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Attached growth fungal system for corn wet milling wastewater treatment

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Attached growth fungal system for corn wet milling wastewater treatment

by

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ABSTRACT

High organic strength food-processing wastewaters are typically treated with conventional aerobic systems such as an activated sludge process that produces substantial quantities of low value bacterial sludge. Treatment and disposal of bacterial sludge place a huge burden on wastewater plants. Industrial wastewaters with high organic content treatment are also often treated with bacterial processes. The research in this dissertation focuses on using fungi to treat food-processing wastewater to produce biomass that is a good source of valuable byproducts (e.g. enzymes, protein, and other bio-chemicals). The recovery of value added products derived from the fungal biomass could generate additional revenue for the industry. However, controlling bacterial domination is critical in non-aseptic fungal wastewater treatment. An attached growth fungal system was employed in this study to prevent the bacterial contamination by maintaining the high fungal density in the reactor. Plastic composite support (PCS) tubes, composed of 50% (w/w) polypropylene (PP) and 50% (w/w) agricultural products, were used as a support medium to grow *Rhizopus oligosporus* on corn wet milling wastewater. The effects of sterile operation, nutrient supplementation, support medium composition, pH, hydraulic retention time (HRT) and airflow rate on PCS biofilm continuous-reactor were evaluated.

The results proved that supplementation of nutrients (nitrogen and phosphorus) under aseptic conditions enhanced the chemical oxygen demand (COD) removal and biomass yield from 50% and 0.11 g(dry-weight)/$gCOD_{removed}$ to 55% and 0.16 g(dry-weight)/$gCOD_{removed}$, respectively. Under non-aseptic operation, total biomass production of 0.32 g volatile suspended solids (VSS)/$gCOD_{removed}$ was obtained with no significant improvement in COD removal (~53%), whereas with nutrient supplementation, COD removal improved significantly to 85% with a high biomass production of 0.56 gVSS/$gCOD_{removed}$. Significantly lower COD removals and biomass yields were observed in the control bioreactors with PP tubes alone and suspended

growth, which confirmed that the PCS medium with agricultural components was essential for better biofilm formation and organic removal.

COD removal and biomass yield were maximal at pH 4.0 with minimal bacterial competition. Highest COD removal of 78% was achieved at a 5 h HRT with a biomass yield of 0.44 $gVSS/gCOD_{removed}$. At 3.75 and 2.5 h HRT, the biomass yield increased to 0.45 and 0.48 $gVSS/gCOD_{removed}$ while COD removal reduced to 76 and 70%, respectively. An HRT of 5 h was most suitable for COD removal because of the longer contact time of wastewater with biomass. Maximum biomass yield was achieved at 2.5 h HRT due to higher substrate availability rate, but the biofilm was more sensitive to wastewater composition changes. Therefore, 3.75 h HRT was recommended as a compromise for bench-scale operation. Competitive bacterial growth was reduced with shorter HRTs. The shortest HRT of 1.25 h led to biomass wash out from the reactor. The wastewater composition proved to have significant effect on the biofilm reactor performance.

Supply of air at a rate of 1.0 $Lmin^{-1}$ (0.8 vvm) was found optimal. Increase in the airflow rates improved COD removal as well as biomass production. *In-situ* dissolved oxygen concentrations indicated an oxygen limiting condition in the reactor. Fungal biomass exhibited better settleability at higher airflows. Detailed study on hydrodynamic properties and mass transfer characteristics in a pilot scale reactor is warranted for better optimization of the aeration system.

The results of this study showed that an attached growth fungal treatment system with PCS medium was effective in treating nutrient supplemented corn wet milling wastewater with simultaneous recovery of high value fungal biomass and suppression of bacterial competition.

1. GENERAL INTRODUCTION

INTRODUCTION

Corn wet milling is a water intensive process that generates about 30 to 48 gallons of high strength wastewater per pound of corn processed (Foster, personal communication, 2005). Pretreatment or complete treatment prior to disposal is essential to meet the wastewater discharge regulations and therefore reduce the sewage surcharges. Conventional biological aerobic treatment of such wastewaters produces large amounts of low value bacterial biomass. The excess bacterial biomass requires additional treatment and disposal, contributing about 60% to the operating cost (Canales et al., 1994). Thus, it places a burden on food processing industries resulting in no other benefits than environmental protection.

On the other hand, fungi are often cultivated in industry as a source of high value byproducts, such as protein, enzymes, alcohols, etc., under aseptic conditions on relatively expensive substrates such as starch or molasses (Barbesgaard et al., 1992). The use of micro-fungi to treat high strength food processing wastewaters is emerging as an attractive option, as it converts the wastewater organics into high value fungal protein and bio-chemicals (Cooke, 1976; van der Westhuizen and Pretorius, 1998; van Leeuwen et al., 2003). Additionally, the high dewaterbility of fungal biomass and the filamentous nature of the fungi permit low cost operation and recovery of biomass (Jin et al., 1998, 1999, 2002; Stevens and Gregory, 1987). However, maintaining a pure fungal culture during wastewater treatment is often challenging, as bacteria originally contained in wastewater compete with fungi for organic substrate and proliferate during treatment. Thus, a suitable selective pressure is required to favor the growth of fungi while suppressing the bacteria. The growth of specific microorganisms can be favored by employing selective pressures based on the physiological and physical properties (such as mass, size, etc.) of microorganisms, and environmental conditions (such as operating pH, temperature, etc.).

Biofilms are a natural form of cell immobilization that results from microbial attachment to solid supports in submerged environments and cell immobilization is one way to enhance cell density in a system (Cotton et al., 2001; Ho et al., 1997a,b,c). The increased cell density of specific microorganism can suppress the growth of competitive microorganisms under mixed culture conditions. Because of their strong affinity to attach on organic or inorganic surfaces, an attached growth system was investigated in this study as a selection mechanism to maintain a dominant fungal culture under non-aseptic conditions. Plastic composite support (PCS) medium, developed at Iowa State University stimulates the biofilm formation (Demirci and Pometto, 1995). The PCS tubes are a high temperature extruded product composed of 50% polypropylene (PP) and 50% agricultural products. The initial phase of this study involved evaluating the organic removal efficiency and fungal biomass production potential of a PCS biofilm continuous-reactor using sterile and non-sterile corn processing wastewater, examining the effect of nutrient supplementation on bioreactor performance, and comparing its performance with that of PP biofilm and suspended growth continuous-reactors as controls.

Fungal selection pressure can further increase by controlling environmental factors, such as pH, temperature, hydraulic retention time, etc. Fungi are known for their ability to grow at low pH, which is a desirable property as it minimizes the bacterial contamination. The specific growth rate, initial microbial concentration in a bioreactor, and available substrate are the primary factors in determining the dominance of a specific microorganism in a continuous system (Harder and Kuenen, 1977). The PCS biofilm continuous-reactor provides the required initial higher fungal cell density and the specific growth rate is directly correlated to dilution rate or hydraulic retention time (HRT). The optimal pH and HRT for organic removal efficiency, fungal biomass/protein production and fungal dominance in a PCS biofilm continuous-reactor were evaluated during the second phase of the study.

Oxygen plays a vital role in biological treatment of wastewater, and because of its low solubility and consequent low transfer rate, a sufficient supply to meet the demand in biological reactors is one of the major limitations (Nicolella et al., 2000). Inadequate oxygen supply results in poor performance of biological systems, where as an excessive

supply adds-up unnecessary capital and operating cost. Optimization of aeration or oxygen supply rate is therefore critical, especially in the aerobic treatment of high organic strength wastewaters. Thus, a preliminary investigation on the effect of airflow rates was conducted in the third phase of the study.

THESIS ORGANIZATION

This dissertation comprises of six chapters that summarize the research and experimental work. The first chapter provides a general introduction to the study. Literature review on various aspects that affect the fungal wastewater treatment and attached growth systems is the topic of second chapter. Part of this will be included in a review paper prepared by Sindhuja Sanakaran for submission to the journal of *Critical Reviews in Environmental Science and Technology*.

The third chapter is a research paper titled "Fungal treatment of corn wet milling wastewater: Effect of reactor configurations and operating conditions," which presents the effect of reactor design (attached growth and suspended growth), attached growth support medium (PCS and PP), nutrient supplementation and operating conditions (aseptic and non-aseptic) on fungal treatment of corn wet milling wastewater. A paper titled "Attached growth fungal treatment of corn wet milling wastewater: Effect of pH and hydraulic retention time" is included as chapter four. This paper reports the effect of pH and HRT on the PCS fungal biofilm continuous-reactor efficiency in treating corn wet milling wastewater. Chapter five is a paper titled "Treatment of corn processing wastewater in an attached growth fungal system: Effect of gas composition and aeration rate" that focuses on optimization of airflow rate to PCS biofilm-reactor without compromising the system performance in terms of chemical oxygen demand (COD) removal and fungal biomass yield.

The above three research papers are prepared for a possible publication in *Biotechnology and Bioengineering* journal. The sixth and final chapter contains general conclusions followed by acknowledgements.

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2. LITERATURE REVIEW

BACKGROUND

As per 2002, there are 61-corn wet milling industries in the United States with a typical capacity of at least 2,500 tons/day (~77,700 bushels/day) and operating nearly for 365 days per year (Rausch, 2002). Corn wet milling plant operation involves separation of starch, protein, fiber and oil for producing valuable commercial co-products, such as corn gluten meal and feed. These plants also provide pure starch products (> 99.5%) for the paper and corrugating industries, modified starches for food ingredients and high fructose corn syrup. Wet milling is a water intensive process and generates about 30 to 48 gallons of high strength wastewater per pound of corn processed (James Foster, 7th June 2005; personal communication, ADM, Clinton, IA). Pretreatment or complete treatment prior to disposal is essential to meet the wastewater discharge regulations and therefore reduce the sewage surcharges.

Aerobic biological wastewater treatment, particularly the activated sludge process is widely adopted for treating municipal, commercial and industrial wastewaters worldwide. In the conventional activated sludge process, organic pollutants are mineralized to carbon dioxide and water with generation of excess bacterial mass known as waste activated sludge (WAS). The activated sludge process generates large quantity of sludge about 0.4 g volatile suspended solids (VSS)/g chemical oxygen demand (COD) (Metcalf and Eddy, 2003). Thus, over half of the removed COD is actually transformed into new bacterial cells.

Sludge processing, treatment and disposal are the major problems facing many environmental engineers and scientists (Weemaes and Verstraete, 1998). In fact, the costs associated with treatment and disposal of excess sludge accounts for up to 60% of the total wastewater treatment plant operating costs (Canales et al., 1994). Traditional sludge disposal options are experiencing several challenges; for example the regulatory restrictions on incineration, surface disposal, and land filling as stipulated in Title 40 of

the Code of Federal Regulations (CFR), Part 503 (Biosolids Rule), Title 40 of the CFR, Part 58 (Landfill Rule), and additional state's regulations have driven-up the costs of these disposal methods considerably (US EPA, 1993). The public outcry of incineration on human health and environmental impacts has made this disposal option even more costly and difficult to undertake lately (US EPA, 1999). Thus, the sludge handling places considerable burden on food processing industries resulting in no other benefits than environmental protection.

FUNGAL WASTEWATER TREATMENT

The ability of fungi to degrade organic matter was recognized by researchers during the late 1950s to mid of 1960s (Guest and Smith, 2002). Application of fungi to improve sludge characteristics and removal of polycyclic aromatic hydrocarbons (PAHs) has been extensively studied. *Aspergillus niger* and *Penicillium corylophilum* proved to have a potential to enhance the sludge settleability and dewaterability in a liquid state bioconversion process, while achieving the highest COD and turbidity removals of 94 and 99%, respectively, in filtrate of treated sludge (Alam and Fakhru'l-Razi, 2003a; Alam et al., 2001, 2003b, 2004; Mannan et al., 2005). Fungal ability to effectively degrade PAHs could offer an improved efficiency of decontaminating systems such as wetlands (Giraud et al., 2001).

Fungi are cultivated commercially as a source of protein and other high value bio-chemicals under aseptic conditions on relatively expensive substrates such as starch or molasses (Barbesgaard et al., 1992). The food processing wastewaters contain almost none of the hazardous and persistent compounds such as those regulated under the U.S. Environmental Protection Agency's (EPA's) Toxic Release Inventory (TRI) listing, and thus serve as an ideal substrate for fungal cultivation (US-AEP, 1997). The fungal treatment system converts the wastewater organics into highly dewaterable fungal biomass (van Leeuwen et al., 2002). The filamentous nature of fungi facilitates easy separation and recovery from the liquid phase, and also the obligatory acidophilic

property suggests that the fungi would not act as opportunistic pathogens (Nigam, 1994; Jin et al., 1999a,b).

Treatment of food processing wastewater by fungi gained much attention recently, due to their inherent ability to effectively degrade complex polymers such as cellulose, hemi-cellulose, and lignin materials (typical components of agro-based food processing wastewater) and produce high value fungal biomass (Cooke, 1976; van der Westhuizen and Pretorius, 1996, 1998; van Leeuwen et al., 2003; Jasti et al., 2005a,b,c). The highly dewaterable fungal biomass can be used as a source of protein and valuable bio-chemicals (Stevens and Gregory, 1987; Barbesgaard et al., 1992; Jin et al., 1998, 1990b,c, 2001a, 2002). The recovery of value added products derived from the fungal biomass could also generate additional revenue for the food processing industry.

FUNGI AS A PROTEIN SOURCE

The human population is growing rapidly, especially in the developing nations, and will continue to grow for another 50 years or so as depicted in Figure 2.1. This results not only in higher protein demand; but also in acute shortages of conventional protein sources, such as seafood, meat and agricultural products. The escalating prices of traditional (animal and plant) protein ingredients for animal feeds such as fish, meat and soybean meals have further intensified this problem in many countries. This predicament urgently demands for a research and technology development into non-conventional sources of protein, such as yeasts and fungi, to supplement the conventional ones.

Fungi have been known for centuries as a source of protein in specialty food (Ravinder et al., 2003). The myco-protein is of a much higher quality than bacterial protein and is expected to be a very valuable supplement in animal feeds and even human diets. *Aspergillus, Rhizopus, Mucor* and *Acitinomucor* species have long been utilized in oriental food fermentations (Wang et al., 1974). *Rhizopus oligosporus* was used for producing 'tempeh' from partially cooked soybeans, a favorite food and staple source of protein in Indonesia for several hundred years (Del Re et al., 2003).

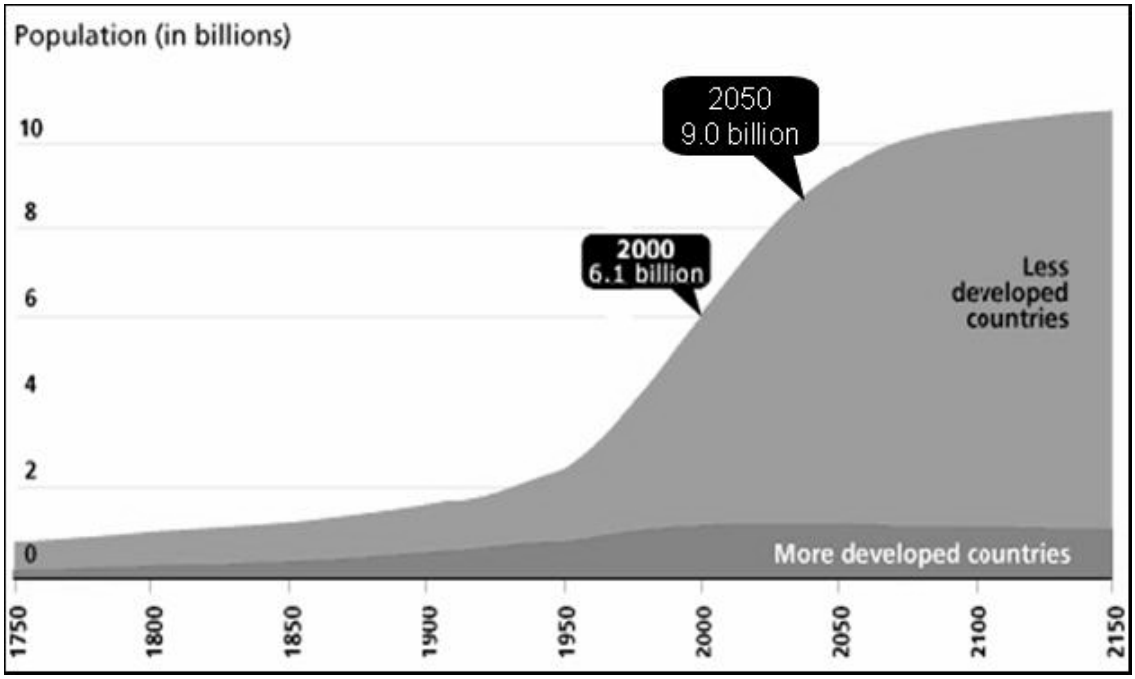


Figure 2.1 World population 1750 – 2150 (Source: United Nations, World Population Prospects, The 1998 Revision; and estimates by the Population Reference Bureau)

Protein of microbial origin is referred to as microbial biomass protein (MBP), if the microorganisms remain combined with the residual organics in the substrate, and as single cell protein (SCP), if the microorganisms are harvested and separated from the substrate. Recombinant strains of *Aspergillus oryzae* were recently found as a source of active human proteins, such as lactoferrin (Ward et al., 1992) and lysozyme (Tsuchiya et al., 1992).

FUNGAL BIOCONVERSION OF AGRO-BASED FOOD WASTES

Large quantities of wastewaters/wastes produced in food processing industries are rich in carbohydrates, lipids and proteins and thus could act as inexpensive substrates for biological conversion to more valuable products. The conversion of wastewater into

valuable by-products and the proper disposal of waste streams is currently a topic of great interest. Several studies have investigated different fungal species in a variety of food processing wastewaters for both pollutant removal and production of fungal protein and valuable enzymes.

Huang et al. (2003) investigated fungal biomass and lactic acid production by *Rhizopus arrhizus* during the treatment of potato processing wastewater. Maximum lactic acid production of 21 g/L with mycelial biomass of 1.7 g/L was obtained with suspended solids (SS) and COD removals of 100 and 90%, respectively, were reported.

Gongronella butleri and *Absidia atrospora* produced the highest level of chitosan (730 mg/L), while reducing 49% COD, 51% total sugar, 43% reducing sugar, 45% protein, 61% total nitrogen and 88% total phosphorus from sweet potato-*schochu* distillery wastewater during a cultivation period of 5 d (Yokoi et al., 1998).

Graham et al. (1976) observed the maximum yields of 1.45 and 1.33 g/L at 37°C and pH ranging from 3.0 to 5.0, respectively, on a synthetic medium for *R. oligosporus.*

Jin et al. (1999c) screened thirty strains of micro-fungi for MBP production and water reclamation from wheat starch processing wastewater. Results from the shake flask tests under non-aseptic conditions established that *A. oryzae, R. oligosporus* and *R. arrhizus* present high enzymatic activity.

When cultivated on wheat starch processing wastewater using a lab-scale external airlift bioreactor for 10 an 12 h, *A. oryzae* and *R. arrhizus* achieved significantly higher COD removal of 95% and biomass yields of 8.45 and 8.06 g/L of wastewater, respectively (Jin et al., 2001a).

A separate study by Jin et al., (1998) to assess the biomass protein and α-amylase production ability of *A. oryzae* from wheat starch processing wastewater resulted in slightly inferior biomass yield of 6.1 g/L with 38% protein with a α-amylase production of 55 EU/mL. Maximum COD, BOD and SS removals of 95, 93 and 98%, respectively, were documented. A pilot-scale external airlift bioreactor study on the same wastewater with *A. oryzae* and *R. oligosporus* demonstrated higher biomass production and protein of 7.5-9.2 g/L and 45%, respectively, while removing 95% of COD and 75% of nitrogen

and phosphorus (Jin et al., 2002). In a different study, treatment with *R. oligosporus* achieved a glucoamylase activity of ~ 4 U/mL during a cultivation period of 14h (Jin et al., 1999b).

Coriolus versicolor, Funalina trogii and *Pleurotus sajor-caju* (all white-rot fungi) showed a better removal potential by minimizing the total sugar, reducing sugar, color, phenolic compound and COD by 73, 77, 73, 93 and 70%, respectively. Higher biomass yields and laccase activities of about 0.95 g/50mL and 5.5 CU/mL were also observed.

The presence of recalcitrant organic compounds such as polyphenols with strong anti-microbial properties makes the treatment of olive mil wastewaters by conventional methods difficult. Fungi, as a means to minimize the toxicity of olive mill wastewaters and biotransformation into valuable products, gained attention of many researchers due its ability to produce enzymes that can degrade aromatic compounds. The excess nutrients in olive mill wastewater in combination with its acidic nature favor fungal growth.

Jaouani et al. (2003) reported a successful decolorization of olive oil mill wastewaters by *Pycnoporus coccineus.* High levels of stable laccase produced by *P. coccineus* at around pH 3.5 and 60°C were found responsible for the aromatic compound degradation and decolorization (Jaouani et al., 2005).

Robles et al. (2000) conducted a series of batch experiments on olive oil industry wastewater using seven strains of *Penicillium* isolated from wastewater disposal ponds. The authors found a maximum biomass yield, COD removal and phenol content reduction of 21.5 g(dry-weight)/$L_{wastewater}$, 39.5 and 45.5%, respectively, after 20 days of cultivation. Yesilada et al. (1999) experimented white and brown rot fungi in shake-flasks for biodegradation of autoclaved olive mill wastewater.

Table 2.1 summarizes the pollutant removals and resultant byproduct yields from fungal treatment of various food processing wastewaters.

Table 2.1 Fungal bioconversion of agro-based food wastewater pollutants into valuable by-products

Substrate	Experimental condition	Fungal species	Pollutant removal	Product yield	Reference
Potato wastewater	Non-aseptic; Shake flask culture	*Rhizopus arrhizus*	COD – 90% SS – 100%	Biomass – 21 g/L Lactic acid – 1.7 g/L	Huang et al., 2003
Sweet potato *schochu*-distillary wastewater	Non-aseptic; Shake test tube culture	*Gongronella butleri*	COD – 49% Total sugar – 51% Reducing sugar – 43% Potato protein – 45% Total nitrogen – 61% Total phosphorus – 88%	Biomass – 6.2 g/L Chitosan – 730 mg/L	Yokoi et al., 1998
Wheat starch processing wastewater	Non-aseptic; External air-lift bioreactor (Lab-scale, batch)	*Aspergillus oryzae* *Rhizopus arrhizus*	COD – 95% SS – 95%	Biomass: *A. oryzae* – 8.45 g/L *R. arrhizus* – 8.06 g/L Protein – 46 to 50%	Jin et al., 2001a
	Non-aseptic; Air-lift bioreactor (Lab-scale, batch)	*Aspergillus oryzae*	COD – 95% BOD – 93% SS – 98%	Biomass – 6.1 g/L Protein – 38% (w/w) α-amylase – 55 EU/mL	Jin et al., 1998
	Non-aseptic; External air-lift bioreactor (Pilot-scale, semi-continuous)	*Aspergillus oryzae* *Rhizopus oligosporus*	COD – 95% Nutrients (N & P) – 75%	Biomass – 7.5 to 9.2 g/L Protein – 45% (w/w)	Jin et al., 2002
	Non-aseptic; External air-lift bioreactor (Lab-scale, batch)	*Rhizopus oligosporus*	COD – 95% SS – 95%	Biomass – 4.5 to 5.2 g/L Protein – 46% (w/w) Glucoamylase ~ 4 U/mL	Jin et al., 1999b

Table 2.1 Fungal bioconversion of agro-based food wastewater pollutants into valuable by-products (continued)

Substrate	Experimental condition	Fungal strain	Pollutant removal	Product yield	Reference
Olive oil mill wastewater	Non-aseptic; Static flask culture – flushed with oxygen for 2 min every 72 h	*Pycnoporus coccineus*	COD – 58% Color – 47%	Biomass – 12.32 g/L Laccase – 48 U/L	Jaouani et al., 2003, 2005
	Aseptic; Shake flask culture	*Penicillium*	COD – 39.5% Phenol content – 45.5%	Biomass – 21.5 g/L	Robles et al., 2000
	Aseptic; Shake flask culture	White-rot fungi (*Coriolus versicolor, Funalina trogii* and *Pleurotus sajor-caju*)	COD – 20% Phenols – 93% Color – 73% Total sugar – 73% Reducing sugar – 77%	Biomass – 1.9 g/100mL Laccase – 5.5 CU/L	Yesilada et al., 1999
Corn wet milling wastewater	Non-aseptic, Continuous reactor with 100 µm micro-screen	*Rhizopus oligosporus*	COD – 80%	Biomass – 0.28 gVSS/gCOD	van Leeuwen et al., 2002, 2003
	Non-aseptic, Continuous stirred PCS* biofilm reactor	*Rhizopus oligosporus*	COD – 85%	Biomass – 0.56 gVSS/gCOD	Jasti et al., 2005a,b,c
	Aseptic, Continuous stirred PCS* biofilm reactor	*Rhizopus oligosporus*	COD – 55%	Biomass – 0.16 gdry-weight/gCOD	Jasti et al., 2005a,b

* PCS – Plastic composite supports – 50% polypropylene , 50% agricultural products

Khiyami et al. (2005) studied the detoxification of corn stover and cornstarch pyrolysis liquors by ligninolytic enzymes produced by *Phanerochaete chrysosporium* in shake flask cultures. Detoxification demonstrated by phenolic compounds reduction was confirmed by subsequent *Lactobacillus casei* growth and lactic acid production in treated liquors. Treatment of corn wet milling wastewater with *R. oligosporus* to produce fungal biomass as a source of valuable byproducts with concomitant organic removal was investigated by van Leeuwen et al. (2002, 2003). The continuous reactor employed with 100 μm micro-screen efficiently reduced 80-90% of COD by retaining larger fungi in the reactor. A consequent biomass yield of 0.28 $gVSS/gCOD_{removed}$ was reported.

USE OF RECOVERED FUNGAL BIOMASS FOR HEAVY METAL REMOVAL

Acute toxicity of heavy metals towards aquatic life and humans prompts for an essential removal from wastewaters. Traditionally, heavy metal ions from wastewaters are removed by chemical precipitation, ion exchange and reverse osmosis processes. Huge amounts of fungal biomass produced as a byproduct of wastewater treatment could be potentially used as a cost effective biosorbant for heavy metal removal from wastewaters.

Kapoor et al. (1999) studied removal of heavy metals by *A. niger* in shake flask cultures using a synthetic medium. Higher lead, cadmium and copper removal capacities were exhibited when the biomass was pretreated by boiling in 0.1N NaOH solution for 15 min. The removals were better that those obtained with granular activated carbon. However, live *A. niger* biomass was found to be more effective in nickel removal. The removal of cadmium from aqueous solutions by using the fungal biomass obtained from starch wastewater treatment was investigated by Yin et al. (1999). The fungal biomass was pretreated with heat and calcium solution. Batch experimental results showed that the removal capacities of the pretreated fungal biomass of *Rhizopus oryzae, R. oligosporus, A. oryzae* and *R. arrhizus* were up to 0.28, 0.35, 0.40 and 0.56 mmol Cd^{2+}/g(dry weight), respectively. Table 2.2 presents the heavy metal removal capacities of various fungi from metal ion aqueous solutions.

Table 2.2 Removal of heavy metals by fungal biomass

Reference	Pretreatment	Fungal strain	Metal removals		
Kapoor et al., 1999	Boiling in NaOH for 15 min	*Aspergillus niger*	Lead	7.24	mmol/g biomass
			Cadmium	3.43	
			Copper	2.66	
			Nickel	0.96	
Yin et al., 1999	Treatment in $Ca(NO_3)_2$ for 2 h followed by heating at 100°C for 24 h	*Rhizopus oryzae*	Cadmium	0.28	mg/g biomass
		Rhizopus oligosporus		0.35	
		Aspergillus oryzae		0.40	
		Rhizopus arrhizus		0.56	

OPTIMIZATION OF FUNGAL WASTEWATER TREATMENT

Several factors including the physiology and physical properties of fungi, environmental factors, and operational conditions influence the treatment efficiency of a fungal wastewater system. Controlling the growth influential factors (e.g. pH, temperature, nutrient availability) and bio-kinetic parameters (hydraulic retention time (HRT) and solids retention time (SRT)) could optimize the fungal bioconversion of organics. Most fungal wastewater treatment systems have been studied under aseptic conditions to prevent bacterial interference. Under non-aseptic operation, bacteria grow well in most wastewaters and compete with fungi for organic substrate. This could adversely affect the byproduct quality as well as system performance. The optimum fungal growth influential factors could be used as selectors to regulate bacterial competition and promote fungal growth in a fungal treatment system. The physiological properties of microorganisms form the basis for "primary" selection stage, while the physical properties can be used as an initial basis for a "secondary" selection stage (Pretorius, 1987). The selection pressure increases with number of selectors applied.

Nutrients

Availability of nutrients greatly impacts fungal growth and survival (Moore-Landecker, 1996). Carbon, nitrogen and phosphorus are the most important macronutrients (Smith and Berry, 1975) and inadequate nitrogen and phosphorus levels are common, especially in the treatment of food-processing wastewaters (Metcalf and Eddy, 2003). Nitrogen is essential to proteins, cell wall components, and nucleic acids during microbial growth (Ammary, 2004). It is stated that the ratio of COD:N:P in the wastewater to be treated should be approximately 100:5:1 for aerobic treatment (Metcalf and Eddy, 2003). An optimization of the wastewater in nutrients is crucial to obtain a good output of pollutants degradations (Coulibaly et al., 2003).

During aseptic submerged fermentations of *A. niger* in rice bran, Oshoma and Ikenebomeh (2005) reported improved dry biomass productions from 0.97 g/L to 2.03 and 1.95 g/L with glucose and nitrogen ($(NH_4)_2SO_4$) supplementation, respectively. Corresponding increase in crude protein from 0.43 to 0.67 g/L was obtained with both glucose and nutrient supplementations.

Miao (2005) observed acceleration in visible *R. oligosporus* growth from 2-15 d to 1 d with nitrogen and phosphorus supplementation at a rate of 150:10:1 (COD:N:P) during corn wet milling wastewater treatment. About 10-20% higher COD removals were achieved in a continuous reactor with micro-screen. Similar effect on fungal growth (30 to 20 h) was noticed in shake-flasks operated with heat-sterilized wastewater to cultivate startup culture for continuous non-aseptic operation. An addition of nitrogen and phosphorus to corn wet milling wastewater treated in aeration pond at a lower ratio of COD:N:P :: 100:5:1 dropped the effluent soluble COD from 400 m/L to an acceptable 100 mg/L (US EPA, 1978). The difference in the required COD:N:P ratio may be due to the fact that the wastewater used in the preceding two studies was collected from different plants and treated with different microorganisms. The wastewater composition differs from plant to plant because of their unique plant operations and thus leads to dissimilar nutrient requirements during the treatment.

By optimizing the olive mill wastewater composition (COD:N:S :: 100:5:2) with $(NH_4)_2SO_4$ addition for *Geotrichum candidum* growth, Assas et al. (2000) obtained a complete degradation of phenols and 70% decolorization. Miranda et al. (1996) maximized the color removal from molasses wastewaters (25 to 69%) with *A. niger* by adjusting the culture medium with co-substrate (sucrose – 10 g/L) and mineral nutrients ($MgSO_4$ – 0.5 g/L; KH_2PO_4 – 1.0 g/L; NH_4NO_3 – 1.8 g/L).

Only slight or insignificant improvement in COD removal, biomass yield and protein content has been reported with nutrient supplementation (N and P), as the starch wastewater treated with *R. oligosporus* (Jin et al., 1999b) and *A. oryzae* (Jin et al., 1998) was originally rich in nutrients. Within the slight improvement observed in protein content and COD removal with phosphorus addition (~5%), K_2HPO_4 was observed to be more effective than KH_2PO_4.

Wu et al. (2005) cultivated *Pleurotus ostreatus*, a white-rot fungus, on pulp mill wastewaters to explore the effect of nitrogen supplementation on its lignin degrading capacity. The highest removal COD and lignin efficiencies of 48 and 71%, respectively, were achieved with 0.2 g/L ammonium tartrate addition. The corresponding removal efficiencies without adding ammonium tartrate were 20 and 23%, respectively. The lignin and COD removal efficiencies decreased with an increase in ammonium tartrate concentration suggesting that a high concentration of nitrogen could possibly repress the production of lignin-degradation enzymes and result in a decrease in lignin degradation. This was supported by a previous study with *P. chrysosporium*, which showed that a low level of nitrogen could promote a high level of inhibition in lignin degradation (Kirk et al., 1978).

Maximum biomass yield of 0.61 gdry-weight and crude protein of 0.3 g per g carbohydrate supplied were documented from potato-processing wastewater treatment with *Cephalosporium eichhorniae* (Stevens and Gregory, 1987). Supply of mono-ammonium phosphate (0.506 g/L), ferric iron (0.1 g/L) and 0.2 N ammonium hydroxide (NH_4OH) were found essential for optimal treatment efficiency. NH_4OH was used to control pH and simultaneously supply nitrogen for biomass protein synthesis. However,

it was observed that fungi preferred to utilize inorganic nitrogen than organic nitrogen from potato protein. The authors suggested a pH control with sodium hydroxide until the depletion of organic nitrogen, followed by automatic titration with NH_4OH to reduce the nutrient supplementation requirement and improve the effluent quality as well.

Truong et al. (2004) utilized *A. oryzae* for the treatment of the cassava starch processing wastewater with high-suspended solids in non-aseptic shake-flask cultures. The effect of wastewater enrichment with various nitrogen supplements (sodium nitrate, ammonium sulfate, urea, ammonium nitrate, peptone) on fungal growth was examined. Enhanced biomass yield of 0.8 g/gCOD was achieved with 1 g/L peptone while the highest total organic carbon (TOC), COD and starch removals of 87, 91 and 94%, respectively, were observed with 1 g/L of ammonium sulfate, after 96 h cultivation. The authors hypothesized that the ability of peptone to serve as both nitrogen and carbon sources as the reason for higher biomass yield.

Santos et al. (2002) examined *Pleurotus* to treat the Kraft bleach plant effluent in a continuous turbulent-flow bioreactor. Improvement in color and lignin/chlorolignin removals from 13.0 and 7.9% to 19.4 and 19.5%, respectively, were noticed with 10 g/L glucose additions. Dilution of 50% was essential due to the observed toxic effect of concentrated effluent on fungal growth.

All the above results support the requirement of nutrient supplementation for optimal bioconversion of wastes, more particularly for food processing wastewaters with high concentrations of complex organics. However, discharge of excess nutrients from nutrient supplemented wastewaters is a major environmental concern. Large part of discharged nutrients is contained within the biomass and an effective removal of sludge from the treated wastewater is important (Slade et al., 2004). Addition of the adequate amount of nutrients by thorough understanding of the microbial growth kinetics and treatment process stoichiometric fundamentals is important for reducing the operating costs and excess discharge to receiving stream as well (Sherrard and Broderick, 1985). Nutrient contributions for the improvement in treatment efficiency and/or product yield on various substrates were demonstrated in Table 2.3.

Table 2.3 Effect of nutrient supplementation on pollutant removal and/or byproduct yield by fungi

Substrate	Fungal species	Added nutrients	Pollutant removal	Product yield	Reference
Filtered aqueous extract of ground rice bran	*Aspergillus niger*	None	NR	Biomass – 0.97 g/L Protein – 0.43 g/L	Oshoma and Ikenebomeh, 2005
		Glucose – 2.0 g/L	NR	Biomass – 2.93 g/L Protein – 0.67 g/L	
		$(NH_4)_2SO_4$ – 2.0 g/L	NR	Biomass – 1.95 g/L Protein – 0.67 g/L	
Olive oil mill wastewater	*Geotrichum candidum*	$(NH_4)_2SO_4$ COD:N:S :: 100:5:2	Maximum phenol – 100% Maximum color – 70%	NR	Assas et al., 2000
Molasses wastewater	*Aspergillus niger*	None	COD – 53% Color – 32%	NR	Miranda et al., 1996
		Sucrose – 10.0 g/L $MgSO_4$ – 0.5 g/L KH_2PO_4 – 1.0 g/L NH_4NO_3 – 1.8 g/L	COD – 75% Color – 69%		
Pulp mill wastewater	*Pleurotus ostreatus*	None	COD – 20% Lignin – 23%	NR	Wu et al., 2005
		Ammonium tartrate – 0.2 g/L	COD – 48% Lignin – 71%		

NR – Not Reported

Table 2.3 Effect of nutrient supplementation on pollutant removal and/or byproduct yield by fungi (Continued)

Substrate	Fungal species	Added nutrients	Pollutant removal	Product yield	Reference
Corn wet milling wastewater	*Rhizopus oligosporus*	$(NH_4)_2SO_4$, KH_2PO_4 COD:N:P :: 150:10:1	COD - 10 to 20% higher with nutrient addition	Biomass - Accelerated growth	Miao, 2005
Potato processing wastewater	*Cephalosporium eichhorniae*	*$NH_4H_2PO_4$ – 0.506 g/L* Fe^{3+} - 0.1 g/L NH_4OH – 0.2 N for N supply and pH control	NR	Maximum biomass – 0.62 g dry-weight/g carbohydrate Maximum crude protein – 0.3 g/g carbohydrate	Stevens and Gregory, 1987
Cassava starch processing wastewater	*Aspergillus oryzae*	None	TOC – 77% COD – 84% Starch – 88%	Biomass – 0.66 g/gCOD	Truong et al., 2004
		Peptone	TOC – 85% COD – 86% Starch – 90%	Biomass – 0.81 g/gCOD	
		$(NH_4)_2SO_4$	TOC – 87% COD – 91% Starch – 94%	Biomass – 0.74 g/gCOD	
Kraft bleach plant effluent	*Pleurotus ostreatoroseus*	None	Color –13.0% Lignin/chlorolignin – 7.9%	NR	Santos et al., 2002
		Glucose – 10 g/L	Color – 19.4% Lignin/chlorolignin – 19.5%		

NR – Not Reported

Growth pH

The pH of wastewater affects the nutrient/metallic ion availability, cell permeability and enzymatic activity (Cooke and Whipps, 1993). Hence, the microbial growth is very sensitive to variation in pH within growth medium (Rafiqul et al., 2005; Riscaldati et al., 2000). Fungi are known for their ability to grow at low pH (mostly ≤ 5.0), which is a desirable property as it not only eliminates the need to increase the pH of many acidic food-processing wastewaters during treatment, but also minimizes the bacterial contamination (Oshoma and Ikenebomeh, 2005). The optimum growth pH is different for each microorganism, even on species and strain level, and therefore could be used as an effective environmental selector.

Literature reports that the variation in pH can affect the fungal morphology significantly (Jin et al., 1999a; Metz and Kossen, 1977; Truong et al., 2004). Fungi exhibit different morphological forms, ranging from dispersed mycelial filaments to intricately interwoven mycelial masses referred to as pellets (Finkelstein and Ball, 1992). Pellet or clumped mycelia forms are considered advantageous compared to dispersed mycelia in industrial processing for various reasons such as better oxygen and nutrient transfer, enzyme production and easier biomass harvesting.

R. oligosporus grown on wheat starch processing wastewater exhibited significant COD removals in a pH range of 3.5 to 5.0 with a maximum removal at 4.0. The activity of glucoamylase produced by fungi was stable between pH 3.0 and 9.0 for 60 min. The optimal stability was achieved at pH 4.5 with an acute drop at pH below 3.0 and above 9.0 (Jin et al., 1999b). Relatively narrow range of optimal growth pH (4.0 to 4.5) was reported as by van Leeuwen et al. (2002) for cultivation on corn wet milling wastewater.

Jin et al. (1999d) studied the effect of pH in a range from 3.0 to 6.0 on yields and morphology of three different species of *A. oryzae* in an airlift bioreactor treating wheat starch processing wastewater. The optimal growth pH for the cultivations of *A. oryzae* DAR 1679 and 3863 was between 4.5 and 5.5. A low growth pH range from 3.5 to 4.5 appeared to be favorable for the cultivation of *A. oryzae* DAR 3699. Pellet formation was noticed at a pH above 4.0 for *A. oryzae* DAR 3863, whereas it occurred in a low pH

range from 3.5 to 4.5 for *A. oryzae* DAR 3699. Contrarily, *A. oryzae* DAR 1679 never formed pellets within the tested pH range. The results of this study proved that the fungal yields and morphology could vary significantly with growth pH, even for different strains of same species. Later studies on *A. oryzae* DAR 3863 reported maximum COD removal of 92.8%, biomass yield of 5.7 gdry-biomass/$L_{wastewater}$, protein content of 38.7% and α-amylase production of 50 EU/mL at chosen optimal growth pH of 5.0 (Jin et al., 1998).

A pH range of 5.0 to 5.5 was found optimal for COD reduction, biomass production and bacterial elimination for the treatment of sugar-furfural effluent by *Aspergillus fumigatus* (van der Westhuizen and Pretorius, 1996). The results were in good agreement with the findings of van der Westhuizen and Pretorius (1998), who reported reduction in *A. fumigatus* yields from 0.44 to 0.38 g/gCOD and one-log increase in bacterial numbers with an increase in pH from 5.0 to 6.1.

Miranda et al. (1996) reported maximum color and COD removals of 64.6 and 75%, respectively, at pH 5.0 (in a trial pH range of 2.0 to 6.0) from molasses wastewaters with *A. niger*. An initial pH adjustment prior to biological treatment removed part of the color by melonoidin precipitation. Maximum melonoidin precipitation was observed at an initial pH of 2.0, but was not recommended as optimum because of an inferior overall color removal and large acid requirements in practical applications.

Three white-rot fungi, *P. chrysosporium, P. ostreatus* and S22, showed exceptional capacity for lignin degradation in an alkaline pH range of 9.0 to 11.0 in pulp mill wastewaters when grown on porous plastic medium (Wu et al., 2005). All three strains could develop a biofilm under alkaline conditions, which typically inhibit the biofilm formation. Over 80% of lignin and 65% of COD were removed from the wastewater medium in the optimum pH range.

Results from cultivation of *R. arrhizus* on potato wastewater indicated that the optimal microbial performance in terms of COD removal and lactic acid production could be achieved in an operating pH range of 4.0 to 7.0, but a control of pH around 5.0 was suggested by to obtain maximum production yield and low operational cost (Huang et al. 2003).

Riscaldati et al. (2000) studied effect of pH (1.85 to 2.9) on itaconate production by *Aspergillus terrus* from glucose-based medium. The differences between substrate consumption based yield coefficients for itaconic acid at pH 2.4 and 2.8 were found statistically insignificant. However, significantly higher biomass based yield coefficients at a pH of 2.8 and 400 $revmin^{-1}$ resulted in maximum itaconate production rate of 0.41 $gL^{-1}h^{-1}$, thus proving it as the optimal condition for treatment efficiency.

Growth pH ranges of 6.5 to 7.5 for *G. butleri* and 5.5 to 8.5 for *A. atrospora* produced higher chitosan levels from sweet potato-*schochu* distillery wastewater. Optimum pH was finalized as 5.0 for a cost-effective operation (Yokoi et al., 1998). During the cultivation of *A. oryzae* on cassava starch processing wastewater with high-suspended solids, superior system efficiency in terms fungal biomass production, and TOC and COD reduction was reported within an initial pH range of 4.0 to 5.0. The formation of pellets in the optimal pH range originated from the adherence of germinated spores to solid particles in the medium (Truong et al., 2004). The results were found in agreement with those documented by Jin et al. (1998, 1999d).

Germination of spores is a factor of importance when spores are used as inoculum for bioreactors. Similar to the growth of fungal mycelia, the spore germination is also sensitive to pH of the medium. Medwid and Grant (1984) studied germination of *R. oligosporus* sporangiospores on synthetic medium and determined the optimal pH range for germination as 3.6 to 4.6, with maximum germination occurring at pH 4.0.

The biosorption of metal ions by fungi is solution pH dependent. Kapoor et al. (1999) found an inhibition in biosorption of lead, cadmium, copper and nickel ions by pretreated *A. niger* at a solution pH of 3.0 and a subsequent sharp increase at a pH above 4.0. Similar observations have been reported by Yin et al. (1999) with an exception that *R. arrhizus* biosorption of cadmium ions was inhibited at pH of 4.0 and reached maximum at around pH of 5.0 and higher. This shows that pH could certainly hamper the functional abilities of fungi, but the condition at which it occurs may be different for each fungus. The optimal growth pH values for various fungi on food processing wastewater were shown in Table 2.4.

Table 2.4 Optimum growth pH for fungi on food processing wastewaters

Reference	Substrate	Fungal strain	Optimum pH		
			Parameter	Range	Value
Jin et al., 1999b	Wheat starch processing wastewater	*Rhizopus oligosporus*	COD removal	3.5 to 5.0	4.0
			Glucoamylase activity and stability	3.0 to 9.0	4.5
Jin et al., 1999d	Wheat starch processing wastewater	*Aspergillus oryzae* DAR 167	Biomass yield	4.5 to 5.5	5.0
		Aspergillus oryzae DAR 3863		4.5 to 5.5	5.0
		Aspergillus oryzae DAR 3699		3.5 to 4.5	4.0
Jin et al., 1998	Wheat starch processing wastewater	*Aspergillus oryzae* DAR 3863	COD removal Biomass yield/protein α-amylase production	4.5 to 5.5	5.0
van der Westhuizen and Pretorius, 1996	Sugar-furfural effluent	*Aspergillus fumigatus*	COD removal Biomass yield Bacterial elimination	5.0 to 5.5	5.0
Miranda et al., 1996	Molasses wastewater	*Aspergillus niger*	COD removal Color removal	–	5.0
Huang et al., 2003	Potato wastewater	*Rhizopus arrihzus*	COD removal Lactic acid production Biomass yield	4.0 to 7.0	5.0
Truong et al., 2004	Cassava starch processing wastewater	*Aspergillus oryzae*	COD removal TOC removal Biomass yield	4.0 to 5.0	–
van Leeuwen et al., 2002, 2003	Corn wet milling wastewater	*Rhizopus oligosporus*	COD removal Biomass yield	4.0 to 5.0	–
Wu et al., 2005	Pulp mill wastewaters	*Phanerochaete chrysosporium*	COD removal Lignin degradation	8.0 to 9.0	–
		Pleurotus ostreatus		9.0 to 11.0	–
		S22		9.0 to 11.0	10.0

Temperature

Temperature is an important environmental factor for growth, selection and survival of microorganisms. It influences the growth rate, metabolism, regulation mechanisms of the enzymatic reactions and cell permeability (Madigan et al., 1997). The optimal temperature is anticipated to produce more biomass and enzymes in pure or dominant cultures. The optimal growth temperature range is typically quite narrow, although most microorganisms can survive above or below the optimal range. Temperatures below the optimum significantly affect the microbial growth rate. In fact, the growth rates double with approximately every 10°C increase in temperature until the optimum temperature is reached (Metcalf and Eddy). Temperature is known to alter the structure and composition of cell cytoplasmic membranes, consequently modifying the nutrition absorption capacities of fungi. Similar to pH, the temperature for optimal growth varies for each microorganism and therefore could determine the dominant species in a mixed culture. Relatively fewer studies were conducted to study the effect of temperature on fungal treatment efficiency of food processing wastewaters.

Jin et al. (1998) studied effect of temperatures in a range form 25 to 45°C on wheat starch processing wastewater treatment by *A. oryzae*. The optimum performance in terms of biomass protein, COD reduction and α-amylase enzyme activity was achieved at growth temperatures that ranged from 30 to 37°C. Temperatures below 28°C and above 40°C adversely affected the treatment efficiency. The α-amylase enzyme activity increased with an increase in temperature from 25 to 37°C, but decreased thereafter. These results are in exact agreement with those obtained by growing *R. oligosporus* on the same substrate for glucoamylase production, with an exception that the best glucoamylase activity was observed in a narrow temperature range of 35 to 37°C (Jin et al. 1998). A temperature of 35°C was chosen optimum in both studies.

Van der Westhuizen and Pretorius (1996) reported that the yield coefficients of *A. fumigatus* were 0.39, 0.52, and 0.51 g/g COD at temperatures of 44, 47 and 50°C, respectively during sugar-furfural effluent treatment. Temperature of fresh sugar-furfural

effluent at 45°C and exergonic nature of biological oxidation were stated as the reasons for the selection of evaluated temperature range.

Germination of *R. oligosporus* sporangiospores on synthetic medium was scored after incubation for 6 h in a temperature range of 26 to 50°C and was most rapid at 42°C (Medwid and Grant, 1984). Table 2.5 presents the effect of temperature on fungal growth and/or enzymatic activity in various substrates.

Table 2.5 Effect of temperature on growth, substrate degradation, and/or enzymatic activity in fungal systems

Reference	Substrate	Fungal strain	Optimum temperature, °C		
			Parameter	Range	Value
Jin et al., 1999b	Wheat starch processing wastewater	*Rhizopus oligosporus*	COD removal	30 to 37	35
			Glucoamylase activity	35 to 37	35
Jin et al., 1998	Wheat starch processing wastewater	*Aspergillus oryzae*	COD removal α-amylase activity	30 to 37	35
van der Westhuizen and Pretorius, 1996	Sugar furfural effluent	*Aspergillus fumigatus*	Biomass yield	–	47
Huang et al., 2003	Potato wastewater	*Rhizopus arrhizus*	COD removal	–	30
			Biomass yield	–	22
			Lactic acid production	–	30
Wang et al., 1974	Aqueous wheat bran solutions	*Rhizopus oligosporus*	Acid protease production	–	25
		Mucor disperses		–	25
		Actinomucor elegans		–	20

R. arrhizus cultivation on potato wastewater at 30°C led to the best lactic acid concentration (~ 16gdm^{-3}) and COD reduction (~ 78%) after 48 h of cultivation. The biomass production, however, decreased from ~ 1.4 to 0.75 gdm^{-3} with a rise in temperature from 22 to 38°C. The lactic acid production was found sensitive to variation in temperature, especially during 36 h cultivation period (Huang et al., 2003).

Wang et al. (1974) investigated acid protease productions on wheat bran by fungi used in soybean food fermentation under aseptic conditions in shake-flask cultures. The optimal conditions for enzyme production of the three fungi grown on wheat bran were: *R. oligosporus*, 25°C for 3 to 4 days; *Mucor dispersus*, 25°C for 3 to 4 days; *Actinomucor elegans*, 20°C for 3 days. All fungi yielded greater amounts of enzyme at temperatures lower than their optimum growth temperatures.

Hydraulic Retention Time (HRT)

HRT is the amount of time during which the wastewater remains in the reactor. It determines the contact time of the biomass with wastewater organics and therefore is an important operating condition for microbial growth and organic removal in a biological reactor. As per Monod growth kinetics, the specific growth rate of fungi is an inverse of HRT (Finkelstein and Ball, 1992). When the HRT exceeds the maximum growth rate, the microorganisms are washed out of the system. The specific growth rate of fungi is typically lower than that of bacteria, which implies that fungi require relatively longer HRTs for optimal growth. Bioreactor operation at an optimal HRT for fungi could result in higher treatment efficiency for fungal systems, provided the bacterial proliferation that occurs at relatively lower HRTs (normally less than 3 h) is controlled using some selection pressure (Van der Westhuizen and Pretorius, 1998). Physical properties of microorganisms can be used as a dynamic selection tool to determine the dominant microorganism when two or more organisms are competing for substrate in a system.

Suspended Growth Reactors with Cross-flow Microscreen for Fungal Selection (Cell Recycle Reactor). Fungal selection in continuous systems based on the difference

in the fungal and bacterial cell sizes was first introduced by Pretorius and Lempert (1993). A cross-flow microscreen was used to retain larger fungal cells in the reactor while washing out the smaller bacterial cells. As a result, two different and independently controllable retention times are established in the reactor. The retention time of wastewater and bacteria that pass through the screen is nothing but HRT, whereas the retention time of the fungi that remain in the reactor is solids retention time (SRT). Harvesting the fungi at a specific rate regulates the SRT in this system. Schematics of a typical continuous reactor with cross-flow microscreen and cell recycle were presented in Figure 2.2.

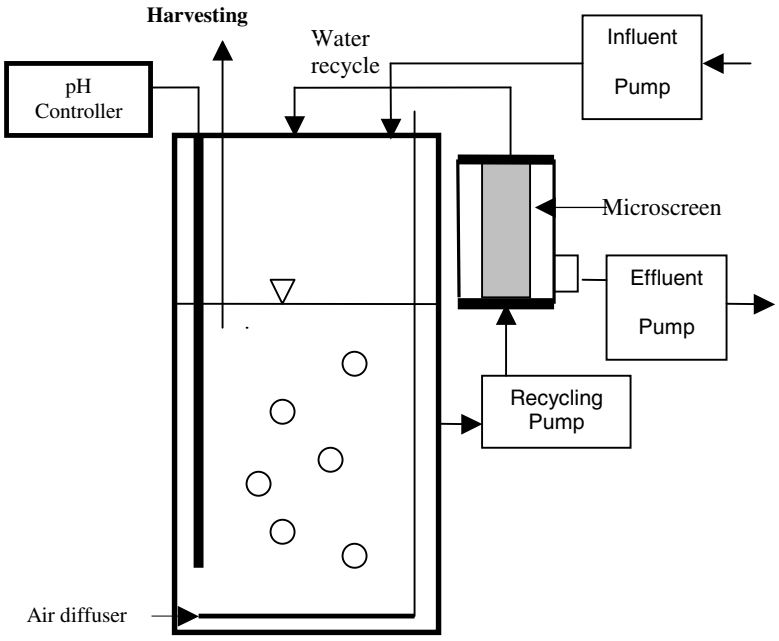


Figure 2.2 Schematics of continuous reactor with a cross-flow microscreen (van Leeuwen et al., 2002)

Van der Westhuizen and Pretorius (1998) employed a continuous reactor with cell recycle to treat non-sterilized sugar furfural effluent with *A. fumigatus*. Best bacterial elimination from the reactor was achieved at an HRT < 4 h and SRT of 10 h. In a similar reactor, van Leeuwen et al. (2002) observed complete fungal domination at above 99% of total biomass at an HRT < 6 h and SRT of 2 d while treating corn-processing wastewater with *R. oligosporus.* A microscreen of 100 µm pore size was employed.

Jin et al. (2001) evaluated an external airlift bioreactor system with 100 µm cross-flow micro-screen for treating wheat starch processing wastewater with *A. oryzae* and *R. arrhizus*. A significant reduction in the performance in terms of COD reduction and fungal biomass productivity was observed at HRTs less than 6.25 h.

Attached Growth Reactors for Fungal Selection (Biofilm Reactor). Biofilms are a natural form of cell immobilization that results from microbial attachment to solid supports in submerged environments and cell immobilization is one way to enhance cell density in a system (Ho et al., 1997a,b,c). The higher cell density of specific microorganism can suppress the growth of competitive microorganisms under mixed culture conditions.

The higher affinity of filamentous fungi to attach on organic or inorganic surfaces can be used as a factor for fungal selection. However, when grown simultaneously the faster growing bacteria could become the dominant species in a mixed culture of fungi and bacteria (Banks and Byers, 1991; Elvers et al., 1998). Therefore, an initial aseptic cultivation of pure fungi may be required to achieve fungal domination during non-aseptic attached growth wastewater treatment. Alternatively, high fungal inoculums to biological reactor may also lead to high fungal cell density. Additionally, the capability of attached growth systems to efficiently operate at smaller HRTs could result in bacterial washout from the reactor.

Attached growth systems were extensively studied and applied in wastewater treatment due to their obvious advantages, such as small reactor size and reduced sludge production. Several attached growth studies were also conducted under aseptic

conditions to enhance the yield of a microbial byproduct. Efficient degradation of complex organics by fungi is possible with the longer SRTs provided by biofilm systems. For example, immobilization of the *P. chrysosporium* mycelia was more effective in promoting cell growth and improving lignin peroxidase production to 8100 U/L for a hypersecreting strain from 75 U/L achieved in conventional stationary liquid culture with wild type (Bonnarme et al., 1993). *Saccharomyces cerevisiae* cultivation on molasses in a packed bed continuous bioreactor resulted in an ethanol productivity of 28.6 $gL^{-1}h^{-1}$ as compared to 3.35 $gL^{-1}h^{-1}$ in a free cell continuous process (Tyagi and Ghose, 1982).

Wu et al. (2005) used porous plastic support media to evaluate the lignin degradation capacities of white rot fungi from pulp mill wastewater. The porous plastic medium proved to be an effective medium by resulting in higher lignin degradations than those achieved by suspended growth systems. Even growth of fungi was observed in side the porous medium (Figure 2.3).

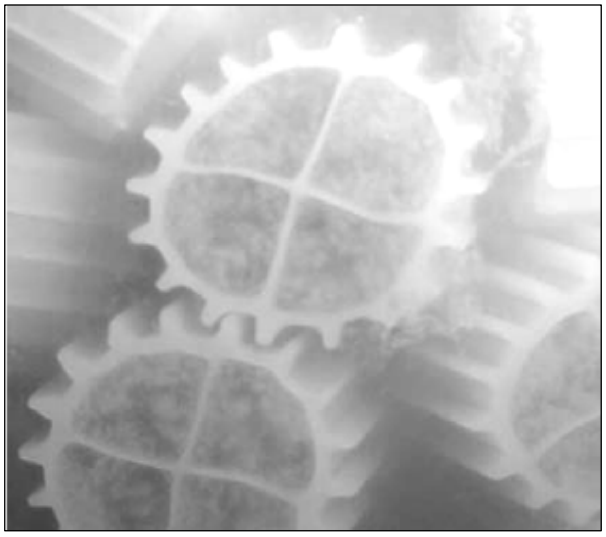


Figure 2.3 Attached growth of *Pleurotus ostreatus* inside porous plastic ring medium (Wu et al., 2005)

Khiyami et al. (2006) investigated the effect the culture conditions on ligninolytic enzyme production by *P. chrysosporium* on a synthetic medium in PCS biofilm stirred tank reactors. Maximum lignin peroxidase productions were achieved on day 6 with continuous aeration, 300 rpm agitation and 3 mM veratryl alcohol addition; where as the fastest productions were achieved with the addition of veratryl alcohol and $MnSO_4$ on day 0 with 300 rpm agitation and 0.005 vvm aeration.

Venkatadri and Irvine (1993) investigated the use of innovative biofilm systems to minimize intensive shear and provide for fungal growth. High levels of lignin peroxidase were produced when a hollow-fiber reactor and a stirred-tank reactor were modified into a unique silicone membrane reactor for the cultivation of *P. chrysosporium*.

ATTACHED GROWTH TREATMENT SYSTEMS: AN OVERVIEW

Attached growth systems are biological processes applied in waste neutralization, in which the microorganisms responsible for the conversion of organic matter in the wastewater are attached to some inert solid surfaces. The first known application of biofilm technology was industrial wastewater treatment by trickling filters in the early 1880s in Wales, Great Britain (Lazarova and Manem, 2000).

Advantages of Attached Growth Systems

The attached growth reactors separate HRT from SRT, which together with high biomass concentrations (up to 30 kg/m^3 compared with 3 kg/m^3 for activated sludge) result in high volumetric loading rates and short liquid residence times (van Loosdrecht and Heijnen, 1993). The attached growth systems offer following advantages over suspended growth activated sludge processes (Ødegaard et al., 1994; Loukidou and Zouboulis, 2001):

- Smaller reactor size due to the availability of biofilm with high specific surface area
- Less dependency of the operational performance on sludge separation in a clarifier

- No requirement for sludge return to the biological reactor
- Co-existence of aerobic and anoxic metabolic activity within the biofilm
- Lower sensitivity to adverse environmental conditions and shock loads

Limitations of Attached Growth Systems

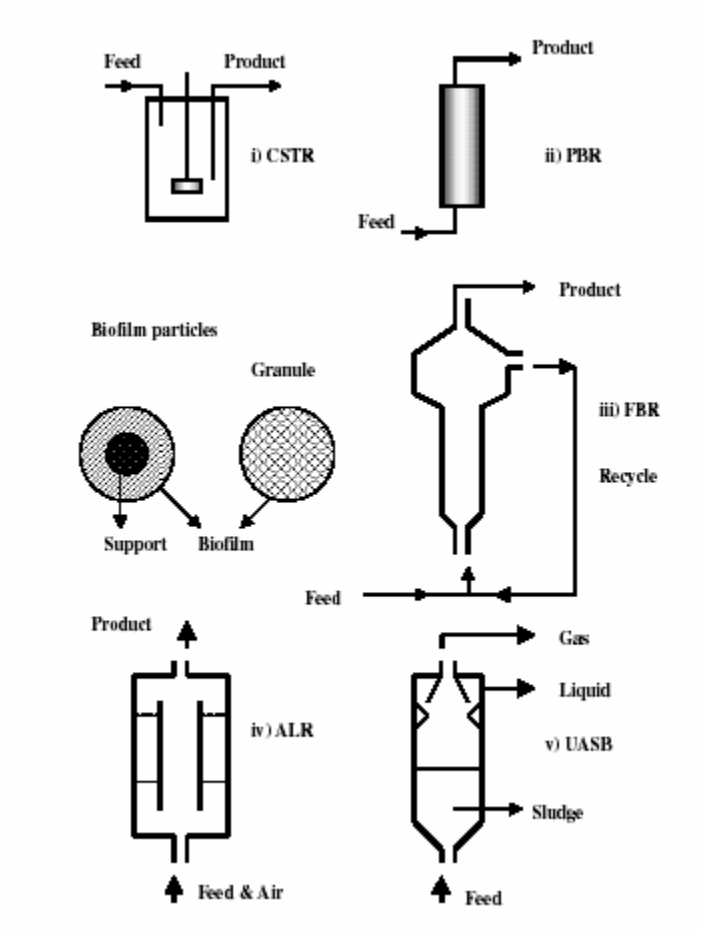
The excessive growth of biofilm is a frequent problem in conventionally aerated biofilm reactors used in wastewater treatment. Also, the performance of the biofilm processes is often diffusion-limited, which is especially important to determine the minimum required dissolved oxygen (DO) concentration level in the bulk liquid. The transport of substrates through a biofilm may become rate limiting, if the biofilm thickness is more than several organisms. The DO concentrations of 2 to 3 mg/L are generally considered satisfactory for activated sludge treatment systems. But such low concentrations can represent oxygen limiting conditions in attached growth system (Metcalf and Eddy, 2003).

Types of Attached Growth Reactors

Biofilm processes in wastewater treatment can be divided as: the fixed medium systems in which the biofilm media are fixed in the reactors and the biological reactions take place in the biofilm developed on fixed media, and the moving medium systems in which the biofilm media are kept continually moving by means of mechanical, hydraulic or air forces (Rodgers and Zhan, 2003). The moving medium systems include rotating biological contactors, moving-bed biofilm reactors, vertically moving biofilm reactors and fluidized bed reactors, while the fixed medium systems include trickling filters and biological aerated filters. The schematics of various types of attached growth reactors were shown in Figure 2.4.

Rotating Biological Contactor. Rotating biological contactors (RBC) consist of a series of discs of about 2-3 m diameter that are mounted on a horizontal shaft positioned above the liquid level (Gavrilescu and Macoveanu, 2000). The discs rotate at right angles

to the flow of wastewater and are only partially submerged in the wastewater. When rotated, the biofilm on the discs is exposed to atmosphere where oxygen is absorbed, and to the wastewater where soluble organic matter is consumed, alternately. Biomass is also sloughed off from the disc at a rate proportional to the thickness of the biofilm.



CSTR – Continuous Stirred Tank Reactor

PBR – Packed Bed Reactor

FBR – Fluidized Bed Reactor

UASB – Upflow Anaerobic Sludge Blanket Reactor

ALR – Air Lift Reactor

Figure 2.4 Schematic diagrams of various types of biofilm reactors and biofilm particles (Qureshi et al., 2005)

Trickling Filter. A trickle filter is a packed bed reactor (PBR) with an upward flow of air and a downward countercurrent flow of liquid waste (Gavrilescu and Macoveanu, 2000). This reactor has been used extensively for decades in wastewater treatment. The wastewater flows in a thin sheet over the packing material and the microorganisms that utilize the organics in the waste form a biofilm.

Fluidized Bed Reactor. In fluidized bed reactors (FBR), solid particles denser than wastewater are suspended in the column by an up flow stream of liquid. The biomass produced during the wastewater treatment attaches to the to the surface and solid particles, forming a biofilm (Gavrilescu and Macoveanu, 2000). The density of a solid particle covered with biofilm decreases with the increase in the biofilm thickness, which consequently leads the bed to expand. In comparison to the other reactors, this reactor is known to provide better mass transfer.

Continuous Stirred Tank Reactor. In a continuous stirred tank reactor (CSTR), wastewater entering the reactor is mixed throughout the tank and diffused into the biofilm along with the oxygen, where it is degraded. The immobilized biomass is maintained in suspension by continuous mixing with the impeller. Shear forces from mixing and attrition (particle to particle or particle to wall contact) control the biofilm thickness on an individual particle (Gavrilescu and Macoveanu, 2000).

A continuous stirred tank reactor with PCS tubes as support media for attached growth was developed at Iowa State University (Ho et al., 1997a,b,c). The PCS tubes, composed of polypropylene, ground soybean hulls, yeast extract and bovine albumin, are attached to reactor agitator shaft (Cotton et al., 2001). The available nutrients in the support medium enhances the biofilm formation. The porosity and roughness of the medium surface reduces the shear forces on biofilm and thereby improves its adhesion. The biofilm thickness is controlled by agitation. The detached biomass enters the mixed liquor surrounding the biofilm and leaves the reactor with effluent.

Air Lift Reactor. Airlift reactors (ALR) contain two concentric tubes, an inner riser and an outer downcomer. Mixing is provided in these reactors by circulating air at the bottom of the reactor. As a result of force applied by air at the bottom of the inner tube, the liquid in the inner tube moves up and overflows downward creating eddies to mix the liquid (Gavrilescu and Macoveanu, 2000). The downcomer is replaced with an external loop to circulate the wastewater in some reactors.

Upflow Anaerobic Sludge Blanket Reactor. As the name suggests, the upflow anaerobic sludge blanket (UASB) reactors are used for anaerobic treatment of wastewater and industrial effluents, and the flow in these reactors is in upward direction. Provisions are made for gases to escape at the top of the reactor and sludge particles to settle to the bottom part of the reactor. The treated effluent is removed from the top of the reactor (Gavrilescu and Macoveanu, 2000).

Biofilm Formation

The biofilm formation is largely dependent on the integration of physical, chemical and biological processes. Initially, some suspended cells are transported from liquid to a carrier surface by physical movement. Then, initial attractive forces (physical and chemical) retain the cells on the carrier surface and further promote stable multi-cellular contacts. The microbial force then makes the attached cells mature. Finally, the biofilms would be shaped by hydrodynamic shear force to form a certain structured community (Liu and Tay, 2001). The factors that influence the biofilm formation and stability are the microorganism involved, support media, chemical composition of wastewater and hydrodynamic conditions in the system (Coelhoso and Rodrigues, 1995; Melo and Vieira, 1999).

Cell Properties. The cell surface charge and presence of regions with hydrophobicity and/or hydrophilicity effects the biofilm formation. The physiological

properties of microorganism influence the both biofilm formation and detachment rates (Qureshi et al., 2005).

Hydrodynamics in the System. The liquid velocities on biofilm system and feed rates create shear forces that influence the biofilm formation as well as cell detachment. Larger feed rates result in serious erosion or sloughing of biofilms (Qureshi et al., 2005).

Support Medium Surface Properties. Microbes are typically attracted to the surfaces with opposite charge. The support media surface properties alter upon the biofilm formation. Shear forces are lower near a rough surface. Therefore, a rough surface with large pores provides an increased surface area for microbial attachment (Qureshi et al., 2005).

Fungal Biofilms

In contrast to the extensive literature describing bacterial biofilms, little attention has been paid to fungal biofilms despite the fact that fungi are excellent colonizers of surfaces (Jones, 1995). The growth of cells in a biofilm is often modeled by Monod type kinetics (Gavrilescu and Macoveanu, 2000). The fungal growth is also modeled by Monod microbial growth kinetics (Finkelstein and Ball, 1992). Hence, the effect of all the factors excluding those related to the physiological functions of microorganism may be similar for fungi and bacteria.

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3. FUNGAL TREATMENT OF CORN WET MILLING WASTEWATER: EFFECT OF REACTOR CONFIGURATIONS AND OPERATING CONDITIONS

(Manuscript will be submitted to '*Biotechnology and Bioengineering*' journal)

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Abstract: An attached growth fungal system with plastic composite support (PCS) medium was examined under both aseptic and non-aseptic conditions for the treatment of corn wet milling wastewater. PCS tubes, composed of 50% (w/w) polypropylene (PP) and 50% (w/w) agricultural products, were used as a support medium to grow fungal species - *Rhizopus oligosporus*. The results showed that supplementation of nutrients (e.g., mineral salts) under aseptic conditions enhanced the chemical oxygen demand (COD) removal and observed biomass yield from 50% and 0.11 g(dry-weight)/$gCOD_{removed}$ to 55% and 0.16 g(dry-weight)/$gCOD_{removed}$, respectively. Under non-aseptic operation, observed yield of 0.32 g volatile suspended solids (VSS)/$gCOD_{removed}$ was obtained with no significant improvement in COD removal (~53%), whereas with nutrient supplementation, COD removal improved significantly to 85% with a high observed yield of 0.56 gVSS/$gCOD_{removed}$. Significantly lower COD removals and biomass yields were observed in the control bioreactors with PP tubes

alone and suspended growth, which confirmed that the PCS medium with agricultural components was essential for better biofilm formation and organic removal.

Keywords: corn processing wastewater; *Rhizopus oligosporus*; attached growth; fungal wastewater treatment; plastic composite support (PCS) medium; biofilm

INTRODUCTION

Corn wet milling is a water intensive process and generates 30 to 48 gallons of high strength wastewater per pound of corn processed (James Foster, 7th June 2005; personal communication, ADM, Clinton, IA). Conventional aerobic biological wastewater treatment, particularly activated sludge processes produce large amounts of low value bacterial biomass. The excess bacterial biomass requires additional treatment and disposal, contributing 40 to 60% to the operating cost (Canales et al., 1994). Thus, it places a burden on corn processing industries resulting in no other benefits than wastewater treatment.

On the other hand, fungi are often cultivated in industry as a source of high value byproducts, such as protein, valuable bio-chemicals, etc., under aseptic conditions on relatively expensive substrates such as starch or molasses (Barbesgaard et al., 1992). The use of micro-fungi to treat high strength food-processing wastewaters is an attractive option since the fungal treatment system converts the wastewater organics into high-value fungal protein in a readily dewaterable biomass that can be used as a source of animal feed and potentially in human diets (Stevens and Gregory, 1987).

Fungi produce a wide range of fine bio-chemicals and enzymes, and are usually more effective in metabolizing complex carbohydrates such as starch than bacteria (van Leeuwen et al., 2003). In addition, the filamentous nature of the fungi permits low cost operation and recovery of biomass (Jin et al., 1998, 1999b). However, maintaining a relatively pure fungal culture during non-aseptic wastewater treatment is often challenging, as bacteria originally contained in wastewater compete with fungi for

organic substrate and proliferate during the treatment. Thus, a suitable selective pressure is required to favor the growth of fungi while suppressing the bacteria.

The growth of specific microorganisms can be favored by employing selective pressures based on the physiological and physical properties (such as mass, size, etc.) of microorganisms, and environmental conditions (such as operating pH, temperature, etc.). Because of their strong affinity to attach on organic or inorganic surfaces, an attached growth system was investigated in this study as a selection mechanism to maintain a dominant fungal culture under non-aseptic conditions. Biofilms are a natural form of cell immobilization, which is one way of enhancing cell density in a biological system (Characklis and Marshall, 1990; Ho et al., 1997a,b,c). Plastic composite support (PCS) medium, developed at Iowa State University stimulates the biofilm formation (Demirci and Pometto, 1995). The PCS tubes are a high temperature extruded product composed of 50% polypropylene (PP) and 50% agricultural products.

The goal of this study was to evaluate the organic removal efficiency and fungal biomass production potential of a PCS biofilm continuous-reactor using sterile and non-sterile corn processing wastewater, to examine the effect of nutrient supplementation on bioreactor performance, and to compare its performance with that of PP biofilm and suspended growth continuous-reactors as controls.

MATERIALS AND METHODS

Fungal Culture

The *Rhizopus oligosporus* strain was selected because of its ability to degrade food- processing wastewater and produce a high yield (Jin et al., 1999a,b). Freeze-dried culture of fungal strain *R. oligosporus* (ATCC #22959) was obtained from American Type Culture Collection (Rockville, MD). This strain is widely used in the industries to produce enzymes, such as amylase, protease and lipase for lowering serum cholesterol level (Sutardi and Buckle, 1985). The culture was rehydrated and revived in yeast-malt (YM) nutrient broth (Difco Laboratories, Sparks, MD) at 24ºC.

Inoculum Preparation and Culture Storage

The revived culture was transferred onto numerous potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) plates and incubated at room temperature (~24ºC) for 6 to 7 days. Fungal spores were harvested from the surface of PDA plates into sterile distilled water containing 0.85% NaCl (w/v) saline solution and 0.5% (v/v) of Tween 80 (Fisher Scientific, Fair Lawn, NJ). The harvested culture was diluted further to achieve a spore count of 10^6 to 10^7 spores/mL, determined by haemocytometer counts. Glycerin (20%, v/v) was added to the spore suspension as a cryoprotectant for ultra-low frozen storage at -75^{o}C in sterile 2 mL cryo-vials for future use as a bioreactor inoculum.

Support Medium for Attached Growth System

PCS tubes composed of 50% (w/w) PP (Quantum USI Division, Cincinnati, OH), 40% (w/w) ground soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (w/w) dried bovine albumin (Proliant, Des Moines, IA), and 5% (w/w) yeast extract (Ardamine Z; Red Star BioProducts, Juneau, WI) were used as support medium for fungal growth. The PCS tubes were fabricated via high temperature extrusion (Cotton et al., 2001) using a twin-screw co-rotating Brabender PL2000 extruder (Model CTSE-V; Brabender Instruments, South Hackensack, NJ), with internal and external diameters of 7.0 and 10.5 mm, respectively. For control studies, PP tubes with 9.0 mm internal and 10.5 mm external diameters were used as a support medium.

Substrate

Corn wet milling wastewater, obtained from the Archer Daniels Midland (ADM) plant at Clinton, IA, was used as feed. The wastewater characteristics are listed in Table 3.1. The wastewater chemical oxygen demand (COD) varied from 1,900 to 3,400 mg/L with relatively low macronutrients (nitrogen and phosphorus) and pH ranged from 5.7 to 10.3, more commonly at lower pH.

Table 3.1 Characteristics of ADM corn processing wastewater*

Parameters	Concentration (mg/L)
Total chemical oxygen demand (TCOD)	1,870 to 3,470
Soluble chemical oxygen demand (SCOD)	1,690 to 3,190
Total suspended solids (TSS)	250 to 300
Volatile suspended solids (VSS)	150 to 200
Biochemical oxygen demand (BOD_5)	1,100 to 2,200
Total Kjeldahl nitrogen (TKN) as N	30 to 78
Total phosphorus (TP) as P	3 to 32
Protein	0.1 to 0.35
Carbohydrate as glucose	400 ± 8
Sulfate as SO_4[#]	340 ± 12
Nitrate as NO_3[#]	ND
Phosphate as PO_4[#]	ND
Chloride[#]	2050 ± 25
Sodium[#]	1260 ± 12
Potassium[#]	115 ± 5
Magnesium[#]	ND
Calcium[#]	95 ± 5

*Based on seven batches of wastewater obtained from ADM plant; ND – Not Detectable (n=7, [#]n=2; where n is the number of wastewater batches used for study/analysis)

Biofilm Reactor

The biofilm reactor design of Cotton et al. (2001) was employed. Six PCS tubes of 60 mm length were bound in a grid-like fashion (Figure 3.1a), and fixed to the agitator shaft of a 2.0 L New Brunswick Bioflo 3000 fermentor (New Brunswick Scientific, Edison, NJ) with a 1.25 L working volume. The ends of PCS tubes were cut at an angle to promote substrate flow through the tubes. The support medium facilitates the formation of biofilm with adequate thickness and surface area to retain sufficient fungal

cell density in the bioreactor. The shear stress on the biofilm is a function of agitation speed in the reactor. Increase in the agitation speed increases the shear stress and biofilm detachment rates (Characklis and Marshall, 1990; Rittmann and McCarty, 2001). Therefore, the biofilm thickness on PCS tubes was controlled by agitation speed. Figure 3.1b shows the schematics of the fungal biofilm continuous-reactor. Graham et al. (1976) reported that the maximum yield of *R. oligosporus* was obtained at a temperature of 37°C and pH ranging from 3.0 to 5.0. Previous studies on suspended growth systems also showed that these parameters are optimal for fungal growth (Jin et al., 1999b; van Leeuwen et al., 2003). Thus, the bioreactor was maintained at a temperature of 37°C, pH of 4.0 using 0.2 N HCl and hydraulic retention time (HRT) of 5 h during this study. Air was constantly supplied at a rate of 1.0 $Lmin^{-1}$ (0.8 vvm).

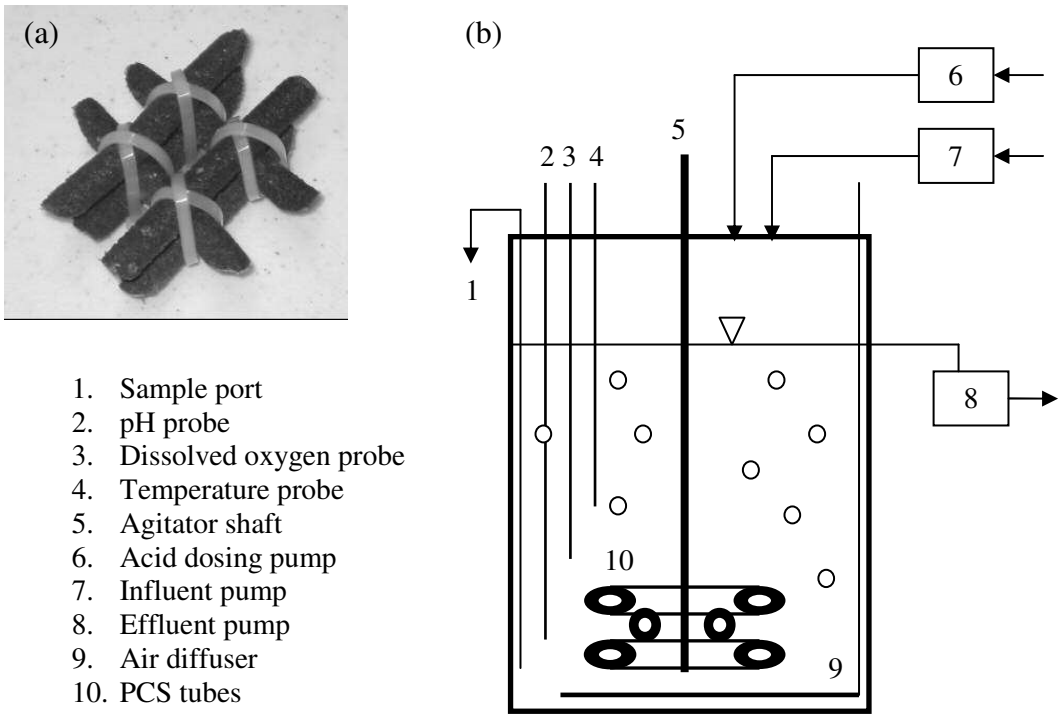

Figure 3.1 (a) Plastic composite support (PCS) medium grid layout; (b) Schematic diagram of attached growth fungal bioreactor

Experimental Methods

Attached Growth System with PCS Medium under Aseptic Conditions. Batch fermentation with sterilized wastewater was carried out for two days to establish a visible biofilm on PCS tubes using 4 mL of R. oligosporus spore suspension. Operation was then switched to continuous with an agitation speed of 500 rpm. Availability of macro- and micronutrients greatly impacts fungal growth and survival (Moore-Landecker, 1996). Biofilms tend to form more readily in the presence of ample nutrients (Cowan et al., 1991). For example, when supplied with sufficient phosphorus microbial cells demonstrate higher tendency to flocculate and adhere due to their increased hydrophobicity (Bücks et al., 1988). Nitrogen is essential to form proteins, cell wall components, and nucleic acids during microbial growth (Ammary, 2004). Since the wastewater had low macro-nutrient contents (N and P), additional N as $(NH_4)_2SO_4$ and P as KH_2PO_4 were added aseptically to the feed to maintain a COD:N:P ratio of 100:5:1, a typical ratio for biological growth (Metcalf and Eddy, 2003). Trace metals such as magnesium, iron and zinc were also supplied as suggested by Graham et al. (1976).

Attached Growth System with PCS Medium under Non-aseptic Conditions. The pH of the wastewater was adjusted to 4.0-4.5 using concentrated hydrochloric acid prior to storage at 4°C during this study. Sterilized potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD) was inoculated with 2 mL fungal spore suspension (10^6 to 10^7 spores/mL) and operated for one to two days under a batch-mode for initial biofilm development. The biofilm maintains a high cell density in the reactor for subsequent continuous operation with a non-sterilized wastewater. The agitation was maintained at 250 rpm. Inadequate nitrogen and phosphorus levels are common in the treatment of food-processing wastewaters (Metcalf and Eddy, 2003). Optimization of the wastewaters in carbon sources or nutrients is extremely vital to obtain significant pollutants degradations (Coulibaly et al., 2003). Several studies on non-aseptic fungal treatment of food-processing wastewaters indicated a superior performance with an additional supplementation of N and P to wastewater (Jin et al., 1999a; Miranda et al., 1996; Mishra et al., 2004; Salmerón-Alcocer et al., 2003; Stevens and Gregory, 1987). The addition of

nitrogen and phosphorus in the ratio COD:N:P of 100:5:1 to corn wet milling wastewater treated in aeration pond dropped the effluent soluble COD from 400 to 100 mg/L (US EPA, 1978). However, the nutrient requirement varies with the wastewater composition. The non-aseptic suspended growth studies conducted on ADM wastewater indicated a higher nitrogen requirement and nutrient supplementation to obtain a COD:N:P ratio of 150:10:1 was found to improve the COD removal and fungal growth (van Leeuwen et al., 2003). Therefore, nitrogen (NH_4HCO_3) and phosphorus (K_2HPO_4) were supplemented to maintain the above ratio in this phase of study.

Attached Growth System with Polypropylene Support Medium. The agricultural components of the PCS tubes perform as slow-releasing nutrient carriers and facilitate the biofilm formation during the initial batch-mode operation (Ho et al., 1997c). Biofilm sustainability and formation with adequate thickness during continuous operation greatly impact the fungal biomass production and COD removal in a biofilm reactor. To assess the importance of agricultural products in PCS tubes to enhance fungal growth and attachment, PP tubes were utilized as a support medium (control).

Suspended Growth System. A suspended growth study (continuous-stirred tank reactor without support medium) was conducted as a control to examine the effect of attached growth on fungal growth and organic removal. The initial batch-mode operation for the development of fungal biomass was carried out for 4-5 days after inoculating the heat-sterilized PDB. About 10-12 mL of fungal spore suspension and one-time adjustment of pH to 4.0 with 15 N NaOH was required to obtain any visible biofilm growth in the reactor. The operating conditions were maintained the same as that of the PCS reactor.

Both PP biofilm and suspended growth reactors were operated under non-aseptic conditions. The wastewater characteristics were observed to be variable for each batch, and continuous-reactor performance was affected by such variation as well. Therefore, a PCS biofilm continuous-reactor was operated simultaneously with the same wastewater

sample that was used for PP biofilm and suspended growth continuous-reactors to ensure the accurate comparison between the three systems.

Quantification of Fungal and Bacterial Biomass

To determine the relative microbial (fungal and bacterial) levels, the reactor sample was filtered through Whatman No. 1 filter paper (Florham Park, NJ) with a pore size of 11 μm to retain the fungal filaments, which are larger than bacteria (~1 to 5 μm). The final filtrate was analyzed for the bacterial concentration as VSS. The fungal concentration was obtained as a difference between the total mg VSS per litre in the reactor sample and the bacterial concentration in the filtrate.

Biomass Harvesting and Sample Collection

A constant biofilm thickness was maintained under steady state conditions (steady COD removal and biomass production) by continuous sloughing of the biomass into the mixed liquor surrounding the biofilm. The biomass in suspension thus leaves the reactor along with the effluent. The reactor sample was collected for analysis on a daily basis from a sample port provided on the top of Bioflo 3000 fermentor. Harvesting of biomass was not considered on bench scale. However on industrial scale, the filamentous form of fungal biomass allows employment of inexpensive harvesting methods such as, settling or screening of biomass from the collected reactor effluent.

Analyses

The wastewater was analyzed for COD, BOD_5, TSS, VSS, TP and TKN as per Standard Methods (APHA/AWWA/WEF, 1998). Total carbohydrate was determined using the phenol-sulphuric method (Dubois et al., 1956); anions and cations were determined using ion chromatography (Model DX 500; Dionex Corporation, Sunnyvale, CA); and protein was determined using Lowry's method (Lowry et al., 1951). Effluent samples were analyzed routinely for COD and VSS until steady-state data were obtained.

The average fungal protein was analyzed using three dried and powdered biomass samples according to the Dumas method (AOAC, 1995). The observed biomass yield was determined as g dry-weight of biomass produced per g COD removed under aseptic conditions and as g VSS produced per g COD removed under non-aseptic conditions. The relative turbidity of bioreactor effluent was determined spectrophotometrically at 620 nm (Abs_{620}) (Unicam Model 4001/4; Spectronic Instruments, Rochester, NY) before fungal biomass settling and was correlated with fungal biomass yield. *In-situ* oxygen uptake rate of the biomass was also measured during each run by stopping the agitation and airflow, and monitoring the rate of dissolved oxygen decrease (respiration rate). Each experiment was conducted twice with different batch of wastewater (n=2).

RESULTS AND DISCUSSION

Attached Growth System with PCS Medium under Aseptic Conditions

The fungal biomass produced under aseptic conditions was strongly attached to the PCS support (Figure 3.2a) and the effluent VSS concentration was less than 5 mg/L. Microscopic observation of the reactor samples showed fungal culture dominance with minimal bacterial contamination (Figure 3.2b). Previous study with bacteria showed that the biofilm thickness could be controlled by the agitation speed (Cotton et al., 2001). However, even with a high agitation speed of 500 rpm, no biofilm was sloughed-off and higher agitation led to considerable loss in working volume due to vortex formation. Thus, only two PCS tubes were used as support medium to minimize excessive fungal biofilm growth.

Aseptic operation of the fermentor using thermally sterilized corn processing wastewater (autoclaved) resulted in average COD removal of about 50%, and observed biomass yield of 0.11 g(dry-weight)/$gCOD_{removed}$ with protein content of 30% (w/w) (Figure 3.3). The average oxygen uptake rate was 0.08 $mgL^{-1}min^{-1}$. With nutrient (N and P) supplementation, COD removal efficiency improved marginally to 55%, whereas, the fungal biomass yield increased by 2-fold with higher fungal protein content of 37% (w/w).

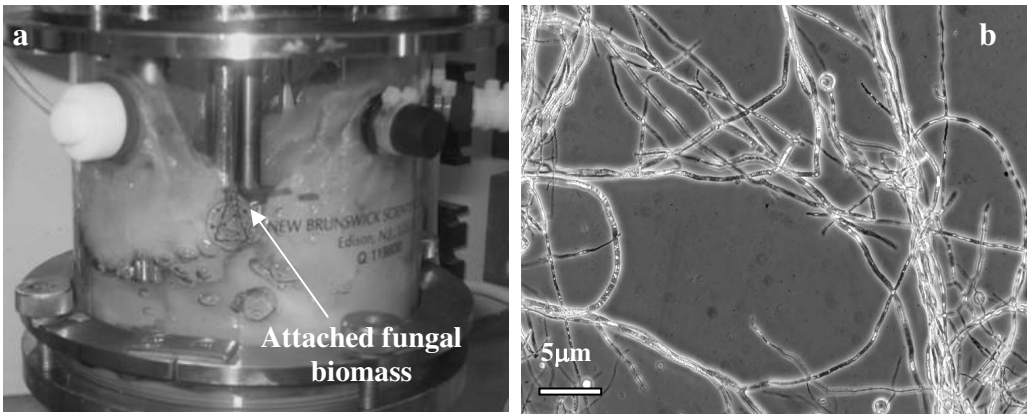

Figure 3.2 PCS biofilm continuous-reactor under aseptic conditions: (a) Accumulation of fungal biomass with nutrient supplementation; (b) Microscopic observation of bioreactor sample (1000X magnification)

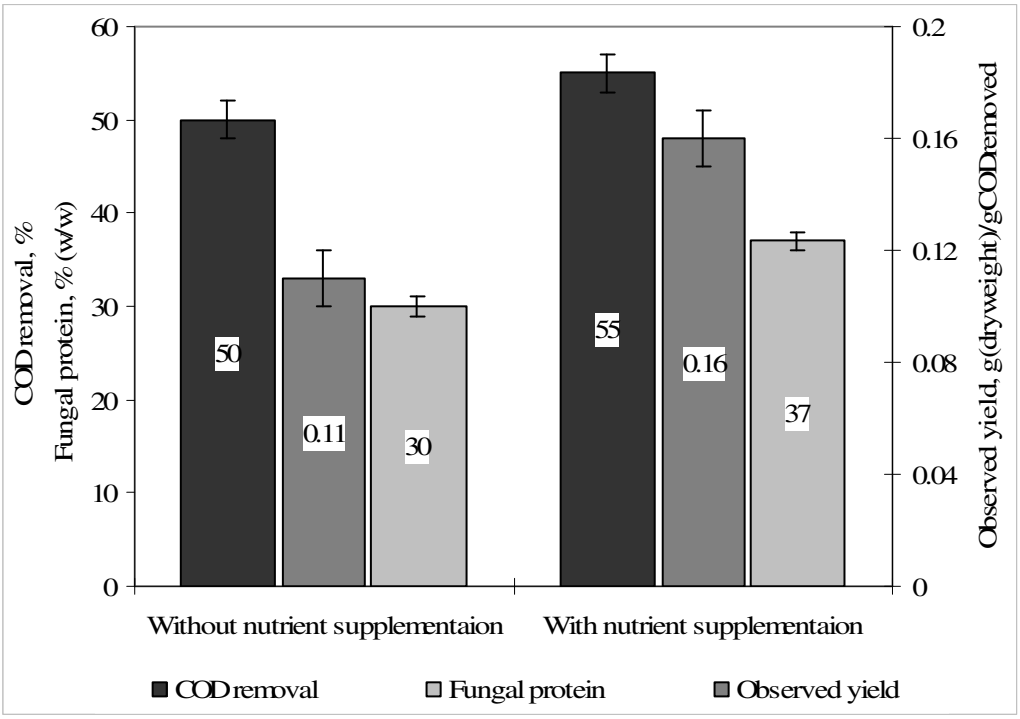

Figure 3.3 Percentage COD removal, observed biomass yield and percentage fungal protein produced under aseptic conditions in a PCS biofilm continuous-reactor (n=2)

The improvement in biomass yield and protein indicates a nutrient limiting condition in the reactor when no additional nutrients were supplemented to the wastewater. This agrees with the aseptic batch study of Oshoma and Ikenebomeh (2005), who reported higher *Aspergillus niger* production with additional supply of N as $(NH_4)_2SO_4$ to rice bran medium. The oxygen uptake rate with nutrient supplementation could not be obtained due to excessive growth of biomass around the dissolved oxygen (DO) probe.

It is important to point out that much of the low biodegradability of the measured COD is most likely resulted from thermal treatment of the soluble corn steep products in wastewater. Sterilization of wastewater was found responsible for the precipitation of the essential micronutrients as witnessed by the formation of white solid deposits, which apparently affected the COD removal efficiency. Further investigation under aseptic conditions could not be performed due to inability of supporting fungal growth with the subsequent autoclaved batches of wastewater obtained from the ADM plant. Precipitation of essential nutrients or production of inhibitory compounds during wastewater sterilization was suspected to be the major reason for the failure of fungal biofilm growth in the reactor.

Attached Growth System with PCS Medium under Non-aseptic Conditions

Initial studies operating with two PCS tubes as support medium at an agitation of 500 rpm resulted in improved COD removal of 70% with corresponding oxygen uptake rate of 0.15 $mgL^{-1}min^{-1}$. Some organic removal may have been contributed by bacterial biodegradation. Dominant bacterial growth was observed in the suspension based on microscopic examination of the reactor effluent indicating a probable significant contribution of bacteria to COD removal. Excessive sloughing of the biofilm from the support medium was also observed at the end of the experiment. This may be attributed to inadequate biofilm density, high shear forces at 500 rpm and probable bacterial infestation of fungal biomass. In the later studies, agitation speed was reduced to 250 rpm to maintain a required biofilm thickness and six PCS tubes were employed to

achieve a higher fungal cell density with minimal bacterial growth in the reactor. This resulted in COD removal of 53% with an observed biomass yield of 0.32 $gVSS/gCOD_{removed}$. The percentage protein content of the fungal biomass was about 34% (w/w). The average *in-situ* oxygen uptake rate of the biomass increased from 0.15 $mgL^{-1}min^{-1}$ to 0.58 $mgL^{-1}min^{-1}$. Dominant fungal growth with a relatively smaller bacterial population was observed during microscopic examination of the bioreactor samples (Figure 3.4a). The scanning electron micrograph (SEM) of biofilm also showed a predominance of fungal biomass in the biofilm attached to the supports (Figure 3.4b).

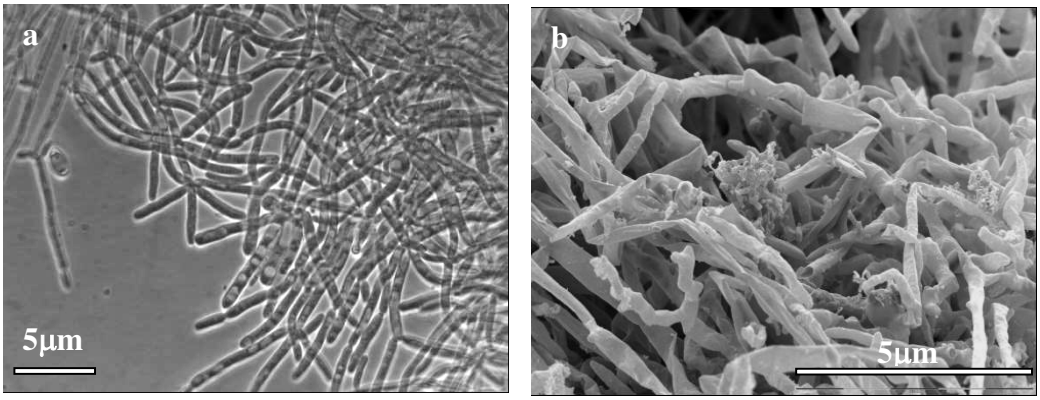

Figure 3.4 PCS biofilm continuous-reactor under non-aseptic conditions: (a) Light microscopic observation bioreactor sample (1000X magnification); (b) SEM of attached fungi in bioreactor (7500X magnification)

Additional nutrient supplementation to the wastewater improved the efficiency of the biofilm reactor in terms of both COD removal and biomass production. The COD removal and observed yield were increased to 85% and 0.56 $gVSS/gCOD_{removed}$, respectively from 53% and 0.32 $gVSS/gCOD_{removed}$ without nutrient supplementation. A slight increase in the fungal protein content of 37% was observed. These results agree with nutrient supplementation studies conducted on other food-processing wastewaters. Stevens and Gregory (1987) reported efficient utilization of supplemental N as

$(NH_4)_2SO_4$ by *Cephalosporium eichhorniae* resulting in an increased microbial biomass production from potato processing wastewater. Truong et al. (2004) also observed 1.3% higher *Aspergillus oryzae* yield with maximum COD removal of 91% from cassava starch processing wastewater supplemented with $(NH_4)_2SO_4$ or peptone. The effect of nutrients on COD removal was not reported. Only slight or insignificant improvement in COD removal, biomass yield and protein content has been reported with nutrient supplementation (N and P), as the starch wastewater treated with *R. oligosporus* and *A. oryzae* was originally rich in nutrients (Jin et al., 1998, 1999b).

Average *in-situ* oxygen uptake rate by the microbial biomass also increased to 1.69 $mgL^{-1}min^{-1}$ suggesting an improved microbial activity with the additional nutrient supplementation. An agitation speed of 250 rpm was effective in maintaining a uniform biofilm thickness and lower agitations resulted in fungal biomass accumulation in the reactor. The fungal biomass was continuously sloughed-off the supports and formed 2-4 mm pellets in suspension (Figure 3.5a). Formation of relatively thin biofilm was observed on the PCS medium (Figure 3.5b). Figure 3.6 demonstrates the effect on nutrient supplementation on COD removal, fungal protein and observed biomass yield.

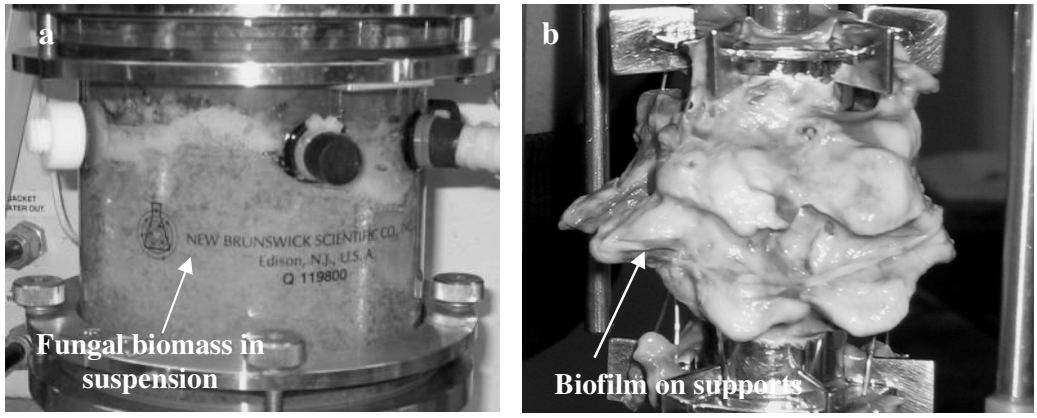

Figure 3.5 PCS biofilm continuous-reactor under non-aseptic conditions: (a) Fungal growth in bioreactor with nutrient supplementation; (b) Attached biofilm on PCS medium

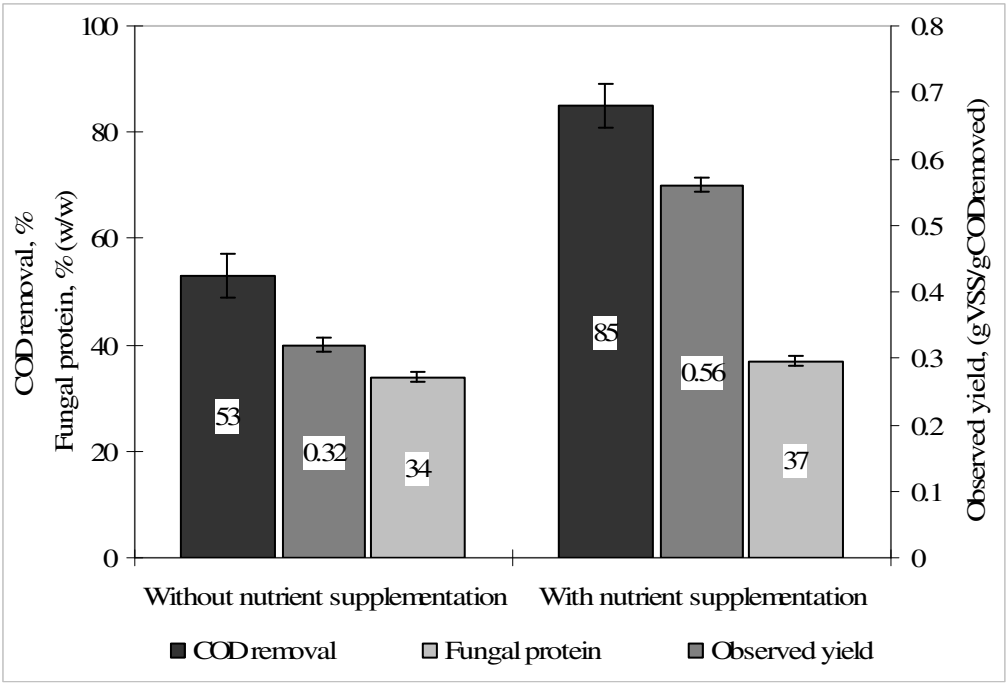

Figure 3.6 Percentage COD removal, observed biomass yield and percentage fungal protein under non-aseptic conditions in a PCS biofilm continuous-reactor (n=2)

Attached Growth System with Polypropylene Support Medium

The bioreactor with PP supports alone showed relatively inferior performance compared to that of a bioreactor with PCS supports. The corresponding percentage COD removal, observed biomass yield, percentage fungal protein and oxygen uptake rate were 28%, 0.19 $gVSS/gCOD_{removed}$, 33% (w/w) and 0.64 $mgL^{-1}min^{-1}$, respectively. The respective values for PCS bioreactor were 78%, 0.44 $gVSS/gCOD_{removed}$, 41% (w/w) and 1.64 $mgL^{-1}min^{-1}$. The study conducted by Wu et al. (2005) on lignin degradation in pulp mill wastewaters by white-rot fungi with porous plastic rings as support media resulted in maximum COD removal of only 48%.

The absorbance values (PCS ~ 0.67 Abs_{620}; PP ~ 0.13 Abs_{620}) correlated with the corresponding biomass yields. The percentage fungal concentration in the total biomass

was inferior in the PP bioreactor with just 67% in comparison to 90% in the PCS bioreactor. Figure 3.7a presents the percentages of COD removal and fungal protein, whereas Figure 3.7b presents the relative microbial concentrations in PCS and PP bioreactors. The observed biomass yield, Abs_{620} and oxygen uptake rate for PCS and PP bioreactors are shown in Figure 3.8.

The PP support tubes failed to develop a complete biofilm during the batch-mode operation. Visual observations illustrated the enhanced fungal growth on PCS tubes in comparison to PP supports alone (Figure 3.9). Cotton et al. (2001) concluded that the presence of agricultural components in PCS stimulates biofilm formation by supplying micronutrients. The surface texture of the support medium plays a vital role in biofilm formation (Gjaltema et al., 1997; van Loosdrecht et al., 1987; Verran et al., 1991). Rough surfaces tend to enhance the biofilm formation offering protection against shear forces (Beeftink and Staugaard, 1986; Characklis and Marshall, 1990; Fox et al., 1990; Heijnen et al., 1992).

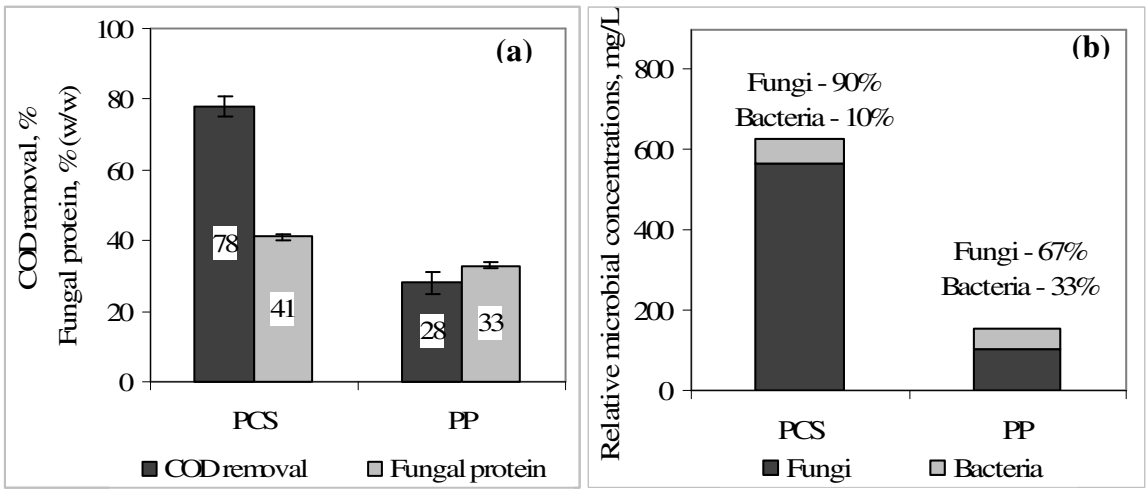

Figure 3.7 PCS and PP biofilm continuous-reactors: (a) Percentage-COD removal and -fungal protein (n=2); (b) Relative microbial concentrations in reactor sample (n=2)

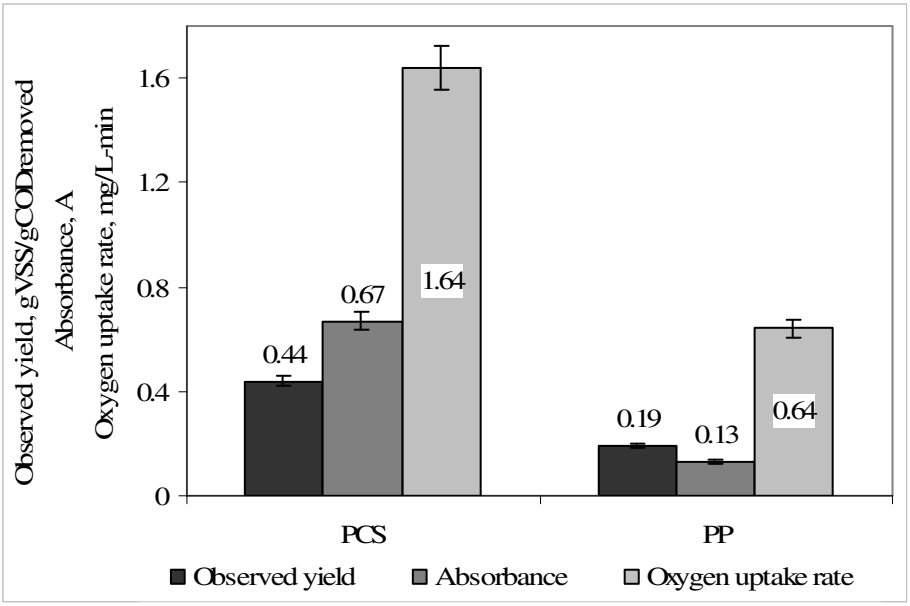

Figure 3.8 Observed biomass yield, absorbance (620 nm) and oxygen uptake rate in PCS and PP biofilm continuous-reactors (n=2)

The soybeans hulls in PCS provide larger rough surface area with regions protected from hydraulic shear forces by forming porous networks (Demirci et al., 1993; Ho et al., 1997a,c). In the absence of a component like soybean hulls, PP provides relatively smoother and therefore smaller surface area resulting in greater shear forces. When shear forces are relatively high only a patchy biofilm develops, as observed in PP biofilm continuous-reactor (Qureshi et al., 2005; van Loosdrecht et al., 1995). Glucose, the main component of PDB, alone is insufficient and additional nitrogen and mineral sources are required to for fungal spore germination and colony formation (Thanh and Nout, 2004). Yeast extract (5% w/w) in PCS supplies nitrogenous compounds, whereas dried bovine albumin (5% w/w) ensures gradual release of these compounds (Ho et al., 1997a,c). Lack of adequate biofilm formation in the PP reactor impaired the COD removal and biomass production in the fungal system. Failure to establish high fungal cell density in the reactor due to insufficient biofilm resulted in higher bacterial contamination.

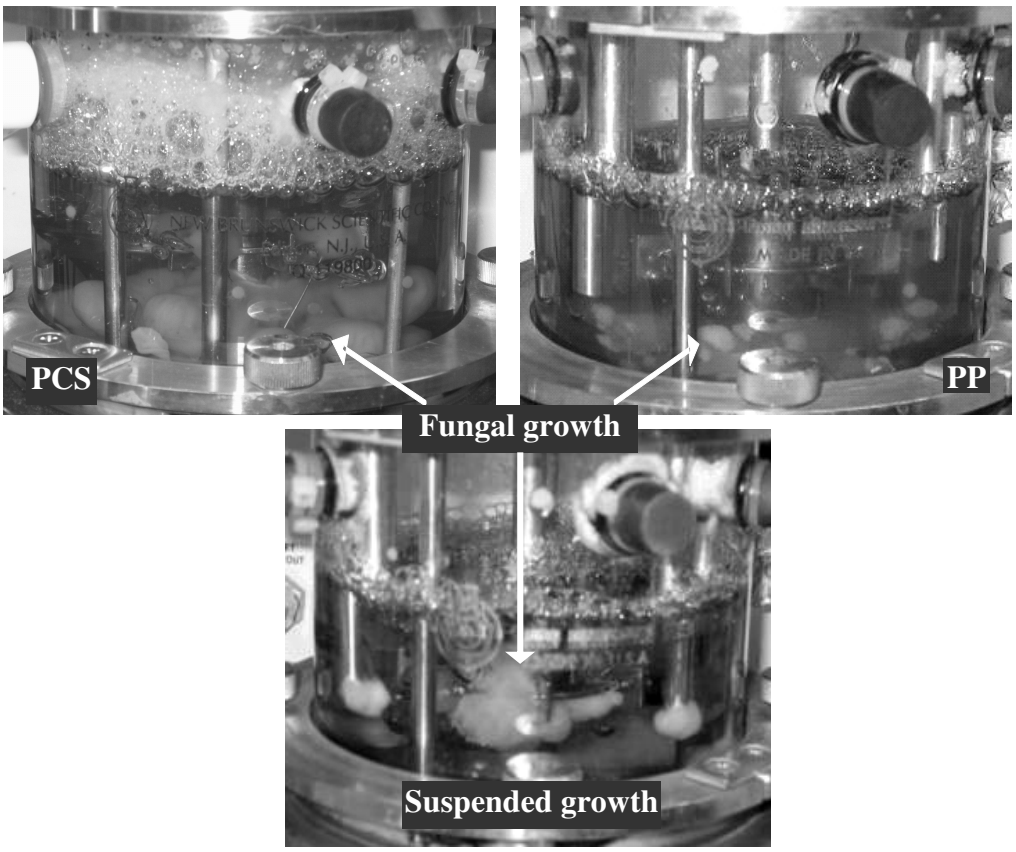

Figure 3.9 Visual fungal growth during batch operation in PCS biofilm, PP biofilm and suspended growth reactors in sterile potato dextrose broth (PDB)

Suspended Growth System

Larger inoculums and longer batch-mode operations were needed to develop a significant fungal biomass in the suspended growth reactor than in a PCS biofilm reactor. This difference was possibly due to the slow release of micronutrients from the PCS that supported the growth of fungal biomass in batch operation. The reactor performance was inferior to the PCS biofilm reactor due to its inability to retain the fungal biomass during

continuous operation. Some attached fungal growth to agitator blades and reactor walls was achieved in the suspended growth reactor during batch-mode operation (Figure 3.9).

Two suspended growth studies (SS1 and SS2) conducted with different batches of ADM wastewater demonstrated a variation in the reactor performance. SSI is a single experiment, whereas SSII is an average of two replicates. The COD removal efficiency and fungal protein in PCS biofilm and suspended growth continuous-reactors are presented in Figure 3.10. The SS1 resulted in a COD removal of 31%, biomass yield of 0.23 gVSS/gCOD$_{removed}$ and fungal percentage in total biomass of 64%. Slightly higher values of COD removal, biomass yield and protein content (e.g., 47%, 0.33 gVSS/gCOD$_{removed}$ and 71%, respectively) were obtained from the SS2. The corresponding values for PCS biofilm continuous-reactor were 78%, 0.46 gVSS/gCOD$_{removed}$ and 90%. Fungal protein was nearly similar for the two suspended growth studies (SS1 ~ 29% (w/w); SS2 ~ 30% (w/w)), whereas for the PCS biofilm continuous-reactor, the value was significantly higher at 41% (w/w). Figure 3.11 includes the biomass yield, Abs$_{620}$ of reactor sample and oxygen uptake rate.

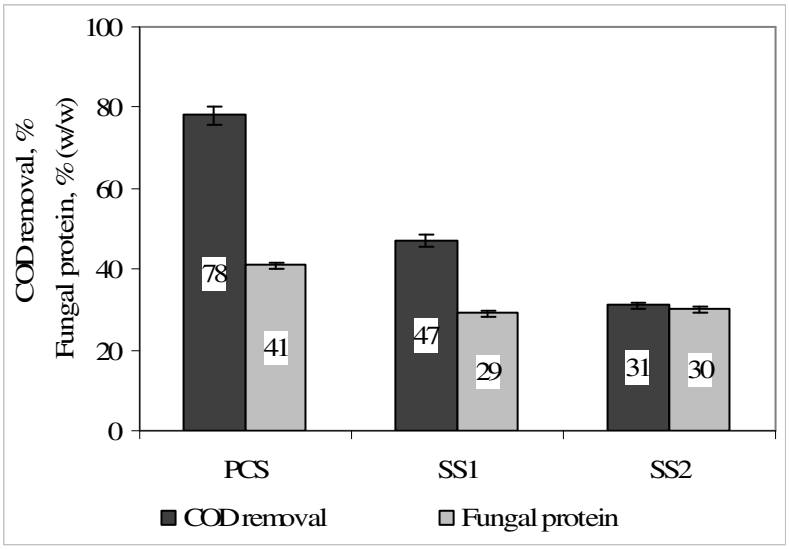

Figure 3.10 Percentage-COD removal and -fungal protein in PCS biofilm (n=2) and suspended growth continuous-reactors SS1 (n=1) and SS2 (n=2)

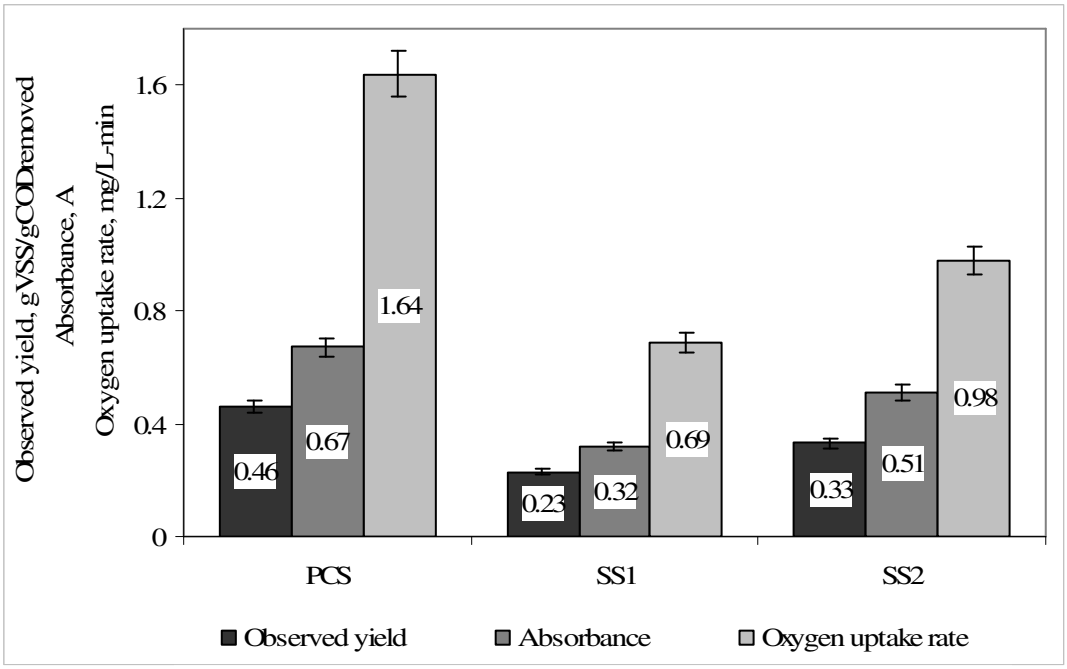

Figure 3.11 Observed biomass yield, absorbance (620 nm) and oxygen uptake rate in PCS biofilm (n=2) and suspended growth continuous-reactors SS1 (n=1) and SS2 (n=2)

Higher COD removal of 84% and biomass yield of 0.42 gdry biomass/$gCOD_{removed}$ were reported from a suspended growth study conducted on corn processing wastewater at ISU (van Leeuwen et al., 2002). This may be attributed to the higher fungal cell density maintained in the reactor by allowing bacterial wash out with effluent through a microscreen (pore size - 100 µm). Oxygen uptake rates and the Abs_{620} values correlated (R^2=0.99) with the biomass yields (PCS ~ 1.64 $mgL^{-1}min^{-1}$ and 0.67 Abs_{620}; SS1 ~ 0.69 $mgL^{-1}min^{-1}$ and 0.32 Abs_{620}; SS2 ~ 0.98 $mgL^{-1}min^{-1}$ and 0.51 Abs_{620}). The fungal percentages in total biomass are shown in Figure 3.12, which again demonstrates that PCS biofilm reactor significantly outperformed the suspended growth reactor. Table 3.2 summarizes the COD removals, observed biomass yields and protein contents in PCS biofilm, PP biofilm and suspended growth continuous-reactors for corn wet milling wastewater treatment by *R. oligosporus*.

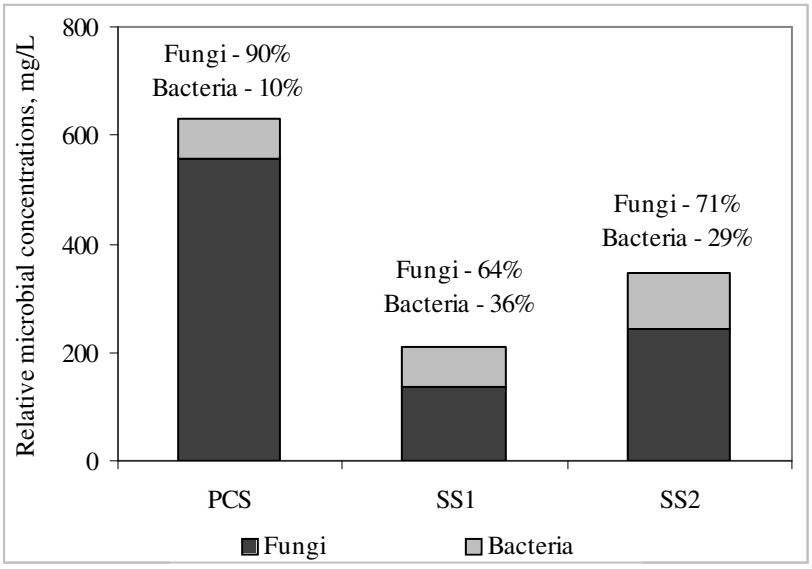

Figure 3.12 Relative bacterial and fungal concentrations in PCS biofilm (n=2) and suspended growth continuous-reactors SS1 (n=1) and SS2 (n=2)

Table 3.2 Results summary for corn wet milling wastewater treatment in attached growth (PCS biofilm and PP biofilm) and suspended growth continuous-reactors (n=2)

Reactor	Condition	COD removal %	Observed biomass yield *	Biomass protein % (w/w)
PCS biofilm	Aseptic [b]	50.1 ± 2.12	0.11 ± 0.010[I]	30.3 ± 0.21
PCS biofilm	Aseptic [a]	55.0 ± 0.42	0.16 ± 0.006[I]	37.0 ± 1.98
PCS biofilm	Non-aseptic [b]	53.2 ± 3.68	0.32 ± 0.025[II]	34.2 ± 2.04
PCS biofilm	Non-aseptic [a]	82.4 ± 4.53	0.51 ± 0.071[II]	38.7 ± 2.90
PP biofilm	Non-aseptic [a]	27.8 ± 2.83	0.19 ± 0.032[II]	32.8 ± 2.92
Suspended growth	Non-aseptic [a]	44.1 ± 11.37	0.30 ± 0.060[II]	29.3 ± 1.27

[a] Nutrient supplementation; [b] No nutrient supplementation

* [I] g dry-weight/gCOD$_{removed}$; [II] gVSS/gCOD$_{removed}$

CONCLUSIONS

The results of this research showed that an attached growth fungal treatment system with PCS medium was most effective in treating nutrient supplemented corn wet milling wastewater with simultaneous recovery of high value fungal biomass. Some of the important findings are summarized below:

- Although aseptic operation resulted in pure fungal biomass production, bioconversion rates were reduced due to suspected inhibitory compound production during thermal sterilization and also harvesting could not be achieved during continuous operation.

- Nutrient supplementation improved the fungal biomass production significantly under both aseptic and particularly non-aseptic conditions. A considerable improvement in COD removal was achieved under non-aseptic conditions. Additional revenue from high biomass yield could be substantial to compensate the nutrient supplementation costs. Discharge of excess nutrients from nutrient supplemented wastewaters is a major environmental concern. Large part of discharged nutrients is contained within the biomass and thus an effective removal of sludge from the treated wastewater is important (Slade et al., 2004). The fungal biomass produced under non-aseptic conditions was easily settleable, which would enhance the fungal biomass harvesting from the wastewater and thus reduce the discharge of excess nutrients in full-scale applications.

- The PP study confirmed that the agricultural products in PCS medium were necessary for biofilm formation during batch-mode cultivation and maintained high fungal cell density during subsequent continuous operation.

- The suspended growth study showed by contrast that the support medium was very effective in retaining fungal biomass and maintaining high fungal cell density in the reactor during continuous operation. Also in comparison to PCS biofilm continuous-reactor, longer start-up batch operation (4-5 days compared to 1 day), significantly higher fungal spore inoculums (10-12 mL compared to 2 mL) and a one-time pH adjustment were observed to be essential for obtaining any visible growth.

- The suspended growth study also showed that variations in the reactor performance might occur depending on the wastewater characteristics.

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4. ATTACHED GROWTH FUNGAL TREATMENT OF CORN WET MILLING WASTEWATER: EFFECT OF PH AND HYDRAULIC RETENTION TIME

(Manuscript will be submitted to '*Biotechnology and Bioengineering*' journal)

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Abstract: Corn wet milling wastewater was treated in an attached growth system using *Rhizopus oligosporus*. Plastic composite support (PCS) tubes, composed of 50% (w/w) polypropylene and 50% (w/w) agricultural products were used as a support medium. The effects of operating pH (3.5, 4.0 and 4.5) and hydraulic retention time (HRT) (5, 3.75, 2.5 and 1.25 h) were evaluated under non-aseptic conditions. COD removal and biomass production were highest at pH of 4.0 with minimal bacterial concentration. Highest COD removal of 78% was achieved at a 5 h HRT with an observed biomass yield of 0.44 g volatile suspended solids (VSS)/g$COD_{removed}$. At 3.75 and 2.5 h HRT, the observed biomass yield increased slightly to 0.45 and 0.48 gVSS/g$COD_{removed}$ while COD removal reduced to 76 and 70%, respectively. An HRT of 5 h was most suitable for COD removal because of the longer contact time of substrate with biomass. Maximum observed biomass yield however was achieved at a shorter HRT of 2.5 h due to higher substrate availability, but the biofilm was more sensitive to wastewater composition

changes. An HRT of 3.75 h was recommended as a compromise for bench-scale operation. In addition, the competitive bacterial growth was reduced with a reduction in HRT. At the shortest HRT of 1.25 h, significant amount of biomass was washed out from the reactor. The wastewater composition proved to have significant effect on the performance of biofilm reactor.

Keywords: corn processing wastewater; attached growth; fungal treatment; plastic composite support (PCS) medium; pH; hydraulic retention time (HRT)

INTRODUCTION

The potential use of fungi for biopurification of wastewaters was recognized during the late 1950s to mid 1960s (Guest and Smith, 2002). Food-processing wastewaters contain readily biodegradable materials and almost none of the hazardous and persistent compounds such as those regulated under the U.S. Environmental Protection Agency's (EPA) Toxic Release Inventory (TRI) listing, and thus constitute an ideal substrate for fungal cultivation (US-AEP, 1997). Several studies reported that food-processing wastewater is amenable to fungal treatment because of their inherent ability to effectively degrade complex polymers such as cellulose, hemi-cellulose, and lignin materials and produce high value fungal biomass (Barbesgaard et al., 1992; Del Re et al., 2003; Huang et al., 2003; Jin et al., 1999a,c, 2001, 2002; Ravinder et al., 2003; Robles et al., 2000; Stevens and Gregory, 1987; van der Westhuizen and Pretorius, 1996; Yokoi et al., 1998). The highly dewaterable fungal biomass can be used as a source of protein and bio-chemicals (Barbesgaard et al., 1992; Jin et al., 1999a,c; Ravinder et al., 2003).

Corn wet milling typically generates about 30 to 48 gallons of high strength wastewater per pound of corn processed (James Foster, 7th June 2005; personal communication, ADM, Clinton, IA). Aerobic biological treatment processes such as activated sludge process generate excess bacterial mass of about 0.4 g volatile suspended solids (VSS)/g chemical oxygen demand (COD) removed (Metcalf and Eddy, 2003). Sludge processing, treatment and disposal present the main treatment challenges and

costs (Weemaes and Verstraete, 1998), as the sludge handling alone accounts for up to 60% of the total wastewater treatment plant operating costs (Canales et al., 1994).

The fungal treatment system on the other hand, converts the wastewater organics into highly dewaterable fungal biomass. Enhancement in sludge settleability and dewaterability was observed with fungal pretreated of domestic wastewater sludge (Alam and Fakhru'l-Razi, 2003; Mannan et al., 2005). The filamentous nature of fungi facilitates easy separation and recovery from the liquid phase (Jin et al., 1999a,c; Nigam, 1994), and also the obligatory acidophilic property suggests that the fungi would not act as an opportunistic pathogen (Mikami et al., 1982). The recovery of value added products (e.g. enzymes, protein, and other bio-chemicals) derived from the fungal biomass could generate additional revenue for the industry.

Maintaining a pure fungal culture under non-aseptic conditions is difficult as most of the fungi are mesophilic and excessive contamination by bacteria occurs in this temperature range. Physiology and physical properties of the fungi, and environmental and operational conditions could be considered as major selection pressures to promote fungal growth and control bacterial competition in a fungal treatment system. During the initial phase of this research, a fungal biofilm reactor with plastic composite support (PCS) medium ensured fungal domination resulting from higher filamentous fungal affinity to surface attachment as selector, thereby successfully reducing the bacterial contamination (Jasti et al., Paper one in Dissertation). The PCS tubes composed of 50% polypropylene and 50% agricultural products were developed at Iowa State University (Demirci and Pometto, 1995; Ho et al., 1997a,b,c).

Fungal selection pressure could be enhanced by controlling environmental and operational factors, such as pH, temperature, hydraulic retention time, etc. Fungi are known for their ability to grow at low pH, which is a desirable property as it not only eliminates the need to increase the pH of many acidic food-processing wastewaters during treatment, but also minimizes the bacterial contamination (Oshoma and Ikenebomeh, 2005). The specific growth rate, initial microbial concentration in a bioreactor, and available substrate are the primary factors in determining the dominance

of a specific microorganism in a continuous system (Harder and Kuenen, 1977). The PCS biofilm continuous-reactor provides the required initial higher fungal cell density and the specific growth rate is directly correlated to dilution rate or hydraulic retention time (HRT).

Therefore, this study focused on evaluating the effect of pH and HRT on organic removal efficiency, fungal biomass/protein production and fungal dominance in a PCS biofilm continuous-reactor.

METHODOLOGY

Materials

Corn wet milling processing wastewater obtained from the Archer Daniels Midland (ADM) plant at Clinton, IA was used as feed. The important wastewater characteristics are listed in Table 4.1.

Rhizopus oligosporus is used for producing 'tempeh' from partially cooked soybeans, a favorite food and staple source of protein in Indonesia for several hundred years (Del Re et al., 2003). The strain was selected to treat corn wet milling wastewater due to its proven ability to degrade food-processing wastewater and produce a high fungal biomass yield (Jin et al., 1999a,b,c, 2002; van Leeuwen et al., 2003). About 10^6 to 10^7 spores/mL of fungal spore suspension prepared from freeze-dried culture obtained from American Type Culture Collection (ATCC #22959, Rockville, MD) was used as an inoculum.

PCS tubes composed of 50% (w/w) polypropylene (Quantum USI Division, Cincinnati, OH), 40% (w/w) ground soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (w/w) dried bovine albumin (Proliant, Des Moines, IA) and 5% (w/w) yeast extract (Ardamine Z; Red Star BioProducts, Juneau, WI) were used as support medium for fungal growth. The PCS tubes, with internal and external diameters of 7.0 and 10.5 mm, respectively, were fabricated with a twin-screw co-rotating Brabender PL2000 high-temperature extruder (Model CTSE-V; Brabender Instruments, South Hackensack, NJ) (Ho et al., 1997a,b,c).

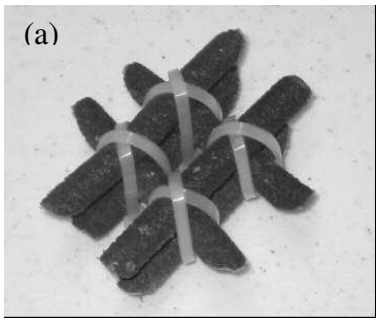
Table 4.1 Characteristics of ADM corn processing wastewater*

Parameters	Concentration (mg/L)
Total chemical oxygen demand (TCOD)	1,870 to 3,470
Soluble chemical oxygen demand (SCOD)	1,690 to 3,190
Total suspended solids (TSS)	250 to 300
Volatile suspended solids (VSS)	150 to 200
Biochemical oxygen demand (BOD_5)	1,100 to 2,200
Total Kjeldahl nitrogen (TKN) as N	30 to 78
Total phosphorus (TP) as P	3 to 32
Protein	0.1 to 0.35
Carbohydrate as glucose	400 ± 8
Sulfate as SO_4[#]	340 ± 12
Nitrate as NO_3[#]	ND
Phosphate as PO_4[#]	ND
Chloride[#]	2050 ± 25
Sodium[#]	1260 ± 12
Potassium[#]	115 ± 5
Magnesium[#]	ND
Calcium[#]	95 ± 5

*Based on seven batches of wastewater obtained from ADM plant; ND – Not Detectable (n=7, [#]n=2; where n is the number of wastewater batches used for study/analysis)

Attached Growth Fungal System Operation

The PCS biofilm reactor design of Cotton et al. (2001) was employed. Six PCS tubes of 60 mm length were bound in a grid-like fashion, as shown in Figure 4.1a, and fixed to the agitator shaft of a 1.25 L New Brunswick Bioflo 3000 fermentor (New Brunswick Scientific, Edison, NJ). Figure 4.1b shows the schematics of the fungal biofilm reactor.



1. Sample port
2. pH probe
3. Dissolved oxygen probe
4. Temperature probe
5. Agitator shaft
6. Acid dosing pump
7. Influent pump
8. Effluent pump
9. Air diffuser
10. PCS tubes

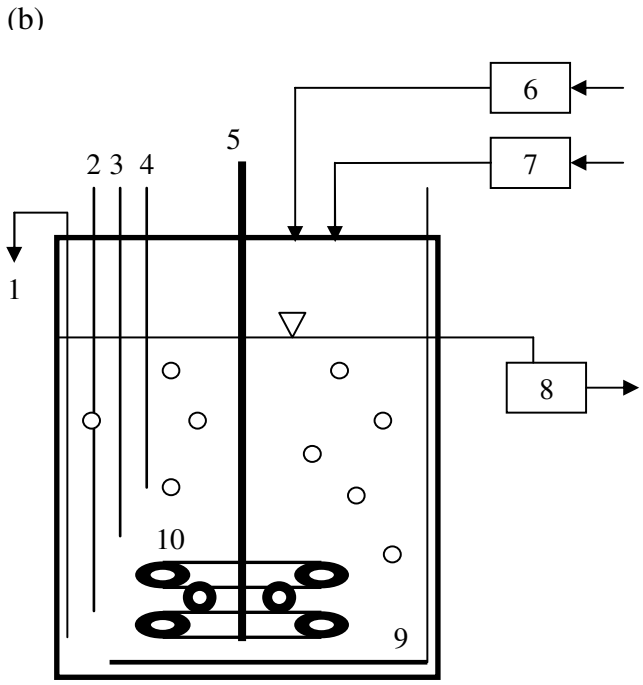


Figure 4.1 (a) Plastic composite support (PCS) medium grid layout; (b) Schematic diagram of attached growth fungal bioreactor

Biofilm thickness on PCS tubes could be controlled by agitation speed. Initial biofilm development on the PCS medium, was achieved in batch mode after inoculating the heat-sterilized potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD) with 2 mL of fungal spore suspension. *R. oligosporus* sporangiospores demonstrate high metabolic activation at 37°C (Thanh and Nout, 2004) and maximum germination at pH 4.0 (Medwid and Grant, 1984). Hence, the batch cultivation was operated at 37°C, pH 4.0 and 250 rpm agitation.

The biofilm formed during batch operation maintains a high fungal cell density in the reactor that can impede the proliferation of the wastewater-borne bacteria during subsequent continuous operation with non-sterilized wastewater. A constant biofilm thickness was maintained under steady state conditions (steady COD removal and

biomass production) by continuous sloughing of the biomass into the mixed liquor surrounding the biofilm. The biomass in suspension thus leaves the reactor along with the effluent. The reactor sample was collected for analysis on a daily basis from a sample port provided on the top of Bioflo 3000 fermentor.

A temperature of 37°C, air supply rate of 1.0 $Lmin^{-1}$ (0.8 vvm) and agitation speed of 250 rpm was maintained throughout the study. It is evident from our previous studies that the nutrient supplementation significantly improved the system performance (Jasti et al., Paper one in Dissertation). Therefore, the wastewater was always supplemented with nitrogen as NH_4HCO_3 and phosphorus as K_2HPO_4 to maintain a COD:N:P ratio of 150:10:1.

Optimization of Process Parameters

Effect of pH. The pH of wastewater affects the nutrient/metallic ion availability, cell permeability and enzymatic activity (Cooke and Whipps, 1993). Hence, the microbial growth is very sensitive to variation in pH within growth medium (Rafiqul et al., 2005; Riscaldati et al., 2000). Graham et al. (1976) observed maximum biomass yields of 1.45 and 1.33 g/L at 37°C and pH ranging from 3.0 to 5.0, respectively, on a synthetic medium for *R. oligosporus.* Therefore, the reactor pH was controlled at 3.5, 4.0 and 4.5 using 0.2 N HCl for pH optimization study. An HRT of 5 h was maintained.

Effect of HRT. In a biofilm reactor, the biomass concentration in suspension is the same as that in the effluent under steady state conditions (Rittmann and McCarty, 2001). At short HRTs, the bacterial biofilm expands relatively fast and sloughs off maintaining uniform biofilm thickness, but increasing the biomass concentration in the suspension or effluent (Characklis et al., 1990; Peyton, 1992). Hence, reduction in HRTs results in increased biomass yields due to higher substrate availability. However, short HRTs could also result in washout of the biomass from the reactor. The main goal was to investigate the lowest possible operating HRT while maintaining the optimal COD removal and biomass production in the reactor. Lower HRTs facilitate faster treatment

rates, which eventually translate to smaller footprint of the bioreactor. The effect of 5.0, 3.75, 2.5, and 1.25 h HRTs on the reactor performance was studied by maintaining the pH at 4.0 with 0.2 N HCl.

Quantification of Fungal and Bacterial Biomass

To determine the relative microbial (fungal and bacterial) levels, the reactor sample was filtered through Whatman No. 1 filter paper (Florham Park, NJ) with a pore size of 11 µm to retain the fungal filaments, which are larger than bacteria (~1 to 5 µm). The final filtrate was analyzed for the bacterial concentration as VSS. The fungal concentration was obtained as a difference between the total mg VSS per liter in the reactor mixed liquor sample and the bacterial concentration in the filtrate.

Analytical Methods

The wastewater was analyzed for COD, BOD_5, TSS, VSS, TP and TKN as per Standard Methods (APHA/AWWA/WEF, 1998). Total carbohydrate was determined using the phenol-sulphuric method (Dubois et al., 1956); anions and cations were determined using ion chromatography (Model DX 500; Dionex Corporation, Sunnyvale, CA); and protein was determined using Lowry's method (Lowry et al., 1951). Effluent samples were analyzed routinely for COD and VSS until steady-state data were obtained.

The average fungal protein was analyzed using three dried and powdered biomass samples according to the Dumas method (AOAC, 1995). The fungal biomass yield was determined as g VSS produced per g COD removed. The relative turbidity of bioreactor effluent was determined spectrophotometrically at 620 nm (Abs_{620}) (Unicam Model 4001/4; Spectronic Instruments, Rochester, NY) before fungal biomass settling and was correlated with fungal biomass yield. *In-situ* oxygen uptake rate of the biomass was also measured during each run by stopping the agitation and airflow, and monitoring the rate of dissolved oxygen decrease (respiration rate).

Experiments at each operating parameter (pH and HRT) were duplicated (n=2). Statistical analysis was conducted using Statistical Discovery 6.0.0 software from Statistical Analysis System (SAS) institute. One-way ANOVA (analysis of variances) was used to determine the mean and standard deviation of the observed parameters (COD removal, biomass yield, biomass fraction, etc.). The effects of variation in pH and HRT on reactor performance were analyzed by Student's t-test considering a statistical significance at 95% confidence interval (probability, $p < 0.05$).

RESULTS AND DISCUSSION

Effect of pH

The highest COD removal of 80% and observed biomass yield of 0.33 $gVSS/gCOD_{removed}$ were attained at pH 4.0 (Figure 4.2 and 4.3). The corresponding values for pH 3.5 and 4.5 were 34%, 0.13 $gVSS/gCOD_{removed}$, and 68%, 0.23 $gVSS/gCOD_{removed}$, respectively. The fungal protein contents were 33, 36 and 31% at pH of 3.5, 4.0 and 4.5, respectively. Thus, a pH of 4.0 was found optimal for fungal biomass production and COD removal. Similar results were reported from the batch studies conducted by van Leeuwen et al. (2002) to optimize pH for *R. oligosporus* suspended growth using the same substrate (corn wet milling wastewater from an ADM plant). Jin et al. (1999a) investigated the treatment of wheat starch processing wastewater by *R. oligosporus* in a bench scale airlift bioreactor. The study revealed that the optimal pH can be selected in a range from 3.5 to 5.0 and concluded 4.0 as the optimum pH for COD removal and biomass yield.

Truong et al. (2004) also found that a pH 4.0 was the most favorable for the treatment of cassava starch wastewater by *Aspergillus oryzae*. However, several studies indicated that the pH for optimum growth might vary depending on the fungal strain and substrate; for example, pH 6.0 for potato chips industry wastewater by *Aspergillus foetidus* and *Aspergillus niger* (Mishra et al., 2004); pH 3.75 for potato processing waste treatment by *Cephalosporium eichhorniae* (Stevens and Gregory, 1987); pH 5.0 for decolorization and phenol degradation from olive mill wastewater by *Geotrichum*

candidum (Assas et al., 2000); pH 3.5-4.0 for aromatic compound degradation from olive oil mill wastewater by *Pycnoporus coccineus* (Jaouani et al., 2003, 2005) and pH 5.0 for lactic acid production from potato processing wastewater by *Rhizopus arrhizus* (Huang et al., 2003). A pH range of 5.0-5.5 was found optimal for COD reduction, biomass production and bacterial elimination for treatment of sugar-furfural effluent by *Aspergillus fumigatus* (van der Westhuizen and Pretorius, 1996).

Average oxygen uptake rate and absorbance at each pH value (pH 3.5 ~ 0.24 $mgL^{-1}min^{-1}$ and 0.17 Abs_{620}; pH 4.0 ~ 0.84 $mgL^{-1}min^{-1}$ and 0.69 Abs_{620}; pH 4.5 ~ 0.53 $mgL^{-1}min^{-1}$ and 0.42 Abs_{620}) correlated (R^2=0.99) with the corresponding biomass yields (Figure 4.3). Higher oxygen uptake rates represent greater microbial activity in the reactor.

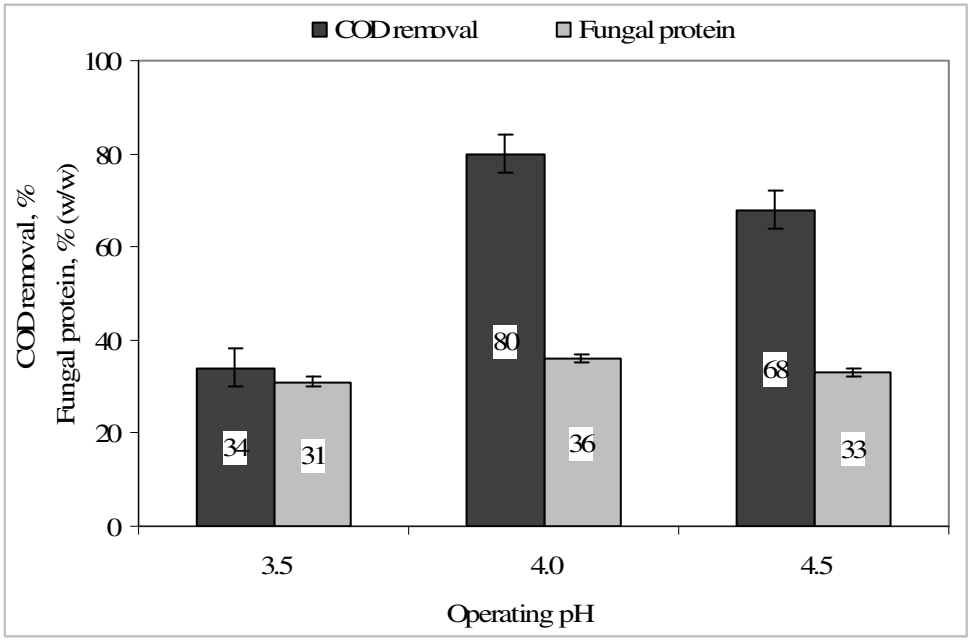


Figure 4.2 Effect of pH on percentage-COD removal and -fungal protein yield in a PCS biofilm continuous-reactor (n=2)

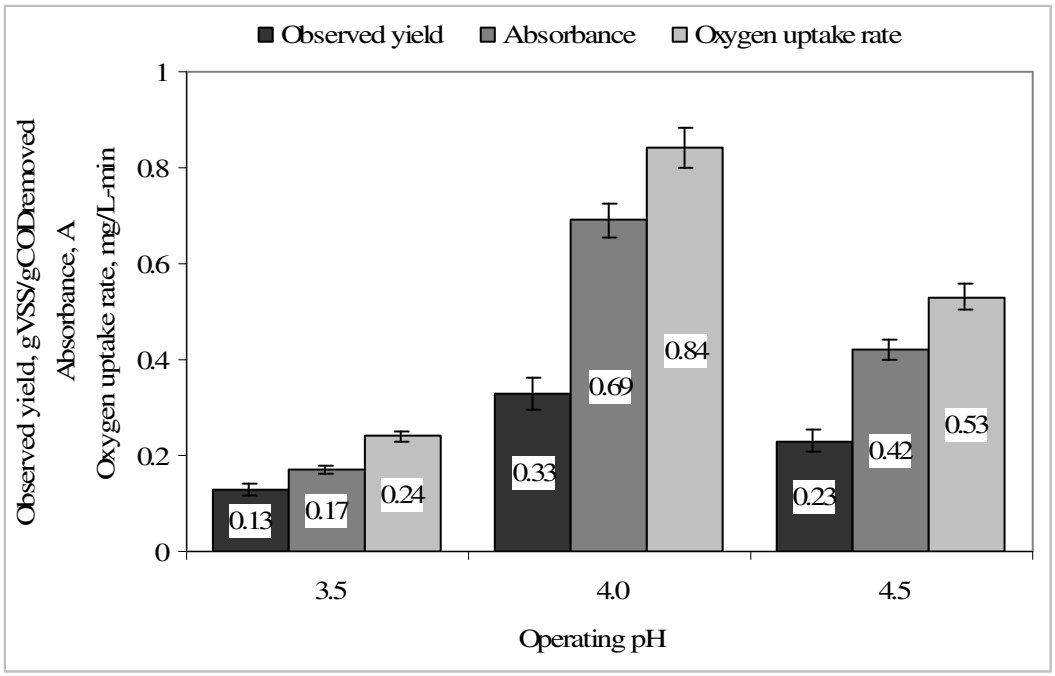


Figure 4.3 Effect of pH on observed biomass yield, absorbance (620 nm) and oxygen uptake rate in a PCS biofilm continuous-reactor (n=2)

Variation in pH influenced the establishment of the relative microbial (fungal and bacterial) population densities in the reactor. The respective mass proportions of fungi in the total biomass concentration at pH 3.5, 4.0 and 4.5 were 73, 93 and 84%. Both bacteria and fungi were inhibited at pH 3.5 and the overall biomass production was nominal. However, depending on the total biomass and fungal fractions depicted in Figure 4.4, it can be derived that the fungi were more affected than bacteria at pH 3.5 and the bacterial competition was greater at pH 4.5. Again, pH 4.0 appeared to be the optimum to reduce the bacterial competition. The results were in good agreement with the findings of van der Westhuizen and Pretorius (1998), who reported reduction in *A. fumigatus* yields from 0.44 to 0.38 g/gCOD and one-log increase in bacterial numbers with an increase in pH from 5.0 to 6.1.

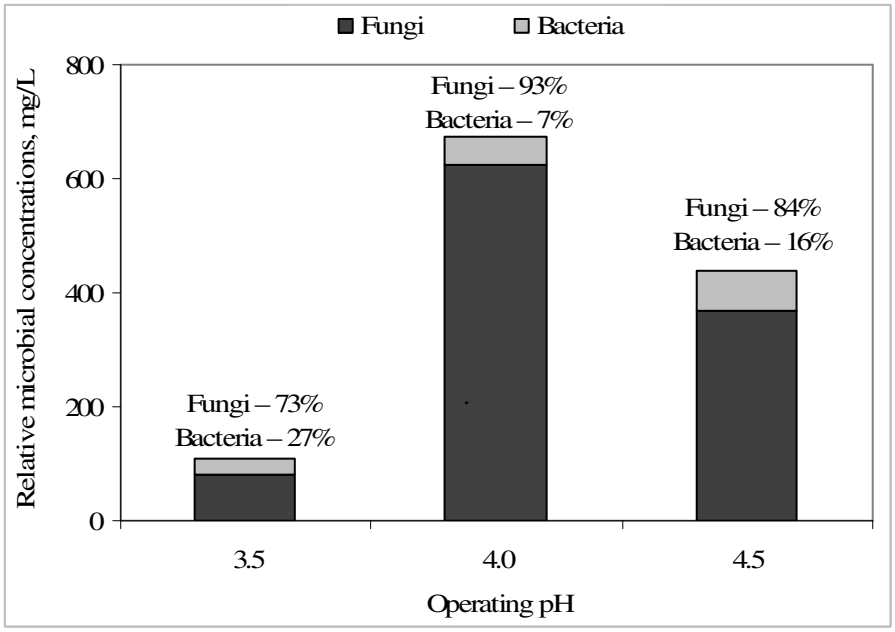


Figure 4.4 Effect of pH on relative bacterial and fungal concentrations in a PCS biofilm continuous-reactor (n=2)

Literature on suspended growth systems reports that the variation in pH can also affect the fungal morphology (Jin et al., 1999c; Metz and Kossen, 1977; Truong et al., 2004). However, no visible variation was observed in this study indicating that a pH change may not affect the fungal morphology in attached growth systems, specifically in the pH range of 3.5 to 4.5.

The COD removal and biomass yield achieved at pH 4.0 were inferior to those obtained during previous nutrient supplementation studies (85% and 0.56 $gVSS/gCOD_{removed}$, respectively) (Jasti et al., Paper one in Dissertation). Both studies were conducted under similar operating conditions using different batches of wastewater. The biofilm sustainability and system performance depend on the type and composition of substrate (Villaseñor et al., 2000). The wastewater composition differed from batch to batch obtained from ADM, which may have affected the efficiency of the system. For example, fungal growth in the sterilized wastewater during the aseptic studies varied

from excellent to none for different batches of wastewater. Relatively high concentrations of total suspended solids in wastewater utilized for pH studies were deposited on the biofilm, as clearly shown in Figure 4.5, and hindered its ability to produce an optimum biomass yield. However, these results demonstrate that the biofilm reactor is capable of handling wastewaters with different compositions. Table 4.2 summarizes the COD removal, observed biomass yield and protein content at each operating pH in the PCS biofilm continuous-reactor.

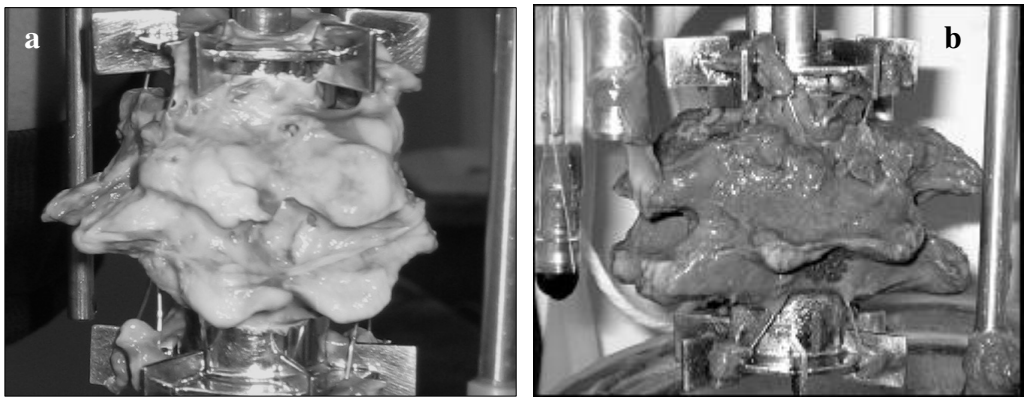


Figure 4.5 PCS biofilm continuous-reactor at pH 4.0: (a) Biofilm from previous nutrient supplementation study; (b) Biofilm covered with wastewater suspended solids from pH study

Table 4.2 Summary of the effect of pH on corn wet milling wastewater treatment in a PCS biofilm continuous-reactor (n=2)

pH	COD removal %	Observed biomass yield $gVSS/gCOD_{removed}$	Biomass protein % (w/w)	Biomass fraction % (w/w) in total biomass	
				Fungi	Bacteria
4.5	68.2 ± 0.71	0.23 ± 0.022	33.4 ± 0.02	83.7 ± 1.10	15.9 ± 0.23
4.0	79.2 ± 1.23	0.33 ± 0.006	36.1 ± 0.14	92.8 ± 1.10	07.2 ± 1.23
3.5	31.2 ± 1.91	0.13 ± 0.003	30.7 ± 0.14	73.1 ± 0.50	27.4 ± 2.58

Effect of HRT at pH 4.0

Reduced operating HRTs resulted in a decline in COD removal. Maximum COD removals of 79, 76, 70 and 38% were obtained at HRTs of 5, 3.75, 2.5 and 1.25 h, respectively (Figure 4.6). This is identical to the trend observed by Pan et al. (2004) at HRTs ranging from 2 to 12 h in a sequential aerobic sludge blanket reactor treating synthetic wastewater with aerobically grown microbial granules. Small, but statistically significant improvement in observed biomass yield was observed between HRTs 5 and 2.5 h, followed by a huge decline at 1.25 h. The corresponding values were 0.44, 0.45, 0.48 and 0.25 gVSS/gCOD$_{removed}$ at 5, 3.75, 2.5 and 1.25 h HRT, respectively. The effect on fungal protein was insignificant between HRTs 5 and 2.5 h (5 h ~ 40% (w/w); 3.75 h ~ 38% (w/w); 2.5 h ~ 39% (w/w)) while a reduction to 35% (w/w) was observed at 1.25 h HRT (Figure 4.6).

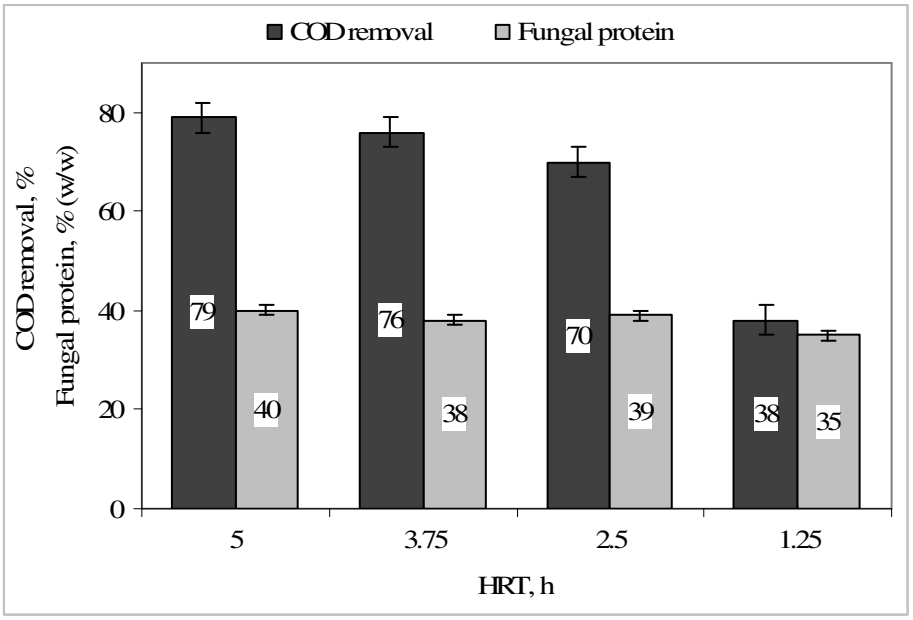


Figure 4.6 Effect of HRT on percentage-COD removal and -fungal protein yield in a PCS biofilm continuous-reactor (n=2)

The oxygen uptake rate increased with the reduction in HRT from 5 to 2.5 h, indicating a greater microbial activity at lower HRTs (5 h ~ 1.64 $mgL^{-1}min^{-1}$; 3.75 h ~ 1.72 $mgL^{-1}min^{-1}$; 2.5 h ~ 1.96 $mgL^{-1}min^{-1}$) (Figure 4.7). A significant decrease to 0.40 $mgL^{-1}min^{-1}$ was found at 1.25 h HRT due to biomass washout.

The substrate contact time with biomass has an important effect on the COD removal. Maximum COD removal was achieved at 5 h due to longer contact time available between wastewater and fungal biofilm. Decrease in HRT enhances the organic loading rate, consequently resulting in faster biomass growth and thicker biofilm (Chen and Chai, 2005; Kwok et al., 1998; Peyton, 1996; Tijhuis et al., 1994; Yang et al., 2004). Hydrophobicity plays an important role in self-immobilization and attachment of microbial cells (Pringle and Fletcher, 1983). Short HRTs were reported to produce bacterial biomass with high cell hydrophobicity (Pan et al., 2004), also resulting in the formation of thicker biofilm. Similar effect could be expected on fungal biomass, as fungi too were known to alter their hydrophobicity as a survival action against adverse environmental conditions (Smits et al., 2003). The biofilm thickness has a linear effect on detachment rates (Lin et al., 2004; Patel et al., 2005). Higher detachments augment the suspended or effluent biomass concentrations. This explains the increased biomass productions from 5 to 2.5 h HRT.

A significant washout of biomass leading to poor bioreactor performance was observed at the shortest HRT of 1.25 h. Pan et al. (2004) also reported similar observation at the shortest HRT of 1.0 h in sequential aerobic sludge blanket reactors with aerobically grown microbial granules. This may imply that at an HRT of 1.0 h is too short to retain biomass in attached growth systems. The strength of biofilm is related to organic loading rate (Nicolella, 2000). Excessive bacterial biofilm detachment rates that lead to eventual washout is commonly observed at higher substrate loadings (Characklis et al., 1990; Chen and Chai, 2005; Peyton, 1992; Tijhuis et al., 1994, 1996). Similar to bacteria, the effect of substrate concentration on fungal growth may be expressed by Monod model for microbial growth kinetics (Finkelstein and Ball, 1992). Thus, the reduced reactor efficiency at 1.25 h HRT could be attributed to the extremely high organic loading rate. An increase in organic loading rate was found to decrease the

DO concentration in bacterial biofilm and impose an adverse effect on the biofilm stabilization (Ong et al., 2004; Zhang et al., 1994).

The absorbance at each HRT (5 h ~ 0.67 Abs_{620}; 3.75 h ~ 0.69 Abs_{620}; 2.5 h ~ 0.69 Abs_{620}; 1.25 h ~ 0.37 Abs_{620}) correlated (R^2=0.98) with the corresponding observed biomass yields (Figure 4.7). The biomass production at an HRT of 2.5 h was about 0.82 $gVSS/L_{WWtreated}$, significantly higher than that at HRTs of 5 h (~0.63 $gVSS/L_{WWtreated}$), 3.75 h (~0.69 $gVSS/L_{WWtreated}$) and 1.25 h (~ 0.20 $gVSS/L_{WWtreated}$). Fungi dominated at all HRTs and the respective mass fractions of fungi/total biomass were 90, 93, 94 and 85% at 5, 3.75, 2.5 and 1.25 h HRT, respectively (Figure 4.8). Effective control of bacterial growth without fungal biomass washout was achieved at HRTs as low as 2.5 h.

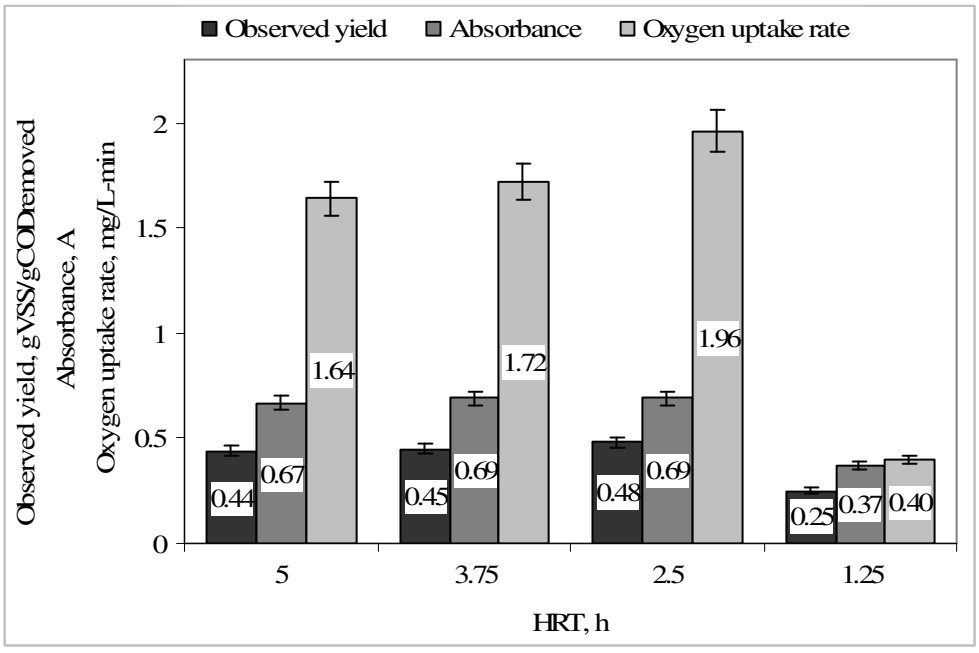


Figure 4.7 Effect of HRT on observed biomass yield, absorbance (620 nm) and oxygen uptake rate in a PCS biofilm continuous-reactor (n=2)

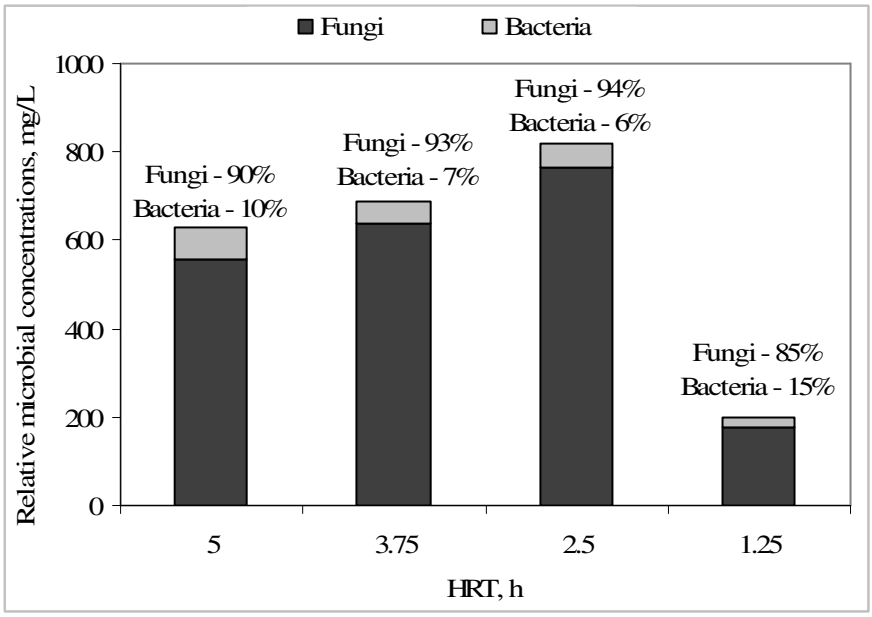


Figure 4.8 Effect of HRT on relative bacterial and fungal concentrations in a PCS biofilm continuous-reactor (n=2)

Van Leeuwen (2003) and van der Westhuizen and Pretorius (1996, 1998) observed an efficient bacterial elimination from a suspended growth fungal system at HRTs less than 6 and 3 h, respectively. In both studies, the rectors were employed with a microscreen to prevent the fungal washout at the lower HRTs required for bacterial control. Jin et al. (2001) reported a significant reduction in the external airlift bioreactor performance in terms of COD reduction and fungal biomass productivity at HRTs less than 6.25 h. In comparison to the bioreactors used in precedent studies, the PCS biofilm continuous-reactor has an obvious advantage of achieving optimal fungal bioconversion and bacterial elimination at relatively lower HRTs.

The above results show that an HRT of 5 h was most suitable for COD removal, whereas an HRT of 2.5 h was optimal for biomass yield. Shorter HRTs are favorable on industrial scale due to higher bioconversion rates and smaller required reactor footprint. However, continuous operation at 5 h HRT until steady state prior to 2.5 h HRT

operation was found essential during the subsequent studies. In one particular experiment, disintegration of biofilm was observed during steady state when a new carboy of wastewater (from same batch) was used as a feed. Change in the composition of the wastewater during storage was suspected to create a stress on the biofilm leading to its disintegration. Inhibited fungal growth was identified with stored potato processing wastewaters (Stevens and Gregory, 1987). The authors observed dense bacterial populations (~10^9 bacteria/mL) and the fungal inhibition might have occurred due to the addition one or more bacterial growth byproducts to wastewater. López et al. (2001) reported a significant influence of wastewater storage on xanthan production from olive-mill wastewater in shake-flask bacterial cultures. The biofilm reactors are more vulnerable to sudden changes in substrate composition, particularly at short retention times (Jeppsson, 1996). This indicates that a longer HRT than 2.5 h may be more suitable on a bench-scale. Optimal and stable performance at 2.5 h HRT may still be achieved on an industrial scale due to the availability of continuous fresh wastewater supply. The effect of HRT on COD removal, biomass yield and protein content in PCS biofilm continuous-reactor is summarized in Table 4.3. This summary makes it clear that an optimization curve would be rather flat, so that different HRTs should not have a large effect on yields and COD removal.

Table 4.3 Summary of the effect of HRT on corn wet milling wastewater treatment in a PCS biofilm continuous-reactor (n=2)

HRT	COD removal	Observed biomass yield	Biomass protein	Biomass fraction	
h	%	$gVSS/gCOD_{removed}$	% (w/w)	% (w/w) in total biomass	
				Fungi	Bacteria
5.0	79.1 ± 0.67	0.44 ± 0.003	40.3 ± 1.21	90.4 ± 0.57	09.6 ± 0.57
3.75	76.3 ± 0.80	0.45 ± 0.002	38.2 ± 0.65	92.8 ± 0.26	07.2 ± 0.26
2.5	69.9 ± 1.47	0.48 ± 0.015	39.0 ± 0.41	93.6 ± 0.29	06.4 ± 0.29
1.25	37.6 ± 0.90	0.25 ± 0.025	35.1 ± 0.15	84.6 ± 0.38	15.4 ± 0.38

CONCLUSIONS AND RECOMMENDATIONS

Optimizing process parameters is crucial for economic and efficient wastewater treatment operation. The optimal pH and HRT for an attached growth fungal treatment system with PCS medium were evaluated in this study. Operating pH of 4.0 was found optimal for both COD removal and biomass yield. Superior fungal dominance of 93% was also obtained at pH 4.0. Reactor performance was found to be highly dependent on the wastewater composition. Higher suspended solids in wastewater resulted in reduced COD removal and biomass production.

COD removal improved with increase in HRT. An HRT of 2.5 h attained maximum biomass production at a high treatment rate, ultimately reducing the required footprint of a bioreactor. However, the biofilm failure to withstand the changing composition of stored wastewater implied a necessity to operate at HRTs longer than 2.5 h on bench-scale. On an industrial scale, continuous supply of fresh wastewater may improve the stability of the system at 2.5 h HRT and achieve the optimal performance. Variation in wastewater composition can be expected on the industrial scale also because of both the nature of the raw product and the production schedule of most plants (US EPA, 1978). But it may or may not have similar effect on the system performance. Therefore, a study with a continuous supply of fresh wastewater is necessary to conclude on optimal HRT.

The attached growth fungal process employed in this study demonstrated an ability to withstand smaller HRTs without fungal biomass washout. The higher treatments rates obviously offer economical advantage on industrial scale with lower capital and operational costs. The process is easily applicable to all food-processing wastewaters to produce relatively pure fungal protein, which could be used as a source of protein for growing populations in developing nations around the globe.

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5. TREATMENT OF CORN PROCESSING WASTEWATER IN AN ATTACHED GROWTH FUNGAL SYSTEM: EFFECT OF GAS COMPOSITION AND AERATION RATE

(Manuscript will be submitted to '*Biotechnology and Bioengineering*' journal)

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Abstract: An adequate oxygen supply is vital for successful operation of biological systems. Optimization of air supply rate without compromising the treatment efficiency is essential for an economical benefit to aerobic wastewater treatment pants. Effects of gas composition and aeration rate on oxygen availability and microbial consumption in an attached growth fungal system with plastic composite support (PCS) medium were studied. *Rhizopus oligosporus* was used to treat corn wet milling wastewater. Supply of air rather than a gas mixture of oxygen (20% (v/v)) and nitrogen (80% (v/v)) at a rate of 0.8 vvm (1.0 $Lmin^{-1}$) was found optimal. Increase in the airflow rates improved chemical oxygen demand (COD) removal as well as biomass production. *In-situ* dissolved oxygen (DO) concentrations indicated an oxygen limiting condition in the reactor at all airflow rates. Fungal biomass exhibited better settleability at higher airflows. Detailed study on

hydrodynamic properties and mass transfer characteristics in a pilot scale reactor is warranted for better optimization of aeration system.

Keywords: fungal wastewater treatment; attached growth; airflow rate; oxygen consumption; off-gas analysis

INTRODUCTION

Oxygen is often the rate limiting substrate/nutrient in aerobic biological treatment of wastewater. Because of its low solubility and consequent low transfer rate, a sufficient supply to meet the demand in biological reactors is one of the major limitations (Nicolella et al., 2000). Most aerobic bioreactors are supplied with sparged air or pure oxygen as small gas bubbles to provide oxygen necessary for microbial growth or maintenance of cellular functions (Metcalf and Eddy, 2003). Energy costs for aeration typically account for 60 to 65% of the total energy consumption cost in a wastewater treatment plant (Dempsey, 1994). Inadequate air/oxygen supply results in poor performance of aerobic biological systems, where as an excessive supply adds-up unnecessary capital and operating costs. Optimization of aeration or oxygen supply rate is therefore critical; especially in the aerobic treatment of high organic strength wastewaters such as corn wet milling wastewater used in this study.

As of 2002, there are 61 corn wet milling industries in the United States with a typical plant processing at least 2,500 tons of corn per day and operating year-round (Rausch, 2002). Corn wet milling plant operation involves separation of starch, protein, fiber and oil for producing commercial co-products, such as corn gluten meal and feed, ethanol, corn oil, etc. These plants also provide pure starch products (>99.5%) for the paper and corrugating industries, modified starches for food ingredients and high fructose corn syrup.

About 30 to 48 gallon of organic rich wastewater per pound of corn processed is generated in wet milling (James Foster, 7th June 2005; personal communication, ADM, Clinton, IA). Huge quantities of bacterial sludge are produced when these wastewaters

are treated with conventional suspended growth aerobic treatment systems. The bacterial sludge produced is of little value and its management alone contributes to 60% of the treatment plant operating expenses (Canales et al., 1994), costing about $ 35-38 per dry ton (US EPA, 1999).

In contrast, the attached growth fungal system employed in this study produces highly valuable fungal sludge with concomitant organic removal (Jasti et al., Paper one and Paper two in Dissertation). The fungal biomass produced is a good source of high value protein and bio-chemicals (e.g. enzymes, organic acids, etc.) (Barbesgaard et al., 1992; Jin et al., 1999a,b,c; Ravinder et al., 2003; Wang et al. 1974). Recombinant strains of fungal species *Aspergillus oryzae* were recently found as a source of active human proteins, such as lactoferrin (Ward et al., 1992) and lysozyme (Tsuchiya et al., 1992). Additionally, the system is less prone to bacterial competition, a common threat in non-aseptic fungal treatment systems, because of strong fungal affinity to surface attachment and steadily maintained high fungal density in the reactor through immobilization. Plastic composite support (PCS) tubes developed at Iowa State University with 50% polypropylene and 50% agricultural products, were used as support medium for fungal attachment (Demirci and Pometto, 1995; Ho et al., 1997a,b,c).

The main goal of this study was to optimize the oxygen supply to PCS biofilm continuous-reactor by evaluating the effect of oxygen percentages (v/v) in an influent gaseous mixture of oxygen and nitrogen and aeration rates.

MATERIALS AND METHODS

Culture Medium

Wastewater obtained from Archer Daniels Midland (ADM) corn wet milling plant located in Clinton, IA was used as substrate. The important wastewater characteristics are shown in Table 5.1. The wastewater pH ranged from 5.7 to 10.3, more commonly at lower pH.

Table 5.1 Characteristics of ADM corn processing wastewater*

Parameters	Unit	Concentration (mg/L)
Total chemical oxygen demand (TCOD)	mg/L	1,870 to 3,470
Soluble chemical oxygen demand (SCOD)	mg/L	1,690 to 3,190
Total suspended solids (TSS)	mg/L	250 to 300
Volatile suspended solids (VSS)	mg/L	150 to 200
Biochemical oxygen demand (BOD_5)	mg/L	1,100 to 2,200
Total Kjeldahl nitrogen (TKN) as N	mg/L	30 to 78
Total phosphorus (TP) as P	mg/L	3 to 32
pH		5.7 to 10.3

*Based on seven batches of wastewater obtained from ADM plant (n=7, where n is the number of wastewater batches used for study/analysis)

The wastewater was relatively low in macronutrients (nitrogen and phosphorus) and previous studies indicated a need for nutrient supplementation to achieve optimal bioconversion (Jasti et al., Paper one in Dissertation). Therefore, the wastewater was supplied with nitrogen as NH_4HCO_3 and phosphorus as K_2HPO_4 to maintain a COD:N:P ratio of 150:10:1.

Fungal Strain and Inoculum Preparation

Rhizopus oligosporus (ATCC #22959) was obtained from American Type Culture Collection (Rockville, MD). This strain is extensively used in Unites States to produce enzymes such as amylase, protease and lipase (Sutardi and Buckle, 1985), and soybean tempeh (Medwid and Grant, 1984). The culture was revived in yeast-malt (YM) nutrient broth (Difco Laboratories, Sparks, MD) at 24ºC, which was then transferred onto numerous potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) plates and incubated at room temperature (~24ºC) for 6 to 7 days. Fungal spores were harvested from the surface of PDA plates into sterile distilled water containing 0.85% NaCl (w/v)

saline solution and 0.5% (v/v) of Tween 80 (Fisher Scientific, Fair Lawn, NJ). The harvested culture was diluted further to achieve a spore count of 10^6 to 10^7 spores/mL, determined by haemocytometer counts. Glycerin (20%, v/v) was added to the spore suspension as a cryoprotectant for ultra-low frozen storage at -75^{o}C in sterile 2 mL cryo-vials for future use as a bioreactor inoculum.

Support Medium for Fungal Biofilm

PCS tubes composed of 50% (w/w) polypropylene (Quantum USI Division, Cincinnati, OH), 40% (w/w) ground soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (w/w) dried bovine albumin (Proliant, Des Moines, IA), and 5% (w/w) yeast extract (Ardamine Z; Red Star BioProducts, Juneau, WI) were used as support medium for fungal growth.

The PCS tubes were fabricated via high temperature extrusion (Cotton et al., 2001) using a twin-screw co-rotating Brabender PL2000 extruder (Model CTSE-V; Brabender Instruments, South Hackensack, NJ), with internal and external diameters of 7.0 and 10.5 mm, respectively (Ho et al., 1997a,b,c).

PCS Biofilm Reactor

New Brunswick Bioflo 3000 fermentor (New Brunswick Scientific, Edison, NJ) with a working volume of 1.25 L was used. Six PCS tubes of 60 mm length were bound in a grid-like fashion, as shown in Figure 5.1a, and fixed to the agitator shaft of the reactor (Cotton et al., 2001). The ends of PCS tubes were cut at an angle to promote substrate flow through the tubes. Figure 5.1b shows the schematics of the fungal biofilm reactor.

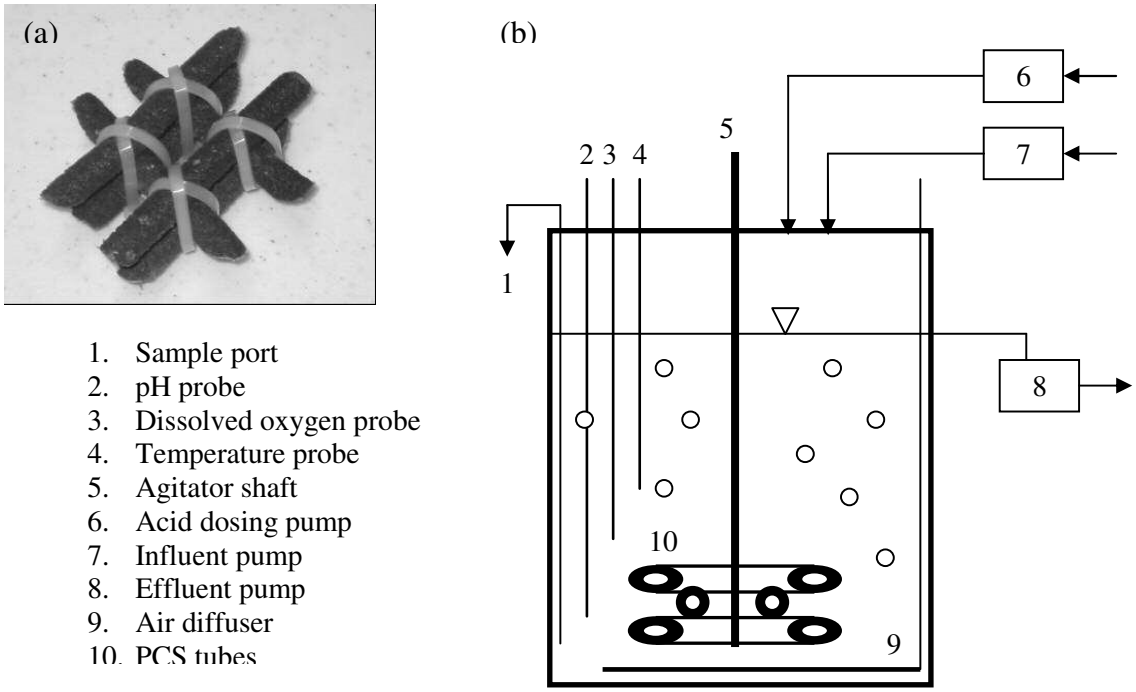

Figure 5.1 (a) Plastic composite support (PCS) medium grid layout; (b) Schematic diagram of attached growth fungal bioreactor

Batch Process. Biofilm formation on the PCS medium was achieved in batch mode after inoculating the heat-sterilized potato dextrose broth (PDB) (Difco Laboratories, Sparks, MD) with 2 mL of fungal spore suspension. The batch cultivation was operated for 1-2 days at a temperature of 37°C for high metabolic activation (Thanh and Nout, 2004) and pH of 4.0 for maximum germination (Medwid and Grant, 1984) of R. oligosporus sporangiospores. Agitation was maintained at 250 rpm. The biofilm formed during batch operation maintains a high fungal cell density in the reactor that can impede the proliferation of the wastewater-borne bacteria during subsequent continuous operation with non-sterilized wastewater.

Continuous Process. Upon adequate biofilm development, the reactor operation was transferred to continuous mode. Temperature of 37°C, pH of 4.0, hydraulic retention time (HRT) of 5 h and agitation speed of 250 rpm were maintained throughout the study. These conditions were found optimal during previous studies (Jasti et al., Paper one and Paper two in Dissertation). A constant biofilm thickness was maintained under steady state conditions (steady COD removal and biomass production) by continuous detachment of the biomass into the mixed liquor surrounding the biofilm. The biomass in suspension thus leaves the reactor along with the effluent. At steady state, the biofilm growth rate equals the biofilm detachment rate (Rittmann and McCarty, 2001). The reactor sample was collected for analysis from a sample port provided on the top of Bioflo 3000 fermentor.

Experimental Methods

Gas flow rates and percent volumes of oxygen in a gas are equally important for adequate oxygen consumption in an aerobic bioreactor (Bischof et al., 1996). The percent volume determines the amount of oxygen available for microbial growth and cell functions, whereas the gas flow rate effects gas hold-up and contact time in the reactor thereby controlling the oxygen transfer to the microorganisms. Optimizing one or both of above parameters could result in considerable reduction in energy costs for oxygen supply.

Effect of Gas Composition. Initially, a gas composition study was performed by assuming that a gas flow rate of 0.8 vvm (1.0 $Lmin^{-1}$) provides enough gas hold-up and contact time in the reactor. The effect of oxygen percentage (v/v) in influent gas mixture of oxygen and nitrogen was investigated at 20.0, 10.0, 5.0 and 2.5 % (v/v) of oxygen. The gases were alternately pulsated into the reactor over a pulsing period of ~2.5 sec. The pulsing period for each gas varies with respective percentage (v/v) in the influent gas. The main aim was to check if an excess of oxygen is being supplied to the reactor. The dissolved oxygen (DO) probe was calibrated to 100% saturation under steady state at

0.8 vvm (1.0 $Lmin^{-1}$) air supply (oxygen ~ 20.9% (v/v); nitrogen ~79.1% (v/v)) and the DO concentration at each percentage (v/v) of influent gas oxygen was monitored. The reactor off-gas oxygen and carbon dioxide concentrations were monitored for each in-gas oxygen percentage (v/v). The analyzers were calibrated with respective influent gas prior to each experiment. Knowing the optimal oxygen percentage (v/v) in influent gas, the actual volume of oxygen required in the reactor could be determined and used as a reference for any future studies that use gaseous mixtures for oxygen supply.

Effect of Aeration Rate. Aeration rate is one of the most important factors that influence the oxygen transfer to the microbial cells. Airflows at 0.8 (1.0), 0.6 (0.75), 0.4 (0.5) and 0.2 vvm (0.25 $Lmin^{-1}$) were evaluated by monitoring the percentages (v/v) of oxygen and carbon dioxide in the reactor off-gas. The off-gas oxygen and carbon dioxide analyzers were calibrated with respective airflow rate before each experiment. At each airflow rate, DO concentration in reactor was monitored with built-in DO meter, and the COD removal and biomass production were analyzed as well.

Reactor Off-gas Analysis

Off-gas oxygen concentration measurement is common in activated sludge plants to estimate the oxygen transfer rate and control the aeration (Tanuma et al., 1981). Spérandio and Paul (1997) observed a close connection between oxygen consumption, substrate degradation and carbon dioxide evolution in a stirred tank reactor seeded with activated sludge. The off-gas oxygen concentration was obtained using microprocessor-based Percent Oxygen Analyzer (Model 3300PA; Teledyne Analytical Instruments, City of Industry, CA). Gas Analyzer (Model EX-2000; New Brunswick Scientific, Edison, NJ) with non-dispersive infrared sensor was used to determine the off-gas carbon dioxide concentration. Both analyzers were equipped with a simultaneous data export to computer.

Analyses

The wastewater was analyzed for COD, BOD_5, TSS, VSS, TP and TKN as per Standard Methods (APHA/AWWA/WEF, 1998). During aeration study, the effluent samples obtained under steady were analyzed for COD and VSS. The fungal biomass production was determined as g VSS produced per L wastewater treated per hour. *In-situ* oxygen uptake rate of the biomass was measured during each run by stopping the agitation and airflow, and monitoring the rate of dissolved oxygen decrease (respiration rate). The results of airflow rate study were an average of two experiments (n =2).

RESULTS AND DISCUSSION

Effect of Gas Composition

The reduction of percentage (v/v) oxygen in influent gas decreased both off-gas oxygen and carbon dioxide (Figure 5.2). Furthermore, rapid decline of DO levels was noticed in the reactor (Figure 5.2). A huge drop from 45 to 9.6% DO was observed between 20 and 10% (v/v) oxygen, with a further decrease to 2.6 and 1.6% DO at 5.0 and 2.5% (v/v) oxygen, respectively. The downward trend of off-gas carbon dioxide along with DO proves that higher percent (v/v) oxygen in influent gas grants relatively higher oxygen availability as well as consumption. It is evident from the results that a mixture of 20% (v/v) oxygen and 80% (v/v) nitrogen is optimal within the tested range of nitrogen and oxygen gas compositions.

When shifted from air to 20% (v/v) oxygen gas mixture supply, the DO concentration dropped by about 50% while the oxygen consumption was almost unchanged. During this study, pure oxygen and nitrogen were supplied to the reactor by alternate pulsing of the gases. The pulsing periods, which were controlled automatically in the fermentor, change linearly with the influent gas percentages (v/v). This could mean a short pulsing period of pure oxygen at long intervals for the influent gas oxygen percentages (v/v) used in this study.

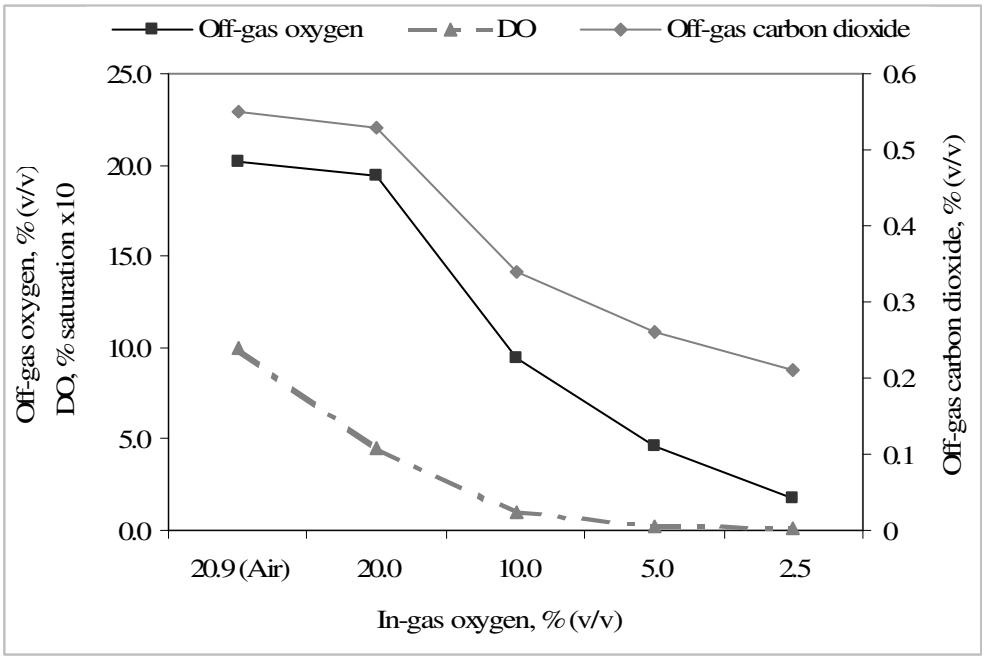

Figure 5.2 Effect of influent gas oxygen percentage (v/v) on off-gas percentages (v/v) of oxygen and carbon dioxide, and DO concentrations in a PCS biofilm continuous-reactor (n=1)

The total oxygen available in the reactor could be lower with the intermittent supply of pure oxygen when compared to that with continuous air supply. At constant microbial oxygen consumption rates, this may lead to lower DO concentration as it is nothing but the excess oxygen available after microbial consumption.

The diffusion of oxygen to the interior parts of biofilm enhances when the reactor is supplied with oxygen rather than air (Casey et al., 1999; Qureshi et al., 2005). Better penetration into biofilm leads to improved oxygen consumption. Higher oxygen uptakes were observed with air than oxygen (in combination with nitrogen) in this study, which could have been due to the fact that a gaseous mixture of oxygen and nitrogen was supplied and the above theory is based on pure oxygen supply. Pure oxygen supply on industry scale is highly expensive. Alternatively, supply of systems with air-oxygen

mixture achieves superior system performance compared to that with air supply due to higher availability of oxygen to penetrate the biofilm. It is also more economical than pure oxygen supply. Significant improvement in fermentation rate and lactic acid yields were reported with supply of air-oxygen mixture at a volumetric ratio of 5:1 to a rotating fibrous bed bioreactor (Tay and Yang, 2002).

For 100% saturation in water, the DO concentration is 6.72 mg/L at 37°C temperature and standard pressure. Thus, the DO concentrations calculated from observed % DO saturations in the reactor were 3.02, 0.64, 0.17, 0.11 mg/L at 20.0, 10.0, 5.0 and 2.5% (v/v) oxygen, respectively. The actual values in the reactor could be even lower due the high viscosity of the mixed liquor containing suspended fungal mycelia. Oxygen limitation affects the metabolic and oxygen utilization rates of a biomass for DO concentrations below 1.6 mg/L (Bailey and Ollis, 1986). Ideally, a minimum of 1 to 2 mg/L of DO concentration is maintained in typical wastewater treatment systems (Metcalf and Eddy, 2003). Therefore, it can be derived that the oxygen limitation occurred in the reactor below percentage oxygen in influent gas of 20% (v/v), which provided a DO level saturation of 45%. Jin et al. (2001) maintained a DO level above 50% of saturation to ensure oxygen supply enough to match the requirement in an external airlift fungal bioreactor. Tay and Yang (2002) postulated that the poor oxygen uptake rates by *Rhizopus oryzae* even at above critical *in-situ* DO concentrations indicate relatively much lesser oxygen concentration in the mycelial layer than that in the reactor medium. Thus, they found it necessary to maintain a high DO level of 90% for optimal oxygen transfer to the mycelial biofilm in a rotating fibrous bed bioreactor.

Minimum of about 20% oxygen (v/v) supply by air or an oxygen-nitrogen gas mixture was proved essential from the highest oxygen consumptions demonstrated at those conditions. As mentioned before in this paper, the gas composition study was conducted by hypothesizing that the flow rate of 0.8 vvm provides sufficient gas hold-up and gas-microbial contact time in the reactor. Bischof et al. (1996) stated that faster rise of air bubbles leads to decrease in oxygen transfer. Aeration rate is one of the factors that control the bubble rise. Hence, the subsequent studies were conducted to investigate

the possibility of optimizing the aeration system by reducing the airflows and thereby providing longer gas hold-up and contact times.

Effect of Aeration Rate

At airflow rates of 0.8, 0.6, 0.4 and 0.2 vvm, the off-gas oxygen readings were 20.26, 20.16, 20.13 and 20.06% (v/v) with corresponding off-gas carbon dioxide readings of 0.52, 0.58, 0.67 and 0.93% (v/v), respectively (Figure 5.3). Evidently, the trend indicates higher oxygen consumption at lower airflow rates. A decrease in airflow rate resulted in reduced COD removal as presented in Figure 5.4 (0.8 vvm ~ 76%; 0.6 vvm ~ 65%; 0.4 vvm ~ 60%; 0.2 vvm ~ 41%). Similarly, higher biomass productions were obtained at higher airflows from 0.2 to 0.6 vvm (0.2 vvm ~ 0.12 gVSS/L-h; 0.4 vvm ~ 0.08 gVSS/L-h; 0.6 vvm ~ 0.06 gVSS/L-h). But, slightly lower biomass production of 0.10 gVSS/L-h was observed at 0.8 vvm (Figure 5.4).

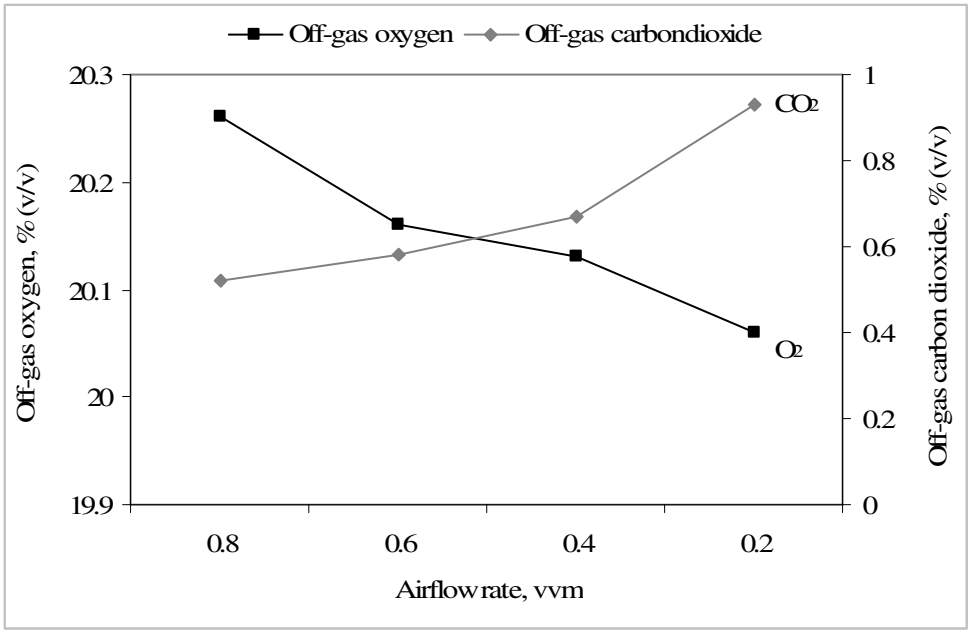

Figure 5.3 Effect of aeration rate on percentages (v/v) of oxygen and carbon dioxide in a PCS biofilm continuous-reactor off-gas using air (n=2)

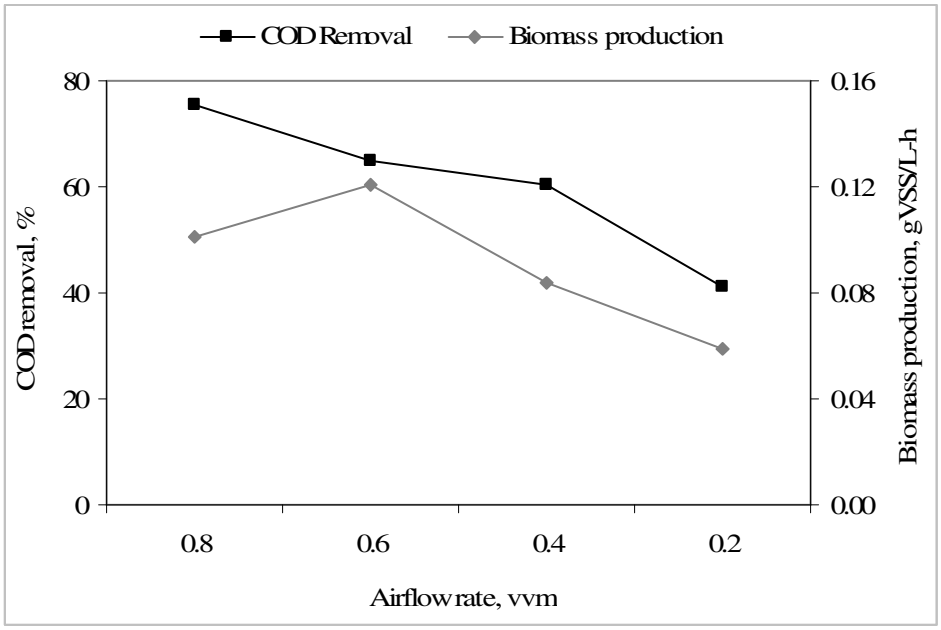

Figure 5.4 Effect of aeration rate on percentage COD removal and biomass production in a PCS biofilm continuous-reactor using air (n=2)

Studies on mass transfer characteristics in fermentations proved that the oxygen transfer rate generally increases with an increase in airflow rate, resulting in an improved biomass production (Ahmad et al., 1994; Jin et al., 2001). The results of this study agree with above reports between the airflows of 0.2 and 0.6 vvm; but found in disagreement with Vanhooren (2001), who reported an insignificant effect of airflows on oxygen transfer. The increase in biomass production between 0.8 and 0.6 vvm airflows may be attributed to biomass wash out from the reactor. The DO concentrations were observed to be close to zero at all airflows except for 0.8 vvm (~ 1 mg/L). Poor oxygen transfer at lower airflows led to oxygen limitation in the reactor inhibiting the microbial growth and weakening the biofilm, with subsequent biomass washout from the reactor.

The possible reason for higher off-gas carbon dioxide measurements at lower airflows is the reduction in fungal growth along with the increase in endogenous decay.

When more oxygen is consumed for cell maintenance and endogenous decay rather than cell growth, the carbon dioxide production increases (Vanhooren, 2001).

Pictorial presentation of reactor samples in Figure 5.5a demonstrates a better settleability of fungal biomass at higher airflows. This is in agreement with Martins et al. (2003), who reported a strong negative effect of low DO concentrations on sludge settleability. At the higher airflows the shear stress on fungal biomass is higher, which could result in fungal clumping and thereby increase settleability (Yang et al., 2004). Settleability is an extremely important factor in fungal biomass harvesting and a poor settleability could increase the operational costs of a fungal wastewater treatment plant. No severe damage to the biofilm was observed at the end of the experiment (Figure 5.5b), showing that the biofilm deterioration was not occurring rapidly. However, prolonged operation at lower aerations could eventually lead to biofilm disintegration. Thus, an airflow rate of 0.8 vvm found to be optimal within the investigated flow rates.

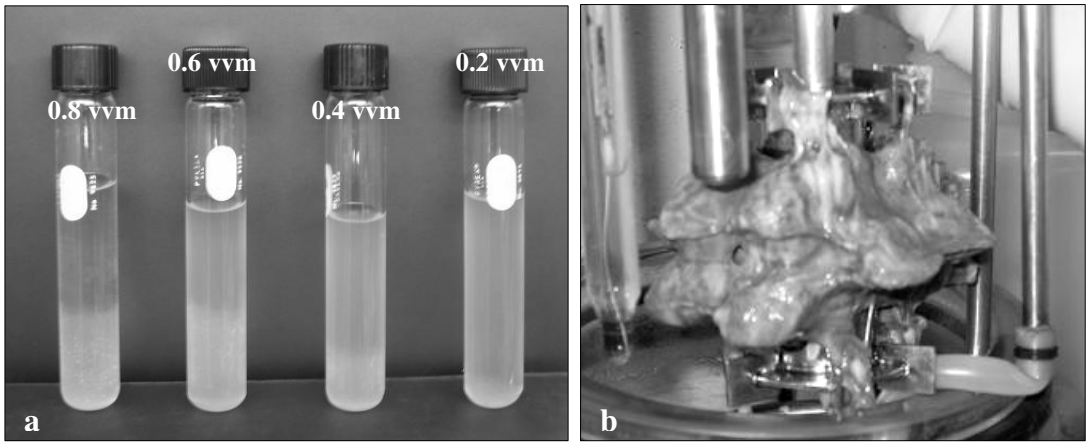

Figure 5.5 (a) Visual comparison of PCS biofilm continuous-reactor samples at different airflow rates; (b) Attached biofilm at the end of aeration study in a PCS biofilm continuous-reactor

CONCLUSIONS AND RECOMMENDATIONS

Both gas composition and aeration rate studies established 0.8 vvm supply as best suited among the examined values for an optimal bioconversion of corn wet milling wastewater organics to highly settleable fungal biomass in a PCS biofilm continuous-reactor. The higher settleability observed at faster airflows could be of engineering significance, as it enables the application of simple and economical harvesting methods such as settling or screening.

The aeration study indicated a possible oxygen limitation in the reactor under steady state (DO ~ 1 mg/L), which prompts for investigation of higher airflow rates to optimize the oxygen supply. However, only a small increase in oxygen transfer was observed by Jin et al., (2001) even at higher airflows of 1.25 to 2.00 vvm in a pilot plant external air-lift bioreactor for fungal biomass production. It was reported that increasing the airflow rate at high fungal biomass concentrations fails to obtain a desired DO level to meet oxygen demand sufficient for fungal growth (Jin et al., 1999b, 2001). Additionally, the higher aerations may lead to extreme turbulence in the PCS biofilm continuous-reactor due to its smaller vessel size (1.25 L working volume) and the consequent increase in shear rates affects the biofilm stability. The gas retention, gas-liquid and gas-biofilm contact times are dependent on the reactor size. Higher hydrostatic pressure in deeper tanks increases the oxygen transfer. This reduces the required air volumes, ultimately resulting in less operation cost (Bischof et al., 1996). Small liquid depths severely reduce the gas hold-up time at higher airflows. Walls effects are more pronounced in smaller reactors as well. Large-scale reactors should be able to meet the high oxygen demands of viscous fungal broth at relatively low aeration rates due to the fact that the aeration efficiency increases with the size of the vessel (Barker and Worgan, 1981).

The most common approach to enhance the oxygen transfer rate is increasing the agitation rate. For some bioreactors, increasing aeration rates alone may not rectify oxygen-limiting conditions (Del Re et al., 2003). Gas dispersion improves with agitation speeds and leads to more mass transfer. Ahmad et al. (1994), Del Re et al. (2003) and

Zhang et al. (2006) reported improved oxygen transfer rates at higher agitation speeds in suspended growth reactors. However, biofilm reactors are comparatively more sensitive to agitations. At critical agitation speeds specific to reactors, biofilm detachment could occur due to increased shear forces limiting the applicability of agitation for enhancing oxygen transfer.

An adequate and efficient contact between gas and liquid phases is critical to obtain high reaction rates (Khare and Niranjan, 1995), particularly in highly viscous liquids. Increasing gas-liquid interfacial surface area by bubble size reduction is another approach to enhance the oxygen transport to microorganisms. Smaller bubbles provide longer gas hold-up or bubble residence time because of their comparatively slow rise in the fluid. Use of microbubble dispersion for oxygen supply increased the transport of oxygen in stirred tank reactor growing *Saccharomyces cerevisiae*, especially when control of agitation to achieve it is limited (Kaster et al., 1990). However, clogging of air diffusers or spargers is a common problem in fungal systems due to dense mycelial growth. Jin et al. (2001) observed clogging of air sparger beyond three days of continuous cultivation of *A. oryzae* and *R. arrhizus* in an external airlift bioreactor.

Further investigation on hydrodynamics and mass transfer aspects to determine and evaluate the factors that affect oxygen transfer (such as liquid properties, gas hold-up, mixing time, oxygen transfer coefficient, biofilm density, surface area and thickness, etc.) is needed. Considering the limitations of bench-scale reactor used in this study and relative advantages of large-scale reactors, a pilot-scale study is warranted for better understanding of hydrodynamics and optimization of aeration rates as well.

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6. GENERAL CONCLUSIONS

The attached growth fungal system with plastic composite support (PCS) medium was highly effective in treating corn wet milling wastewater with simultaneous recovery of high value fungal biomass.

Aseptic treatment was found impractical despite of the pure fungal biomass production. Fungal growth and bioconversion rates were severely reduced due to suspected inhibitory compound production during thermal sterilization. Also, harvesting could not be achieved because of fungal accumulation in the reactor during continuous operation.

Nutrient supplementation significantly improved the system performance under both aseptic and particularly non-aseptic conditions. Additional revenue from improved biomass yield could offset the nutrient supplementation costs. The fungal biomass in the effluent under non-aseptic conditions was easily settleable, which enhances the harvesting of fungal biomass from the wastewater in operational applications.

The suspended growth studies showed by contrast that the support medium was very effective in retaining fungal biomass and maintaining high fungal cell density in the reactor during continuous operation. The control studies with polypropylene confirmed that the agricultural products in PCS medium act as slow releasing nutrient carriers and therefore were necessary for biofilm formation during batch-mode cultivation and high fungal cell density maintenance during subsequent continuous operation.

Maximum system efficiency in terms of COD removal, biomass yield and biomass protein was achieved at pH of 4.0. Superior fungal dominance of 93% (of total biomass) was obtained at pH 4.0 as well. COD removal improved with increase in hydraulic retention time (HRT). The attached growth system with PCS supports demonstrated a remarkable ability to successfully eliminate bacterial competition without washing out fungal biomass at lower HRTs. Maximum biomass production at a high

treatment rate was achieved at an HRT as low as 2.5 h, ultimately reducing the required footprint of a bioreactor. This HRT was lower than that required in suspended growth systems employed with microscreen to prevent fungal washout (van der Westhuizen and Pretorius, 1996, 1998; van Leeuwen, 2003). The next lower HRT of 3.75 h was recommended as a compromise for bench-scale operation as the biofilm failed to withstand the changing composition of stored wastewater at 2.5 h HRT. However, a continuous fresh wastewater supply on an industrial scale may achieve stable system performance at an HRT of 2.5 h. This could be confirmed by a pilot-scale study at a corn wet milling plant.

Effect of wastewater composition on reactor performance was observed during both suspended growth and HRT studies. Storage and high-suspended solid content of wastewater resulted in reduced COD removal and biomass production.

The aeration study indicated a possible oxygen limitation in the reactor under steady state (DO ~ 1 mg/L) even at the highest aeration rate of 0.8 vvm (1.0 L/min) and thus prompts for further investigation to optimize the air supply for better oxygen transfer. The reactor was operated at optimal operating conditions (pH of 4.0, temperature of 37°C and agitation of 250 rpm). Increasing aeration and/or agitation to improve the oxygen transfer may increase the shear rates leading to extreme turbulence in the PCS biofilm continuous-reactor and ultimately affecting the biofilm stability. The smaller vessel size (1.25 L working volume) could be a limitation for oxygen transfer because of smaller gas holdup and contact times. Further investigation on hydrodynamics and mass transfer aspects to determine and evaluate the factors that affect oxygen transfer (such as liquid properties, gas holdup, mixing time, oxygen transfer coefficient, biofilm density, surface area and thickness, etc.) is recommended. Considering the limitations of bench-scale reactor used in this study, a pilot-scale study is warranted for better understanding of hydrodynamics and optimization of aeration rates as well.

Rhizopus oligosporus, the fungal strain used for the treatment, is widely known for its application in food fermentation. Efficient reduction of bacterial contamination in

the PCS biofilm continuous-reactor at optimal pH and HRT led to relatively pure fungal biomass production. Thus, the recovered fungal biomass could be potentially used as a protein source in animal feed. The system operation could be easily modified and/or optimized for the production of other high value fungal fermentation byproducts (e.g. enzymes, organic acids etc.). The fungal process developed in this study is easily applicable to all food-processing wastewaters to produce relatively pure fungal protein, which could be used as a source of protein for growing population in developing nations around the globe.

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APPENDIX A

BIOREACTOR SET UP

Figure A.1 Experimental set up of PCS biofilm continuous-reactor

APPENDIX B

EFFECT OF INOCULUM SIZE

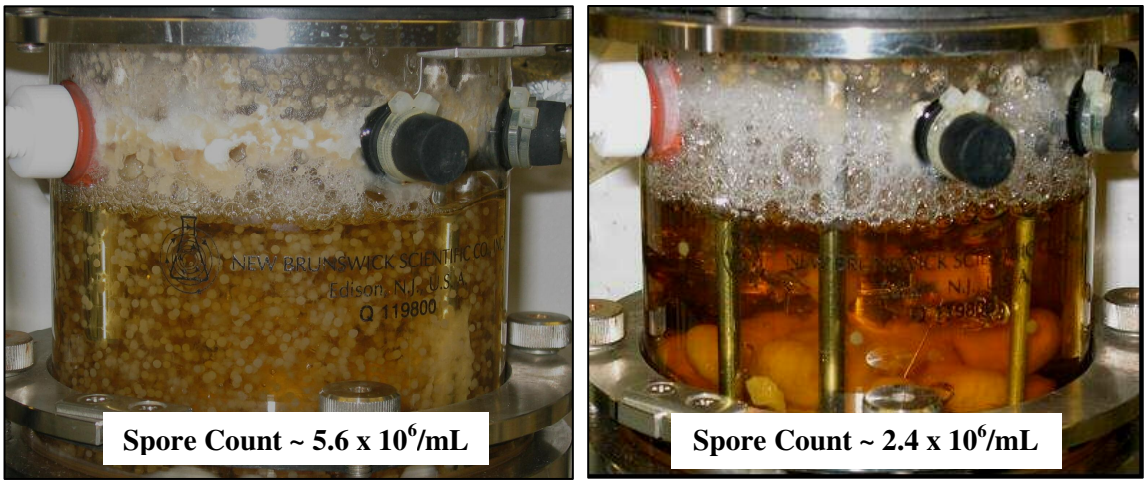

Figure B.1 Effect of inoclum size on fungal morphology during batch operation

Note: Suspended pellets along with biofilm on PCS medium were observed with higher inoculums during batch operation with heat sterilized potato dextrose broth. However, no significant effect on the reactor performance during subsequent continuous operation with non-aseptic wastewater was noticed.

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