludge. The excess sludge is a burden and the disposal cost could be as much as 60% of the total operating cost of wastewater treatment plants (Canales et al., 1994; Wei et al., 2003). Although an anaerobic treatment generates methane, the process is capital intensive and methane is a low-value by-product (Tchobanoglous et al., 2003). On the other hand, a biorefinery concept, which aims at optimizing the use of resources by producing high-value co-products from the residues/wastes and concomitantly minimizing waste generation, could address the concern over biofuel residues disposal and enhance the economic viability and the long-term sustainability of biofuel industries. Fungal fermentation can be easily integrated into an existing biofuel facility with minimal additional unit processes and operations; thus, it is one of the promising approaches for

high-value co-product generation. Fungal processing of biofuel residues could potentially improve the economics of biofuel industries because a protein-rich fungal biomass can be formulated to produce high-value animal feeds, providing a market value close to $467-$472 or $1,395-$1,450 per dry ton (US) as compared to commercial protein source for animal feed such as soybean meal or Menhaden fish meal, respectively (USDA, 2012). This process will also likely to lessen the environmental impacts from biofuel waste/residues since fungi are capable of producing wide variety of enzymes, which facilitate degradation of complex organic matters present in the residue stream (Silva and Nocoli, 1983; Ceccto-Antonini and Tauk, 1994; Shojaosadati et al., 1999). Consequently, the effluent following fungal fermentation can be discharged to the environment with minimal treatment.

The filamentous nature of fungi offers the benefit of fungal biomass separation and recovery. The growth of filamentous fungi in submerged fermentation has been reported to depend on a wide range of parameters, including cultivation medium, agitation intensity, and shear stress, which result in the varying fungal morphology (Cui et al., 1998a; Wang et al., 2005). Fungal morphology has an effect on rheological property of fermentation broth thereby affecting both fungal growth and product formation. Generally, three types of fungal morphology: free filamentous mycelia (suspended mycelia), clumps, and pellets are formed (Wang et al., 2005). The formation of free filamentous mycelia or clumps in a bioreactor may have several issues such as increase in medium viscosity, fungal growth around impellers, thereby reducing fungal growth, productivity, and reactor performance (Wang et al., 2005; Liu et al., 2008). Considering industrial-scale submerged fermentation process, fungal pellet formation is desirable as it provides good mass transfer (oxygen and nutrients), minimizes adverse effect on reactor performance, and does not increase the fermentation broth viscosity.

Rhizopus microsporus (var. *oligosporus*) has been given generally recognized as safe (GRAS) status by the U.S. Food and Drug Administration (FDA) (Schauer and Borriss, 2004). The food-grade fungus is widely used as a starter culture for producing

tempeh, a traditional Indonesian delicacy. Thus, an edible fungal biomass could potentially serve as an excellent protein source for animal feed. *R. oligosporus* has been successfully cultivated on thin stillage from corn-ethanol industries and the fungal biomass product contained high protein (~ 43% dry weight) with various essential amino acids suitable for animal feed applications (Rasmussen et al., 2007). Moreover, a significant reduction in organic matter (up to 46% COD) and inorganic matters was achieved within 3-5 days of cultivation. However, corn-ethanol stillage contains relatively high amounts of protein and other nutrients compared to vinasse. Therefore, in this research, the study of fungal technology as a cost effective and environment-friendly approach in utilizing the wastes/residues from biofuel industries, vinasse, as a sole substrate for food-grade fungal protein production was investigated.

Specifically, the successful application of fungal technology could potentially provide a unique opportunity for sugar-based ethanol production in Hawaii, one of the world's most isolated archipelagos. The state is heavily dependent on foreign oil and extremely vulnerable to its price fluctuation. Nearly all of its energy need comes from imported fossil fuels (EIA, 2012a). To enhance Hawaii's energy security, the government has been promoting the use of renewable energy and thus launched a policy that aims to encourage energy self-sufficiency requiring at least 85% of gasoline sold in the state to be blended with at least 10% ethanol by volume (E10) by 2008 (Brown et al., 2007). With the advantage of tropical climate, Hawaii has a unique opportunity for sugarcane ethanol production. Not only the production of an inexpensive food-grade fungal protein offers an alternative option for vinasse utilization, but the fungal product, which has an immediate application for animal/aquatic feed, can potentially reduce the state's dependency on imported feed ingredients, lowers the production cost, and increases an economic viability and sustainability of local aquaculture farming. Last but not the least, Hawaiian sugarcane-ethanol refineries could address a concern over state's dependency on imported petroleum fuels and improve the state's economy by creating jobs in bio-based economy.

1.2 Objectives of the Study

Based on the above rationale, the overarching goal of this research was to investigate the feasibility of protein-rich food-grade fungus, *Rhizopus oligosporus* production on sugar-ethanol derived vinasse for animal feed applications. Specifically, the objectives of this study were to:

1. Optimize fungal growth conditions on vinasse for maximizing fungal biomass production.

2. Investigate the effect of substrate concentrations on fungal growth pattern.

3. Evaluate fungal cultivation on vinasse in a bioreactor system for process scale-up.

4. Determine the chemical composition and nutritional value of vinasse-derived fungal biomass as an animal feed ingredient.

CHAPTER 2: LITERATURE REVIEW

2.1 Sugar-based Ethanol Production and Residues

2.1.1 Background

The dependence of the global economy on fossil-derived fuels coupled with political instability in oil producing countries has pushed petroleum prices near an all-time high. Oil prices have also been fluctuating significantly and are expected to rise from $79 per barrel in 2010 to $145 per barrel in 2035 (EIA, 2012b). The rapidly increasing energy demands and dwindling reserves of petroleum fuel coupled with an environmental devastation resulting from energy-derived greenhouse gases (GHGs) emission has raised concerns over the use of non-renewable fuels. Moreover, energy security concerns due to the heavy dependency on imported petroleum oils demand for the development of sustainable, affordable, and environment-friendly energy sources. Bioethanol, derived from renewable resources, is one of the promising alternative liquid transportation fuels and possibly the world most used biofuel (Azadi et al., 2012). It is produced from fermentable sugars, especially six-carbon sugar derived from plants-based feedstocks, via yeast fermentation. Currently, the majority of global ethanol comes from first-generation feedstocks, such as corn in the U.S. and sugarcane in Brazil (Stephen et al., 2012). Sugars in sugar-based feedstocks can be readily converted into ethanol by *Saccharomyces cerevisiae*. As the world second largest ethanol producer, Brazil produced about 7.2 billion gallons (27.3 billion liters) of ethanol from over 50% of the domestic sugarcane in 2011 (UNICA, 2012). Sugarcane is considered to be the most efficient feedstock for ethanol production (Macedo et al., 2008) and Brazilian ethanol derived from sugarcane alone represents about one third of global ethanol production (Souza et al., 2012). Moreover, sugarcane ethanol production is also predicted to be developed in the United States (OECD, 2012).

2.1.2 Sugar-based Ethanol Production

Bioethanol is primarily produced from the fermentation of sugarcane- and sugar beet-derived sugar/molasses and starch-derived sugar obtained from corn, cassava, and sorghum (Bothast and Schlicher, 2005; Sánchez and Cardona, 2008). Although the classifications of bioethanol feedstocks vary significantly, the feedstocks can be broadly placed into three categories: sugar-, starch-, and lignocellulosic-based feedstocks. The use of feedstocks differs from country to country depending on availability, feedstock cost, and biofuel conversion technology. The following sections cover the generation of sugar-based bioethanol and its residues from different feedstocks along with their characteristics.

Sugar-based feedstocks (e.g., sugarcane, molasses, sweet sorghum, and sugar beet) are widely used globally for bioethanol production. For example, in Brazil, the world second largest biofuel producer, ethanol is primarily produced from cane sugar (Zanin et al., 2000). Sugarcane molasses, another source of fermentable sugars derived from sugar processing, are the major feedstock for ethanol production in India (Ghosh and Ghose, 2003), whereas sugar beet is the main crop used in Europe (Berg, 2004). Of the many crops being investigated for ethanol production in China, sweet sorghum is considered to be one of the most promising feedstocks (Lookhart and Bean, 2000; FAO, 2002). Sugar-based feedstocks mainly consist of the carbohydrates in the form of disaccharide primarily as sucrose. The sugars in these feedstocks can be readily converted into ethanol by the yeast, *S. cerevisiae*.

In the ethanol production process, sugar crops are passed through an extractor/expeller to produce juice; a sugar-rich solution. Molasses, the liquid by-product from the sugar industry that contains ~ 49% sucrose, can be directly fermented into ethanol. Dilutions may sometimes be required to obtain optimal sugar concentrations to avoid the effect of osmolality (Morimura et al., 1997). Ethanol production from sugar-based feedstocks is illustrated in Figure 2.1. The conversion process, however, generates residues/by-products along with bioethanol. Residues and by-products derived from

sugar-based ethanol production process differ significantly in quality depending on types of raw material used. However, they can be broadly placed into two categories: solid residue, bagasse, and liquid residue, vinasse/stillage/distillery spent wash.

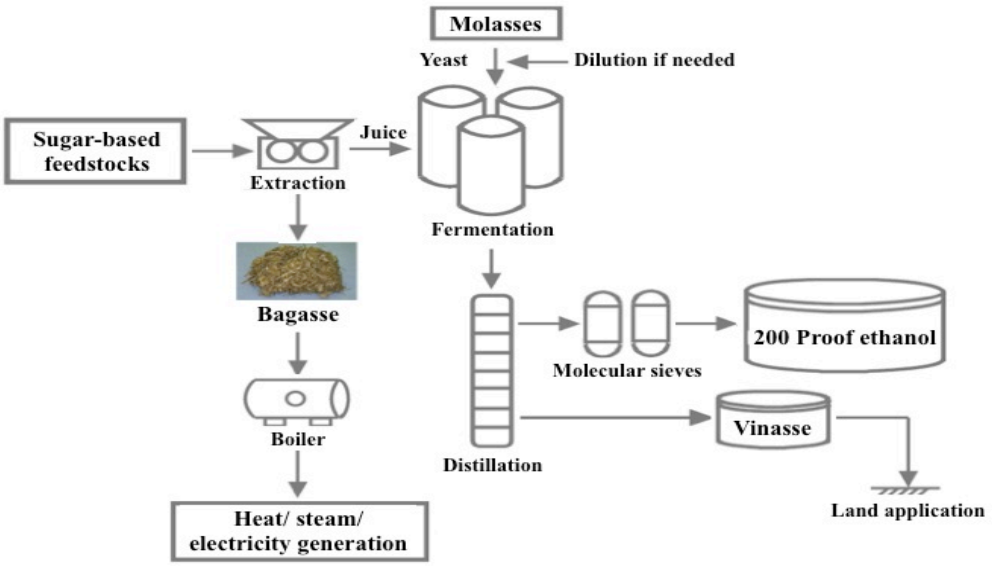


Figure 2.1 Schematic diagram of ethanol production process from sugar-based feedstocks

2.1.3 Sugar-based Ethanol Residues

The composition of residues varies depending on the feedstocks that are used in the ethanol production. In general, bagasse and vinasse are the two major residues generated from the sugar-based ethanol plants. The fibrous residue, bagasse, is obtained after crushing and juice extraction. The liquid stream obtained after ethanol recovery is known as vinasse (also called stillage or distillery spent wash).

Although the composition of bagasse varies according to its variety, maturity, cultivation, harvesting, and juice extraction process, the major constituents of bagasse remain fairly similar. Sugarcane and sweet sorghum bagasses mainly consist of cellulose,

hemicellulose, and lignin. The compositions of sugarcane and sweet sorghum bagasses are showed in Table 2.1.

Table 2.1 Compositions of sugarcane and sweet sorghum bagasses

Constituents	Bagasse composition (%)	
	Sugarcane	**Sweet sorghum**
Moisture	45-50	54
Cellulose	19-27	17
Hemicellulose	11-15	12
Lignin	7-13	9
Others	2	8

Sources: Adapted from Pandey et al. (2000); Katyal et al. (2003); Gnansounou et al. (2005)

Each gallon of ethanol is produced from 120 lbs of harvested sugarcane with concomitant production of approximately 31 lbs of bagasse (~ 45-50% moisture content) (da Rosa, 2005). Bagasse is primarily used to meet an internal energy demand of the ethanol plant. It is usually burned to produce heat/steam and electricity for in-plant use. The surplus bagasse is used as a raw material in pulp and paper, acoustic board, pressed wood, cattle feed, and ethanol production (Paturau, 1989; Dominguez et al., 1996; Pessoa et al., 1997). Beet pulp, which is the residue left after sugar beet juice extraction, contains high amounts of pectin rather than lignin (Dinand et al., 1996). Beet pulp is mainly composed of cellulose (20%), hemicellulose (25%), and pectin (25%) together with small amounts of protein, lignin, and ash (Schweizer and Wursch, 1979; Bertin et al., 1988; Michel et al., 1988; Liu et al., 2007).

Along with the bagasse, vinasse (also known as stillage or distillery spent wash) is generated as a leftover at the bottom of distillation column following ethanol recovery

process. It mainly consists of organic matter and soluble inorganics including potassium at an extremely high level. Sugarcane-to-ethanol process generates about 8-15 gallons of vinasse per gallon of ethanol produced (Saha et al., 2005). The amount and compositions of vinasse are highly variable and dependent on the feedstocks and various aspects of the ethanol production process. Production of a gallon of ethanol from cane juice, cane molasses, and sweet sorghum generates around 16.3, 14, and 16 gallons of vinasse, respectively (de Menezes, 1989; Wilkie et al., 2000).

Vinasse is typically dark colored, with high total solids and organic matter contents, and strong acidic properties (Goldemberg et al., 2008; Nandy et al., 2002). Vinasse characteristics of various feedstocks are shown in Table 2.2. As can be seen from the table, beet vinasse is higher in protein content in which the major nitrogen sources are betaine (9-41%) and glutamic acid (15-26%) (Goldemberg et al., 2008).

A direct utilization of vinasse, known as land application or fertirrigation, has been investigated as an alternative vinasse disposal method. However, due to the vinasse characteristics, this disposal method can cause groundwater contamination and a serious detrimental impact on soil properties (such as nutrients imbalance and reduction of alkalinity), soil salification, increasing soil loss, and decreasing plant cover (Goldemberg et al., 2008; Tejada et al., 2009). Land application of vinasse can also cause metal leaching, phytotoxicity, and odor nuisance (Navarro et al., 2000). Thus, vinasse treatment is necessary prior to discharge. Several methods have been examined to reduce vinasse volume, including recirculation and concentration of vinasse, but they are not cost effective and the vinasse recirculation has an adverse effect on ethanol yield (Moura and Medeiros, 2008). Therefore, by finding diverse applications of the residues and by-products or by converting them into higher value products, ethanol plants can potentially generate higher revenue and subsequently improve their long-term sustainability.

The following sections primarily highlight the bioconversion of sugar-to-ethanol vinasse into bio-based products and valuable biochemicals.

Table 2.2 Characteristics of vinasse from sugar-based feedstocks

Parameters	Sugarcane juice[1]	Sugarcane molasses[1]	Sugar beet molasses[1]	Sweet sorghum[2]
pH	4.0 ± 0.5	4.5 ± 0.4	5.4 ± 1.0	4.5
Chemical oxygen demand (g/L)	30.4 ± 8.2	84.9 ± 30.6	91.1 ± 38.9	80
Biochemical oxygen demand (g/L)	16.7 ± 3.4	39.0 ± 10.8	44.9 ± 21.7	46
Total solids (%)	-	10	-	30
Total nitrogen (mg/L)	628 ± 316	1,229 ± 639	3,569 ± 2,694	800
Total phosphorus (mg/L)	130 ± 110	187 ± 350	163 ± 66	2,000
Sulfate (g/L)	1.4 ± 1.4	3.5 ± 2.5	3.7 ± 2.0	-
Potassium (g/L)	2.0 ± 1.2	5.1 ± 3.1	10.0 ± 6.3	-

Mean value ± standard deviation

Sources: Modified from [1]Wilkie et al. (2000); [2]de Menezes (1989); [3]Harada et al. (1996)

2.2 Value-added Processing of Sugar-based Ethanol Vinasse

2.2.1 Background

With the increasing sugarcane ethanol production, considerable amount of vinasse would be generated from the ethanol plants. Given the characteristics of vinasse, treatment is often required prior to its disposal to minimize the environmental impact. Energy consumption for the treatment could be considerable. A substantial amount of organic matter and some essential micronutrients present in the vinasse could serve as a potential low-cost cultivation medium for growing microorganism to produce microbial biomass or valuable co-products. Thus, value-added processing of the vinasse may serve as a viable alternative that not only reduces the impact on the environment, but also generates additional revenue source for ethanol industries, thereby enhancing the profitability and the economic sustainability of sugar-based ethanol facilities.

This research summarizes the-state-of-the-art of value-added products generation from sugar-based ethanol vinasse.

2.2.2 High-value Products Generation from Sugar-based Ethanol Vinasse

The long-term sustainability of emerging biofuel industries is primarily governed by the value-added processing of residues or low-value co-products. For example, distillers dried grains with solubles (DDGS) from dry-grind corn ethanol plants contribute up to 20% of the total revenue in these plants, which make the corn-based ethanol economically competitive to petroleum fuels (Khanal, 2008). However, sugarcane-to-ethanol plants currently lack suitable options for value-added processing of low-value residue stream, especially vinasse.

Vinasse is a distillery effluent generated following ethanol recovery in sugar-based ethanol plants. Due to high chemical oxygen demand (COD) of the vinasse (Table

2.2), anaerobic fermentation has been adopted for its treatment and biogas generation (Driessen et al., 1994; Fitzgibbon et al., 1995; Kida et al., 1995; Van Haandel and Van Lier, 2006; Satyawali and Balakrishnan, 2008). This approach represents an opportunity to produce alternative gaseous fuels for in-plant heat/steam and electricity productions. Biomethane could potentially replace natural gas and coal used in ethanol facilities. With this opportunity, ethanol industries could significantly reduce the production cost, as the equivalent methane energy produced from anaerobic digestion of vinasse is estimated to be around 6,700-12,600 Btu per gallon of ethanol produced.

Regarding the moderate amounts of nitrogen and phosphorus in vinasse, it could be a potential alternative nutrient-rich medium for producing high-value products. However, the vinasse bioconversion process could be impeded by several inhibitory compounds such as phenolic compounds, melanoidin, and vanillic and gallic acids (Akin and Rigsby, 1985; Borneman et al., 1986; Martin and Akin, 1988; Buswell and Eriksson, 1994; Fitzgibbon et al., 1995). Melanoidin, a brown polymer (from Maillard reaction of sugars with proteins) present in vinasse, is difficult to degrade by microorganisms and also has antioxidant properties, which renders it toxic to many microorganisms (Kitts et al., 1993). Moreover, the high potassium content in vinasse also has a negative impact on the growth of microorganisms (Saha et al., 2005). Consequently, dilution may become essential for effective microbial utilization of vinasse.

The utilization of vinasse as a low-cost substrate for microbial cultivation has been investigated. The goal is the production of microbial biomass, which can subsequently be used as a protein-rich animal feed. Azzam and Heikel (1989) studied two-step aerobic cultivation of *Candida utilis* (yeast) and *Paecilomyces variotii* (fungus). The authors reported the production of high-value microbial biomass containing high leucine, lysine, and threonine with concomitant significant reduction in COD. White-rot fungi have an advantage of utilizing vinasse as they produce a wide array of enzymes which could essentially degrade lignolytic compounds, melanoidins, and phenolic compounds that are difficult to degrade by other microorganisms (Benito et al., 1997). It

has been shown that filamentous fungi such as *Geotrichum candidum, Coriolus versicolor, Phanerochaete chrysosporium, Mycelia sterilia,* and *Pleurotus sajor-caju* were capable of growing on vinasse (Fitzgibbon et al., 1998; Ferreiza et al., 2011). It is evident from these studies that considerable decolorization of vinasse is also possible by fungal cultivation. Thus, fungal treatment can be used to reduce organic matter in vinasse, and at the same time to obtain valuable product such as protein-rich fungal biomass as an animal feed. Moreover, Ferreiza et al. (2011) showed the feasibility of vinasse biodegradation by *Pleurotus sajor-caju*, lactic acid producing fungal species, with the reduction in vinasse toxicity. Therefore, high-value organic acid can be produced from the low-cost raw substrate, vinasse.

Chitin and its derivatives (e.g., chitosan, chitin-oligosaccharides, chitosan-oligosaccharides) are other high-value products that could be derived from fungal biomass. Fungal cell walls and septa of *Ascomycetes, Zygomycetes, Basidiomycetes,* and *Deuteromycetes* are mainly composed of chitin (Hon, 1996; Wu et al., 2005a). Wu et al. (2005a) reported that, on dry weight basis, *Aspergillus niger* and *Mucor indicus* (obsolete *Mucor rouxii)* contain crude chitin of 24% and 13.3%, respectively. *Rhizopus microsporus* (var. *oligosporus)* is also capable of producing chitosan. When cultivated on nutrient medium under aseptic conditions, 3.2% chitosan was produced on dry weight basis (Tan et al., 1996). Thus, fungal biomass could serve as a potential source of several high-value products.

With the relatively high protein content in beet vinasse, it is generally used as an additive for animal feed and as a fertilizer. A study also reported the use of beet vinasse for pig and cattle feeds (Goldemberg et al., 2008). Because of the high potassium and betaine contents, vinasse was recommended for use in low proportions in animal feed to prevent diarrhea in animals. Betaine composition in beet vinasse possesses amphoteric surfactant properties, which present industrial applications in toiletries and personal care products (Giacobello et al., 2000). Similarly, molasses vinasse could possibly be fed to animals but special attention has to be placed on its high potassium level and non-protein

content (betaine) (Olbrich, 1963).

2.3 Fungal Fermentation as a Value-added Processing of Sugar-based Ethanol Vinasse for Animal Feed Applications

2.3.1 Background

Due to vinasse characteristic (high organic content), anaerobic digestion to produce biogas could be considered as a vinasse treatment method because the process is capable of handling waste stream with very high concentration of organic matter (Tchobanoglous et al., 2003). However, the anaerobic treatment of vinasse generates low-value methane and the capital cost is also high that could make sugar-based ethanol production uneconomical (Khanal, 2008). Value-added processing of vinasse via fungal fermentation offers an opportunity of producing high value product with concomitant wastewater remediation.

Moreover, because of the acidic range of optimal pH for fungal growth, fungal fermentation is less susceptible to contamination by other microorganisms. Because fungi are eukaryotes, they can produce numerous extracellular enzymes facilitating the degradation of recalcitrant compounds in the waste; thus they have better adaptation to the waste treatment process and greater ability to resist the inhibitory compounds in the waste stream than those of bacteria (Guest and Smith, 2002). For example, whit-rot fungi can degrade lignin, phenolic compounds and various xenobiotic pollutants (Elisa et al., 1991; Boyle et al., 1992; Yesilada et al., 1999). Fungi remove pollutants including heavy metal and recalcitrant organic compounds via the biosorption through the cell walls (Kapoor and Viraraghavan, 1995) and non-specific oxidative system (Jaouani et al., 2005). Simple process for fungal biomass separation can be adopted if filamentous fungi are used thereby lowering the operating cost for fungal process. Because fungal biomass contains relatively high protein content, the primary purpose of fungal process is to produce single cell protein (SCP) for food/feed applications. Literature complied by

Thrane (2007) suggested the use of numerous fungal species including filamentous fungi and yeast for SCP production in food and feed applications, and some fungi are capable of utilizing wastewater for SCP production (Sankaran et al., 2010) (Table 2.3).

However, the use of fungal biomass (also known as mycoprotein) has some drawback as pathogenic fungi produce toxins called mycotoxins through secondary metabolic process (Adams, 2001). Therefore, the cultivation of edible fungi could potentially eliminate this concern. In fact, fungal biomass production for human consumption has been commercialized. The fungal protein product named Quorn® resulted from the fungal species, *Fusarium venematum* (formerly known as *Fusarium graminearum*), is available in the market and is used as a meat analog which contains ~ 45% protein, 14% fat, and 26% fiber (on dry weight basis) with essential amino acids comparable to eggs, and protein digestibility comparable to beef and soybean (Anderson and Solomons, 1984; Miller and Dwyer, 2001; Rodger, 2001; Wilson, 2001). Furthermore, fungi are used to produce food products worldwide, such as tempeh (*Rhizopus oligosporus*), Roquefort or blue cheese (*Penicillium roqueforti*), pehtze or sufu (*Actinomucor repens*, *Actinomucor taiwanensis*, *Mucor circinelloides*, *Mocur hiemalis*, *Mocur racemosus*, and *Rhizopus microsporus* (var. *microsporus*)), miso, sake, and soy sauce (*koji/Aspergillus* sp.) (Fujita, 2003; Han et al., 2004; Nout, 2007). This research focuses particularly on the use of an edible filamentous fungal species, *Rhizopus microsporus* var. *oligosporus* (shortened *R. oligosporus*), for animal feed applications and wastewater remediation, and is presented in a greater detail.

2.3.2 *Rhizopus microsporus* var. *oligosporus*

The filamentous fungal species, *R. oligosporus,* belongs to the phylum *Zygomycota*, order *Mucorales*, and family *Mucoraceae* (Schipper and Stalpers, 1984). The colonies of *R. oligosporus* are pale brownish-gray and dark pigmented sporangiophores. According to the American Type Culture Collection (ATCC),

Table 2.3 Single cell protein (SCP) production from fungi for food and feed, and biochemical applications

Fungal species	Substrate	Reference
Actinomucor elegans	Beet waste	Hang, 1976
Aspergillus awamori	Apple distillery wastewater	Friedrich, 1987
Aspergillus foetidus	Potato chips industry wastewater	Mishra and Arora, 2004
Aspergillus fumigatus	Cassava	Reade and Gregory, 1975; Khor et al., 1977
Aspergillus niger	Starch wastewater from wheat milling, Potato chips industry wastewater, Beet waste, Cassava (flour and gari), Corn cob, Maize and Cotton stalk	Christias et al., 1975; Hang, 1976; El-Saadany et al., 1988; Singh et al., 1991; Oboh et al., 2002; Mishra and Arora, 2004
Aspergillus oryzae	Cassava starch processing wastewater, Beet waste	Hang, 1976; Jin et al., 1998; Truong et al., 2004
Candida lipolytica	Glucose, N-paraffin fraction of crude oil, n-alkanes C:7-C:17	Achermowicz et al., 1977
Candida tropicalis	Glucose, N-paraffin fraction of crude oil, n-alkanes C:7-C:17, Sulfite waste liquor	Christias et al., 1975; Achermowicz et al., 1977; Guven and Cansunar, 1989

Table 2.3 Single cell protein (SCP) production from fungi for food and feed, and biochemical applications (continue)

Fungal species	Substrate	Reference
Candida utilis	Apple pomace	Villas-Boas et al., 2003
Chaetomium cellulolyticum	Cellulosic waste	Singh, 1998
Chaetomium globosum	Beet waste	Hang, 1976
Chrysonilia sitophilia	Lignin	Rodriguez et al., 1997
Fusarium graminearum	Starch hydrolysate	Singh, 1998
Fusarium moniliforme	Carbohydrate (carob aqueous extract)	Christias et al., 1975; Drouliscos et al., 1976
Fusarium oxysporum	Cassava starch	Sukara and Doelle, 1989
Fusarium venenatum	Carbohydrate (glucose)	Anderson and Solomons, 1984; Trinci, 1992
Geotrichum candidum	Orange peel extract	Robinson and Smith, 1984; Ziino et al., 1999
Paecilomyces variolii	Sulfite liquor	Singh, 1998

Table 2.3 Single cell protein (SCP) production from fungi for food and feed, and biochemical applications (continue)

Fungal species	Substrate	Reference
Penicillium cyclopium	Whey	Kim and Lebeault, 1981
Pestalotriopsis westerdijkii	Beet waste	Hang, 1976
Phanerochaete chrysosporium	Vinasse	Cardoso and Nicoli, 1981
Rhizopus oligosporus	Cassava starch, Food-processing wastewater	Sukara and Doelle, 1989; van Leeuwen et al., 2003
Saccharomyces cereviceae	Molasses, Stillage	Singh, 1998
Thielavia terrestris	Sugar beet pulp, Potato processing waste	Bajon et al., 1985; Stevens and Gregory, 1987
Trichoerma reesei	Apple distillery wastewater	Friedrich, 1987
Trichoerma viride	Beet waste, Rice straw	Hang, 1976; Youssef and Aziz, 1999

Sources: Adapted from Anupama and Ravindra (2000); Sankaran et al. (2010); Thrane (2007)

R. oligosporus has been classified as a biosafety level 1 (BSL-1), which is not known to cause disease in immunocompetent adult humans. In spite of the fact that some texa of *Rhizopus* are human pathogens, *R. oligosporus* is not associated with the production of any potentially harmful metabolites (Jennessen et al., 2005). Moreover, the fungus has been given generally recognized as safe (GRAS) status by the U.S. Food and Drug Administration (FDA) (Schauer and Borriss, 2004). Therefore, although, the food-grade fungus, *R. oligosporus* is not found in nature (Pitt and Hocking, 1999), it is widely used by human to produce tempeh, the traditional Indonesian fermented food (Hesseltine et al., 1963; Steinkraus et al., 1983; Sharma and Sarbhoy, 1984; Nout and Kiers, 2005). During the process of producing tempeh, a fungus forms white mycelium that bound soybeans together forming a cake and releases enzymes making the product more digestible to humans (Beuchat, 1987).

Further to the growth, which produces cell biomass, *R. oligosporus* also excretes a wide range of biochemicals and enzymes for complex nutrients degradation (van Leeuwen et al., 2003). The enzymes such as lipase (Nahas, 1988; Nakashima et al., 1990) and protease (Wang and Hesseltine, 1965; Wang et al., 1974) from *R. oligosporus* have been produced at an industrial level. The phytase produced by *R. oligosporus* displayed physicochemical characteristics for potential used in the animal feed industry (Sabu et al., 2002; Casey and Walsh, 2004). The *R. oligosporus* is capable of utilizing a wide range of protein as an energy source for its growth. Handoyo et al. (2006) investigated the assimilation of allergenic buckwheat proteins by *R. oligosporus*. The study reported the use of *R. oligosporus* in hypoallergenic buckwheat flour production for noodle making application. Additionally, the fungus can also produce secondary metabolites which are valuable for nutritional food production and pharmaceutical applications. Several vitamins, including riboflavin, niacin, and pyridoxine, were reported to be produced by *R. oligosporus* (Murata et al., 1967; Keuth and Bisping, 1993; Wiesel et al., 1997). The production of ergostadientriols by the fungus, which is useful in lowering serum cholesterol levels, was patented (Zilliken, 1980).

R. oligosporus is also extremely valuable from environmental perspectives. With the filamentous structure of the fungus, *R. oligosporus* has been potentially used as a biosorbent for industrial wastewater treatment. The fungus can significantly remove COD of up to 90% from wheat milling and corn wet milling wastewater streams (Jin et al., 1999; Jasti et al., 2006). Moreover, the fungal biomass has been reported to be effective as an adsorbent for Cu (II) ion removal (Beolchini et al., 2003). Ozsoy et al. (2008) studied the use of food processing wastewater as a substrate for the *R. oligosporus* biosorbent production. The study indicated the reduction of fungal cultivation cost for producing a Cu (II) ion biosorbent. Othman and Akil (2008) also indicated the ability of *R. oligosporus* to enhance the carbon-based gas adsorption.

2.3.3 Fungal Growth and Growth Requirements

2.3.3.1 Fungal Growth

The growth of single-celled fungi, such as yeast, in liquid culture follows a typical bacterial growth curve, including six stages− lag, acceleration, exponential, deceleration, stationary, and decline phases− as shown in (Figure 2.2) (Moore-Landecker, 1990). The lag phase (Stage I) occurs when organisms are inoculated into growth medium. The organisms adapt themselves to the new environment resulting in no cell division. Following the lag phase, there is the acceleration phase (Stage II), where cell division begins and transition between dormancy and active growth occurs. The cells then go to Stage III, exponential or log phase, where cell division reaches and maintains a uniform rate until the depletion of nutrients in growth medium and accumulation of wastes or inhibitory compounds. Under this stage, number of cells increases exponentially and the greatest number of cells is reached. However, the cell division then begins to decline in the next stage, the deceleration phase (Stage IV). Due to a growth-limiting factor in the stationary phase (Stage V), there is no additional growth. The production of new cell is balanced by the death of old cells. Finally, the decrease in viable cell number occurs in

the decline phase (Stage VI).

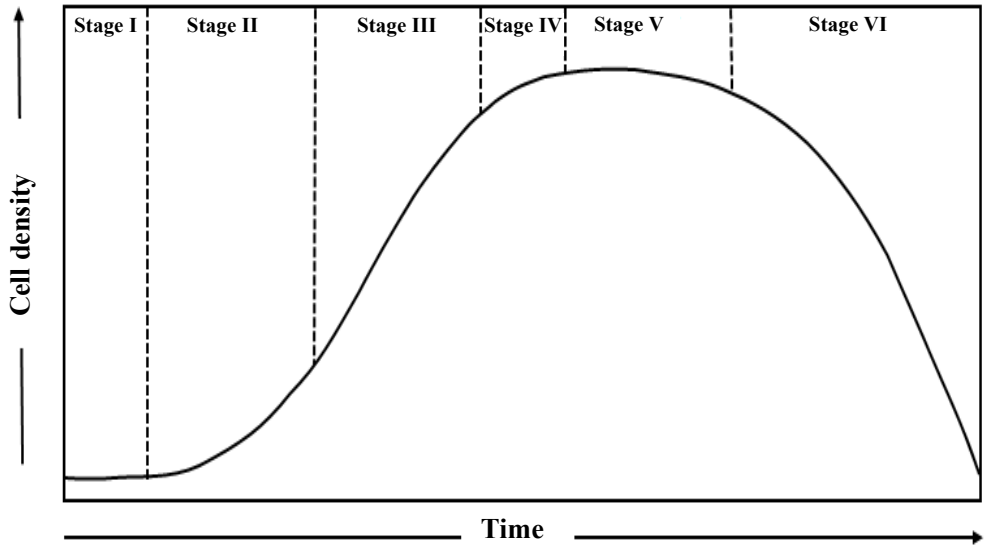


Figure 2.2 A typical bacterial growth pattern

Source: Moore-Landecker (1990)

The cell growth of filamentous fungi, however, does not follow this growth pattern. Filamentous fungi lack cell division from each individual cell into two cells. The growth of filamentous fungi is restricted only to the hyphal tip, which grows at a constant linear rate (Moore-Landecker, 1990). However, if the entire branching mycelium is considered, the exponential growth at the early stages can be observed. In later growth stages, the growth in the center of the colony declines while the marginal hyphae maintain their linear growth rate. Generally, growth of filamentous fungi can be broadly classified into three stages: Stage I (lag phase) with no apparent growth; Stage II (linear phase) with rapid and approximately linear growth; and Stage III (decline phase) with a

decline in cell dry weight due to cell autolysis (Figure 2.3).

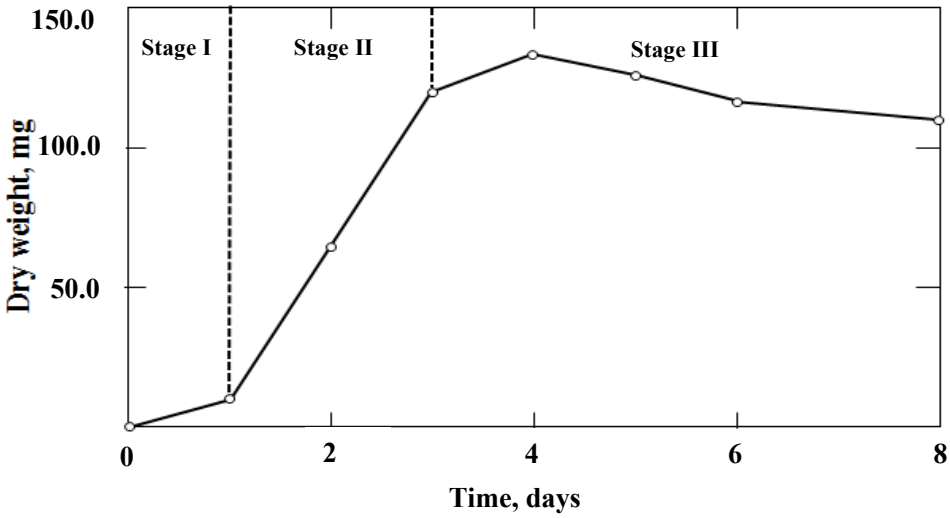


Figure 2.3 A typical growth pattern of filamentous fungi

Source: Moore-Landecker (1990)

2.3.3.2 Growth Requirements for Fungi

Nutrients

Fungi are heterotrophic osmotrophs, which excrete digestive enzymes to breakdown complex nutrients and absorb digested soluble nutrients through their cell walls via active and passive transport (Dick, 1997). Thus fungi require external nutrient source. Fungi produce a series of extracellular hydrolytic enzymes which can degrade wide variety of organic materials such as cellulose and lignocellulose, starch, cheese whey, spent sulfite liquor, molasses, and sugar beet pulp (Satyawali and Balakrishnan, 2008; Sankaran et al., 2010). For example, extracellular enzymes produced from white-rot fungi have potential for treating wastewater containing lignin and cellulose in pulp and paper mill and cork-boiling industries and *Phanerochaete chrysosporium* is the most

effective species of the white-rot fungi (Friedrich et al., 1986; Mendonca et al., 2004; Raghukumar et al., 2004; Guimarães et al., 2005; Wu et al., 2005b; Shrestha et al., 2007). Carbohydrates in the form of simple sugars are the excellent carbon source for fungal growth. Sorenson and Hesseltine (1996) reported that common sugars including glucose, fructose, galactose, xylose, and maltose, were the excellent carbon source to support the growth of *R. oligosporus*. However, the fungus did not use sucrose and raffinose as its growth substrate. Sugar alcohols such as sorbitol, glycerol, and mannitol may be utilized but are less preferable with respect to simple sugars (Moore-Landecker, 1990). Other than carbohydrates, lipids and fatty acids including oleate and palmitate can also be a carbon source for fungal growth (Sorenson and Hesseltine, 1996; Walker and White, 2005). Nitrogen source can be both organic (yeast extract, peptone, and amino acids) and inorganic (ammonium, nitrate, and nitrite). However, *Blastocladiales* sp., *Saprolegniaceae* sp., *R. oligosporus*, few types of yeast, and higher basidiomycetes do not consume nitrate (Whitaker, 1976; Liu and Sundhesim, 1996; Sorenson and Hesseltine, 1996; Siverio, 2002). Carbon to nitrogen [C/N] ratio of a balance fungal medium should be about 10:1 for high protein content of the fungal biomass, whereas the ratio exceeding 50:1 favors the accumulation of secondary metabolites, alcohols, lipids, or extracellular polysaccharides (Carlile and Watkinson, 1994). However, the nutrient requirement is a case sensitive and also depends on growth conditions. Sulfur is usually supplied in the culture medium in the form of sulfate ion (SO_4^{2-}) to support fungal growth (Moore-Landecker, 1990). However, Blastocladiales cannot reduce the sulfate ion; therefore reduced sulfur in form of cysteine, glutathione, or methionine has to be supplied in the growth medium. Phosphorus, potassium, and magnesium are required at a minimal concentration of about 1-4 mM (Moore-Landecker, 1990).

pH

The optimal pH for most fungi is below 5.0, which is within an acidic range. Sorenson and Hesseltine (1966) showed that the growth of *R. oligosporus* is possible at a pH as low as 2.6-3.0. pH affects the growth rate resulting from enzyme activity, cell

permeability, and metal ion availability. Moreover, pH also has an effect on microbial competition for substrate in a mixed culture (Van der Westhuizen and Pretorius, 1998; Jasti et al., 2006). However, it is possible for fungi having two optimal pHs. Moore-Landecker (1990) reported that the lower pH increases iron availability whereas the higher pH improves enzyme activity.

Temperature

Temperature affects growth rate, nutritional requirements, cell permeability, enzyme activity, and metabolism. The major mechanism affected by temperature is the structure and the composition of cytoplasmic membranes which determines the rate of substrate utilization (Madigan et al., 2000). The optimal temperature for fungal growth is in the rage of 20-40^{o}C since most fungi suitable for wastewater treatment are mesophiles. Nout and Kiers (2005) suggested that *R. oligosporus* prefers a temperature range of 30-40^{o}C for its growth. However, some fungi, capable of growing at a higher temperature, are known as thermophilic fungi. Maheshwari et al. (2000) studied the use of thermophilic fungi with cultivating temperature above 20^{o}C to ~ 62^{o}C. The study showed a higher growth rate and better waste degradation measured as soluble-protein fractions of the thermophilic fungi compared to that of mesophilic fungi. In contrast, the psychrophiles grow at a much lower temperature with the optimal temperature generally about 10^{o}C (Moore-Landecker, 1990).

Oxygen

Although some fungi are facultative anaerobes, typically, the growth of fungi requires oxygen since most fungi are obligate aerobes. The obligate aerobes require free molecular oxygen to support their growth. Limited oxygen in the culture media can have adverse effect on fungal growth resulting from increasing nutritional demand (Tabak and Cooke, 1968; Moore-Landecker, 1990). Oxygen level also affects the effective utilization of nutrients, such as carbon and nitrogen. For example, under oxygen limiting condition, *Mucor rouxii* is able to use only hexose as a carbon source and poorly consumes amino

acids as a nitrogen source whereas nitrate or ammonium ions serve as a better nitrogen source (Bartnicki-Garcia and Nickerson, 1962).

Light

The growth of most fungi is independent of light; however, strong light intensity may inhibit the fungal growth (Moore-Landecker, 1990). Interestingly, light can act as both stimulator and inhibitor on the vegetative structure of individual fungus. The growth of *Candida albicans* was inhibited by light whereas the synthesis of carbohydrate was enhanced (Saltarelli and Coppola, 1979).

2.3.4 Protein-rich Fungal Biomass for Animal Feed Applications

One of the most critical parameters in animal feeds is protein content. Soybean meal and fishmeal have been commercially used as major protein supplements for animal feeds. With the high demand for meat and fish products, rapid increase in feed ingredient prices has been affecting their economic viability. Taking a closer look in particular at an aquaculture industry, the world fastest growing food production sector, with the annual growth rate of 8-10% (Tacon et al., 2011), it has been pushing the demand for feed ingredients perpetually. Although global aquatic feed production is expected to grow continuously at a similar rate to 71 million metric tons by 2020, the major dietary source of protein is largely dependent on capture fisheries for fishmeal production. Fishmeal is expensive (currently ~ $1,395-$1,450 per dry ton (US) (USDA, 2012)) and continues to increase with a significant raise in its demand. This results in increasing the cost of feed and ultimately increasing the cost of meat and fish products. Consequently, there is a greater need of a more sustainable feed source. The use of cost-effective dietary fishmeal replacement especially a single cell protein (SCP) derived from low-cost feedstock or residues has gained significant attention. Among the SCPs for feed applications, fungal SCP is favorable over bacterial SCP (Anupama and Ravinda, 2000). For example, fungi can produce sulfur-containing amino acids, cysteine and methionine, which are often the

limiting amino acids in feeds, whereas bacteria have no such ability. Methionine is considered as an essential amino acid thus it cannot be synthesized in the animal body and has to be supplied in feed. Similarly, cysteine is a semi-essential amino acid and can be synthesized only from methionine. There are, however, some issues with the fungal SCP, such as mycotoxins and high content of non-protein nitrogen, that have to be taken into account.

The use of SCP for food/feed applications is not new. Fungi grow rapidly under optimal growth conditions and are able to utilize diverse organic substrates (Sumbali, 2005). Stevens and Gregory (1997) suggested that fungal wastewater treatment is a low-cost and simple process for animal feed production. Fungal biomass has high protein content which can replace the use of major supplements in animal feeds such as soybean meal and fishmeal (Anupama and Ravinda, 2000; Sumbali, 2005; Thrane, 2007). However, fungal protein also has relatively high non-protein nitrogen content, such as nucleic acids and chitin, and mycotoxins thereby causing concerns of its application for feedstuffs. Although, dietary nutritional requirements for animals differ greatly depending on type of animals, their growth stage, and environment, the following section aims to emphasize the use of filamentous fungal biomass, *R. oligosporus*, as a protein ingredient for feed applications.

Although the gross energy content of protein (23.6 kJ/g) is much greater than that of carbohydrate (17.4 kJ/g for starch), dietary protein is not used efficiently as a source of energy. Dietary protein in feeds is generally converted into body protein; however, if energy is limiting, it will be used as a source of energy resulting in excreting nitrogen (N) in the forms of urea (mammals), ammonia (fish), or uric acid (birds). Generally, fish requires less energy, but high protein; therefore, fish feeds contain significantly high protein compared to other animal feeds (Table 2.4) (Miller, 2004). Consequently, supplying adequate and suitable energy source in fish feed formula is critical, because if protein is utilized as an energy source, ammonia excretion from protein catabolism will have adverse effect on the environment. In addition, protein, a polymer composed of

chains of various monomers, namely amino acids, makes up the most of the animal bodies including, muscle, skin, and wool. The amount of protein in feed is unfortunately not what it is available to animals. One of the critical parameters in animal feed is its digestibility (Bregendahl, 2008). Protein digestibility is dependent on animal species and stages of animal growth (Miller, 2004). For example, carnivores lack ability to digest fibrous feed resulting in higher protein content requirement if the feed contains fibrous material. Considering protein content and its digestibility, the use of cell biomass produced from *R. oligosporus* for feed application is promising. The fungal biomass contains crude protein of nearly 50% (dry weight) with 84% *in vitro* digestibility when cultivated on starch processing wastewater, which is comparable to soybean meal (Jin et al., 2002). The protein digestibility largely depends on harvesting and drying technologies. Heat affects protein digestibility and quality as it changes amino acids structure resulting in loss of some available essential amino acids (Miller, 2004). Other compositions including fiber and/or phytate can reduce protein digestibility (Mwachireya et al., 1999). The composition of fungal biomass is presented in Table 2.5.

For feed purpose, the amino acid constituent in the protein is critically important other than available protein quantity. Some amino acids, known as essential or indispensable amino acids, are required as the body cannot synthesize and must be provided in the diet. It is important to note that the requirement of protein and essential amino acids varies depending on organism, their growth stage, and growth environment. For example, arginine is an essential amino acid for birds, fish, and young mammals but not for adult mammals (Tapiero et al., 2002). Utilizing urea cycle, mammals can synthesize arginine but not in sufficient amount as it also breaks down to release urea. Similarly, glycine and serine can be synthesized but not in sufficient quantities in young animals. These amino acids are also known as conditionally essential/indispensable amino acids. The essential amino acids can also be the starting material for synthesizing the remaining amino acids. For example, cysteine and tyrosine, semi-essential/indispensable amino acid, can be synthesized only from methionine and phenylalanine, respectively. Commonly, lysine and methionine (or methionine + cystine)

Table 2.4 Typical dietary crude protein (CP) and percentage protein energy

Animals	Growth state		Crude protein (g/kg)	Protein energy (%)
PIG	Starter 3 week weaning	5-10 kg	240	29
	Starter 5 week weaning	10-20 kg	210	26
	Grower	20-60 kg	165	22
	Finisher	60-90 kg	140	19
	Sow lactating		176	24
	Sow pregnant		130	18
BIRDS	Broiler starter	0-2 weeks	230	31
	Broiler grower	2-4 weeks	210	27
	Broiler finisher	4-7 weeks	190	24
	Rearing pullets	0-6 weeks	210	31
		6-12 weeks	145	21
		12-18 weeks	120	18
	Laying hens		160	24
	Turkey starter	0-6 weeks	300	40
	Turkey grower	6-12 weeks	260	35
	Turkey finisher	12 + weeks	180	24
	Breeding turkeys		160	24
DOGS	Growth/Lactation		250	30
	Maintenance		130-20	17-29
CATS	Growth/Lactation		>310	33
	Maintenance		>220	27
FISH	Salmonids fry, fingerlings		550	70
	Salmonids fry, smolt		400-460	58
	Catfish		320-360	58

Source: Modified from Miller (2004)

Table 2.5 Composition of single cell protein derived from fungi

Parameters	Percentage composition (dry weight basis)
True proteins	30-70
Total nitrogen (protein + nucleic acids)	35-50
Lysine	6.5-7.8
Methionine	1.5-1.8
Fats/Lipids	5-13
Carbohydrates	Not available
Bile pigment and Chlorophyll	Not available
Nucleic acids	9.70
Mineral salts	6.6
Amino acids	54
Ash	Not available
Moisture	4.5-6.0
Fiber	Not available

Source: Anupama and Ravinda (2000)

are the first two limiting amino acids in all feeds (Miller, 2004; Craig and Helfrich, 2009).Tryptophan and threonine are also limiting amino acids in non-ruminant diets (Cheeks, 2005). Moreover, some amino acids play significant role in feed acceptance especially in aquaculture as the feeds must attract fish or crustaceans prior to palatable acceptability (Anupama and Ravinda, 2000). For example, carnivorous fish prefer glycine, proline, taurine, and valine whereas omnivorous fish prefer aspartic and glutamic acids (NRC, 1993). Table 2.6 summarizes the percentage of amino acid composition (% protein basis) of *R. oligosporus* and the requirement from the Food and Agriculture Organization of the United Nations (FAO).

Table 2.6 The amino acid composition (%) of *Rhizopus oligosporus* as compared to the FAO standard

Amino acids	Percentage composition (protein basis)	
	***Rhizopus oligosporus*[1]**	**FAO standard[2]**
Lysine	4.07	4.2
Histidine	1.52	2.4
Arginine	2.47	2.0
Aspartic	4.82	Not available
Threonine	2.56	2.6
Serine	2.95	Not available
Glutamic	4.72	Not available
Proline	1.96	Not available
Glycine	2.68	Not available
Alanine	2.77	Not available
Cystine	1.03	Not available
Valine	3.41	4.2
Methionine	0.89	2.2
Isoleucine	3.22	4.2
Leucine	4.61	4.8
Tyrosine	1.85	2.8
Phenylalanine	2.89	2.8
Tryptophan	0.50	1.4
Total amino acid (T)	48.92	Not available
Total essential amino acid (E)	25.03	Not available
E/T	0.51	Not available

Sources: [1]Stillings and Hackler (1995); [2] FAO/WHO (1991)

The non-protein nitrogen in fungal biomass may result in overestimation of protein quantity in the cell biomass. Fungal cell component is composed of various non-protein nitrogen substances, such as nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)), chitin and chitosan (cell wall material), and nonessential metabolites, resulting in only approximately 60-70% of total nitrogen of the fungal biomass as available protein (Moore-Landecker, 1990). The non-protein nitrogen compounds are nutritionally unavailable for nonruminants and mostly have an adverse effect on animals (Moore-Landecker, 1990; Sadler, 1994; Miller, 2004).

Nucleic acid content in feed is also a concern as the uric acid, final product from nucleic acid degradation, can accumulate in the body causing kidney stone formation and gout (Calloway, 1974). The DNA content of fungi is relatively low (0.15-0.30%), which is below the recommended maximum limit of 2% for humans (Thrane, 2007). Moreover, the author suggested that heat-shock of the fungal biomass at 64^{o}C for 20 min can significantly reduce nucleic acid content in the fungal cell to the safe level even for human consumption. Other chemicals containing nitrogen present in fungal cell composition are chitin and chitosan, which are polysaccharides derived from biosynthesis of cell wall material. Chitin and chitosan have a high viscosity causing an increase in water fraction of poultry gut content and reduce digestibility by impeding mucosal nutrient uptake, and they serve as dietary fiber resulting in lower digestibility (Cheeke, 2005). However, fortunately, *R. oligosporus* consisted of only ~ 3% chitosan (Rhodes et al., 1961; Tan et al., 1996).

Fungal protein derived from *R. oligosporus* has several advantages including the production of large variety of valuable organic compounds, including vitamins (riboflavin, niacin, and pyridoxine) (Murata et al., 1967; Keuth and Bisping, 1993; Wiesel et al., 1997) and antioxidant, such as ergostadientriols, which is useful in lowering serum cholesterol levels (Zilliken, 1980). Further, fungi have an ability to produce long-chain fatty acids (namely palmitic, oleic, and linoleic acids), phospholipids, and sphingolipids (Kendrick, 2000). Apart from valuable biochemical, on the other hand,

fungi can also produce toxins, known as mycotoxins, typical secondary metabolites (Bennett and Keller, 1997). Although the presence of mycotoxins is a major hindrance to the use of fungal biomass in feed applications, *R. oligosporus* does not produce any harmful metabolites and is considered safe for feed applications (Jennessen et al., 2005).

In conclusion, the economic viability and the long-term sustainability of biofuel plants are not only dependent on generation of biofuel: but also on the production of other high-value co-products from low-value residue streams. Considerable amounts of residues/by-products generated during biofuel production bring about not only environmental concerns, but also high treatment costs. Therefore, it makes the production of biofuel costly and uncompetitive with the petroleum fuel, if biofuel is the only product. Consequently, adaption of biorefinery concept is critically important for a long-term sustainability of biofuel plants.

The protein production on sugarcane vinasse via fungal fermentation provides a plausible solution. However, attention must be placed in maximizing fungal biomass yield and product recovery. The composition of fungal biomass depends significantly on harvesting and dewatering processes. For example, heat treatment results in lowering digestibility and destroys some essential amino acids. Moreover, the occurrence of undesirable microbial contamination during cultivation would change the nutritional composition of fungal biomass. Consequently, this affects the quality of fungal biomass as an animal feed.

CHAPTER 3: MATERIALS AND METHODS

3.1 Vinasse Samples

Three different types of vinasses, rum-vinasse, molasses-vinasse, and cane-vinasse, were examined in the fungal fermentation studies. Vinasse sample was collected from Haleakala Distillers (Kula, HI, USA), defined here as rum-vinasse, and was used directly without further treatment. Molasses-vinasse and cane-vinasse samples were prepared in the laboratory by the yeast fermentation of molasses and sugarcane juice, respectively, obtained from Hawaiian Commercial & Sugar Company (HC&S) (Puunene, HI, USA). After growth optimization studies, cane-vinasse was selected for further experiments. The cane-vinasse sample was prepared in the laboratory using sugarcane syrup obtained from HC&S. The sugarcane syrup was diluted to obtain a desired concentration for ethanol fermentation (approximately 15 degree Brix or $g_{sucrose}/100\ g_{sample}$). Ethanol fermentation was carried out using yeast, *Saccharomyces cerevisiae,* for 72 h. The fermentation was performed in a batch-mode using a stirred tank bioreactor (5.5 L working volume) (BioFlo 110, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at a temperature of 30^{o}C and a pH of 4.0. The first 6 h of fermentation was operated aerobically at an agitation speed of 300 rpm and at an aeration rate of 0.5 vvm ($volume_{air}/volume_{liq}$/min) then followed by an anaerobic fermentation at an agitation speed of 100 rpm. The reaction was terminated by autoclaving the fermentation broth at 121^{o}C for 20 min (HICLAVE™ HVE-50, Hirayama, Amerec Instruments Inc., Lafayette, CA, USA). Ethanol was recovered using a rotary evaporator operating at 50^{o}C and 120 rpm (Rotavapor R-215, Büchi Labortechnik AG, Flawil, Switzerland), and a vacuum pump was employed to maintain a pressure of 93 kPa (Welch® 202501 Self-cleaning dry vacuum system™, Gardner Denver Welch Vacuum Technology, Inc., Monroe, LA, USA). The ethanol recovery process was conducted for 30 min.

In order to evaluate the feasibility of fungal biomass production on vinasse in sugarcane-ethanol industries, vinasse sample, known as Brazil-vinasse, obtained from a

commercial sugarcane-to-ethanol facility in Brazil was used.

All vinasse samples were kept refrigerated at 4^{o}C until further use. The characteristics of the vinasse samples for fungal growth optimization studies and other experiments are summarized in Table 3.1.

3.2 Fungal Culture and Fungal Mycelia Inoculum Preparation

Food-grade fungal species, *Rhizopus microsporus* (var. *oligosporus*), was used in this research. There were two fungal cultures derived from two different sources, including a tempeh starter obtained from a fungal spore powder of a tempeh starter (The Farm's Tempeh Lab Inc., Summertown, TN, USA) and an ATCC culture obtained from the American Type Culture Collection (ATCC # 22959, Rockville, MD, USA). The tempeh starter culture was used in growth optimization studies whereas the ATCC fungal culture was used in later experiments. The freeze-dried samples were both reactivated for fungal spore suspension preparation. While the tempeh starter was reactivated in yeast mold (YM) broth (Difco Laboratories, Sparks, MD, USA) at 30°C, an optimal temperature for the culture, the ATCC freeze-dried fungal culture was rehydrated and revived using sterile water following the instruction provided by ATCC. In brief, about 5 ml of sterile water was transferred into the freeze-dried culture. The mixture was allowed to rehydrate for a minimum of 2 h before transferred into a solid medium. The fungal suspensions from both fungal cultures were transferred into potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, USA), and the plates were incubated at 30°C (for tempeh starter culture) and 24^{o}C (for ATCC culture) for 5-7 days. To prepare fungal spore suspension, the fungal spores were harvested and kept in a solution containing 0.1% (w/v) peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). Glycerol (20% v/v) was added to the harvested spore solution prior to storage at -30^{o}C (R 404 freezer, Summit commercial freezer, Bronx, NY, USA). Harvested fungal spore suspension contained approximately 10^{6}-10^{7} spores/ml, based on hemacytometer count (Hausser Scientific, Horsham, PA, USA).

Table 3.1 Characteristics of various vinasse samples

Parameters	Fungal growth optimization experiments			Other experiments	
	Rum-vinasse	Molasses-vinasse	Cane-vinasse	Vinasse (sugarcane)	Brazil vinasse
pH	4.30 ± 0.07	3.96 ± 0.27	3.88 ± 0.11	4.25 ± 0.32	4.15 ± 0.04
Total solids (TS) (%)	11.82 ± 0.14	5.14 ± 0.15	2.46 ± 0.04	2.62 ± 0.12	3.68 ± 0.00
Volatile solids (VS) (%)	73.77 ± 1.00	66.54 ± 1.00	71.95 ± 0.20	65.47 ± 1.16	64.67 ± 0.00
Total suspended solids (TSS) (%)	1.86 ± 0.12	1.16 ± 0.11	0.59 ± 0.02	0.76 ± 0.05	0.67 ± 0.03
Volatile suspended solids (VSS) (%)	77.96 ± 0.70	77.59 ± 2.70	89.83 ± 1.10	90.14 ± 1.88	77.61 ± 0.20
Total chemical oxygen demand (COD) (g/L)	126.57 ± 6.01	78.61 ± 6.47	85.79 ± 8.38	64.48 ± 2.31	42.99 ± 1.54
Soluble chemical oxygen demand (SCOD) (g/L)	123.17 ± 4.84	68.60 ± 3.83	68.35 ± 7.48	55.55 ± 4.15	37.11 ± 0.81
Total Kjeldahl nitrogen (TKN) (mg/L)	965.84 ± 8.60	550.40 ± 20.54	340.00 ± 4.07	365.86 ± 80.90	748.04 ± 32.92

Table 3.1 Characteristics of various vinasse samples (continue)

Parameters	Fungal growth optimization experiments			Other experiments	
	Rum-vinasse	Molasses-vinasse	Cane-vinasse	Vinasse (sugarcane)	Brazil vinasse
Ethanol (g/L)	9.53 ± 0.53	15.75 ± 0.56	20.43 ± 0.30	29.43 ± 3.87	2.33 ± 0.00
Glycerol (g/L)	-	-	-	6.80 ± 0.00	3.48 ± 0.01
Lactic acid (g/L)	7.08 ± 0.37	3.51 ± 0.19	4.15 ± 0.10	7.43 ± 0.00	3.91 ± 0.01
Acetic acid (g/L)	0.75 ± 0.00	-	-	0.78 ± 0.00	0.89 ± 0.00
Potassium (K) (g/L)	10.67 ± 0.29	5.20 ± 0.20	1.53 ± 0.15	1.73 ± 0.19	4.45 ± 0.14
Calcium (Ca) (g/L)	2.30 ± 0.10	1.21 ± 0.01	0.34 ± 0.00	0.56 ± 0.13	1.17 ± 0.01
Magnesium (Mg) (g/L)	1.32 ± 0.01	0.61 ± 0.01	0.17 ± 0.01	0.38 ± 0.04	0.43 ± 0.02
Phosphorus (P) (mg/L)	118.32 ± 2.90	38.80 ± 0.98	5.07 ± 0.04	28.58 ± 2.61	31.20 ± 2.83
Sodium (Na) (mg/L)	890.00 ± 1.76	651.79 ± 0.94	374.20 ± 0.36	954.40 ± 252.91	91.11 ± 68.72

Table 3.1 Characteristics of various vinasse samples (continue)

Parameters	Fungal growth optimization experiments			Other experiments	
	Rum-vinasse	Molasses-vinasse	Cane-vinasse	Vinasse (sugarcane)	Brazil vinasse
Iron (Fe) (mg/L)	74.02 ± 0.88	30.84 ±0.42	0.64 ±0.03	1.84 ± 0.22	11.63 ± 0.02
Manganese (Mn) (mg/L)	40.07 ± 0.08	14.02 ± 0.05	1.45 ± 0.01	3.12 ± 0.42	3.56 ± 0.01
Zinc (Zn) (mg/L)	5.40 ± 0.02	1.13 ± 0.01	0.64 ± 0.00	0.80 ± 0.14	0.55 ± 0.29
Copper (Cu) (mg/L)	4.02 ± 0.07	0.93 ± 0.03	0.16 ± 0.01	0.15 ± 0.09	0.20 ± 0.08
Boron (B) (mg/L)	1.26 ± 0.01	0.76 ± 0.01	0.18 ± 0.00	0.48 ± 0.14	0.26 ± 0.16
Molybdenum (Mo) (mg/L)	0.27 ± 0.01	-	-	-	-
Aluminum (Al) (mg/L)	13.77 ± 0.10	-	-	-	-
Silicon (Si) (mg/L)	46.54 ± 1.34	-	-	-	-

Mean value ± standard deviation (sample size (n) ≥ 6)

To prepare mycelia inoculum, 0.5% (v/v) spore suspensions were inoculated into YM media of 100 ml (for experiments conducted using 250-ml Erlenmeyer flasks) and 500 ml (for experiment conducted in bioreactors). The mycelia starter cultures were incubated in an incubator shaker (Inova™ 4230 and Excella E25, New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and 30°C for 2 days for experiments conducted using the fungal tempeh starter culture whereas the ATCC fungal culture was incubated at 150 rpm and 37°C for 3 days (Table 3.2). Regarding the fungal mycelia inoculum prepared using tempeh starter, the culture was then decanted by centrifugation at 15,557 × g for 15 min and inoculated into vinasse samples (Eppendorf 5810R, Brinkmann Instruments Inc., Westbury, NY, USA). The other set of mycelia inoculum prepared from ATCC culture was either decanted by passing through the USA Standard Test Sieve with 250-µm nominal opening size (ASTM E-11, No. 60, Fisher Scientific, Fair Lawn, NJ, USA) and inoculated into vinasse samples (for experiment conducted in shaker flasks) or used directly without decanting the medium as a starter for fungal cultivation on vinasse in bioreactors (Table 3.2).

3.3 Fungal Cultivation and Fungal Biomass Yield Determination

3.3.1 Fungal Growth Optimization Studies

The fungal mycelia prepared from the tempeh starter were cultivated in a series of 250-ml Erlenmeyer flasks containing 100 ml of the substrates (e.g., rum-vinasse, molasses-vinasse, and cane-vinasse). The fungal mycelia were incubated in the incubator shaker at 150 rpm and 30°C for 3 days. The pH of the growth media was adjusted on a daily basis to maintain a desired pH, using 1 N sodium hydroxide (NaOH) and 1 N sulfuric acid (H_2SO_4), except for the control sample (without pH adjustment) during pH optimization studies. Fungal growth conditions, including vinasse concentration, pH, nutrient supplementation, and sterilization, were optimized in a sequential order. The optimal conditions obtained from the preceding experiments were used to obtain the optimal conditions for the following experiments. A detailed experimental procedure is

summarized in Figure 3.1. The cultures were centrifuged at 15,557 × g for 15 min after 3 days of cultivation. The solid fraction was dried at 70°C until constant weight was obtained (Lindberg Blue M MO1450A-1, Thermo Scientific, Waltham, MA, USA). The fungal biomass weight was calculated by subtracting the dried solid fraction in the culture sample from the dried solids in vinasse under the same centrifugation conditions. Because all the experiments were performed in a batch-mode, the fungal biomass yields were calculated based on initial fungal biomass weight in order to account for variation in the starting amount of fungal mycelia inoculums in each batch. The fungal biomass yield in this research was defined as $g_{\text{biomass increase}}/g_{\text{initial biomass}}$. The optimization experiments were conducted in duplicate with cultivation of cultures in triplicates in each set of experiment.

3.3.1.1 Concentration Optimization Study

Fungal cultivation experiments were conducted at various vinasse concentrations of 10, 25, 50, 75, and 100% (v/v). The fungal cultures were inoculated at a pH of 5.0, a temperature of 30°C, and a mixing intensity of 150 rpm for 3 days.

3.3.1.2 pH Optimization Study

Under an optimal vinasse concentration, the experiments were conducted at five different pH conditions, pH 4.0, 4.5, 5.0, 5.5, and a control (without pH adjustment). The pH of cultivation media was adjusted to the desired pH on a 24-h basis.

3.3.1.3 Nutrient Supplementation Study

Ammonium sulfate ($(NH_4)_2SO_4$) and potassium dihydrogen phosphate (KH_2PO_4) as sources of nitrogen (N) and phosphorus (P), respectively, were supplemented into the vinasse samples to maintain a SCOD:N:P ratio of 100:5:1. The optimal vinasse concentration and pH obtained from previous experiments were fixed, and the pH of the samples was adjusted on a daily basis. Nutrient supplementation optimization

experiments were conducted with N supplementation, P supplementation, and both N and P supplementation. A control experiment was conducted without nutrient supplementation.

3.3.1.4 Vinasse Sterilization Study

This experiment was conducted under the optimal growth conditions for each vinasse sample from the previous experiments. Vinasse samples were sterilized by autoclaving at 121°C for 20 min.

Table 3.2 Fungal cultures and cultivation conditions for mycelium inoculum preparation

Fungal culture	Experiment	Fungal mycelium inoculum size	Fungal spore inoculum size	Cultivation conditions	Fungal mycelia harvesting process
Tempeh starter	Fungal growth optimization	100 ml Yeast Mold (YM) broth in 250-ml Erlenmeyer flask	0.5% (v/v)	150 rpm, 30°C 2 days	Centrifuged at 15,557 × g for 15 min
ATCC # 22959	Other experiments	100 ml Yeast Mold (YM) broth in 250-ml Erlenmeyer flask	0.5% (v/v)	150 rpm 37°C 3 days	Decanted using sieve screen
		500 ml YM broth in 1-L Erlenmeyer flask			No decanting process

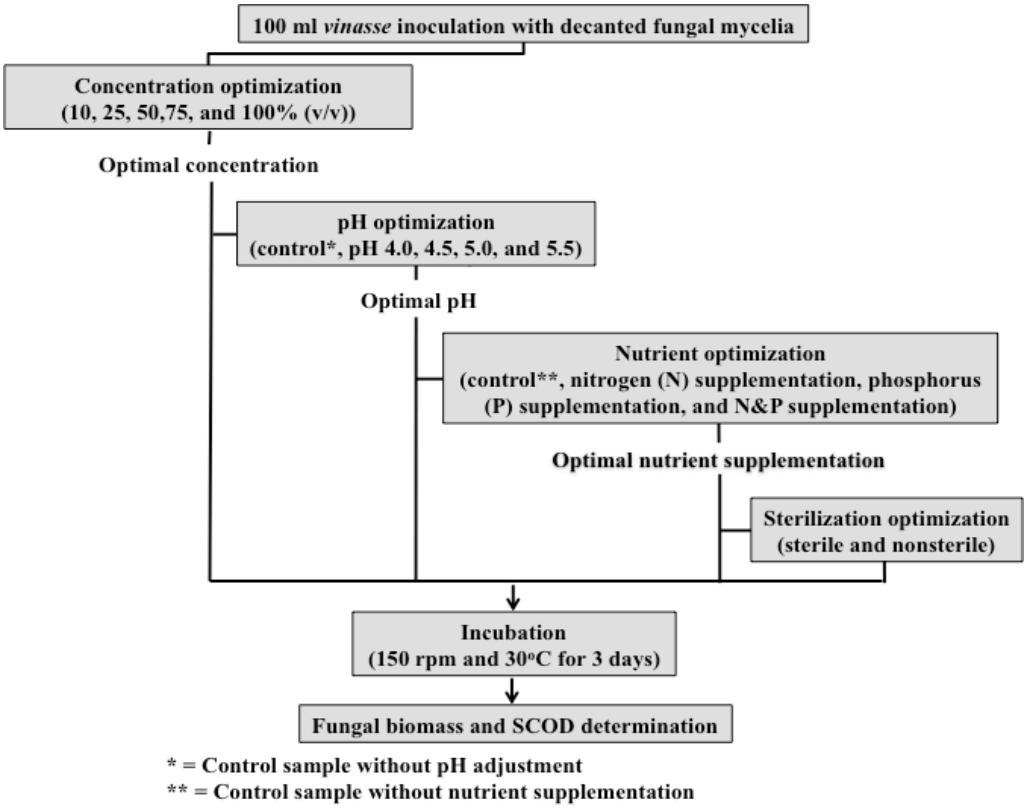


Figure 3.1 Summary of experimental plan for fungal growth optimization studies

3.3.2 Fungal Cultivation for Evaluating the Effect of Substrate Concentration on Fungal Growth Pattern

The fungal mycelia prepared from the ATCC fungal culture were cultivated in a series of 250-ml Erlenmeyer flasks containing 100 ml of various concentration of vinasse; 25, 50, 75, and 100% (v/v). The vinasse used in this study was prepared in the laboratory using sugarcane syrup as a substrate for ethanol fermentation (section 3.1). The pH of the growth media was adjusted on a daily basis to maintain a desired pH level of 5.0, using 1 N NaOH and/or 1 N H_2SO_4. Nutrient was supplemented to maintain a SCOD:N:P ratio of 100:5:1 using $(NH_4)_2SO_4$ and KH_2PO_4 as sources of N and P,

respectively. The cultures were maintained in the incubator shaker at 150 rpm and 37°C for 3 days. Samples were taken at 6-h intervals for the first 2 days and 12-h interval for the rest of cultivation period. In order to harvest fungal biomass, the cultures were passed through the USA Standard Test Sieve with 250-µm nominal opening size and dried at 70^{o}C for 24 h until constant weights were obtained. Fungal biomass, COD, and reducing sugar concentrations (g/L) were used to evaluate the growth pattern at different substrate concentrations.

3.3.3 Fungal Mycelia Preparation for Morphology Study

The effect of inoculum size on fungal morphology and biomass yield was investigated by inoculating various concentration of fungal spore suspensions (0.2, 0.5, 1.0, and 2.0% (v/v)) prepared from the ATCC culture into YM broth (100 ml). The fungal cultivation was conducted in an incubator shaker at 150 rpm and 37^{o}C for 3 days.

3.3.4 Effect of Ethanol on Fungal Biomass Yield

Fungal cultivations were performed in a series of 250-ml Erlenmeyer flasks containing 100 ml of YM media. Absolute ethanol (99.5+%; Acros Organics, Fair Lawn, NJ, USA) at different concentrations of 0.5, 1.0, 3.0, 5.0, and 7.0 % (v/v) were added into YM media. The cultures were incubated at 150 rpm and 37°C for 3 days. Fungal biomass was harvested using the USA Standard Test Sieve with 250-µm nominal opening size and dried at 70^{o}C until constant weight was obtained. Fungal biomass concentration in g/L was used to determine the effect of ethanol on fungal biomass production with respect to control sample (without ethanol).

3.3.5 Fungal Cultivation in Bioreactors

Fungal cultivations were conducted in two 2.5-L working volume airlift and bubble column bioreactors. A fermentation broth containing 2 L of sterile vinasse sample

was inoculated with 500 ml fungal mycelia inoculum. Fungal cultivation was conducted at a pH of 5.0 and a temperature of 37^{o}C with nutrient supplementation. These conditions were found to be optimal for fungal growth based on our previous study. The pH was controlled to maintain a desired pH level using 2% NaOH and 2% H_2SO_4 solutions. Nutrient supplementation was maintained at a SCOD:N:P ratio of 100:5:1. Ammonium sulfate ($(NH_4)_2SO_4$) and potassium dihydrogen phosphate (KH_2PO_4) were supplemented to the vinasse as sources of N and P, respectively. The bioreactors were operated at different aeration rates of 0.5, 1.0, 1.5, and 2.0 vvm. The fungal biomass samples were harvested following 3 days of fermentation using the USA Standard Test Sieve with 250-µm nominal opening size, washed with tap water, and dried at 70^{o}C for 24 h until constant fungal biomass weight was obtained. The dry weight of fungal biomass samples was determined to calculate the fungal biomass yield defined as $g_{\text{biomass increase}}/g_{\text{initial biomass}}$.

3.4 Bioreactor Configurations

Two types of bioreactor configurations including airlift and bubble column reactors with 2.5 L working volume were used in this research. A 3.5-L internal loop airlift bioreactor of cylindrical geometry with an inner diameter of 14 cm and a height of 40 cm, was fabricated (Figure 3.2). The bioreactor and draft tube were made of clear acrylic plastic with a thickness of 0.5 cm. The draft tube had inner diameter of 10 cm and height of 16 cm. The top and bottom clearances were 17 cm and 3.5 cm, respectively. The ratio of the downcomer and riser cross-sectional areas (A_d/A_r) was about 1. Air was supplied through porous air diffusers at the bottom of the riser section. Inlet air was passed through a polytetrafluoroethylene (PTFE) membrane filter (0.1-µm pore size) (Whatman, FlorhamPark, NJ, USA) to prevent contamination. Similarly, the airlift bioreactor without draft tube was used in order to conduct experiments with the bubble column reactor.

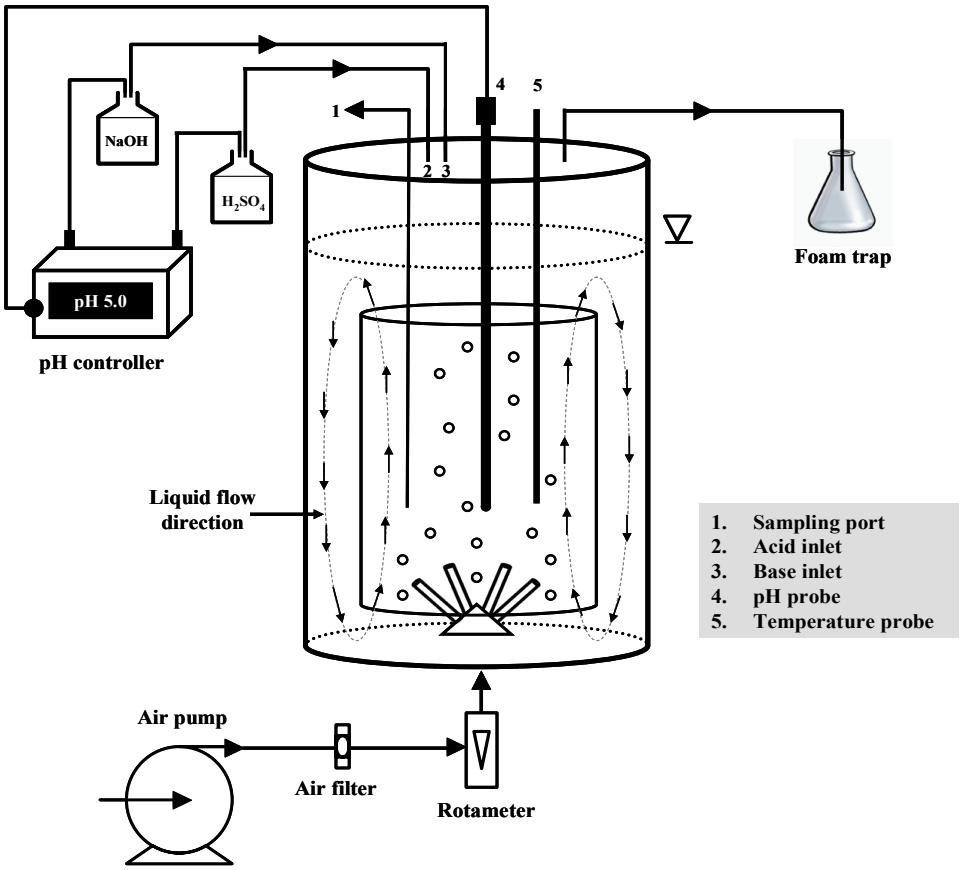


Figure 3.2 Schematic diagram of an airlift bioreactor system

3.5 Vinasse Characterization

All analyses were performed at a minimal of triplicate. For mineral analysis, the vinasse samples were sent to the Agricultural Diagnostic Service Center (ADSC) at the University of Hawai'i at Mānoa (Honolulu, HI, USA). The mineral analyzed were aluminum (Al), boron (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), silicon (Si), and zinc (Zn).

3.5.1 pH

The pHs of the samples were measured with a pH meter (accumet* AB15+, Fisher Scientific, Fair Lawn, NJ, USA) equipped with the pH probe (accuTupH* # 13-620-183A, Fisher Scientific, Fair Lawn, NJ, USA). The pH meter was calibrated with pH reference buffers (pH 4.00, 7.00, and 10.01) regularly.

3.5.2 Solids

Vinasse samples were characterized for solids content, such as total solids (TS), total suspended solids (TSS), volatile solids (VS), and volatile suspended solids (VSS) following the procedures outlined in the Standard Methods (APHA/AWWA/WEF, 2005) as discussed below:

3.5.2.1 Total Solids (TS)

The total solids (TS) was determined following method 2540 B (APHA/AWWA/WEF, 2005). Generally, about 5 ml of well-mixed sample was transferred into a preweighed evaporating dish preheated at 550^{o}C for 1 h in a muffle furnace (Isotemp® Muffle Furnace, Fisher Scientific, Fair Lawn, NJ, USA). The sample was then evaporated to dryness in an oven at ~ 95^{o}C (the recommended temperature for evaporation to prevent splattering (Lindberg Blue M MO1450A-1, Thermo Scientific, Waltham, MA, USA). Then, the evaporated sample was dried in an oven at temperature of 103-105^{o}C for at least 1 h. After that, the dish was cooled in a desiccator (Dry Keeper™, Sanplatec corp., Osaka, Japan) to balance temperature for approximately 20 min and weighed using analytical balance (Mettler Toledo AL 104, Mettler-Toledo Inc., Columbus, OH, USA). Cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained or until weight change was less than 4% of previous weighing or 0.5 mg, whichever was less.

3.5.2.2 Total Suspended Solids (TSS)

The total suspended solids (TSS) was analyzed following method 2540 D (APHA/AWWA/WEF, 2005). In brief, a glass-fiber (GF) filter disk (GF/C glass microfiber filter Whatman # 1822047, Whatman, Florham Park, NJ, USA) was washed under vacuum with three successive 20 ml of deionized water and dried in an aluminum weighing dish preheated at 550^{o}C for 1 h. After drying, the dish containing the filter was cooled in a desiccator (~ 20 min) and weighed. Cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained or until weight change was less than 4% of previous weighing or 0.5 mg, whichever was less. Then, the filter disk was placed on the filtering apparatus (Glass filter holder for 47 mm disc filter, © EMD Millipore Corporation, Billerica, MA, USA) and wet with small volume of deionized water under vacuum. A stirred sample of 5 ml was transferred onto the preweighed seated GF filter to complete filtration and then washed with three successive 10 ml volumes of deionized water, allowing complete drainage between washing, and continue suction for ~ 3 min after completing the filtration. The GF filter disk was transferred into aluminum weighing dish and dried for at least 1 h at 103-105^{o}C, cooled in a desiccator (~ 20 min), and weighed. Cycle of drying, cooling, desiccating, and weighing was repeated until a constant weight was obtained or until weight change was less than 4% of previous weighing or 0.5 mg, whichever was less.

3.5.2.3 Volatile Solids (VS) and Volatile Suspended Solids (VSS)

Volatile solids (VS) and volatile suspended solids (VSS) were measured according to method 2540 G (APHA/AWWA/WEF, 2005). Briefly, the dried samples from section 3.5.2.1 and 3.5.2.2 were placed into a cool muffle furnace. The furnace was heated to 550^{o}C and the samples were ignited for 1 h then cooled in a desiccator (~ 20 min) and weighed. Igniting (30 min), cooling, desiccating, and weighing steps were repeated until a constant weight was obtained or until weight change was less than 4% of previous weighing or 0.5 mg, whichever was less.

Calculation

$$\%\ Total\ solids = \frac{A-B}{C-B} \times 100 \quad \text{---------- (3.1)}$$

$$\%\ Total\ suspended\ solids = \frac{D-E}{F} \times 100 \quad \text{---------- (3.2)}$$

$$\%\ Volatile\ solids = \frac{A-G}{A-B} \times 100 \quad \text{---------- (3.3)}$$

$$\%\ Volatile\ suspended\ solids = \frac{D-H}{D-E} \times 100 \quad \text{---------- (3.4)}$$

Where,

A = weight of residue + dish before ignition (mg)

B = weight of dish (mg)

C = weight of wet sample + dish (mg)

D = weight of filter + dried residue before ignition (mg)

E = weight of filter (mg)

F = equivalent weight of wet sample for determination

G = weight of residue + dish after ignition (mg)

H = weight of residue + filter after ignition (mg)

3.5.3 Total Chemical Oxygen Demand (COD) and Soluble Chemical Oxygen Demand (SCOD)

Total chemical oxygen demand (COD) and soluble chemical oxygen demand

(SCOD) were determined by means of two methods including Standard Methods (APHA/AWWA/WEF, 2005) and the US Environmental Protection Agency (USEPA) reactor digestion method (# 10212; HACH Company, Loveland, CO, USA). For SCOD determination, the sample was subjected to filtration through a Whatman cellulose nitrate membrane filter (0.45-μm pore size) prior to analysis (Whatman, Florham Park, NJ, USA).

3.5.3.1 Standard Method for COD Determination

The determination of COD and SCOD were performed according to a standard method for determination of chemical oxygen demand (manganese III oxygen demand) of water (designation: D66697-01) (APHA/AWWA/WEF, 2005). The standard method is based on the oxidation of sample organic matter by manganese III and subsequent measure of the organic matter equivalent. An analysis of mixture at a sample:potassium dichromate ($K_2Cr_2O_7$) digestion solution:sulfuric acid (H_2SO_4) reagent ratio of 5:3:7 was transferred in a screw cap test tube 20 × 150 mm. The $K_2Cr_2O_7$ digestion solution (16.67 mM) contained 4.903 g of $K_2Cr_2O_7$ (primary standard grade), 167 ml of concentrated H_2SO_4, and 33.3 g of $HgSO_4$ in 1 L total volume. The sulfuric acid reagent was prepared by adding Ag_2SO_4 into concentrated H_2SO_4 (5.5 g Ag_2SO_4/kg H_2SO_4). The sample was capped tightly and inverted several times to completely mix. The sample was then placed in an oven preheated to 150^{o}C for 2 h. After heating (reflux), sample was cooled to room temperature before subjected to titration. Titration was performed using ferroin indicator and standard ferrous ammonium sulfate (FAS) titrant (~ 0.10 M). The end point was a sharp color change from blue-green to reddish brown, although the blue-green reappeared within minutes. The ferroin indicator (100 ml) was composed of 1.485 g of 1,10-phenanthroline monohydrate and 695 mg of $FeSO_4 \cdot 7H_2O$. The standard ferrous ammonium sulfate (FAS) titrant (1 L) contained 39.2 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 20 ml concentrated H_2SO_4. Concentration of FAS titrant was standardized daily against a standard $K_2Cr_2O_7$ digestion solution. Molarity of FAS titrant was calculated using eq. (3.5). The blank was prepared in the same manner but containing the reagents and a

volume of deionized water equal to that of the sample.

Calculation

$$Molarity\ of\ FAS = \frac{K_2Cr_2O_7\ (ml) \times 0.10}{FAS\ (ml)} \quad \text{---------- (3.5)}$$

$$COD\ (mg_{O_2}/L) = \frac{(A - B) \times M \times 8000}{sample\ (ml)} \quad \text{---------- (3.6)}$$

Where,

A = volume of FAS titrant for blank (ml)

B = volume of FAS titrant for sample (ml)

M = molarity of FAS

3.5.3.2 The US Environmental Protection Agency (USEPA) Reactor Digestion Method for COD Determination

The analysis was carried out using COD TNTplus™ 823 reagent set following the method 10212 (HACH Company, Loveland, CO, USA). The sample of 0.3 ml was transferred into a vial (previously mixed). The test vial was inverted several times before placing into the preheated heat block reactor at 150^{o}C for 2 h (DRB200, HACH Company, Loveland, CO, USA). After heating, the sample was cooled in a reactor for 20 min and inverted several times. The sample was kept outside the reactor to cool to room temperature before taking the COD measurement by a spectrophotometer (HACH DR5000, HACH Company, Loveland, CO, USA). The HACH spectrophotometer read the barcode on the test vial and the resulting COD measurement was presented in mg/L.

3.5.4 Total Kjeldahl Nitrogen (TKN) Analysis

The Nessler method was used to obtain nitrogen concentration as total Kjeldahl nitrogen (TKN) (# 8075; HACH Company, Loveland, CO, USA). The sample was digested using peroxymonosulfuric acid prior to colorimetric analysis (HACH et al., 1985; Hach et al., 1987). In brief, the sample was subjected to digestion (using Digesdahl digestion apparatus, HACH Company, Loveland, CO, USA) with a concentrated sulfuric acid (3 ml) at a temperature of 440^{o}C for 4 min. After that, 10 ml of 50% hydrogen peroxide was added and the digestion was performed at the same temperature for additional 1 min. A digested sample was made up to the volume of 100 ml by deionized water. Approximately 1 ml of sample was selected for further colorimetric analysis, Nessler method (HACH method # 8075; HACH Company, Loveland, CO, USA). The absorbance of analyzed sample containing nitrogen in yellow color was measured using the HACH spectrophotometer. The HACH program: 2410 nitrogen, TKN was selected for the measurement of absorbance at 460 nm. The instrument was set to zero using blank prepared in the same way without the sample. The resulting TKN value displayed on the screen as mg/L was used for TKN calculation using eq. (3.7) given by HACH:

$$ppm\ TKN = \frac{75 \times A}{B \times C} \quad \text{---------- (3.7)}$$

Where,

A = TKN value displayed on the screen (mg/L)

B = g or ml of sample taken for digestion

M = analysis volume of digested sample (ml)

3.5.5 Alcohols, Glycerol, and Organic Acids Analyses

The ethanol, methanol, glycerol, lactic acid, and acetic acid concentrations were analyzed using a Waters high performance liquid chromatography (HPLC) system

(Waters model 2695, Waters, Milford, MA, USA). The HPLC was equipped with a Waters model 410 differential refractometer. A Waters model 2695 separate module composed of a column heater, auto-sampler, and computer controller was used in the system. A Rezex ROA organic acid column (Phenomenex, Inc., Torrance, CA, USA) was used with 5 mM sulfuric acid solution as the mobile phase at a flow rate of 0.6 ml/min, an injection volume of 40 µl, and a column temperature of 65^{o}C.

3.5.6 Phosphorus Analysis

Phosphorus measured as PO_4^{3-} was determined by the ascorbic acid method (# 10210) using TNTplus™ 845 vials (HACH Company, Loveland, CO, USA). A measurement in mg/L PO_4 was obtained by the HACH spectrophotometer. The instrument read a barcode on the test vial then selected and performed the correct test therefore no blank was required.

3.5.7 Potassium Analysis

Potassium (as K) was determined by the tetraphenylborate method (# 8049; HACH Company, Loveland, CO, USA). After completion of chemical reaction, the potassium as K was measured at a wavelength of 650 nm. The instrument was set to zero using blank prepared in the same manner without the sample.

3.5.8 Sugar Analysis

Sugars in vinasse sample were determined by means of colorimetric and chromatographic methods. A modified dinitrosalicylic acid (DNS) colorimetric method was employed for reducing sugar analysis. Using the standard protocol of the National Renewable Energy Laboratory (NREL), the acid hydrolyzate sample was analyzed by HPLC.

3.5.8.1 Modified Dinitrosalicylic Acid (DNS) Colorimetric Method

Vinasse was analyzed for reducing sugar concentration using a modified DNS colorimetric method (Miller, 1959). A sample size of 100 µl was mixed thoroughly with 1 ml of DNS reagent. The DNS reagent of 1 L contained 1 g of 3,5-dinitrosalicylic (DNS) acid powder, 300 g of potassium sodium tartrate, and 16 g of sodium hydroxide. The mixture of sample and DNS reagent was heated to 100^{o}C for 10 min and then cooled in an ice bath. The absorbance of the sample was measured at a wavelength of 570 nm using a spectrophotometer. Reducing sugar concentrations were calculated from the standard calibration curve obtained using standard solutions of D-glucose.

3.5.8.2 High Pressure Liquid Chromatography (HPLC) Method

Sugar concentrations were determined in acid hydrolyzates prepared following a standard protocol developed by NREL (Sluiter et al., 2008). The acid hydrolyzates were filtered through a 0.22-µm nylon filter (Fisherbrand* Syringe filter, Fisher Scientific, Fair Lawn, NJ, USA) and analyzed using HPLC. The peaks were compared to six standard monomeric sugars: glucose, fructose, galactose, mannose, arabinose, and xylose. A Rezex RPM Monosaccharide column (00H-01350K0) (Phenomenex, Inc., Torrance, CA, USA) was used with deionized water as the mobile phase at a flow rate of 0.8 ml/min, an injection volume of 40 µl, and a column temperature of 85°C.

3.6 Specific Oxygen Uptake Rate (SOUR) Determination

In order to quantify microbial contamination during fermentation in an airlift bioreactor, specific oxygen uptake rate (SOUR) was determined as per the method outlined in Standard Methods (method 2710 B; APHA/AWWA/WEF, 2005). The experiment was conducted over 3 days in a bioreactor at an aeration rate of 0.5 vvm without fungal inoculation. The measurements of dissolved oxygen (DO) concentration and volatile suspended solids (VSS) were conducted at 8-h intervals for the first 24 h and

24-h intervals for up to 72 h of fermentation. DO concentrations were measured using an oxygen probe (HQ40d, HACH Company, Loveland, CO, USA). Volatile suspended solids were determined according to method listed in section 3.5.2.3. The SOUR was then calculated using the following equation:

$$SOUR\ (mg\ O_2/g\ VSS - hr) = \frac{Oxygen\ consumption\ rate\ (mg\ O_2/L - min) \times (60\ min/hr)}{Biomass\ concentration\ (g\ VSS/L)} \quad \text{----- (3.8)}$$

3.7 Fungal Biomass Characterization

The vinasse-derived fungal biomass from various conditions were freeze-dried and characterized for chemical composition and nutritional value. All analyses were conducted in triplicates except for crude lipids and essential amino acid profiles which were carried out in duplicates. For essential amino acids, crude lipid, and fatty acids analyses, the samples were sent to Aquatic Feeds and Nutrition Department, Oceanic Institute (Waimanalo, HI, USA).

3.7.1 Moisture Content (MC) and Dry Matter (DM) Determinations

Moisture content of fungal biomass was determined according to the method 934.01 outlined in Standard Methods (AOAC, 2005). Moisture content determination was carried out based on weight loss by heating the sample at 95-100^{o}C under vacuum (pressure ≤ 100 mm Hg) for 5 h (Fisher Isotemp vacuum oven 281, Fisher Scientific, Fair Lawn, NJ, USA). Moisture content and dry matter were calculated using the following equation:

$$\%\ (w/w)\ Moisture = \frac{Weight\ loss\ after\ drying\ (g)}{Weight\ of\ test\ portion\ (g)} \times 100 \quad \text{---------- (3.9)}$$

$$\%\ (w/w)\ Dry\ matter = 100 - Moisture\ (\%)$$ ---------- (3.10)

3.7.2 Crude Protein

Crude protein concentrations of the samples were measured and calculated using multiplying factor of 6.25. The samples were analyzed for TKN (section 3.5.4) and the nitrogen contents as TKN were used to calculate crude protein content in samples.

$$\%\ (w/w)\ Crude\ protein = Nitrogen\ content\ (\%) \times 6.25$$ ---------- (3.11)

3.7.3 Ash

Ash contents were determined following AOAC official method 942.05 (AOAC, 2005). Ashings were conducted in a muffle furnace at a temperature of 600°C for 2 h. Ash contents were calculated using the following equation:

$$\%(w/w)\ Ash = \frac{Weight\ of\ test\ portion\ (g) - Weight\ loss\ on\ ashing\ (g)}{Weight\ of\ test\ portion\ (g)} \times 100$$ ------ (3.12)

3.7.4 *In vitro* Protein Digestibility

The *in vitro* protein digestibility was determined using pepsin and pancreatin enzymes system (Akeson and Stahman, 1964). The samples (100 mg protein equivalent) were incubated with 15 ml of 0.1 N hydrochloric (HCl) solution containing 2.0 mg of enzyme pepsin (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) at 37°C for 3 h prior to neutralize with 0.2 N sodium hydroxide (NaOH). A 7.5-ml phosphate buffer (0.1 M and pH 8.0) containing 5 mg of pancreatin (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) was added followed by addition of 1 ml toluene. The samples were incubated for additional 24 h at 37°C. An enzyme blank was prepared in the same way without the sample. After 24 h, the enzyme was inactivated by precipitating undigested protein with

10 ml of 10% trichloroacetic acid (TCA) solution. Finally, the volume was made up to 50 ml and sample was centrifuged at 3,214 × g for 20 min. The resulting supernatant was determined for protein content by Kjeldahl method (section 3.5.4). The TCA soluble protein at 0 h was also determined and used for calculation. The same amount of TCA solution was added into 100 mg (protein equivalent) sample. The sample was stirred by a magnetic stirrer bar allowing TCA soluble protein to precipitate prior to making up final volume to 50 ml, centrifuged, and analyzed for TKN. The digested protein relative to the total protein was expressed as % protein digestibility which was calculated using the following equation:

$$\%\ Protein\ digestibility = \frac{Protein\ in\ supernatant - Protein\ in\ enzyme\ blank}{Protein\ in\ sample - TCA\ soluble\ protein\ at\ zero\ h} \times 100 \quad \text{---------- (3.13)}$$

3.7.5 Tryptophan

The trytophan content was analyzed by means of spectrophotometry using an acid ninhydrin method (Pintér-Szakács and Molnán-Perl, 1990). One gram of freeze-dried fungal sample was mixed with 10 ml of 75 mM NaOH solution. The sample was continuously mixed using a magnetic stirrer bar for 30 min prior to centrifuge at 3,214 × g for 10 min. The resulting supernatant of 0.5 ml was mixed with 5 ml ninhydrin reagent prepared freshly by adding 1.0 g of ninhydrin into 100 ml of a mixture containing concentrated HCl and concentrated (96%) formic acids at a HCl:formic acid ratio of 2:3. The reaction solution was then incubated at 35^{o}C for 2 h, cooled to room temperature, and made up the final volume to 10 ml with diethyl ether. The solution was well mixed with vortex and filtered through a glass-fiber filter disk under vacuum. The absorbance of the resulting clear solution obtained after filtration, was taken at a wavelength of 380 nm by the spectrophotometer. Tryptophan concentration in the sample was determined against a tryptophan standard curve prepared in the same way using standard tryptophan (0-100 µg).

3.8 Statistical Analysis

The sample analyses for various parameters were performed in triplicates. The results were statistically analyzed by Predictive Analytics SoftWare (PASW) Statistics version 18 (SPSS Inc., Chicago, IL, USA). The statistical differences were determined using a one-way analysis of variance (ANOVA) followed by a Duncan's comparison. The values reported are mean values with standard deviation. The resulting statistical analyses with significantly difference/same are presented with superscript letters.

CHAPTER 4: RESULTS AND DISCUSSION: FUNGAL GROWTH OPTIMIZATION STUDIES

4.1 Rationale

The presence of chemicals/compounds in vinasse may inhibit fungal growth. Additionally, considerably high amount of organic matters in the vinasse could constrain the fungal growth. Consequently, examining the ability of fungus in utilizing vinasse as a sole substrate to support its healthy growth is critical. Furthermore, fungal growth is affected by several factors including pH, nutrients availability, and organic content of the substrate. Therefore, optimization of fungal growth conditions is critically important to maximize the fungal biomass yield. Investigating the fungal growth conditions will provide useful data for evaluating the efficacy of fungal protein production on vinasse.

4.2 Concentration Optimization

The fungal biomass yields obtained from fungal cultivations on three vinasse samples at various concentrations using shaker flask experiments are summarized in Table 4.1. It is important to note that the letters in superscript were used to correlate statistical analyses within each vinasse sample. Prolific fungal growth was observed in all three types of vinasses. The highest fungal biomass yields of 0.91 ± 0.03, 0.61 ± 0.05, and 0.62 ± 0.01 ($g_{biomass\ increase}/g_{initial\ biomass}$) were obtained from 25% (v/v) rum-vinasse, 100% (v/v) molasses-vinasse, and 100% (v/v) cane-vinasses, respectively (Table 4.1).

Dilution was needed for rum-vinasse sample as it contained significantly higher amounts of organic matter, solids, and chemical constituents, especially potassium in comparison to molasses- and cane-vinasses (Table 3.1). The significantly high potassium concentration in rum-vinasse of about twice and 6-folds higher than the potassium content in molasses-vinasse and cane-vinasse, respectively, could be detrimental to the fungal growth. Although potassium ion plays an important role in controlling the apical

growth and stimulating cell mass production (Gow, 1990), it can inhibit the growth at a high concentration (Allaway and Jennings, 1970). Jones and Jennings (1965) reported that potassium ion at a low concentration (0.39 g/L) activated the glucose degradation thus increased cell dry weight of marine fungus, *Dendryphiella salina*. Similarly, Peñaloza et al. (1991) studied effect of potassium ion on the growth of *R. oligosporus*, and showed that addition of potassium salts at concentration up to 0.01 g K^+/L increased the production of fungal mycelium linearly regardless of type of potassium added. The authors also pointed out that the improvement of fungal biomass production was not further observed after certain amount of potassium addition (which was not identified), and the fungal dry weights of media supplemented with potassium at levels of 0.04 and 0.39 g K^+/L were similar. However, at a high concentration (~ 7.80 g/L), potassium inhibited the transportation of glucose into fungal cell (Allaway and Jennings, 1970). The lower limit of potassium ion concentration causing inhibitory effect could vary significantly depending on fungal species. However, because the presence of ions affects the ability of fungi to retain nutrients inside the mycelium, marine fungi, which are surrounded by the environment with high ions concentration, would have a tolerance to high ions level. The extremely high potassium content of rum-vinasse (10.67 g/L), possibly inhibited by the nutrients transported into cells thus dilution was necessary. Furthermore, rum-vinasse contained considerably higher amounts of sodium and calcium in comparison to other vinasse samples. These cations also have adverse effects on fungal growth. A high concentration of calcium reduces the permeability of sugars into fungal cells via passive transport, whereas a high concentration of sodium inhibits glucose degradation resulting in reducing glucose uptake by the fungal cells and eventually reducing the production of important metabolites and the growth rate (Allaway and Jennings, 1970).

Fungal fermentation of corn-ethanol thin stillage, however, required no dilution. Thin stillage had low organic and potassium contents and high nitrogen content compared to that in rum-vinasse. The organic content and total nitrogen concentration of thin stillage were 80–100 g COD/L and 6 g/L, respectively (Rasmussen et al., 2007; Khanal,

2008), whereas the organic content and nitrogen concentration of rum-vinasse were 127 g COD/L and 0.97 g TKN/L, respectively (Table 4.1).

Table 4.1 Fungal biomass yields for various vinasse samples at different concentrations

Vinasse samples	Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)				
	10% (v/v)	**25% (v/v)**	**50% (v/v)**	**75% (v/v)**	**100% (v/v)**
Rum-vinasse	0.26 ± 0.04^{a}	0.91 ± 0.03^{b}	0.78 ± 0.03^{c}	0.41 ± 0.03^{d}	0.34 ± 0.03^{e}
Molasses-vinasse	0.09 ± 0.01^{a}	0.31 ± 0.07^{b}	0.49 ± 0.02^{c}	0.55 ± 0.03^{d}	0.61 ± 0.05^{e}
Cane-vinasse	0.45 ± 0.01^{a}	0.36 ± 0.02^{b}	0.49 ± 0.01^{c}	0.60 ± 0.01^{d}	0.62 ± 0.01^{e}

Mean value ± standard deviation (sample size (n) = 6)
Superscript letters represent statistical analyses within each vinasse sample.

4.3 pH Optimization

The fungal biomass yields on all substrates under various pH conditions showed a similar trend. With respect to fungal biomass yields, three groups: 1) control; 2) pH 4.0, 4.5, and 5.5; and 3) pH 5.0 were derived from statistical analyses as indicated by superscript letters (Table 4.2). Fungal growths in all samples under the fixed pH of 4.0, 4.5, and 5.5 were not significantly different at a 95% confidence level. Based on statistical analyses, the optimal pH was found to be 5.0 for all vinasse samples. The maximal fungal biomass yields were 0.92 ± 0.04, 0.67 ± 0.05, and 0.61 ± 0.01 ($g_{biomass\ increase}/g_{initial\ biomass}$) for rum-, molasses-, and cane-vinasse, respectively. However, the optimal pH for fungal fermentation in corn-ethanol residue was reported to be 4.0 (Rasmussen et al., 2007; Jasti et al., 2008). This could be due to different chemical attributes of these substrates affecting enzyme activity, cell permeability, and metal ion availability. It is important to point out that the desired pH of 4.0, 4.5, 5.0, and 5.5 were

not always possible to maintain precisely in the shaker flasks. Therefore, the actual optimal pH could be slightly different from the optimal pH obtained in this study. The bioreactor experiments, however, provided a better pH control, as it was possible to maintain a desired pH more accurately.

Table 4.2 Fungal biomass yields for various vinasse samples at different pHs

Vinasse samples	Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)				
	Control*	**pH 4.0**	**pH 4.5**	**pH 5.0**	**pH 5.5**
Rum-vinasse	0.80 ± 0.04^{a}	0.48 ± 0.03^{b}	0.45 ± 0.04^{b}	0.92 ± 0.04^{c}	0.48 ± 0.03^{b}
Molasses-vinasse	0.28 ± 0.03^{a}	0.47 ± 0.02^{b}	0.47 ± 0.05^{b}	0.67 ± 0.05^{c}	0.48 ± 0.02^{b}
Cane-vinasse	0.25 ± 0.05^{a}	0.34 ± 0.02^{b}	0.35 ± 0.06^{b}	0.61 ± 0.01^{c}	0.50 ± 0.06^{b}

* = Control sample without pH adjustment

Mean value ± standard deviation (sample size (n) = 6)

Superscript letters represent statistical analyses within each vinasse sample.

4.4 Effect of Nutrient Supplementation on Fungal Biomass Production

Significant improvement in fungal biomass yields was observed for all three vinasse substrates supplemented with both nitrogen (N) and phosphorus (P) in the growth media. With respect to the control, at the optimal vinasse concentration and pH, 52%, 223%, and 87% improvements in fungal biomass yield were derived from N and P supplemented rum-, molasses-, and cane-vinasse samples, respectively (Table 4.3). This finding closely agrees with the aseptic batch study of Jasti et al. (2008) who reported an increase in fungal biomass yield in N and P supplemented corn wet-milling effluent compared to non-nutrient supplemented samples.

Table 4.3 Fungal biomass yields for various vinasse samples from nutrient supplementation study

Vinasse samples	Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)			
	Control*	**Nitrogen (N)**	**Phosphorus (P)**	**N and P**
Rum-vinasse	0.87 ± 0.02^{a}	0.92 ± 0.02^{b}	1.07 ± 0.04^{c}	1.32 ± 0.02^{d}
Molasses-vinasse	0.61 ± 0.02^{a}	1.13 ± 0.13^{b}	1.31 ± 0.11^{c}	1.97 ± 0.05^{d}
Cane-vinasse	0.60 ± 0.01^{a}	0.68 ± 0.02^{b}	0.75 ± 0.04^{c}	1.12 ± 0.07^{d}

* = Control sample without nutrient supplementation

Mean value ± standard deviation (sample size (n) = 6)

Superscript letters represent statistical analyses within each vinasse sample.

Interestingly, the improvement in fungal biomass yield obtained from nutrients supplemented molasses-vinasse was significantly higher compared to that of other vinasse samples. Because the amount of nutrients supplemented, was kept constant at a SCOD:N:P ratio of 100:5:1 for all vinasse samples, the considerably higher fungal biomass production on molasses-vinasse could be due to the nature of molasses-vinasse substrate such as types of organic compound available, and the amount of N and P present in the sample. Under optimal concentration, the initial organic contents of rum-, molasses-, and cane-vinasses were approximately 31, 67, and 68 g SCOD/L, respectively. The lower amount of organic concentration in rum-vinasse may result in lower substrate availability for fungal biomass production. However, it is important to mention that fungal biomass yield obtained from rum-vinasse without nutrient supplementation (control) was higher than that of molasses-vinasse and cane-vinasse. Molasses- and cane-vinasse samples had lower N and P, thus nutrient supplementation would significantly improve fungal biomass production. On the contrary, rum-vinasse contained considerably high amount of N and P. Therefore, nutrient supplementation would improve fungal biomass yield at a much lower level when compared to molasses- and cane-vinasses. Regarding molasses- and cane-vinasse control samples with similar amounts of organic

content and fungal biomass yield, significant improvement in the fungal biomass yields on nutrient supplemented samples would be due to the limitation of N and P in both vinasse samples. The SCOD:N:P ratios of molasses-vinasse and cane-vinasse used in this study were 100:0.80:0.06 and 100:0.50:0.01, respectively. Although, it is evident that cane-vinasse had lower N and P contents in comparison to molasses-vinasse, fungal biomass yields of both control samples were not different. The SCOD reductions in both samples were, however different. The net SCOD removals of 10.68 ± 1.37 and 27.87 ± 0.55 g/L were obtained for molasses- and cane-vinasse control samples, respectively. The higher SCOD reduction in cane-vinasse suggested the use of organic content to support other metabolisms other than fungal growth. This finding becomes more apparent as the SCOD removals of fungal cultivations on cane-vinasse under all conditions (control, N supplementation, P supplementation, and both N and P supplementation) were statistically insignificant at 95% confidence level (Figure 4.1). Furthermore, the significant improvement in fungal biomass yield on molasses-vinasse could be due to availability of other trace elements and the type of substrate available for cell synthesis.

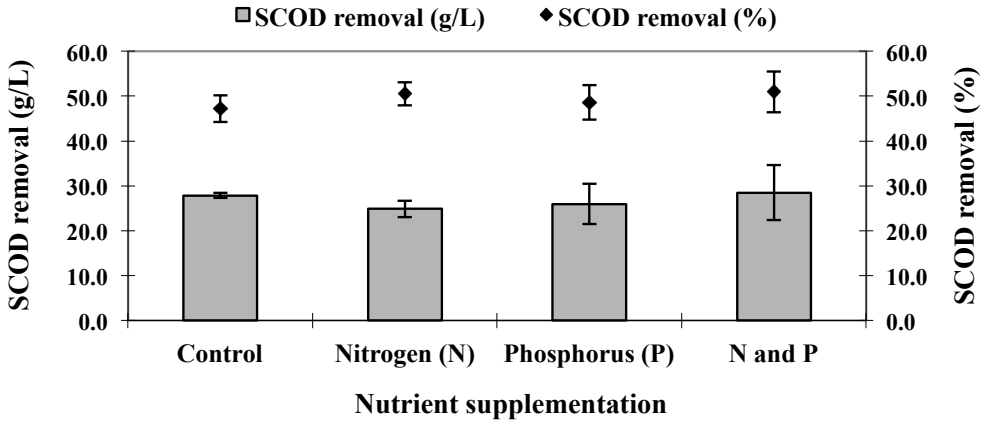


Figure 4.1 Soluble chemical oxygen demand (SCOD) removal of fungal cultivations on cane-vinasse sample at various nutrient supplementation conditions

Note: The bar graph represents net SCOD removal in g/L and the scatter plot graph represents percentages of SCOD removal.

Optimization experiments showed the feasibility of vinasse utilization as a low-cost substrate for fungal protein production. Prolific fungal growths were observed with the yields of 1.32 ± 0.02, 1.97 ± 0.05, and 1.12 ± 0.07 ($g_{biomass\ increase}/g_{initial\ biomass}$) in rum-vinasse, molasses-vinasse, and cane-vinasse, respectively. The optimal fungal cultivation conditions for three vinasses are summarized in Table 4.4.

Table 4.4 Optimal fungal growth conditions for different vinasse samples

Substrates	Concentration (%, v/v)	pH	Nutrient supplementation
Rum-vinasse	25	5.0	Nitrogen and Phosphorus
Molasses-vinasse	100	5.0	Nitrogen and Phosphorus
Cane-vinasse	100	5.0	Nitrogen and Phosphorus

4.5 Effect of Vinasse Sterilization on Fungal Biomass Production

Optimal fungal growth requires a slightly acidic condition, which also minimizes the growth of undesirable bacterial cells that typically prefer neutral pH range. Thus, fungal fermentation could possibly be operated under non-aseptic condition. The fungal biomass yields and SCOD removals from three different vinasse samples under sterilized and nonsterilized conditions are shown in Figure 4.2. The results showed that both fungal biomass yield and SCOD removal in sterile and nonsterile rum- and cane-vinasses were statistically insignificant at 95% confidence level. The fungal biomass yields of 1.32 ± 0.03 and 1.15 ± 0.06 ($g_{biomass\ increase}/g_{initial\ biomass}$) with SCOD removals of 59.49 ± 1.64 and 48.91 ± 4.75%, respectively, were obtained for fungal cultivation in nonsterile rum- and cane-vinasse samples. Thus, sterilization was not necessary for fungal cultivation on rum- and cane-vinasses under the cultivation conditions stated here.

A significant effect of sterilization however, was observed for molasses-vinasse. Fungal biomass yields of 1.93 ± 0.06 and 0.41 ± 0.03 ($g_{biomass\ increase}/g_{initial\ biomass}$) with

SCOD removals of 21.85 ± 1.59 and 14.38 ± 1.74% were achieved for fungal cultivation on sterile and nonsterile molasses-vinasse samples, respectively. The observed differences between the types of vinasses are likely associated with the process of vinasse preparation. Rum-vinasse had been subjected to sterilization as it had undergone a distillation process and sugarcane juice, used for cane-vinasse production, had been subjected to heat treatment (~ 70°C) during the sugar production process whereas the molasses-vinasse was obtained after low temperature evaporation of ethanol at 50°C after fermentation.

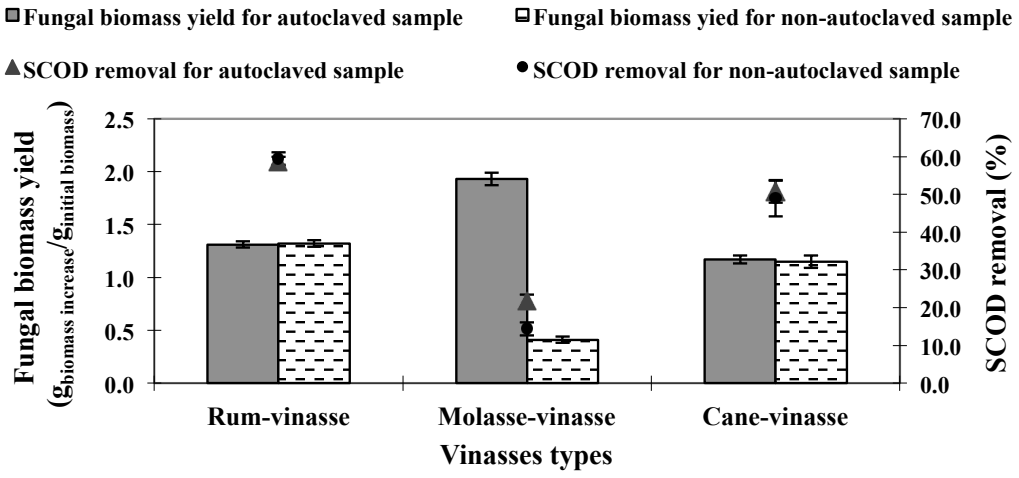


Figure 4.2 Fungal biomass yield and SCOD removal for various vinasse samples

Note: The bar graph represents fungal biomass yield and the scatter plot graph represents SCOD removal.

The relationship between fungal growth and SCOD reduction, however, was not linear (as mentioned previously). With respect to fungal cultivation on sterile molasses-vinasse, the lower fungal biomass productions in rum- and cane-vinasse samples had higher SCOD removals. The reduction in organic content was not directly attributed to the fungal growth. Microorganisms degrade organic content in the substrate for various purposes including growth, respiration, and secondary metabolite production. Krzywonos

and Przemyslaw (2012) studied the decolorization of sugar beet molasses vinasse by lactic acid bacteria and found that there was no correlation between color removal and COD reduction.

This study evaluated the feasibility of sugar-to-ethanol-derived vinasse utilization for protein-rich fungal biomass production. Three vinasses substrates were selected as they were locally available in Hawaii. The vinasses were used as liquid substrates to support fungal growth. Although *R. oligosporus* grew rapidly on a solid substrate (PDA), particularly one day for mycelium formation and two days for spore production, the dry weight of fungal biomass produced on solid medium was low. Moreover, as an aerobic microbe, the fungus grew mostly on the surface where it was exposed to the air. Consequently, fungal cultivation on solid medium requires large surface area and would not be an economical approach as a value-added processing of biofuel residues. Microbial growth in submerged fermentation operated in a bioreactor can achieve high microbial biomass production (García-Soto et al., 2006). Therefore, fungal cultivations on liquid medium, vinasse, were carried out for the production of fungal biomass in this study.

Among all vinasse samples, molasses-vinasse was the best substrate for fungal growth. The optimal fungal growth conditions were sterile and undiluted molasses-vinasse at a pH of 5.0 and a temperature of 30°C with nutrients supplementation. Dilution was found to be unnecessary for fungal cultivation on molasses-vinasse under these conditions. The elimination of vinasse dilution could possibly save the cost for fungal protein production by reducing the costs of clean water use and wastewater remediation. The organic content in vinasse reduced approximately by 50% (as SCOD) suggesting the use of fungal-treated effluent for land application such as fertirrigation.

Although fungus was successfully cultivated in a laboratory-scale experiment using 250-ml Erlenmeyer flasks containing 100 ml sample, fungal fermentation in a bioreactor provides better control of operating conditions such as pH, mixing, and air supply. Therefore, further study on fungal protein production in a bioreactor system is

critically important for obtaining reliable data on fungal biomass yields and organic removal for process scale-up.

However, fungal growth could also be limited by other factors and presence of inhibitory compounds which were not investigated in these optimization studies. Ethanol, for example, is inevitably present in the vinasse and could be detrimental to fungal growth. Therefore, the fungal growth pattern on various substrate concentrations was evaluated. Further, prior to the fungal cultivation in bioreactor study, the effect of ethanol on fungal growth and the effect of spore inoculum size on the mycelia starter morphology were also examined.

CHAPTER 5: RESULTS AND DISCUSSION: FUNGAL CULTIVATION ON VINASSE IN BIOREACTOR SYSTEM FOR POTENTIAL LARGE-SCALE PROTEIN-RICH FUNGAL BIOMASS PRODUCTION

5.1 Rationale

Fungal protein production in a bioreactor system is critical in obtaining reliable data on fungal biomass yield and organic removal for process scale-up. An airlift bioreactor in which mixing is facilitated by rising air bubbles, provides high mass transfer efficiency with low energy consumption. Eliminating extensive mechanical shear during mixing will enhance the fungal pellets formation, a desired fungal morphology in industrial-scale submerged fermentation processes. The pellets formation does not contribute to the increase in medium viscosity and at the same time it eliminates the possibility of fungal growth around impellers. Therefore, fungal growth in the form of pellets provides good substrate/nutrients and oxygen transfer, and minimizes adverse effect on bioreactor performance. Additionally, pellet formation is often credited to efficient settling and recovery of fungal biomass

The growth of filamentous fungi in submerged fermentation could be complicated and depends on a wide range of factors, such as cultivation medium, inoculum size, temperature, agitation intensity, and shear stress, which result in varying fungal morphology (Cui et al., 1998a; Gibbs et al., 2000; Wang et al., 2005). Among parameters influencing pellet formation, inoculum size is generally recognized as one of the most critical factors. As mentioned earlier, the growth of filamentous fungi in submerged fermentation, however, is restricted only to the hyphal tip (Moore-Landecker, 1990). Regarding the fungal pellet, the active zone where the growth occurs is around the surface of pellets. Therefore, fungal inoculation with a small pellet size would be beneficial in providing large surface area for the active growth.

Although previous optimization studies showed that molasses-vinasse provided the highest fungal biomass yield, cane-vinasse was selected as a substrate, representing

sugarcane-to-ethanol residue, for fungal biomass production. Because there is no sugarcane ethanol facility in Hawaii, the vinasse sample was prepared in the laboratory using sugarcane syrup as mentioned in section 3.1. The vinasse characteristics are summarized in Table 3.1. It is also important to mention that *R. oligosporus* culture used in these studies was derived from the ATCC and the fungus was cultivated at 37^{o}C, an optimal temperature recommended by the ATCC.

5.2 Fungal Growth Pattern under Various Substrate Concentrations

Although the optimal substrate concentration for fungal growth was obtained previously, the use of new substrate might change the fungal yield. Moreover, because no direct relationship between fungal biomass production and organic reduction (as SCOD) was observed in the previous study, evaluating fungal growth pattern on different vinasse concentrations would be important and data could be useful for process scale-up.

Fungal biomass production on vinasse at different concentrations of 25, 50, 75, and 100% (v/v) were examined. Figure 5.1 shows the similar fungal growth pattern for all vinasse concentrations. The fungal growth pattern can be divided into three stages. During the first 24 h of cultivation, the fungus grew rapidly with relatively constant growth rate, and the fungal biomass concentrations increased about 64-79% from the starter cultures. Then, the fungal biomass production increased at a slower growth rate and reached a plateau by 60 h of cultivation. At the end of cultivation, fungal biomass concentrations of 8.51 ± 0.16^{a}, 7.81 ± 0.50^{a}, 10.09 ± 0.69^{b}, and 10.92 ± 0.51^{b} g/L were obtained at vinasse concentrations of 25, 50, 75, and 100% (v/v), respectively. Statistical analysis, represented by superscript letters, indicated that fungal growths on 25% and 50% vinasse concentrations were not significantly different at 95% confidence level. Similarly, at 75% and 100% vinasse concentrations, fungal biomass concentrations were found to be statistically insignificant at 95% confidence level. The growth pattern of *R. oligosporus* closely agrees with the growth of filamentous fungus, *R. nigricans*, in submerged fermentation using a bubble column reactor (García-Soto et al., 2006). The

authors used digital image analysis technique to characterize growth of the filamentous fungus. The core pellet and hairy mycelia, surrounding the pellet which was metabolically active, were distinguishable. It is also important to mention that the results in this study were obtained based on biomass dry weight, which therefore represents both structures. García-Soto et al. (2006) observed the exponential growth of core and hairy mycelia up to the first 16 h, which was 24 h in this study. At the end of fermentation (48 h), the authors found that hairy mycelia zone reduced whereas core pellet without mycelia grew continuously.

The organic content reduction, however, did not follow the same trend. COD reduced rapidly within the first 6 h at all substrate concentrations (Figure 5.2). The COD concentrations continued to decrease gradually until the end of cultivation. However, interestingly, COD concentrations reduced regardless of fungal biomass production. Table 5.1 shows a comparison between fungal biomass concentration and COD concentration reduction at all vinasse concentrations. The net COD reductions at the end of fungal cultivation were not significantly different at 95% confidence level. This finding is in close agreement with the previous optimization studies in which direct relationship between fungal biomass yield and SCOD reduction was not observed. The fungal growth was, however, related to reducing sugar present in vinasse. Reducing sugar reduced rapidly and reached nondetactable level after 24 h where the end of exponential growth was observed (Figure 5.3). This suggests that fungus primarily used sugars to support its growth. However, it can also use other unidentified organic compounds in the vinasse.

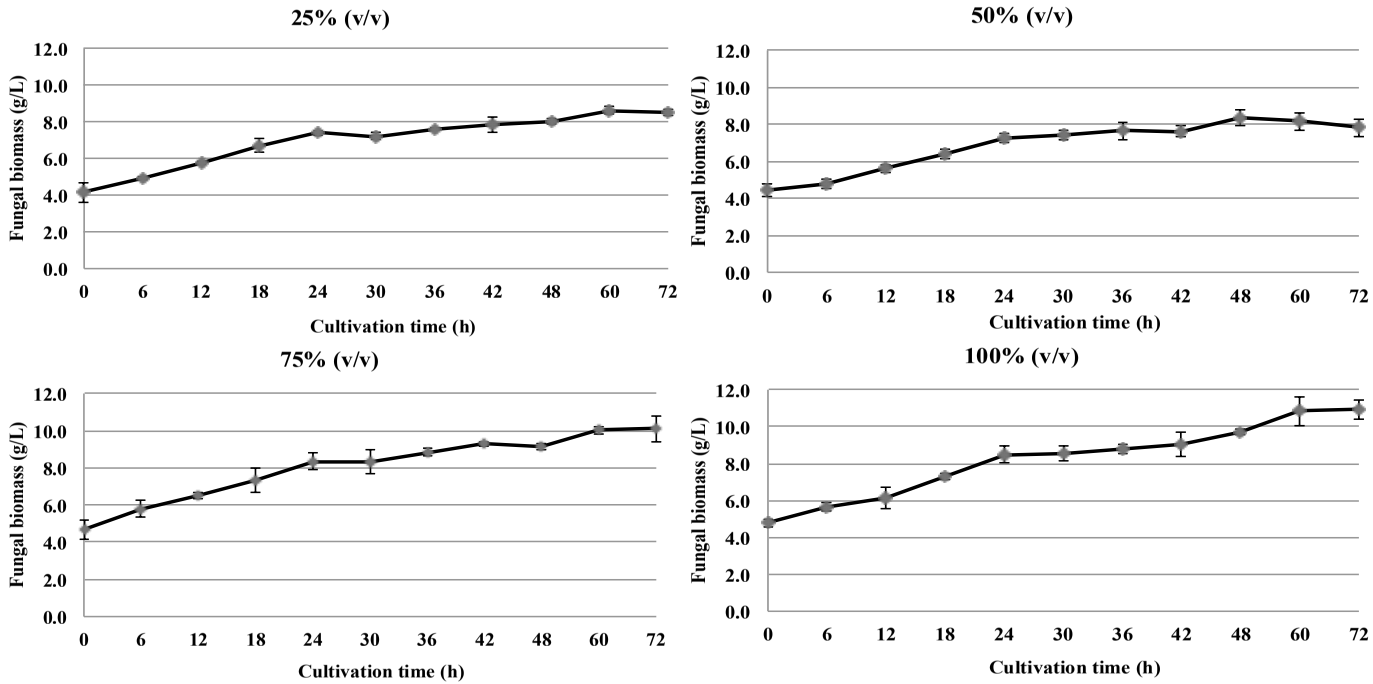


Figure 5.1 Fungal biomass concentrations in cultivation media at various vinasse concentrations

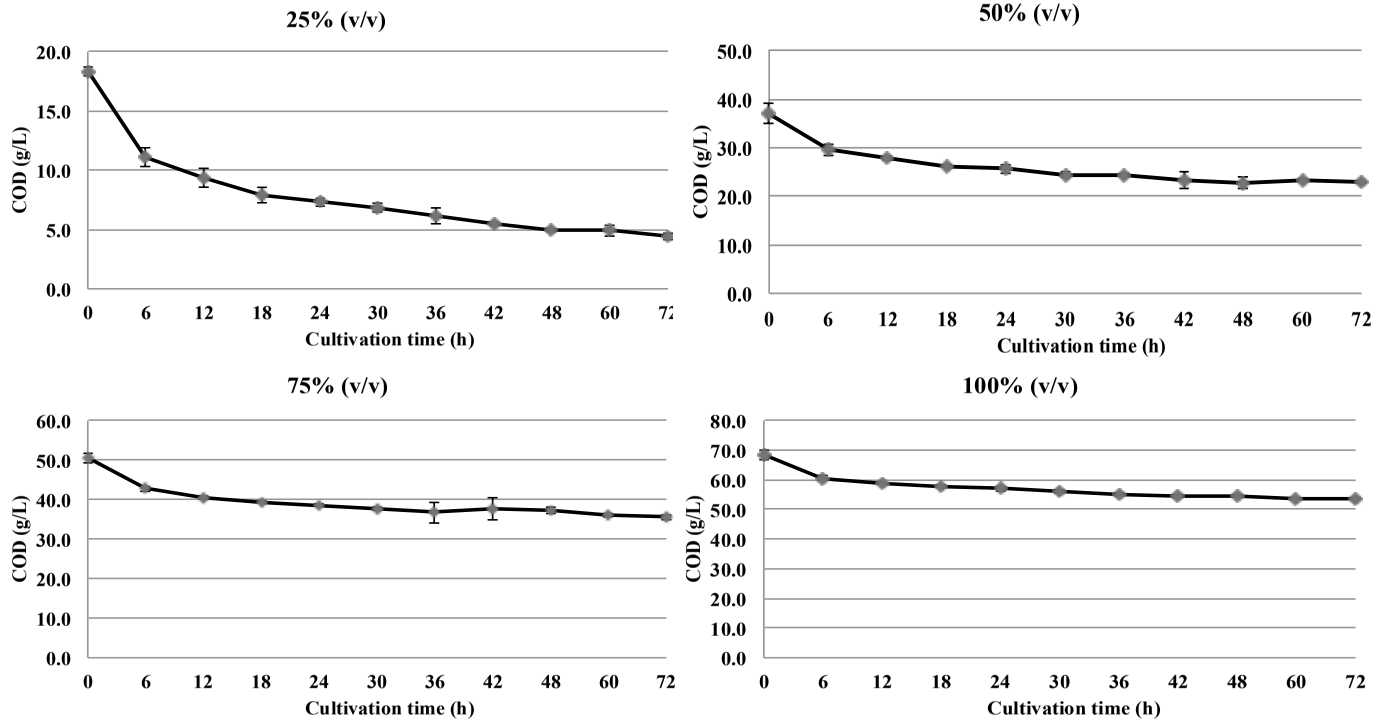


Figure 5.2 Chemical oxygen demand (COD) concentrations of fungal cultivation media at different vinasse concentrations

Table 5.1 Fungal biomass concentrations and chemical oxygen demand (COD) reductions of fungal cultivation media at various vinasse concentrations (25, 50, 75, and 100% (v/v))

Cultivation time (h)	Fungal biomass concentration (g/L)				COD reduction (g/L)			
	25%	50%	75%	100%	25%	50%	75%	100%
0	4.15 ± 0.54	4.41 ± 0.33	4.67 ± 0.51	4.78 ± 0.21				
6	4.92 ± 0.08	4.76 ± 0.23	5.80 ± 0.46	5.65 ± 0.21	7.27 ± 0.76	7.44 ± 1.19	7.64 ± 0.78	7.86 ± 0.73
12	5.76 ± 0.05	5.61 ± 0.22	6.49 ± 0.15	6.12 ± 0.57	8.97 ± 0.80	9.31 ± 0.34	9.80 ± 0.33	9.75 ± 0.08
18	6.72 ± 0.36	6.39 ± 0.25	7.32 ± 0.64	7.27 ± 0.16	10.44 ± 0.65	10.76 ± 0.29	10.86 ± 0.35	10.70 ± 0.24
24	7.39 ± 0.16	7.26 ± 0.24	8.36 ± 0.46	8.50 ± 0.46	11.05 ± 0.33	11.40 ± 0.90	11.65 ± 0.30	11.52 ± 0.75
30	7.22 ± 0.18	7.42 ± 0.30	8.32 ± 0.63	8.55 ± 0.40	11.52 ± 0.35	12.57 ± 0.48	12.78 ± 0.38	12.49 ± 0.57
36	7.64 ± 0.07	7.64 ± 0.48	8.81 ± 0.20	8.80 ± 0.27	12.22 ± 0.66	12.54 ± 0.31	13.61 ± 2.74	13.36 ± 0.44
42	7.81 ± 0.42	7.62 ± 0.32	9.29 ± 0.13	9.04 ± 0.65	12.83 ± 0.16	13.65 ± 1.78	12.63 ± 2.92	14.02 ± 0.39
48	8.05 ± 0.16	8.35 ± 0.44	9.12 ± 0.16	9.70 ± 0.13	13.39 ± 0.18	14.30 ± 1.11	13.01 ± 0.89	13.89 ± 0.45
60	8.63 ± 0.21	8.15 ± 0.49	10.02 ± 0.20	10.84 ± 0.79	13.42 ± 0.47	13.71 ± 0.31	14.19 ± 0.51	14.83 ± 0.31
72	8.51 ± 0.16	7.81 ± 0.50	10.09 ± 0.69	10.92 ± 0.51	13.90 ± 0.29	14.11 ± 0.19	14.73 ± 0.68	14.78 ± 0.19

Mean value ± standard deviation (sample size (n) = 6)

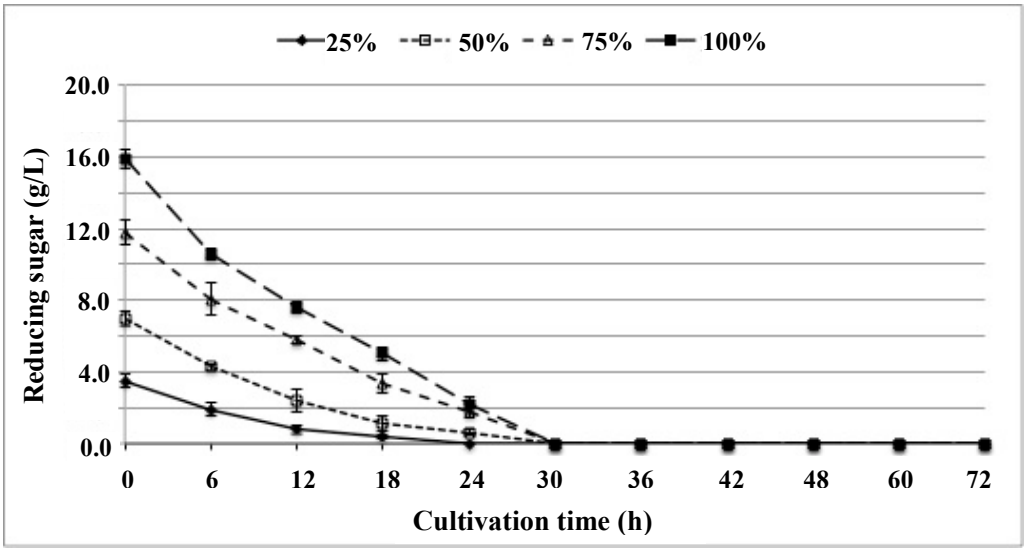


Figure 5.3 Reducing sugar profiles of fungal cultivation media at various vinasses concentrations (25, 50, 75, and 100% (v/v))

5.3 Effect of Inoculum Size on Fungal Mycelial Morphology

Among parameters influencing pellet formation, inoculum size is generally recognized as one of the most critical factors. Fungal mycelia starters were prepared with different spore inoculum sizes of 0.2, 0.5, 1.0, and 2.0% (v/v). Different fungal mycelial morphologies under various spore inoculum sizes were observed (Figure 5.4). Fungal pellets were obtained at low spore inoculum levels of 0.2% and 0.5%. The fungal pellets were, however, different in sizes. Pellets of increasing sizes were formed as the inoculum level was reduced. Similar findings were also reported by others (Metz and Kossen, 1977; Nielsen and Carlsen, 1996). However, at higher spore inocula (1% and 2% (v/v)), fungal pellets with radial hyphae were observed after first day of cultivation. The hairy edge of fungal pellets resulted in the connection between pellets thereby causing fungal biomass agglomeration. The agglomeration of fungal biomass and pellets were observed at 1.0% spore inoculum whereas fungal biomass clump was found at 2.0% spore

inoculum.

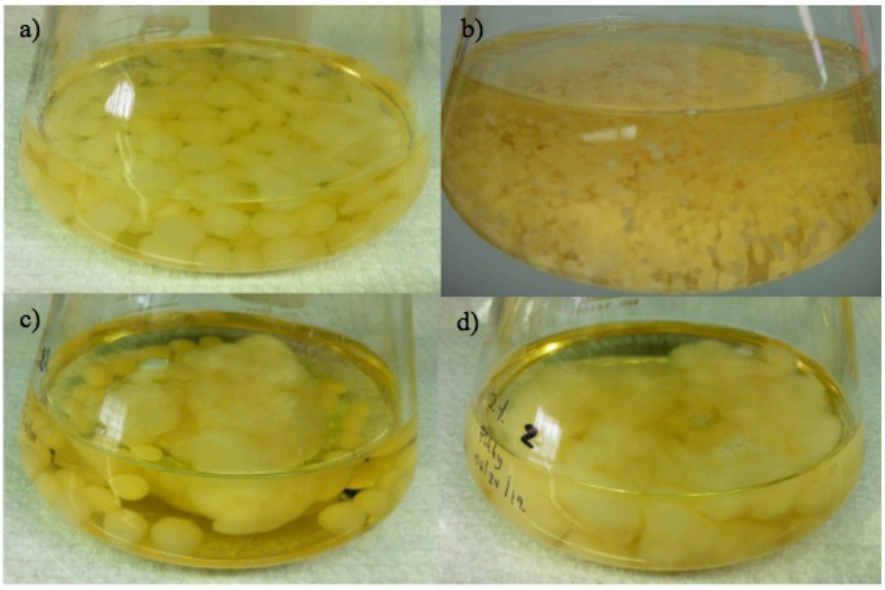


Figure 5.4 Fungal mycelia starters at different spore inoculum sizes (% (v/v)): a) 0.2; b) 0.5; c) 1.0; and d) 2.0

Fungal cultivation in submerged fermentation is highly dependent on the initial mycelial morphology used as an inoculum. Fungal mycelia starter in the pellet form is preferable for submerged fermentation because the surface area per volume ratio (A/V) of pellets is higher than A/V of other mycelial forms if suspended mycelial growth is not considered. However, the limitation of intraparticle mass transfer causes the problem of substrate availability inside the microenvironment of pellets when large pellets are formed (Cui et al., 1998b). Consequently, the spore inoculum level of 0.5% (v/v) was suitable for further bioreactor experiments. It is important to note that fungal mycelial morphology depends greatly on 1) genetic material of fungi and its interaction with 2) chemical factors (culture media composition), and 3) physical factors (pH, temperature, and mechanical forces) (Papagianni, 2004).

5.4 Effect of Ethanol on Fungal Biomass Yield

Vinasse derived after ethanol recovery process typically contains residual ethanol. The amount of ethanol present in vinasse depends on the efficiency of ethanol recovery process. Therefore, the effect of ethanol on fungal biomass production is critically important for vinasse concentration determination in a fungal fermentation process. Fungal biomass concentrations of samples containing 0.5, 1.0, 3.0, 5.0, and 7.0% (v/v) ethanol are presented in Figure 5.5. Ethanol at concentration less than 3.0% (v/v) had no adverse effect on fungal biomass production. However, the fungal biomass concentrations decreased significantly when the cultivating media contained 5.0% and 7.0% (v/v) ethanol. Ethanol at concentration higher than 3.0% (v/v) adversely affected fungal mycelial morphology as well (Figure 5.6). Pellets agglomeration was observed in the samples with ethanol concentrations of 5.0% and 7.0% thereby resulting in decrease in the fungal biomass concentration. As mentioned in the previous section, the agglomeration of fungal biomass creates microenvironment at the center of the clump, which results in nutrient transport limitation and consequently lowering the fungal growth. However, the mechanism by which ethanol affects the fungal mycelial morphology is still unknown.

Although, vinasse prepared in the laboratory contained ~ 3.73% (v/v) ethanol, the low biomass concentration of fungal cultivation on 100% vinasse was not observed (Figure 5.1). Therefore, it could be assumed that fungus developed tolerance to ethanol at a concentration of 3.73% (v/v), but ethanol at a concentration of 5.0% (v/v) had adverse effect on fungal biomass production. However, the maximum limit of tolerance of ethanol concentration on fungal growth on vinasse was not identified in this study. Vinasse derived from real ethanol industries generally have lower ethanol level due to efficient ethanol recovery process employed by the industries. Typically, vinasse from ethanol plants contains approximately 0.44% (v/v) ethanol (Silva et al., 2005). The vinasse obtained from the ethanol facility in Brazil used in the later experiment contained ~ 0.30% (v/v) ethanol (Table 3.1).

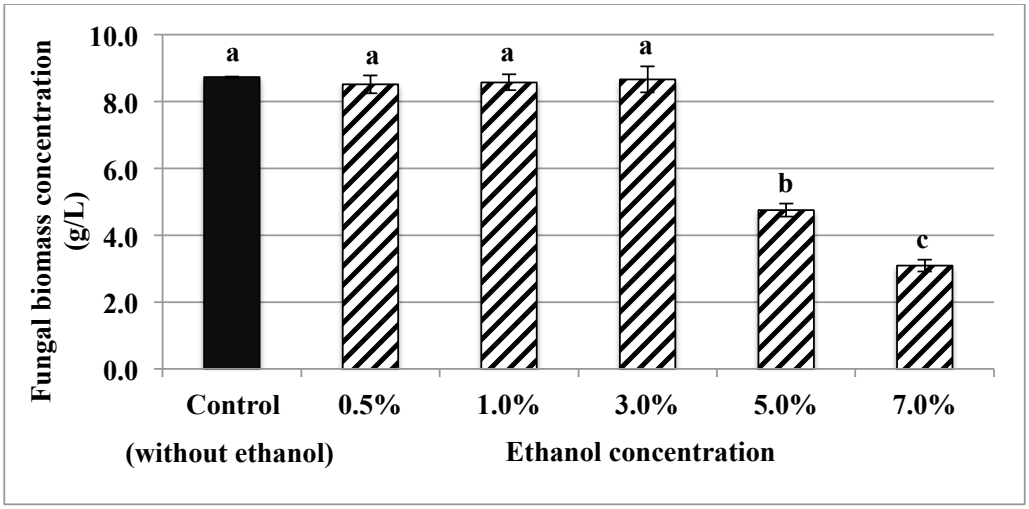


Figure 5.5 Effect of ethanol concentrations (% (v/v)) on fungal biomass concentrations (sample size (*n*) = 3)

Note: The letters in the bar graphs correlate statistical analyses.

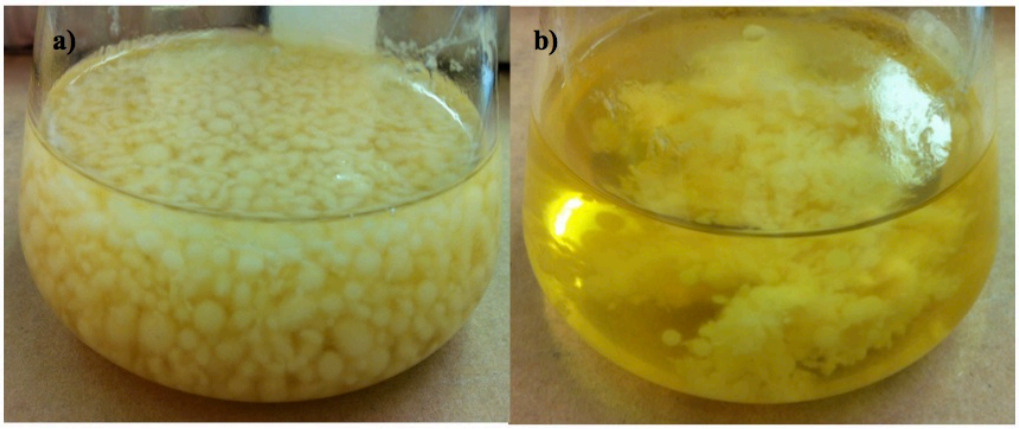


Figure 5.6 Fungal pellets at different ethanol concentrations: a) 3.0% (v/v) ethanol and b) 5.0% (v/v) ethanol

5.5 Fungal Fermentation on Vinasse in an Airlift Bioreactor

One of the most critical parameters influencing fungal pellet formation in the reactor is agitation. Although high agitation rate is necessary to provide adequate mixing and mass transfer, it can also damage the mycelium structure. Moreover, mechanical shear affects the fungal morphology by promoting free filamentous mycelia formation, thus ultimately decreasing the fungal growth (Cui et al., 1998a). Therefore, to avoid high shear stresses on fungal biomass, reactor configurations that minimize mechanical forces would be favorable for fungal pellet formation.

An airlift bioreactor system with the aid of a draft tube creates a homogeneous mixing pattern, resulting in improved oxygen transfer into the liquid phase (Chisti and Moo-Young, 1987). In the airlift bioreactor, rising air bubbles from the porous diffusers promotes agitation in the liquid phase. Consequently, the use of an airlift bioreactor for fungal cultivation eliminates the need of mechanical devices for mixing within the fermentation system. The recirculating liquid flow patterns generated by the bioreactor system can be conducive to fungal pellets formation; thereby simplifying the fungal biomass separation and recovery. The fungal growth, however, depends on dissolved oxygen level in the aqueous phase, which in turn is governed by the oxygen mass transfer rate. Therefore, this study was aimed at investigating the effects of aeration rates on fungal biomass yield and organic removal.

5.5.1 Fungal Biomass Yield at Different Aeration Rates

Fungal cultivations in an airlift bioreactor (2.5-L working volume) were performed under the optimal growth conditions based on growth optimization studies (Chapter 4). The fungal fermentation was conducted at a pH of 5.0 and a temperature of 37^{o}C for 3 days. Vinasse derived from sugarcane syrup fermentation was supplemented with nutrients (N and P) and used as a substrate for fungal cultivation in the airlift bioreactor. Because ethanol fermentation was performed in a batch-mode generating approximately 4.7-4.8 L of vinasse per batch, vinasse characteristics varied from batch to

batch. Initially, the effect of ethanol on fungal growth was determined and it was found that ethanol concentration of up to 3.73% (v/v) had no adverse effect on fungal biomass production. However, due to the variation in vinasse characteristics from batch to batch, ethanol concentration in vinasse sample reached as high as 4.22% (v/v). Although the maximum concentration of ethanol that causes no negative impact on fungal biomass production was not determined, ethanol concentration of 5.0% (v/v) was detrimental to fungal growth. Therefore, to avoid the adverse effect of ethanol on fungal fermentation, 75% (v/v) vinasse concentration which resulted in the same fungal biomass yield as indicated in section 5.2, was selected for cultivating fungus in this study.

Based on visual observations, the fungal biomass morphology in submerged fermentation depended on aeration rate, which was found to contribute to different fungal biomass yield. Among the aeration rates examined, 0.5, 1.0, and 1.5 vvm resulted in the formation of spherical pellets (~ 5-8 mm in diameter) within a day of inoculation (Figures 5.7a and 5.7b). During cultivation, the pellets grew larger in size with a more pronounced extension zone (hairy surface structure). Physical networking between fungal pellets was observed together with individual pellets. In the second day, a mixture of spherical pellets and chunks of fungal biomass settled at the bottom of the bioreactor. The attachment of clumps of fungal biomass at the annulus section between the draft tube and the external column was also observed during fungal fermentation at aeration rates of 1.0 and 1.5 vvm. The clumps of fungal biomass attached at the downcomer section when operating the reactor at the aeration rate of 1.5 vvm were bigger than those observed at 1.0 vvm. The presence of large fungal biomass chunks at the bottom of the reactor at a low aeration rate (e.g., 0.5 vvm) suggested that the aeration rate was insufficient to maintain circulatory movement of biomass within the bioreactor. Although this also occurred at the higher aeration rates of 1.0 and 1.5 vvm, the fungal biomass chunks at the downcomer section were occasionally circulated in the reactor in spite of their larger size due to higher aeration rates. The fungal biomass yields at different aeration rates are presented with the superscript letters representing the correlation based on statistical analyses. The maximum fungal biomass yield of 8.04 ± 0.80^{c} $g_{biomass\ increase}/g_{initial\ biomass}$ was obtained at

an aeration rate of 1.5 vvm. The lower fungal biomass yields of 3.73 ± 0.47[a] and 5.86 ± 0.40[b] $g_{biomass\ increase}/g_{initial\ biomass}$ were obtained at aeration rates of 0.5 and 1.0 vvm, respectively. Figure 5.7 shows the difference in fungal mycelial morphologies, during fungal fermentation at 1.5 vvm for 3 days.

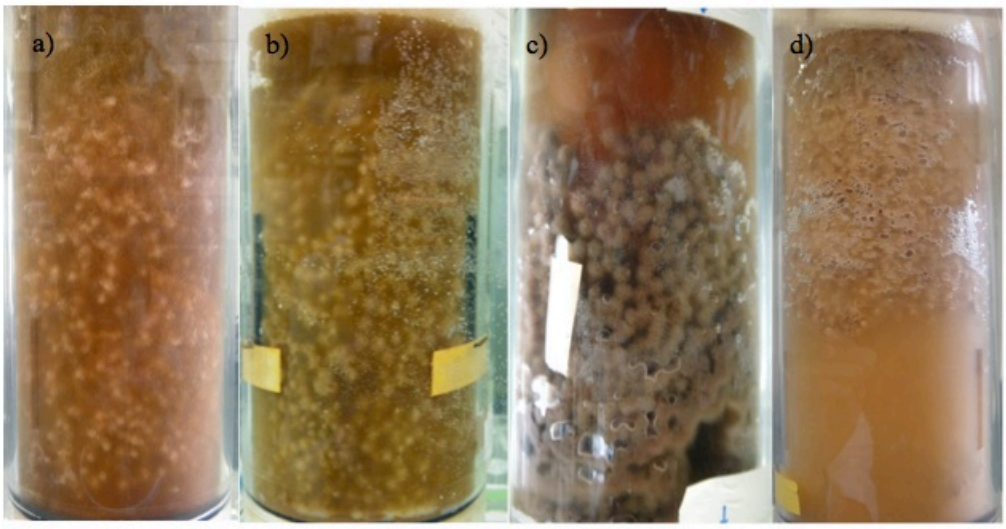


Figure 5.7 Changes in fungal mycelial morphologies during fungal fermentation at 1.5 vvm: a) 16 h; b) 24 h; c) 48 h; and d) 72 h

Increasing superficial gas velocities resulted in improving the volumetric mass transfer coefficient (k_La), gas holdup, and liquid circulation velocity (Sivasubramanian and Prasad, 2009). It is also important in aerobic processes to maintain oxygen levels above a critical level to provide the mass transfer rate equal to or exceeding the oxygen uptake rate by the microorganisms. Moreover, enhancing the aeration rate provides better mass transfer, which could eliminate the accumulation of metabolic gases, an inhibiting factor for fungal growth (Issarapayup et al., 2009). Consequently, higher aeration rates resulted in higher fungal biomass production. Figure 5.8 shows dissolved oxygen (DO) concentration during fungal cultivation at various aeration rates. Only at the aeration rate of 1.5 vvm, it was possible to maintain DO concentrations of 2.0 mg/L or higher in the bioreactor, which is the lowest concentration required in the suspended growth biological

wastewater treatment process (Tchobanoglous et al., 2003). The findings clearly represent that an insufficient DO concentration was a major reason for low fungal biomass production in the fungal fermentation at low aeration rates.

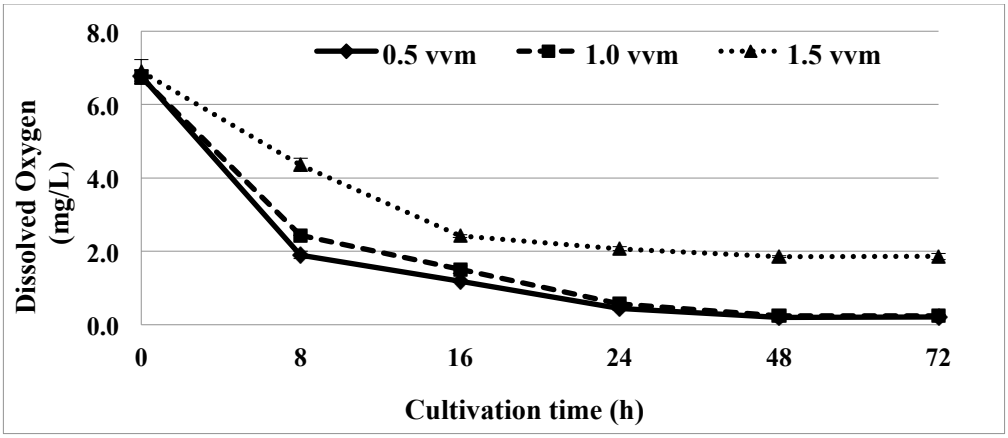


Figure 5.8 Dissolved oxygen (DO) concentration during fungal cultivation at various aeration rates (0.5, 1.0, and 1.5 vvm)

The fungal biomass yield, however, decreased to 5.14 ± 0.40^{b} $g_{biomass\ increase}/g_{initial\ biomass}$ at an aeration rate of 2.0 vvm. Increasing the air supply rate beyond 1.5 vvm neither addressed the recirculation problem nor improved the mass transfer efficiency. Luo et al. (2011) reported that the superficial gas velocity did not affect the k_La value significantly. In fact, the k_La improvement with increasing superficial gas velocity was dependent on interfacial area (a), which was strongly related to the gas holdup (Luo et al., 2011). Although gas holdup increases with the superficial gas velocity, high superficial gas velocity beyond the critical value results in heterogeneous flow regime. Accordingly, the intensive turbulent flow occurred in the liquid phase in the riser area. The excessive shear force affected the form of fungal growth. Erosion on microorganisms' surface occurs as a consequent of an excessive velocity gradient generated over their surfaces (García-Soto et al., 2006). This finding was further evidenced by the absence of fungal pellets formation in the reactor. Instead of spherical fungal pellets, the thread-like mycelia structure was observed, which could significantly increase the apparent viscosity

of fermentation broth (Figure 5.9). Couto and Toca-Herrera (2007) reported that the rheology of the fermentation broth resulting from different morphological growth forms of the filamentous fungi significantly affected the performance of the bioreactors. Further, García-Soto et al. (2006) concluded that superficial air velocity and thus hydrodynamic regime had a significant effect on the morphological development of fungi. The turbulent regimes led to the transformation of fungal pellets into disperse mycelia.

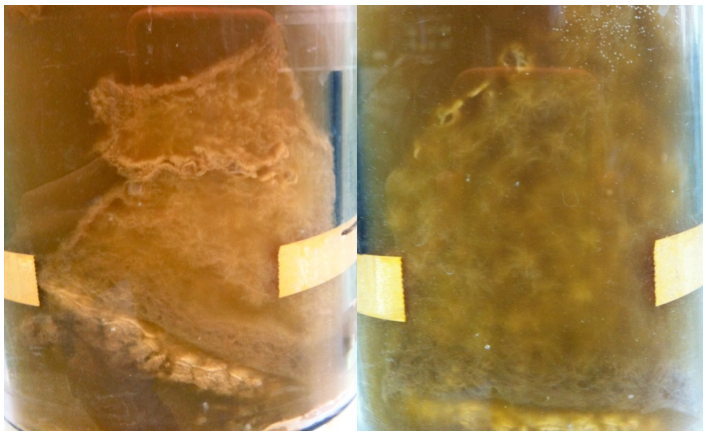


Figure 5.9 Fungal biomass in airlift bioreactor at 2.0 vvm

5.5.2 Organic Removal at Different Aeration Rates

Enhancing the aeration rate is likely to improve the mass transfer of oxygen and nutrients resulting in rapid substrate consumption (as discussed earlier). In this study, the organic matter (measured as SCOD) in vinasse was determined during fungal fermentation. The SCOD concentrations in the fungal bioreactor at various aeration rates are presented in Figure 5.10. Different SCOD reductions at various aeration rates were observed during the first day of fungal fermentation, and the SCOD concentrations reduced more rapidly at a higher aeration rate. However, after the second day of fermentation, SCOD concentrations remained fairly the same at all aeration rates.

Although fungal cultivations at different aeration rates yielded different fungal biomass yields, SCOD reductions at the end of fungal fermentation were not significantly different. Fungal cultivation at 1.5 vvm, the aeration rate that yielded the highest fungal biomass production, resulted in SCOD removal of 77.60 ± 3.73% and the effluent SCOD concentration was 7.43 ± 0.52 g/L. Lower aeration rates resulted in lower oxygen solubility in the fermentation broth. Consequently, the fungal growth was negatively impacted that led to the lower fungal biomass yields at 0.5 and 1.0 vvm in comparison to fungal biomass yield at 1.5 vvm. However, at high aeration rate (2.0 vvm), significantly lower fungal biomass yield was observed and SCOD removal remained nearly the same as at other aeration rates. This became apparent that part of SCOD removed was not directly associated to fungal growth and most likely consumed by other microbes in the bioreactor. Therefore, further experiments were conducted to evaluate microbial contamination during fungal fermentation.

5.5.3 Evaluation of Microbial Contamination during Fungal Fermentation

As discussed earlier, organic reduction was not in direct proportional to the fungal biomass yield. Experiments conducted in small volume (100 ml) without air supply suggested that the reduction of COD remained fairly constant after 48 h of fungal cultivation in all cases. However, during fungal fermentation in an airlift bioreactor, organic content reductions continued until the end of fermentation at all aeration rates. For aeration rates of 0.5 and 1.0 vvm, this could not be associated with a suitable aerobic condition for the fungal growth in bioreactor as DO levels went below the lowest level required in the suspended growth biological wastewater treatment process. Consequently, a series of experiments were conducted in an airlift reactor at an aeration rate of 0.5 vvm without fungal inoculation to investigate the SCOD removal by other microbes contaminated during fermentation. It was found that SCOD reduced regardless of fungal inoculation (Figure 5.11). This was due to the growth of undesired microbial contamination in the course of fermentation. Consequently, the specific oxygen uptake rate (SOUR), representing biological activity, and the SCOD reduction of fermentation

medium without fungal inoculation were determined to verify the growth of undesired microbes (non-fungal) in the bioreactor (Figure 5.12).

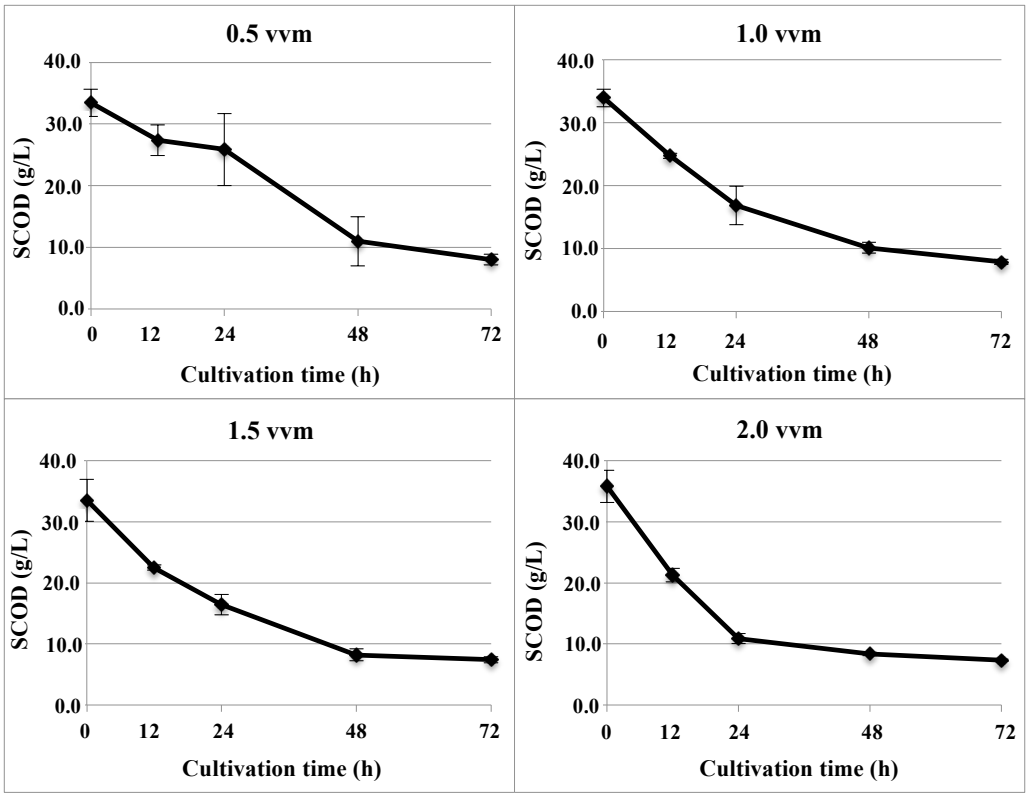


Figure 5.10 Soluble chemical oxygen demand (SCOD) concentrations during fungal fermentation at various aeration rates (0.5, 1.0, 1.5, and 2.0 vvm)

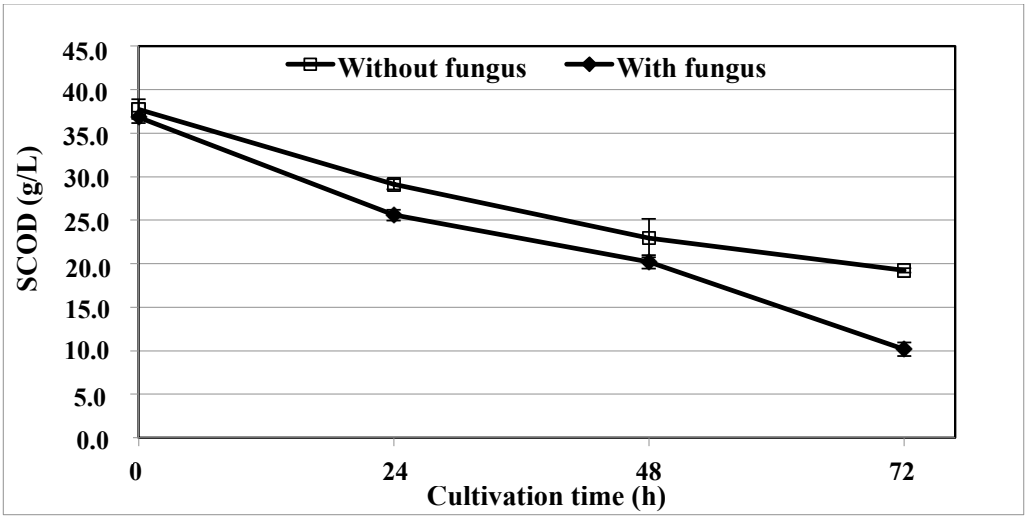


Figure 5.11 Soluble chemical oxygen demand (SCOD) concentrations of the fermentation media with and without fungal inoculation at an aeration rate of 0.5 vvm

The SCOD reduction along with SOUR suggested that the contamination had occurred during the fermentation process. The SOUR had a similar trend as typical bacterial and yeast growth patterns. The rapid SCOD reduction observed on the first day of cultivation was in close agreement with the highest SOUR at the same period. Low SOUR of about 3.8 mg O_2/g VSS-hr was observed at the end of cultivation suggesting the exhaustion of readily degradable organic matter in the growth medium. SCOD removal with fungal inoculum on day 2 to the end of cultivation was higher with respect to the SCOD removals of the sample without fungal inoculum while lower SOUR was obtained from the fermentation without fungal inoculum. This suggests that the SCOD reduction during that period was predominantly from the fungal growth.

Because the reactor was disinfected with 10% bleach (freshly prepared) and all connecting tubes were sterilized by autoclaving at 121^{o}C for 20 min, the contamination likely existed in the vinasse substrate. Sterilization of vinasse sample was performed by

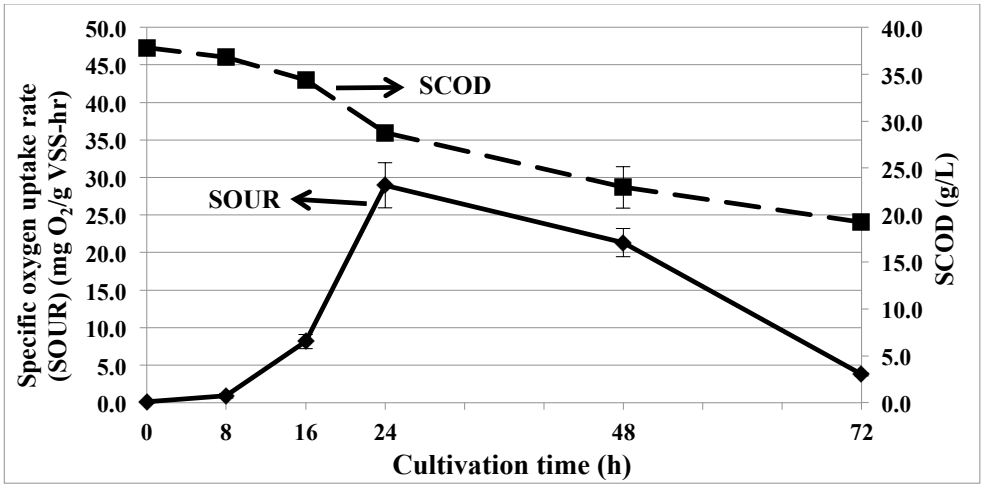


Figure 5.12 Soluble chemical oxygen demand (SCOD) concentrations and specific oxygen uptake rate (SOUR) during fermentation without fungal inoculation at an aeration rate of 0.5 vvm

autoclaving vinasse at 121^{o}C for 20 min, a standard practice for microbial decontamination. However, the further investigation using microbial screening technique suggested the presence of microbes in vinasse sample even though sterilization was carried out. A single type of microbial growth was observed from PDA plate inoculated with vinasse substrate and incubated at 37^{o}C for 3 days (Figure 5.13a). The contamination could be from yeast as the contaminated microbial colony appearance was identical to the yeast used for ethanol fermentation. Figure 5.13b shows microbial growth on PDA plate inoculated with effluent after fungal fermentation. There were two types of microbes grown on the plate. The first one was identical to that observed on a PDA plate inoculated with vinasse substrate and the other one was white fungal mycelia covered with black colored spores which was assumed to derive from *R. oligosporus*. It was apparent that no other microbes contributed to contamination because no other types of microbial growth was found on a solid medium.

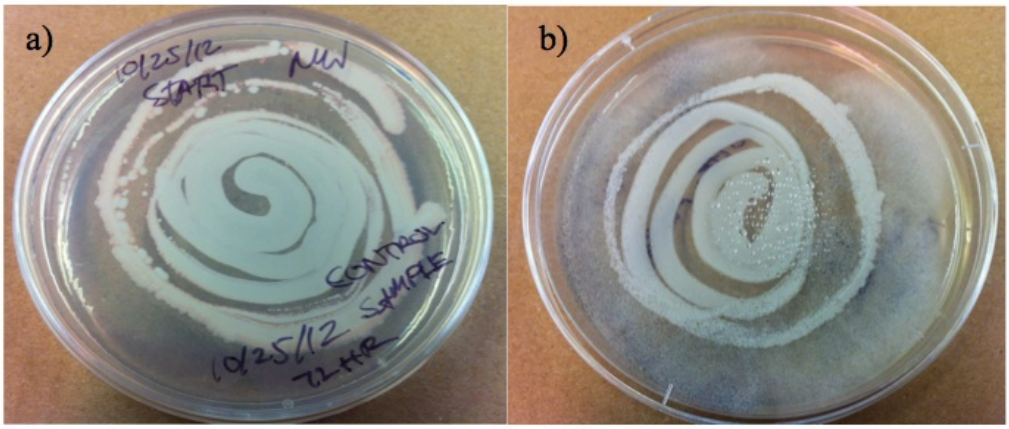


Figure 5.13 Microbial growth on solid media: a) vinasse substrate after autoclaving at 121^{o}C for 20 min and b) effluent after 3 days of fungal fermentation on vinasse at an aeration rate of 1.5 vvm

Yeast cells in the vinasse sample were expected to be beneficial to fungal protein production as the yeast cells attached to the fungal biomass could improve the protein content in fungal biomass product. However, ethanol at a concentration of higher or equal to 5.0% (v/v) negatively impacted the fungal mycelial morphology and thus the overall fungal biomass production. During the initial period of fungal fermentation which was under aerobic condition, yeast cells consumed oxygen for their cell synthesis that resulted in decrease in the DO concentration in vinasse substrate. The depletion of DO in vinasse would definitely limit the growth of fungus. Moreover, anoxic condition was observed after 24 h of fungal fermentation (Figure 5.8). Yeast shifts its metabolic pathway to fermentation in the absence of adequate DO. Yeast fermentation generates ethanol that would again inhibit fungal growth. Consequently, a better technique to control viable yeast cells in vinasse and subsequent fungal fermentation is necessary. However, vinasse from actual ethanol plants is generally derived following distillation at high temperature that essentially inactivate yeast cells. Thus, this issue should not be a concern if the fungal protein production is integrated into the existing ethanol industries.

5.6 Fungal Fermentation on Vinasse in a Bubble Column Bioreactor

Because the opening between the annulus section of the draft tube and the reactor wall in this bioreactor design was 2.0 cm, the agglomerated fungal biomass was not able to flow freely downward to generate a recirculation pattern. To address this issue, future bioreactor design should consider optimizing the A_d/A_r ratio such that it is greater than 1. A series of experiments without the draft tube in the airlift reactor (known as a bubble column reactor) to determine fungal growth were also conducted. Interestingly, the bubble column reactor resulted in elimination of fungal biomass chunk formation (Figure 5.14a). Significantly higher of fungal biomass yields were obtained when cultivating fungus in a bubble column reactor at the aeration rates of 0.5, 1.0, and 1.5 vvm in comparison to an airlift reactor. Fungal biomass yield improved by 33% (11.18 ± 0.13 $g_{\text{biomass increase}}/g_{\text{initial biomass}}$) in comparison to the yields in an airlift reactor at an aeration rate of 1.5 vvm, the optimal aeration rate for fungal fermentation (Figure 5.15). At a higher aeration rate (2.0 vvm), the fungal biomass yield was, however lower than fungal biomass yield obtained at 1.5 vvm aeration rate. Moreover, at 2.0 vvm, the fungal biomass yields obtained from airlift and bubble column reactors were not significantly different at 95% confidence level. The lower fungal biomass production at 2.0 vvm in the bubble column reactor was also resulted from excessive shear force due to high superficial velocity in liquid phase, which disrupted fungal pellets structure and a thread-like mycelia structure was observed (Figure 5.14b). The organic content (measured as SCOD) reductions for all aeration rates were not significantly different at 95% confidence level and the percentages of SCOD reductions were 75.63 ± 0.62^a, 76.48 ± 0.45^a, 76.28 ± 4.17^a, and 78.63 ± 1.70^a, respectively, at aeration rates of 0.5, 1.0, 1.5, and 2.0 vvm.

Moreover, other than superficial gas velocity and reactor geometry, sparger type is also a critical factor because it affects the gas holdup, liquid circulation velocity, and the mass transfer coefficient in an airlift reactor system. Thus, future research should examine how these parameters affect the hydrodynamics and mass transfer in the

bioreactor.

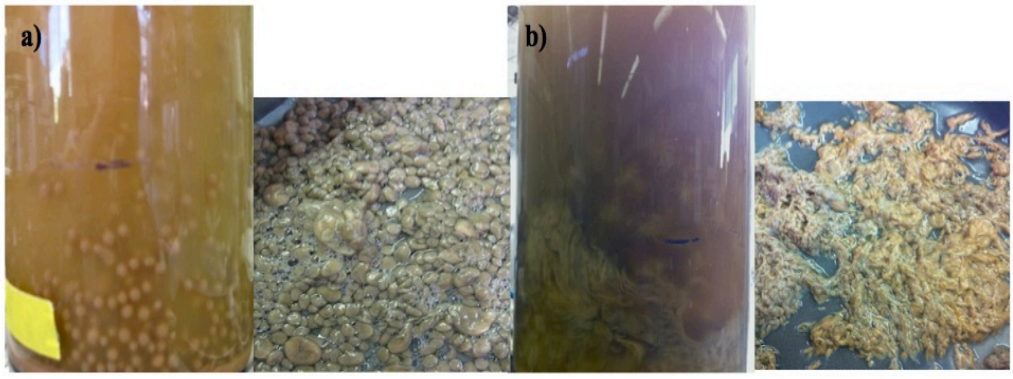


Figure 5.14 Fungal biomass in a bubble column reactor at aeration rates of: a) 1.5 vvm and b) 2.0 vvm

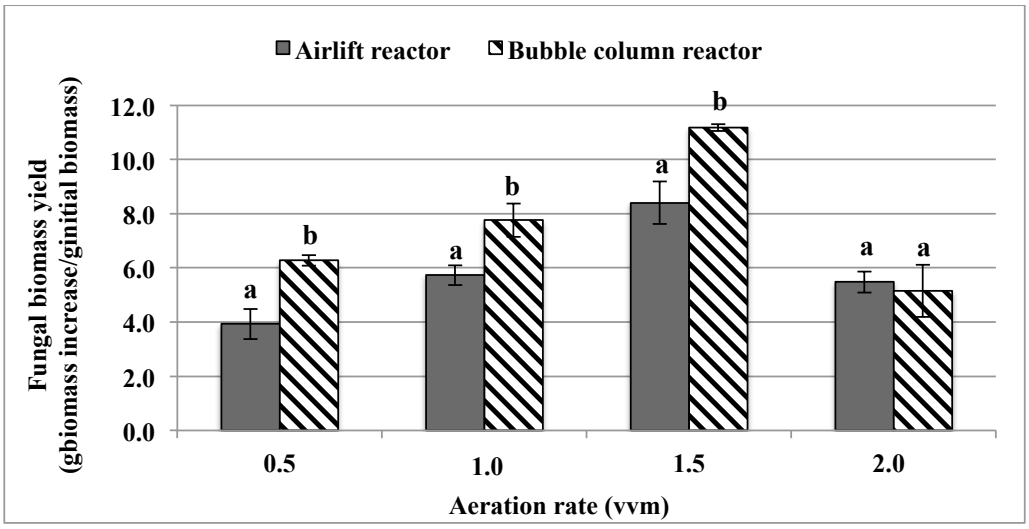


Figure 5.15 Fungal biomass yields in airlift and bubble column bioreactors at various aeration rates (0.5, 1.0, 1.5, and 2.0 vvm) (sample size (*n*) = 3)

Note: The letters in the bar graphs correlate statistical analyses within each aeration rate.

5.7 Fungal Cultivation on Sugarcane-ethanol Derived Vinasse

Because of lack of existing sugarcane ethanol facility in the United States, vinasse used in the study was prepared from yeast fermentation of sugarcane syrup in the laboratory. However, characteristics of vinasse obtained from a commercial sugarcane-ethanol facility may be different from that produced in our laboratory. Thus, the fungal cultivation on a sugarcane-ethanol derived vinasse obtained from a commercial sugarcane-ethanol facility in Brazil was examined. The vinasse characteristics from the commercial facility are summarized in Table 3.1. Brazil vinasse contained considerably high solids, TKN, potassium, calcium and iron; but was low in organic content (expressed as COD) and sodium in comparison to the laboratory-prepared vinasse. Significantly high amounts of sodium in laboratory-prepared vinasse were due to the use of NaOH solution (2%) for controlling the pH of the fermentation medium. Because Brazil vinasse had significantly lower organic and ethanol contents compared to laboratory-prepared vinasse, the Brazil vinasse was used without dilution for fungal fermentation. The experiment was conducted under an optimal aeration rate of 1.5 vvm. The result showed that fungal biomass yields on Brazil vinasse and laboratory-prepared vinasse were not significantly different at 95% confidence level and the fungal cultivation in Brazil vinasse resulted in the fungal biomass yield of 8.52 ± 2.44 $g_{biomass\ increase}/g_{initial\ biomass}$. The organic content of the effluent was about 13.44 ± 0.58 g SCOD/L, which was equivalent to 59.57% or 19.80 g/L of SCOD reduction. Other inorganic matters were reduced by 67.79% nitrogen (as TKN) and 79.73% phosphorus (as PO_4^{3-}). This finding clearly showed that the results obtained from laboratory-prepared vinasse can be potentially applied to vinasse obtained from a commercial sugarcane-ethanol plant. The elimination of vinasse dilution could possibly save costs for fungal protein production by reducing clean water use and wastewater remediation.

5.7.1 Evaluation of Microbial Contamination in Fungal Fermentation on Sugarcane-ethanol Derived Vinasse

A study on microbial contamination during fungal fermentation on sugarcane-ethanol derived vinasse was performed. Brazil vinasse after sterilization by autoclaving at 121^{o}C for 20 min and the effluent from fungal fermentation at 1.5 vvm were inoculated into PDA plates. The plates were incubated at 37^{o}C for 3 days. Figure 5.16 shows the resulting PDA plates after three days of incubation. No microbial growth was observed on Brazil vinasse substrate (Figure 5.16a). Moreover, the plate inoculated with effluent from fermentation was found to contain only *R. oligosporus* mycelia along with its spores (Figure 5.16b). As indicated previously, ethanol facilities recover yeast cells in the fermentation process to save the material and production costs. Consequently, yeast contamination was not found when using Brazil vinasse for fungal fermentation.

The study of fungal fermentation on sugarcane-ethanol derived vinasse suggested a feasibility of integrating fungal processing into ethanol industries for the production of high-value fungal protein from their residue, vinasse.

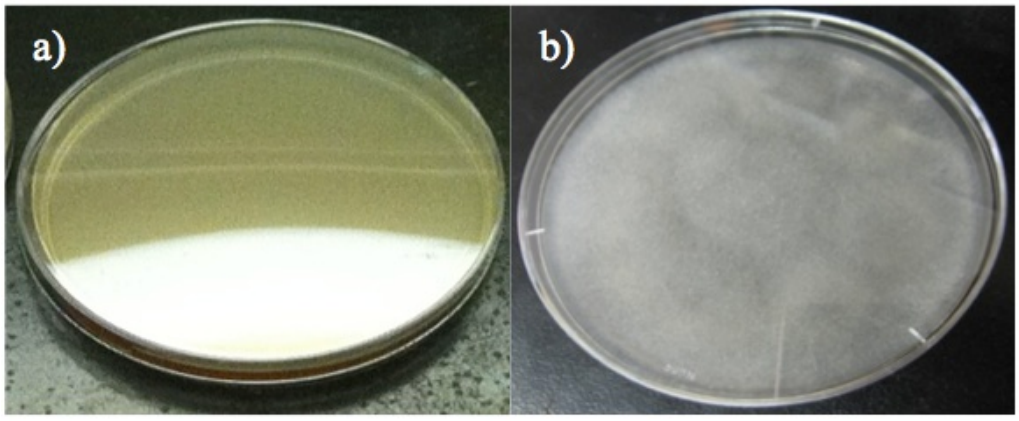


Figure 5.16 Microbial growths on solid media: a) Brazil vinasse substrate after autoclaving at 121^{o}C for 20 min and b) effluent after 3 days of fungal fermentation on Brazil vinasse at an aeration rate of 1.5 vvm

CHAPTER 6: RESULTS AND DISCUSSION: VINASSE-DERIVED FUNGAL PROTEIN AS AN ANIMAL FEED INGREDIENT

6.1 Rationale

Protein and its amino acid contents are essential chemicals, which attribute to healthy growth of animals. Therefore, they are considered to be one of the most critical factors in animal feeds. Presently, soybean meal and fishmeal are commercially used as major protein supplements for animal feeds and prices of which are increasing perpetually because of the rising demands. The fungal protein can replace the use of expensive commercial protein sources (fishmeal and soybean meals), if it contains high protein and balanced essential amino acid profiles with a good digestibility. Producing value-added product such as protein-rich fungal biomass from low-cost biofuel residue, vinasse would enhance the sustainability of biofuel industries by generating additional revenue and simultaneously lessening their impacts on the environment. Inexpensive protein ingredient for animal feed production would eventually reduce the production cost of meat and fish products.

Nutritional requirements of feeds vary greatly depending on the species and age of animals. Typically, the requirements of protein contents for swine, poultry, fish, and shrimp feeds are 13–24%, 12–30%, 32–45%, and 25–42% (dry weight basis), respectively (Miller, 2002; Miles and Chapman, 2009). The protein content of *R. oligosporus* cultivated on thin stillage from corn-ethanol industries was relatively high (~ 43% dry weight) with various essential amino acids suitable for animal feed applications (Rasmussen et al., 2007). However, it is important to note that the fungal biomass product also contained residual solids particle from corn-ethanol stillage, which had relatively high amounts of protein compared to vinasse. Vinasse-derived fungal biomass might result in lower protein content and consequently the essential amino acids. Therefore, in this research, the chemical composition and nutritional value of vinasse-derived fungal

biomass were determined with a particular emphasis on the possibility of its use for protein supplementation in animal feed ingredient.

6.2 Effect of Fungal Fermentation at Different Aeration Rates on Crude Protein and Crude Lipid Contents of the Fungal Biomass Product

The crude protein contents in fungal biomass obtained at 1.5 vvm were not significantly different with respect to crude protein contents of fungal protein cultivated at other aeration rates. Under the optimal aeration rate (i.e., 1.5 vvm), the fungal biomass contained approximately 49.57 ± 0.34% crude protein (dry weight basis). The protein contents of fungal biomass obtained in this research were independent of the fungal mycelial morphology. The protein production, however, depended on fungal morphology (Xu et al., 2000). It is important to mention that fungal morphology is greatly dependent on a wide range of factors and its genetic material; therefore, this finding was applied only to the fungal species, *R. oligosporus*, and under the growth conditions stated in this research.

Nevertheless, the crude lipid content in the fungal biomass was dependent on the aeration rate. The crude lipid content improved with the increase in aeration rate up to 1.5 vvm. The crude lipid content then decreased at an aeration rate of 2.0 vvm. The highest crude lipid content was 5.27 ± 0.96% (dry weight), and was obtained at an aeration rate of 1.5 vvm. The improvement of lipid content in fungal biomass at higher aeration rates was likely attributed to oil accumulation in fungal cells due to nitrogen limiting conditions. Fungal growth was more rapid at higher aeration rates and resulted in faster consumption of nutrients. Lipid accumulation in microbial cells occurs when an element, especially nitrogen in the medium becomes limiting while the carbon source is present in excess (Beopoulos et al., 2009). When the carbon source is in excess, nitrogen becomes limiting and it results in the preferential channeling of carbon flux toward lipid synthesis (Beopoulos et al., 2009). Consequently, triacylglycerols produced from lipid synthesis are accumulated in lipid bodies of the microbial cells. However, the low crude lipid content

of fungal biomass cultivated at 2.0 vvm was in close agreement with low fungal biomass yield indicating that the fungal growth did not result in nitrogen limitations in the fermentation medium. The lower crude lipid in fungal biomass cultivated at 2.0 vvm might be resulted from the shift in its metabolic pathway or through changes in fungal morphology. However, because the change in cell physiology of filamentous fungi varies significantly, it is quite difficult to explain whether the variation in final crude lipid content was caused by the change in the fungal morphology. The crude protein and lipid contents of the fungal biomass at various aeration rates are summarized in Table 61.

Table 6.1 Crude protein and lipid contents of fungal biomass at various aeration rates (0.5, 1.0, 1.5, and 2.0 vvm)

Aeration rates (vvm)	Compositions (% dry weight)	
	Crude protein	Crude lipid
0.5	50.26 ± 0.43^{a}	2.41 ± 0.14^{a}
1.0	50.31 ± 0.63^{a}	3.21 ± 0.34^{b}
1.5	49.57 ± 0.34^{a}	5.72 ± 0.66^{c}
2.0	48.90 ± 0.46^{a}	1.34 ± 0.07^{d}

Mean value ± standard deviation (sample size (n) = 2)
Superscript letters represent statistical analyses

6.3 Chemical Composition of Vinasse-derived Fungal Biomass

The chemical compositions of vinasse-derived fungal biomass obtained when cultivating the fungus at the optimal aeration rate of 1.5 vvm, are presented in Table 6.2. Crude protein content of vinasse-derived fungal biomass was of about 50%, which was in close agreement with protein content of fungal biomass derived from starch processing wastewater (Jin et al., 2002). However, with respect to corn stillage-derived fungal biomass, vinasse-derived fungal biomass had significantly higher crude protein content;

but it had lower crude lipid content. The lower crude protein in fungal biomass produced from corn stillage could be due to the lower protein content in residual corn kernels in the stillage with respect to the fungal biomass. This was further confirmed by a much higher protein content (43%) in fungal biomass cultivated on settled thin stillage supernatant which did not contain suspended solids (Rasmussen et al., 2007). Corn stillage-derived fungal biomass had a large amount of particles that acted as nuclei for agglomeration of fungal biomass and contributed to the higher crude lipid content in comparison to vinasse-derived fungal biomass. Moreover, as discussed previously, aeration rate in fungal fermentation process significantly affected the crude lipid content of the fungal biomass. Also several studies reported the variation of protein production resulted from different fungal morphology in submerged fermentation (Xu et al., 2000; García-Soto et al., 2006).

Table 6.2 Chemical compositions of vinasse-derived fungal biomass, corn stillage-derived fungal biomass, soybean meal, and fishmeal

Compositions (% dry weight)	**Vinasse-derived fungal biomass[1]**	**Corn stillage-derived fungal biomass[2]**	**Soybean meal[3]**	**Fishmeal[4]**
Crude protein	49.57 ± 0.34	28.20	48.10	73.23
Crude lipid	5.72 ± 0.66	33.10	1.43	10.51
Ash	3.46 ± 0.07	5.70	6.21	17.23

[1]Mean value ± standard deviation (sample size (n) = 2)

[2]Rasmussen et al. (2007); [3]Swick (2001); [4]Øverland et al. (2009)

However, when compared to soybean meal, the vinasse-derived fungal biomass had higher protein and lipid contents. Whereas, crude protein, crude lipid, and ash in the fungal biomass from vinasse fermentation were lower than that from fishmeal. It is important to note that the harvested fungal biomass was washed several times before it

was subjected to freeze dried for analysis. The yeast cells in vinasse could possibly improve protein and amino acid profiles of the fungal biomass. Consequently, the protein content in the vinasse-derived fungal biomass product could likely be improved by eliminating the washing step for fungal biomass recovery.

Low ash content in vinasse-derived fungal biomass will have significant merits for aquatic feed applications. In aquaculture feed, phosphorus is required and its digestibility is inversely related to ash content (NRC, 1993). The diet containing fishmeal has surprisingly low phosphorus digestibility and the digestibility increases when the fishmeal is replaced with soybean meal containing lower ash.

6.4 Nutrition Value of Vinasse-derived Fungal Biomass

Further analysis of fungal biomass was conducted to determine the essential amino acids for aquaculture feed applications. As shown in Figure 6.1, the overall essential amino acids of vinasse-derived fungal biomass were comparable to fishmeal and soybean meal, the commercial protein ingredients containing excellent amino acid profiles as high quality protein sources for animal feed applications (Lim and Akiyama, 1992; Miller, 2002; Miles and Chapman, 2009). However, the fungal biomass had low methionine and phenylalanine. Importantly, fungal-lysine content (~ 8% on protein basis) was relatively higher than the commercial protein sources. Lysine is the most critical required amino acid in aquatic feed as it is often the first limiting amino acid in the feed ingredients (Miller, 2002; Cheng et al., 2003; Craig and Helfrich, 2009). Wilson (1989) also indicated that lysine is critical for optimal growth of fish. Therefore, high lysine content in fungal protein would have significant merits for aquatic feed applications. Inherently, fungi contains low amount of sulfur-containing amino acids that result in low fungal-methionine content (Rhodes et al., 1961; Gregory et al., 1976). Whereas, high tryptophan contents in fungal-protein is beneficial to aquatic feed applications since it reduces the aggressive behavior and controls juvenile cannibalism in fish (Winberg et al., 2001; Hseu et al., 2003). For non-ruminant diets, the main limiting amino acids are

lysine, methionine, tryptophan, and threonine (Cheeke, 2005). Consequently, considerably high threonine contents in the fungal product would benefit the use of fungal biomass as a protein source in non- ruminant feeds. Methionine supplementation might be necessary to improve animal growth. Santos and Gómez (1983) suggested supplementation of 0.3% methionine into diet produced from fungal biomass, *A. fumigatus*, grown on cassava significantly improved the growth of rats and pigs. However, it is not necessary to meet the ideal balance for all amino acids. Increasing the amount of protein could also meet the amino acid requirements in the diet (Carpenter and De Muelenaere, 1965; Boorman, 1992).

To address a concern over low methionine and phenylalanine contents, co-feeding of the fungal protein with commercial protein could be a desirable option for maintaining adequate amino acid requirements for animal feed applications. Moreover, it is important to mention that fungal biomass also had a good *in vitro* protein digestibility of 83.65 ± 2.21% which is quite comparable to soybean meal and fishmeal with overall digestibility of about 85% (Jasti et al., 2002; Miller, 2002). The digestibility of *R. oligosporus* cultivated on vinasse was very close to that of fungal biomass grown on starch processing wastewater (84% digestibility) (Jasti et al., 2002).

Supplementation of low-cost fungal protein into commercial protein could significantly cut down the cost of animal feed since the major cost of animal feed comes from protein and it accounts for nearly 30-50% of the variable cost of fish/shrimp feed production (Miles and Chapman, 2007).

Analysis of fungal biomass for fatty acid profiles showed that vinasse-derived fungal protein is a promising alternative for aquatic feed ingredients. The fatty acid composition is one of the critical parameters in aquatic feed as it has a significant influence on the tissue fatty acid composition in the fish (Watanabe, 1982; Sargent et al., 1989). The vinasse-derived fungal biomass contained those essential fatty acids (based on dry fungal biomass weight), linoleic acid (1.35%), linolenic acid (0.32%), eicosapentaenoic acid (EPA) (0.12%), and docosahexaenoic acid (DHA) (0.11%).

Linoleic acid, C18:2n-6, and linolenic acid, C18:3n-3, cannot be synthesized by fish and thus they must be supplied in a diet (NRC, 1993). However, the requirement of fatty acid content in feed varies significantly depending on the metabolic pathway for modifying and synthesizing fatty acids in the animals. For instance, freshwater fish species generally require either dietary linoleic or linolenic acid, or both, whereas marine fish species require dietary EPA, C20:5n-3, and/or DHA, C22:6n-3, (NRC, 1993). EPA deficiency leads to various symptoms and diseases, such as fin rot, shock syndrome, and myocarditis. Moreover, it results in the reduction in growth rate and feed efficiency with an increase in mortality (Castel et al., 1972; Takeuchi et al., 1980; Satoh et al., 1989).

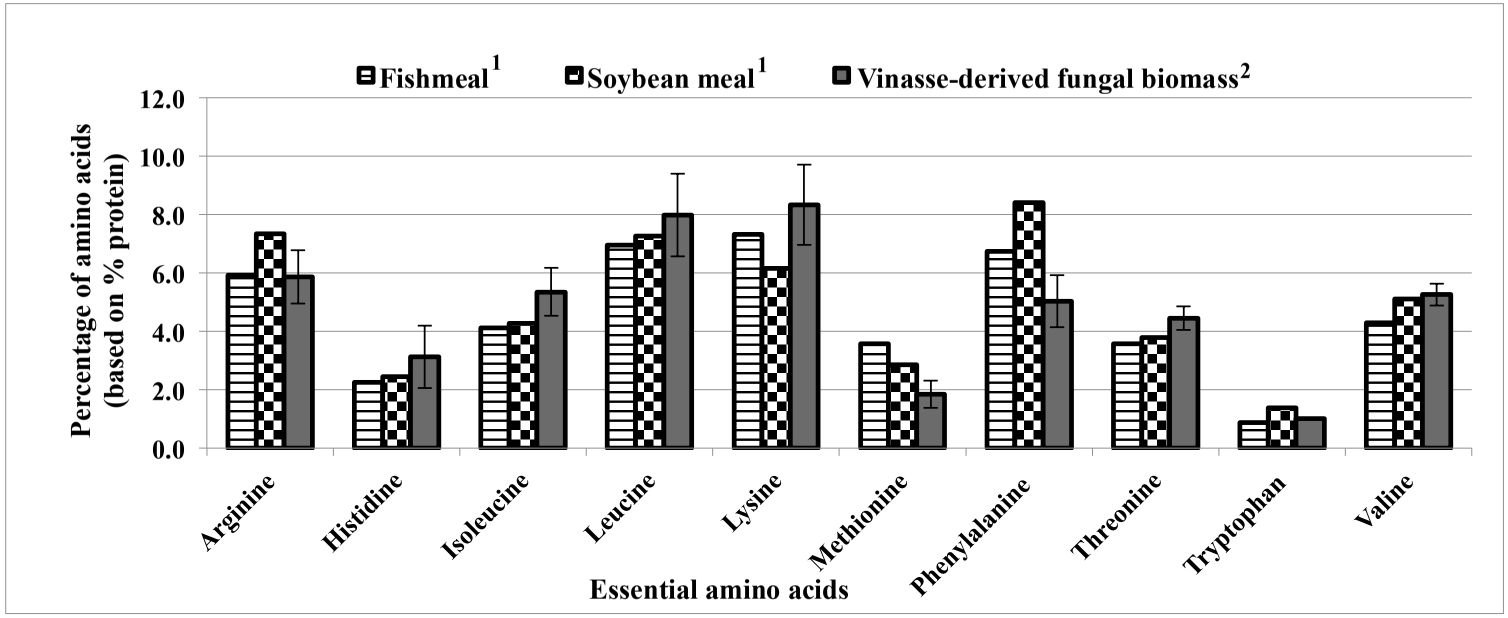


Figure 6.1 The percentage essential amino acids (% protein basis) of fishmeal, soybean meal, and vinasse-derived fungal biomass cultivated at an optimal aeration rate (1.5 vvm)

[1] Modified from NRC (1993)

[2] Experimental analysis (sample size (n) = 4)

CHAPTER 7: ENGINEERING IMPLICATIONS

The extremely high organic matter and nutrient content of vinasse poses a serious environmental concern if discharged without treatment and the necessary treatment costs lead to increased costs of bioethanol production. Since fungal process reduces the organic and inorganic contents of the vinasse, the effluent can potentially be used as process water for in-plant use or for land application. Moreover, fungal biomass has a comparable protein content to commercial protein ingredient for animal feeds, good essential amino acids composition, and invaluable essential fatty acids, which suggests its application in animal or aquatic feeds. An edible fungal biomass could also potentially serve as a human dietary supplement containing several biochemicals, including vitamins and antioxidant. Consequently, fungal fermentation offers a plausible and sustainable solution for sugarcane-to-ethanol industries. The fungal technology could provide protein for animal/aquatic feeds at a cheaper price than the typical commercial protein sources, soybean meal and fishmeal and generates additional revenue to the bioethanol industries.

However, economic viability is a crucial factor to justify its commercialization. A bench-scale optimization provides reliable information and insight for the design and development of full-scale fungal fermentation. The data can be employed to conduct techno-economic analysis as well. Although, no data exists on the fungal biomass production cost, due to the simple operations with minimal additional unit operations/processes and low feedstock cost, the production cost of fungal biomass is expected to be relatively low. However, some aspects, including bioreactor design and improvement in gas (oxygen) solubility in the liquid phase, would have to be further investigated for process commercialization.

Nutrients supplementation to support the maximum fungal growth is one of the most critical factors affecting the production cost of fungal protein. Therefore, to lower the cost of fungal protein production, the experiment without nutrient supplementation

under the optimal air supply rate of 1.5 vvm was conducted. Impressively, the fungal biomass yield on the vinasse without nutrient supplementation was not significantly different than that of fungal biomass yield with nutrient supplemented vinasse and Brazil vinasse samples. The yield of 7.80 ± 0.74 ($g_{biomass\ increase}/g_{initial\ biomass}$) was obtained from fungal fermentation on vinasse under aeration rate of 1.5 vvm without nutrient supplementation. This finding suggests that nutrient supplementation in fungal cultivation in a bioreactor system can be completely eliminated. Moreover, as mentioned in the previous section that vinasse dilution was not necessarily important for fungal fermentation on vinasse obtained from a commercial sugarcane-ethanol facility (Brazil vinasse), the elimination of vinasse dilution could possibly save the cost for fungal protein production by reducing the costs of clean water consumption and wastewater remediation. Figure 7.1 shows an overall schematic diagram of annual fungal protein production from 1 million gallon per year (MGY) sugarcane-ethanol plant.

This study shows that the fungal fermentation technology can potentially be applied to commercial sugarcane-ethanol facilities. An operational cost of fungal protein production comes mainly from air supply system and is estimated to be $262,518 annually. The high aeration cost of fungal cultivation could be reduced by optimizing reactor design and aeration system. Fungal biomass production seems to depend on dissolved oxygen (DO) level in the liquid phase. Therefore, a high aeration rate of 1.5 vvm might not necessary if a better air diffuser is further investigated. Consequently, the cost for air supply could be reduced. Moreover, selection of suitable and high efficiency air generation unit would also considerably reduce the aeration cost for fungal cultivation. With the current reactor configuration and operating condition, the final fungal biomass product provides additional revenue of about $155,462 to 464,389 per year. The calculation was carried out based on fungal biomass production on Brazil vinasse. However, it is important to note that the additional revenue generated from fungal biomass was calculated regardless of other production costs such as pH and temperature controls, and fungal biomass recovery.

Fungal-derived product also has several applications such as protein supplement in human diet. This will have significant implication in curtailing the malnutrition in developing/least developed countries.

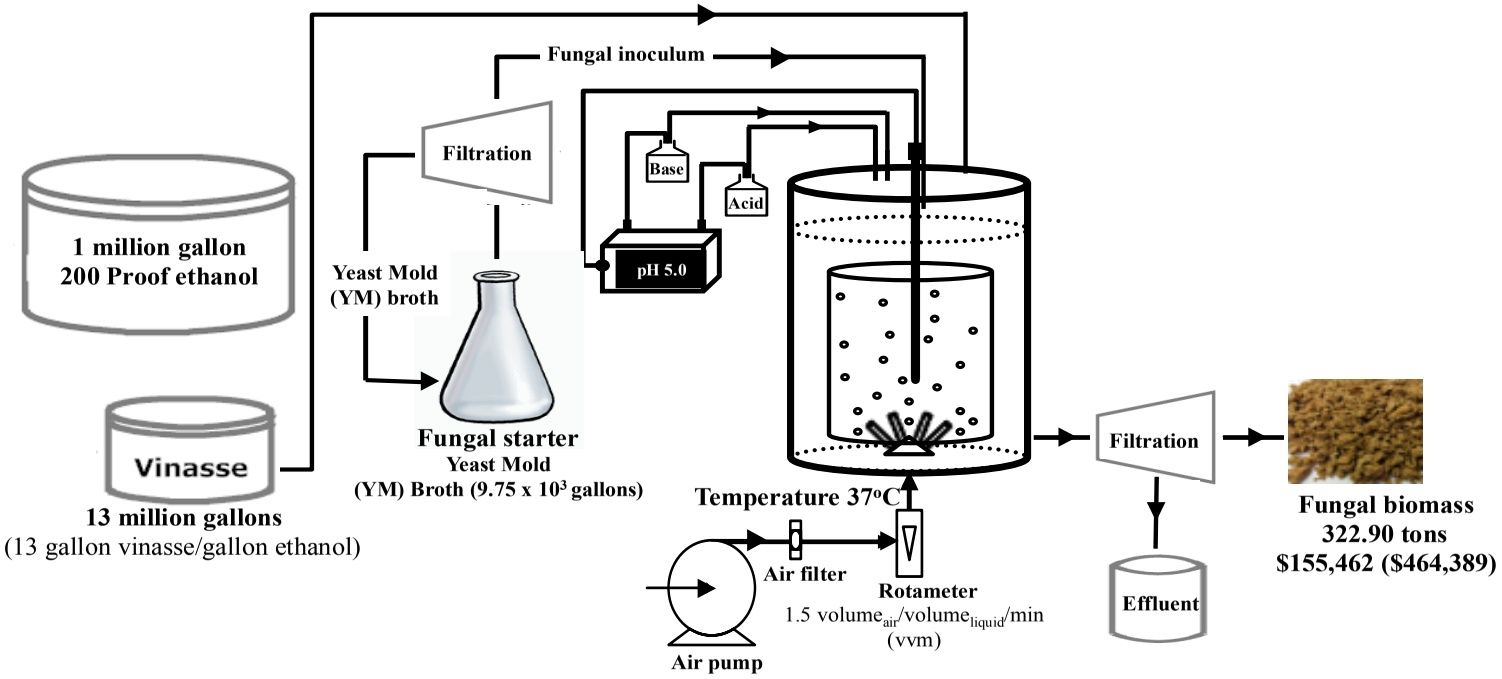


Figure 7.1 Schematic diagram showing inputs and outputs of fungal protein production in sugarcane-to-ethanol industry

CHAPTER 8: CONCLUSIONS

The rapid increase in bioethanol production has dramatically stimulated the search for value-added products from the residues/by-products. The characteristics of residues/by-products have several inherent merits such as their availability at nearly no cost, rich in organic contents and macro/micro nutrients, and their amenability to microbial process. This research evaluated the feasibility of sugar-to-ethanol-derived vinasse utilization for protein-rich fungal biomass production for animal feed applications.

Based on this research, the following conclusions can be drawn.

1. The optimization studies using shaker flask suggested the use of organic content in vinasse to support the growth of *R. oligosporus* with concurrent waste remediation. The studies showed prolific fungal growth at a pH of 5.0 and a temperature of 30°C (for tempeh starter culture) on vinasse with nutrients (nitrogen and phosphorus) supplementation. The molasses-vinasse resulted in the highest fungal biomass yield of 1.97 ($g_{biomass\ increase}/g_{initial\ biomass}$).

2. *R. oligosporus* grew exponentially during the first 24 h using reducing sugar as a major carbon source to support its growth. Reducing sugar reduced to non-detectable level thereby resulting in the slower growth rate after 24 h and the fungal biomass concentration reached a plateau by 60 h of cultivation. The organic reduction was, however, not directly correlated to fungal growth as it continued to decrease until the end of cultivation.

3. Fungal spore inoculum size significantly affected the fungal mycelial morphology which in turn impacted the fungal biomass production in a submerged fermentation experiments. When the medium was inoculated with spores, agglomeration of fungal biomass was observed at high spore inoculum

level (1.0% and 2.0%), while pellets of increasing size were formed as the inoculum level was reduced to 0.5% and 0.2%. Based on this study, the optimal spore inoculum was found to be 0.5% (v/v) which provided pellets of smaller sizes.

4. Ethanol at a concentration higher than 5.0% (v/v) adversely affected fungal mycelial morphology. At this concentration, a transformation of fungal pellets into agglomerated biomass was observed thereby resulting in significant reduction in the fungal biomass production.

5. Airlift bioreactor studies suggested the feasibility of fungal biomass production on sugarcane vinasse. The fungal biomass yield was found to be dependent on aeration rate and the optimal aeration rate for fungal protein production was 1.5 vvm that resulted in the highest fungal biomass yield of 8.04 ($g_{biomass\ increase}/g_{initial\ biomass}$). Significant organic reduction of nearly 80% as SCOD was achieved independent on aeration rate. Thus, the fungal-treated effluent can be land applied, which will have minimal impact on the environment. The organic reduction independent on aeration rates further suggested the growth of other undesirable microbes during fermentation, which was later identified as yeast present in the vinasse.

6. The fungal fermentation in an airlift bioreactor suffered significantly due to agglomeration of fungal biomass in the opening between the annulus section of the draft tube and the reactor wall. Fungal pellets agglomeration resulted in the formation of large clumps of fungal biomass formation, which created a microenvironment within the clumps and might have resulted nutrient deficient conditions within the cells. Fungal cultivation in bubble column bioreactor could overcome the issue. With respect to fungal fermentation in an airlift bioreactor, the use of bubble column bioreactor resulted in the higher fungal biomass yield at all aeration rates except at 2.0 vvm in which the air velocity was beyond the critical limit and thus resulted in fungal mycelia growth instead

of pellets.

7. The vinasse-derived fungal biomass could potentially serve as a protein-rich ingredient for animal feeds since the crude protein content (~ 50% dry weight) and its digestibility (~ 84%) were comparable to that of commercial protein sources. The high-value fungal biomass product contained high percentages of lysine, which is a rate-limiting amino acid in aquatic feeds. Moreover, fungal biomass contained essential fatty acids such as linoleic acid, linolenic acid, EPA, and DHA which could benefit the use of fungal biomass as aquatic feeds.

8. Co-feeding the fungal protein with commercial protein source would address the low methionine and phenylalanine content present in the fungal biomass and reduce the overall cost of protein ingredient for feeds.

9. Fungal fermentation technology is promising for sugarcane-to-ethanol industries as illustrated by fungal biomass production on commercial sugarcane-ethanol derived vinasse (Brazil vinasse). Successful application of fungal protein production would provide a unique sustainable solution to the existing sugar-based ethanol plants. The integration of innovative fungal technology into a sugar-based ethanol plant would not only provide an opportunity for producing food-grade fungal protein for animal feed applications, but it also provides an opportunity for water reclamation.

CHAPTER 9: FUTURE RESEARCH

This research investigated the utilization of low-value sugarcane-to-ethanol residue, vinasse, to produce a high-value fungal biomass. Effort has been made to investigate as many aspects as possible regarding fungal fermentation process optimization. Further studies on fungal fermentation should examine the following aspects:

- Many parameters have been reported to impact the formation of fungal mycelial morphology in submerged fermentation. Some of the parameters include DO, pH, medium composition, and agitation intensity. The change in fungal morphology significantly affects the fungal metabolic activity resulting in different types and amount of products formation. Therefore, for maximizing the quantity of fungal biomass production and the quality of the fungal biomass product, a detailed study on mycelial morphology change associated with the growth of fungus needs to be further investigated.

- The improvement in gas solubility (DO) in the liquid phase through better air diffuser and bioreactor design could enhance the fungal growth thus increase fungal biomass production and lower the production cost.

- Further investigation on the application of fungal biomass as an animal feed ingredient is important. Developing fungal-based diet formulations and conducting feeding trials on animal would provide an insight into potential applicability of the fungal biomass product as animal feed ingredients.

- Fungal fermentation offers a sustainable solution for sugarcane-to-ethanol plants. However, economic viability is a crucial factor to justify its commercialization. A techno-economic analysis of the fungal biomass production on sugarcane-derived vinasse needs to be further investigated.

APPENDIX A: STATISTICAL ANALYSES OF FUNGAL GROWTH OPTIMIZATIONS STUDIES

Fungal biomass yields and organic content removal (as percentages of SCOD removal) were statistically analyzed using a one-way analysis of variance (ANOVA) followed by DUNCAN's comparison at 95% confidence level. The mean values of sample size (*n*) of six were expressed in tables.

Concentration Optimization

Rum-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	1.976	4	.494	618.813	.000
	Within groups	.020	25	.001		
	Total	1.996	29			
SCOD removal	Between groups	6506.882	4	1626.720	51.443	.000
	Within groups	790.549	25	31.622		
	Total	7297.431	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05				
		1	2	3	4	5
10	6	.2607				
100	6		.3400			
75	6			.4095		
50	6				.7814	
25	6					.9087
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05				
		1	2	3	4	5
100	6	17.5361				
75	6		30.1091			
50	6			39.7538		
25	6				47.7797	
10	6					60.6150
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Molasses-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	1.072	4	.268	124.651	.000
	Within groups	.054	25	.002		
	Total	1.125	29			
SCOD removal	Between groups	7391.716	4	1847.929	149.775	.000
	Within groups	308.452	25	12.338		
	Total	7700.168	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05				
		1	2	3	4	5
10	6	.0924				
25	6		.3100			
50	6			.4923		
75	6				.5488	
100	6					.6143
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05		
		1	2	3
100	6	21.8679		
75	6	23.9492		
10	6		35.3434	
50	6			55.8912
25	6			59.3376
Sig.		.315	1.000	.102

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Cane-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	1.302	4	.325	2244.251	.000
	Within groups	.004	25	.000		
	Total	1.305	29			
SCOD removal	Between groups	3349.507	4	837.377	95.705	.000
	Within groups	218.738	25	8.750		
	Total	3568.246	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05				
		1	2	3	4	5
10	6	.0449				
25	6		.3634			
50	6			.4835		
75	6				.5959	
100	6					.6150
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Concentration (% (v/v))	N	Subset for alpha = 0.05	
		1	2
100	6	42.1650	
75	6	43.3729	
50	6	44.3548	
25	6		63.5796
10	6		65.9509
Sig.		.237	.177

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

pH Optimization

Rum-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	1.139	4	.285	204.029	.000
	Within groups	.035	25	.001		
	Total	1.173	29			
SCOD removal	Between groups	515.439	4	128.860	6.555	.001
	Within groups	491.473	25	19.659		
	Total	1006.912	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

pH	N	Subset for alpha = 0.05		
		1	2	3
4.5	6	.4533		
5.5	6	.4778		
4.0	6	.4814		
Control	6		.7984	
5.0	6			.9215
Sig.		.230	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

pH	N	Subset for alpha = 0.05	
		1	2
4.0	6	46.8327	
5.5	6	49.0366	
Control	6		54.6894
4.5	6		54.6917
5.0	6		58.1897
Sig.		.397	.208

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Molasses-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.444	4	.111	79.699	.000
	Within groups	.035	25	.001		
	Total	.479	29			
SCOD removal	Between groups	38.924	4	9.731	1.322	.289
	Within groups	183.958	25	7.358		
	Total	222.882	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

pH	N	Subset for alpha = 0.05		
		1	2	3
Control	6	.2763		
4.0	6		.4647	
4.5	6		.4658	
5.5	6		.4790	
5.0	6			.6608
Sig.		1.000	.539	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

pH	N	Subset for alpha = 0.05
		1
Control	6	20.0174
5.5	6	21.6027
5.0	6	22.3213
4.5	6	22.8077
4.0	6	23.2819
Sig.		.072

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Cane-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.487	4	.122	57.483	.000
	Within groups	.053	25	.002		
	Total	.540	29			
SCOD removal	Between groups	190.095	4	47.524	1.330	.286
	Within groups	893.194	25	35.728		
	Total	1083.289	29			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

pH	N	Subset for alpha = 0.05			
		1	2	3	4
Control	6	.2476			
4.0	6		.3430		
4.5	6		.3494		
5.5	6			.4956	
5.0	6				.6081
Sig.		1.000	.810	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

pH	N	Subset for alpha = 0.05
		1
4.0	6	44.3665
4.5	6	45.3967
Control	6	46.8252
5.5	6	48.6165
5.0	6	51.4837
Sig.		.075

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Effect of Nutrient Supplementation

Rum-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.741	3	.247	351.921	.000
	Within groups	.014	20	.001		
	Total	.755	23			
SCOD removal	Between groups	18.071	3	6.024	.503	.684
	Within groups	239.450	20	11.973		
	Total	257.521	23			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05			
		1	2	3	4
Control	6	.8732			
Nitrogen	6		.9197		
Phosphorus	6			1.0650	
Nitrogen and phosphorus	6				1.3240
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05
		1
Control	6	59.3011
Phosphorus	6	60.2681
Nitrogen and phosphorus	6	60.3121
Nitrogen	6	61.7338
Sig.		.278

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Molasses-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	5.672	3	1.891	253.850	.000
	Within groups	.149	20	.007		
	Total	5.821	23			
SCOD removal	Between groups	9.906	3	3.302	.322	.809
	Within groups	205.168	20	10.258		
	Total	215.074	23			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05			
		1	2	3	4
Control	6	.6144			
Nitrogen	6		1.1308		
Phosphorus	6			1.3054	
Nitrogen and phosphorus	6				1.9740
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05
		1
Nitrogen and phosphorus	6	20.6128
Control	6	21.5252
Nitrogen	6	22.0492
Phosphorus	6	22.2860
Sig.		.418

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Cane-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.959	3	.320	167.854	.000
	Within groups	.038	20	.002		
	Total	.997	23			
SCOD removal	Between groups	54.664	3	18.221	1.443	.260
	Within groups	252.571	20	12.629		
	Total	307.235	23			

Fungal biomass yields ($g_{biomass\ increase}/g_{initial\ biomass}$)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05			
		1	2	3	4
Control	6	.6040			
Nitrogen	6		.6801		
Phosphorus	6			.7472	
Nitrogen and phosphorus	6				1.1238
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

SCOD removal (%)

Duncan[a]

Nutrient supplementation	N	Subset for alpha = 0.05
		1
Control	6	47.1884
Phosphorus	6	48.5887
Nitrogen	6	50.5133
Nitrogen and phosphorus	6	50.9352
Sig.		.108

Means for groups in homogeneous subsets are displayed.

[a]Uses Harmonic Mean Sample Size = 6.000.

Effect of Vinasse Sterilization

Rum-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.000	1	.000	.290	.602
	Within groups	.006	10	.001		
	Total	.006	11			
SCOD removal	Between groups	1.673	1	1.673	.785	.396
	Within groups	21.312	10	2.131		
	Total	22.985	11			

Molasses-vinasse

ANOVA

		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	6.867	1	6.867	3147.623	.000
	Within groups	.022	10	.002		
	Total	6.889	11			
SCOD removal	Between groups	167.376	1	167.376	60.100	.000
	Within groups	27.850	10	2.785		
	Total	195.226	11			

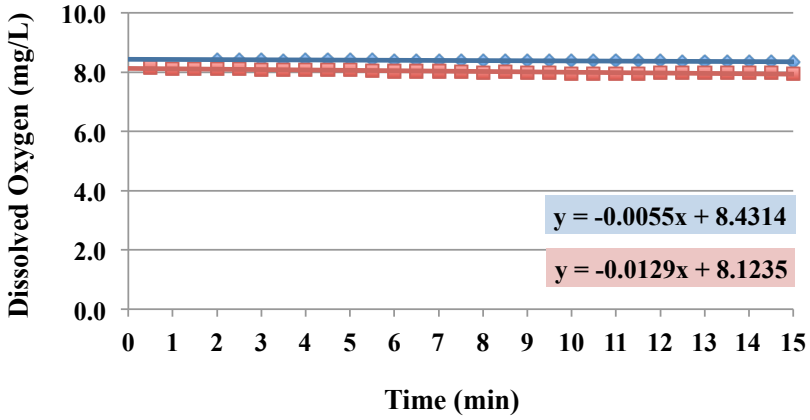
Cane-vinasse

ANOVA

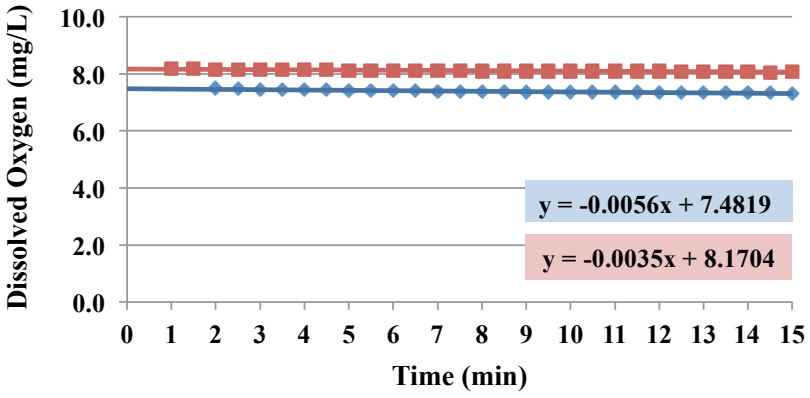
		Sum of squares	df	Mean square	F	Sig.
Fungal biomass yields	Between groups	.002	1	.002	.763	.403
	Within groups	.026	10	.003		
	Total	.028	11			
SCOD removal	Between groups	10.806	1	10.806	.683	.428
	Within groups	158.114	10	15.811		
	Total	168.920	11			

APPENDIX B: SPECIFIC OXYGEN UPTAKE RATE (SOUR) DURING FERMENTATION WITHOUT FUNGAL INOCULATION AT AN AERATION RATE OF 0.5 VVM

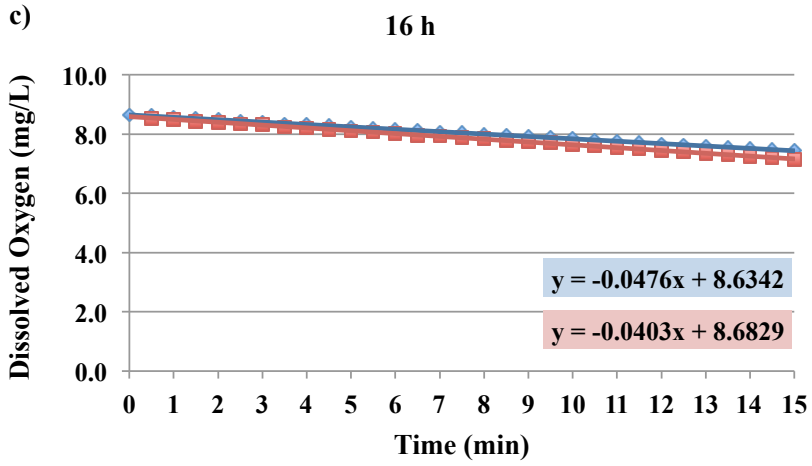
a)



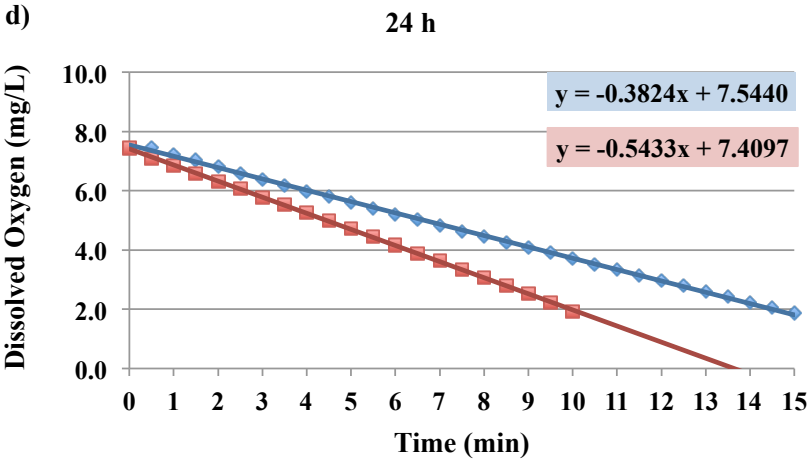
b)



c)


16 h
Dissolved Oxygen (mg/L)
10.0
8.0
6.0
4.0
2.0
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)
y = -0.0476x + 8.6342
y = -0.0403x + 8.6829

d)


24 h
Dissolved Oxygen (mg/L)
10.0
8.0
6.0
4.0
2.0
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)
y = -0.3824x + 7.5440
y = -0.5433x + 7.4097

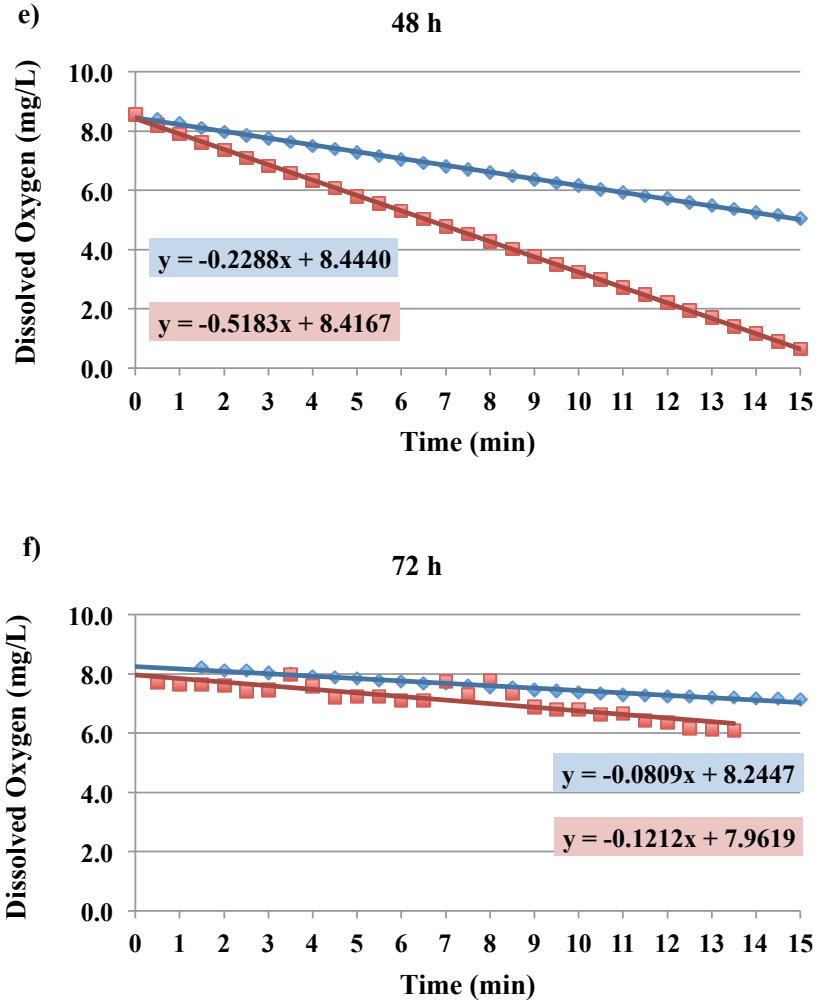


Figure B.1 Dissolved oxygen (DO) reduction of fermentation without fungal inoculation at an aeration rate of 0.5 vvm during various fermentation times: a) 0 h; b) 8 h; c) 16 h; d) 24 h; e) 48 h; and f) 72 h

Table B.1 Specific oxygen uptake rate (SOUR) of fermentation without fungal inoculation at an aeration rate of 0.5 vvm

Time (h)	Reading	Slope (mg O_2/L-min)	Volatile suspended solids (g/L)	Specific oxygen uptake rate (SOUR) (mg O_2/g VSS-hr)		
					Average	Standard deviation
	1	0.0055	2.854	0.116		
0	2	0.0129	3.480	0.222	0.17	0.08
	1	0.0035	0.298	0.705		
8	2	0.0056	0.298	1.128	0.92	0.30
	1	0.0479	0.323	8.898		
16	2	0.0403	0.323	7.486	8.19	1.00
	1	0.3824	0.846	27.121		
24	2	0.5433	1.064	30.637	28.88	2.49
	1	0.2288	0.605	22.691		
48	2	0.5183	1.528	20.352	21.52	1.65
	1	0.0809	1.252	3.877		
72	2	0.1212	1.950	3.729	3.80	0.10

APPENDIX C: ESSENTIAL AMINO ACIDS AND FATTY ACID PROFILES OF VINASSE-DERIVED FUNGAL BIOMASS

Table C.1 Essential amino acids composition of vinasse-derived fungal biomass

Essential amino acids	Values (% protein basis)
Arginine	5.86 ± 0.91
Histidine	3.12 ± 1.07
Isoleucine	5.35 ± 0.82
Leucine	7.99 ± 1.42
Lysine	8.33 ± 1.37
Methionine	1.84 ± 0.47
Phenylalanine	5.03 ± 0.89
Threonine	4.45 ± 0.40
Tryptophan	1.02 ± 0.02
Valine	5.26 ± 0.38

Mean value ± standard deviation (sample size (n) = 4)

Table C.2 Fatty acids composition of vinasse-derived fungal biomass

Fatty acids	Codes	Values (g/kg $_{DM}$)
Tetradecanoic (Myristic)	C14:0	0.29 ± 0.02
Pentadecanoic	C15:0	0.13 ± 0.01
Hexadecanoic (Palmitic)	C16:0	14.90 ± 5.12
Hexadecenoic (trans-Palmitilaidic)	C16:1n-9	-
Hexadecenoic (Palmitoleic)	C16:1n-7	0.40 ± 0.39
Hexadecadienoic	C16:2n-4	2.63 ± 0.85
Hexadecatrienoic	C16:3n-4	0.04 ± 0.01
Heptadecanoic	C17:0	0.13 ± 0.12
Octadecanoic (Stearic)	C18:0	9.68 ± 3.58
Oleic acid	C18:1n-9	23.68 ± 9.69
Octadecenoic	C18:1n-7	0.26 ± 0.12
Octadecadienoic (Linoleic)	C18:2n-6	13.51 ± 4.21
Octadecatrienoic (Gamma Linolenic)	C18:3n-6	0.05 ± 0.04
Octadecatrienoic	C18:3n-4	-
Linolenate (ALA)	C18:3n-3	3.15 ± 0.99
Octadecatetraenoic (Steradonic)	C18:4n-3	0.65 ± 0.52
Eicosanoic (Arachidic)	C20:0	0.47 ± 0.42

Table C.2 Fatty acids composition of vinasse-derived fungal biomass (continue)

Fatty acids	**Codes**	**Values (g/kg $_{DM}$)**
Eicosenoic	C20:1n-9	0.74 ± 0.12
Eicosatrienoic	C20:3n-3	0.10 ± 0.00
Eicosatetraenoic	C20:4n-6	0.04 ± 0.01
Eicosatetraenoic (Arachidonic)	C20:4n-3	0.05 ± 0.02
Eicosapentaenoate (EPA)	C20:5n-3	1.19 ± 0.27
Docosapentaenoate	C22:5n-6	0.03 ± 0.06
Docosapentanoic	C22:5n-3	0.06 ± 0.04
Docosahexaenoate (DHA)	C22:6n-3	1.09 ± 0.59
Lignocerate	C24:0	0.04 ± 0.03
Nervonate	C24:1	0.19 ± 0.36

Mean value ± standard deviation (sample size (n) = 4)

APPENDIX D: INTEGRATING FUNGAL FERMENTATION TECHNOLOGY INTO SUGAR-BASED ETHANOL FACILITY

Fungal biomass production was calculated based on laboratory yield of fungal biomass production on Brazil vinasse without dilution. The nutrient supplementation cost was not included as the insignificant difference on fungal biomass production on vinasse with and without nutrient supplementations were observed.

Table D.1 Calculation of an annual production of fungal biomass from 1 MGY (million gallon per year) ethanol facility

Fungal biomass production	
Fungal biomass concentration (g/L substrate)	4.910
Fungal biomass concentration (g/L vinasse)	6.138
Vinasse available for fungal biomass production from 1 million gallon ethanol production capacity (x10^6 L)	49.205
Fungal biomass production (dry ton (US))	332.895
Price comparison with soybean meal (minimum of $ 467 per dry ton (US))	**$155,462**
Price comparison with fishmeal (minimum of $ 1,395 per dry ton (US))	**$464,389**

The electricity cost for air supply in producing fungal biomass at 1.5 vvm was calculated based on the fungal cultivation in reactor of cylindrical geometry with a diameter of 3 m and a height of 8 m with 80% capacity (~ 45.24 m^3). Electricity cost was calculated based on commercial electricity cost of the US (Total) (10.55 cents per kWh).

Table D.2 Electricity cost estimation for air supply in fungal fermentation at 1.5 vvm

Operating cost with a consideration of air supply		
Ethanol production capacity (x10^6 gallons)		1
Vinasse co-generation (13 gal/gal ethanol) (x10^6 gallons)		13
Vinasse co-generation (x 10^3 m^3)		49.21
Vinasse available with 300 working day (m^3 per day)		164.02
With this reactor configuration, 4 reactors are required per day		
Vinasse volume (m^3 per reactor)		41.00
Blower capacity of 44 m^3/min, 14.4 kWh		
Aeration rate requirement (1.5 vvm)		61.51
Blower needed (per reactor)		2
Considering fungal cultivation in batch mode for 3 days with continuously operation		
	12 reactors	298 days
Reactor requirement	8 reactors	2 days
	4 reactors	2 days
	24 blowers	298 days
Blower requirement	16 blowers	2 days
	8 blowers	2 days
	8294.4	298 days
Power requirement (kWh per day)	5529.6	2 days
	2764.8	2 days
Power requirement (kWh)		2488320
Electricity prices (10.55 cents per kWh[1])		**$262,518**

[1] EIA (2012c)

APPENDIX E: PUBLICATIONS

Refereed Journals

Nitayavardhana, S., Issarapayup, K., Pavasant, P. Khanal, S.K., 2012. Fungal cultivation on vinasse in airlift bioreactor for potential large-scale protein-rich fungal biomass production for aquatic feed applications. Bioresource Technology, accepted.

Takara, D., Nitayavardhana, S., Munasinghe, P., K.C., S., Khanal, S.K., 2012. Sustainable bioenergy from biofuel-derived residues. Water Environmental Research 18, 1568–1585.

Nitayavardhana, S., Khanal, S.K., 2012. Biofuel residues/wastes: Ban or boon?. Critical Reviews in Environmental Science and Technology 42, 1–43.

Nitayavardhana, S., Khanal, S.K., 2011 Biodiesel-derived crude glycerol bioconversion to animal feed: A sustainable option for a biodiesel refinery. Bioresource Technology 102 (10), 5808–5814.

Takara, D., Nitayavardhana, S., Pinowska, A., Khanal, S.K., 2010. Sustainable bioenergy from biofuel residues and wastes, Water Environment Research 82, 1694–1719.

Nitayavardhana, S., Khanal, S.K., 2010. Innovative biorefinery concept for sugar-based ethanol industries: production of protein-rich fungal biomass on vinasse as an aquaculture feed ingredient. Bioresource Technology 101, 9078–9085.

Nitayavardhana, S., Shrestha, P., Rasmussen, M., Lamsal, B.P., van Leeuwen, J., Khanal, S. K., 2010. Ultrasound improved ethanol fermentation from cassava chips in cassava-based ethanol plants. Bioresource Technology 101, 2741–2747.

Book Chapters

Rasmussen, M., Shrestha, P., Nitayavardhana, S**.**, Khanal S.K., van Leeuwen, J., 2010. Value-added processing of residues from biofuel industries. Chapter 17, In: Khanal, S.K., et al., (Eds.), Biofuel and Bioenergy from Biowastes and Biomass, 389–410. The American Society of Civil Engineers, Reston, VA, USA.

Khanal, S.K., Takara, D**.,** Nitayavardhana, S., Lamsal, B.P., Shrestha, P., 2010. Ultrasound applications in biorenewables for enhanced bioenergy and biofuel production. Chapter 14, In: Sharma, S.K., Mudhoo, A., (EDs.), Green Chemistry for Environmental Sustainability, 303–313. CRC Press, Taylor & Francis Group, Boca Raton, FL, USA.

Attended Conferences

International

Nitayavardhana, S., Issarapayup, K., Pavasant, P., Khanal, S.K., 2012. Biofuel residues conversion into aquatic feed via fungal fermentation. International Conference on Challenges in Environmental Science and Engineering (CESE) 2012, Melbourne, Australia (Podium presentation).

National

Nitayavardhana, S., Khanal, S.K., 2012. Large-scale fungal protein production on vinasse in airlift bioreactor for aquatic feed applications. 24th Annual CTAHR Student Research Symposium, University of Hawai'i at Mānoa, Honolulu, HI, USA (Podium presentation).

Nitayavardhana, S., Devappa, R., Khanal, S.K., 2011. Bioconversion of biofuel residues into aquatic feed. Biofuels co-products workshop, Aquatic feed and nutrition department (AFN), Oceanic Institute, Waimanalo, HI, USA (Invited speaker).

Nitayavardhana, S., Khanal, S.K., 2011. Biodiesel-derived crude glycerol bioconversion to animal feed: A sustainable option for a biodiesel refinery. American Society of Agricultural and Biological Engineers (ASABE) 2011 Annual International Meeting, Louisville, KY, USA (Podium presentation).

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