NUTRIENT UTILIZATION FROM ANAEROBIC DIGESTER EFFLUENT THROUGH ALGAE CULTIVATION

by

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ABSTRACT

Nutrient Utilization from Anaerobic Digester Effluent Through Algae Cultivation

by

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Nutrients present in digested animal waste can be utilized for algae cultivation under suitable conditions. Algal growth, however, depends on the chemical forms and speciation of these nutrients. In this study a chemical equilibrium model was first used to describe nutrient speciation and predict conditions that enhance the solubility of nutrients in anaerobic digester effluent. Dilution with water and separation of large particulates greatly improved nutrient availability and light penetration – conditions favorable for algal cultivation. Algae growth was tested using three strains – *Scenedesmus dimorphous* (UTEX # 417), *Chlorella vulgaris* (UTEX# 265), and an algal isolate (designated as LLAI and later identified to be closely related to *Chlorella vulgaris*) from the wastewater treatment lagoons in Logan, UT. All tested strains could be adapted to the effluent to enhance the utilization of native nutrients present in both organic and inorganic forms. There was a marked improvement in growth rates (up to 4.8-fold) and biomass production (up to 8.7-fold) of algal cultures after they adapted to the effluent. Also, effluent-adapted

strains were able to switch from phototrophy to heterotrophy to prolong the growth when light availability became limited. However, an increase in irradiance levels in light-limited cultures led to resumption of phototrophic growth. It was found that this approach of light supplementation prolonged growth and increased biomass production (up to 2.7-fold) in algal cultures. Of all the strains tested, the isolate from the wastewater treating lagoons grew to highest culture densities and produced the highest concentration of intracellular triacylglycerides (TAG). This culture also grew best in non-sterile, native effluent and could reach biomass concentration of up to 4.5g/L with TAG content of approximately 10% (w/w). Culture densities were lower when this organism was grown in sterilized effluent or in sterile artificial media, suggesting that this organism symbiotically associated with other microbes in digested animal waste. Findings of this research study suggest that microalgae can be grown efficiently on inexpensive natural substrates in non-sterile growth conditions. When commercially implemented, biodiesel production from such systems could be more cost effective and sustainable.

(144 pages)

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CONTENTS

Page

ABSTRACT iii

ACKNOWLEDGMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER

1. INTRODUCTION 1

Research Overview 1
Progression of Research and Background Study 1

2. SPECIATION OF DAIRY WASTE TREATING ANAEROBIC DIGESTER EFFLUENTS AND AVAILABILITY OF NUTRIENTS FOR BIOLOGICAL UTILIZATION AND CHEMICAL RECOVERY PROCESS 9

Introduction 10
Materials and Methods 12
Results and Discussion 15
Conclusions 20

3. ADAPTATION OF MICROALGAE TO ANAEROBIC DIGESTER EFFLUENT FOR ENHANCED UTILIZATION OF ORGANIC NUTRIENTS AND HIGH BIOMASS PRODUCTION 29

Introduction 30
Materials and Methods 32
Results and Discussion 35
Conclusions 44

4. MAXIMIZING ALGAL GROWTH IN BATCH REACTORS USING SEQUENTIAL CHANGE IN LIGHT INTENSITY 54

Introduction 55
Materials and Methods 57
Results and Discussion 61
Conclusions 66

5. MAXIMIZING ALGAL GROWTH IN ANAEROBIC DIGESTER EFFLUENT USING LIGHT AND NITROGEN SUPPLEMENTATION 74

Introduction 75
Materials and Methods 77
Results and Discussion 79
Conclusions 85

6. IDENTIFICATION OF AN ISOLATED ALGAE STRAIN AND INTRACELLULAR TAG AND CN RATIO AT DIFFERENT LIGHT INTENSITIES USING ANAEROBIC DIGESTER EFFLUENT AS SUBSTRATE 92

Introduction 93
Materials and Methods 95
Results and Discussion 98
Conclusions 105

7. SUMMARY AND FUTURE WORK 112

Summary of the Research Work 112
Suggestions for Future Work 114

REFERENCES 117

APPENDIX 130

CURRICULUM VITAE.....132

LIST OF TABLES

Table Page

2-1 Concentrations of major elements in the influent and effluent of IBR 21

2-2 Mass balance calculations of major elements present in the effluents of IBR 22

2-3 Distribution of C, N and P in organic and inorganic forms in the IBR effluent 23

2-4 Other nutrient speciation-relevant characteristics of IBR effluents 24

2-5 Inputs for VM to predict solubilization of ortho-P after dilution with water 25

3-1 Biomass yield and growth rate of log phase in different algal cultures grown on IBR effluent in different generations of adaptation study 47

4-1 Yields and NO_3-N consumption at the end of different levels of growth in schemes I and II 67

4-2 Total moles of incident photons, N_{SL} supplied to the culture at different stages of the experiment 68

5-1 Biomass production and dCOD removal in the algal cultures grown with and without light supplementation in the IBR effluent 87

5-2 Biomass production and nutrient uptake by the three algal cultures grown in real and artificial IBR effluent 88

6-1 Nutrient removed by Logan lagoon algal isolate grown under different levels of irradiance in the IBR effluent 106

LIST OF FIGURES

Figure Page

2-1 Comparison between VM-predicted and experimentally determined precipitation of major ionic species in the IBR effluent 26

2-2 Changes in TSS and dissolved ortho-P on diluting the IBR effluent with water. Symbols indicate average experimental values and the solid line represents VM prediction of solubilized ortho-P. 27

2-3 Relative fraction of major precipitates in the effluent of IBR at different dilutions as predicted by chemical speciation modeling 28

3-1 Distribution of different elements in dissolved and particulate phases of the whole (untreated) IBR effluent [A] and distribution of C, N, and P in organic and inorganic forms in the whole (untreated) IBR effluent [B] 48

3-2 Penetration of light through the IBR effluent at different levels of dilution: [A] Whole effluent and [B] Effluent with suspended solids removed 49

3-3 Decrease in TSS concentration and dissolution of ortho-P on increasing the dilution of IBR effluent 50

3-4 Growth curves and dCOD removal from three algal strains grown in sterile and unsterile environment in the IBR effluent 51

3-4 N and P removal by the algal strains grown in sterile and unsterile IBR effluent 52

3-5 dCOD and Organic-N removal by algal cultures during growth in sterile and unsterile IBR effluent [A] Logan Lagoon Algal Isolate, [B] *S. dimorphous*, [C] *C. vulgaris* 53

4-1 Schematic diagram of the reactor and illustration of light levels 69

4-2 Biomass accumulation at different incident light intensities with constant light intensity throughout the growth 70

4-3 Algal growth and NO_3-N uptake during studies under (A) illumination scheme I (sequentially illuminated with light intensities of 91.2, 177.8, 273.1 µmoles/m^2/s) and (B)

illumination scheme II (sequentially illuminated with light intensities of 177.8 and 273.1 µmoles/m^2/s) 71

4-4 Comparison between lower (first) light level growths under illumination schemes I and II. Photon flux densities during this stage of growth were 91.2 and 177.8 µmoles/m^2/s, respectively, for the two illumination schemes 72

4-5 Comparison between higher (second) light level growths under illumination schemes I and II. Photon flux densities during this stage of growth were 177.8 and 273.1 µmoles/m^2/s, respectively, for the two illumination schemes 73

5-1 Growth curves of algal cultures grown in IBR effluent with light supplementation 89

5-2 Comparison between the nutrient removal by three algal cultures grown with and without light supplementation 90

5-3 N and P removal by algal cultures grown in ammonia-N supplemented IBR effluent 91

6-1 Light micrographs of Logan lagoons isolate algal cells 107

6-2 Growth curves of Logan lagoons algal isolate grown under different levels of irradiance in the IBR effluent 108

6-3 Growth curves of Logan lagoons algal isolate grown under different levels of irradiance in the IBR effluent 109

6-4 Typical relationship between growth and TAG accumulation in Logan lagoons algal isolate culture in the IBR effluent 110

6-5 TAG accumulation and intracellular C and P content at different light intensities in LLAI cells grown at different light intensities in IBR effluent 111

CHAPTER

INTRODUCTION

Research Overview

The current research study describes algal biomass production through utilization of nutrients present in anaerobic digester effluent. Characterization of effluent from the Induced Bed Reactor (IBR) anaerobic digester treating dairy waste was done using experimental and theoretical (equilibrium chemical modeling) analyses to determine the speciation of major elements and physico-chemical conditions affecting the speciation. Pretreatment strategies were then devised to improve the penetration of light and availability of nutrients for phototrophic growth. Three algal strains - *Scenedesmus dimorphous*, *Chlorella vulgaris*, and an isolate from Logan City Wastewater Reclamation Facility, Logan UT (designated as LLAI) were grown in the pretreated effluent. Biomass concentration, nutrient utilization, and intracellular triacylglyceride (TAG) accumulation (in case of LLAI) was monitored during growth studies. Finally, 23S rRNA genomic sequence analysis and morphological characterization was performed to identify the isolated alga, LLAI.

Progression of Research and Background Study

Research work performed in this study has been described sequentially in Chapters 2 - 6 of the present dissertation. A summary illustrating the major findings of this research and future hypotheses based on current findings and general approaches to test those hypotheses are presented in Chapter 7 of the dissertation.

Chapter 2: Speciation of Dairy Waste Treating Anaerobic Digester Effluents and Availability of Nutrients for Biological Utilization and Chemical Recovery Processes

Anaerobic digesters are commonly used manure treatment systems that effectively reduce volatile particulate content, mitigate pathogens and odor, and provide containment of the greenhouse gas, methane, as biogas [1]. In addition to pollution control, this method of treatment also allows waste materials to be converted to value-added products such as biofuels and biofertilizers [2]. Although anaerobic digestion greatly reduces the organics content through their breakdown to gases such as carbon dioxide and methane [3], the nutrient content of the manure is not altered significantly [4, 5]. As a result, anaerobically digested dairy wastes retain the high concentrations of nutrients (primarily N and P) originally present in the animal waste and could be of concern since leachates or runoff (if effluents are land applied) from these effluents may cause environmental pollution [6].

The study described in Chapter 2 involved performing mass balances on the major elements in solid and liquid phases and determining the relative distribution of organic and inorganic C, N and P in the effluent of the IBR. The chemical equilibrium model, Visual MINTEQ 2.53 (VM), was then used to describe the speciation of the nutrients (especially ortho-P) in IBR effluents and predict P dissolution upon dilution of the medium.

The results from the speciation study in Chapter 2 showed that nearly 95% of ortho-P present in digested dairy waste from the IBR reactor was insoluble and was likely precipitated with Ca^{+2} as hydroxyapatite. Most of the NH_3-N was soluble. Also, inorganic C (as CO_3^{-2} and HCO_3^{-1}), constituted about 12% of the total-C and was responsible for the high alkalinity of the effluent. Results also showed that the speciation of nutrients is primarily dependent on concentrations of Ca^{+2}, Mg^{+2} and alkalinity along with pH and

ionic strength of the effluent. Phosphate speciation and availability were accurately predicted through chemical equilibrium modeling with as low as 5 input parameters.

Chapter 3: Adaptation of Microalgae to Anaerobic Digester Effluent for Enhanced Utilization of Organic Nutrients and High Biomass Production

Biodiesel production from algae grown on inexpensive and locally available anaerobic digester effluents could be cost effective through utilization of nutrients and other substrates in such sources. However biomass production and intracellular lipid accumulation in such a process could be limited by bioavailability and speciation of native nutrients, indigenous microorganisms, and the presence of other chemical constituents [7-10].

The study in Chapter 3 involved determining the bioavailability of substrates essential for algal growth in the IBR effluent based on the analyses described in Chapter 2. Effluent samples were also analyzed for the penetration of light at different levels of dilution, and the results were used to determine the pretreatments necessary to support phototrophic algal growth in the effluent. Further, three algae strains - *S. dimorphous*, *C. vulgaris* and isolate LLAI were grown in the IBR effluent to investigate the suitability of the effluent to support algal growth. These strains were first grown for multiple generations to adapt them to the effluent environment. Thereafter, biomass production and nutrient utilization during growth on sterile and unsterile effluent was monitored to determine growth rates and uptake of specific nutrients under each condition.

Results from the study showed that growth rates and biomass production improved as cultures became progressively adapted to the effluent. Also, strains acclimated to the effluent utilized significant amounts of organic N and P, and C besides utilizing inorganic

nutrients. Uptake of C and organic N and P did not occur until later stages of growth when light availability to the culture likely became limiting suggesting that the algal cultures switched to heterotrophic growth when photosynthetic growth was inhibited. Of the three strains, highest uptake of organic substrates as well as biomass production occurred in LLAI cultures suggesting that this organism had best adapted to the effluent. As a result, LLAI used native substrates more effectively than the other strains and grew for longer periods of time.

Chapter 4: Maximizing Algal Growth in Batch Reactors Using Sequential Change in Light Intensity

Algal growth requires optimal irradiance. In photobioreactors, optimal light requirements change during the growth cycle. At low culture densities, a high incident light intensity can cause photoinhibition, and in dense algal cultures, light penetration may be limited. Insufficient light supply in concentrated algae suspensions can create zones of dissimilar photon flux density (PFD) inside the reactor, which can cause suboptimal algal growth. However, growth of dense cultures can also be impaired due to photoinhibition if cells are exposed to excessively high light intensities. In order to simultaneously maintain optimal growth and photon use efficiency, strategies for light supply must be based on cell concentrations in the culture.

In the study described in Chapter 4, a lipid-producing microalgal strain *Neochloris oleoabundans* [11, 12] was grown in externally illuminated stirred tank reactors. A step increase in illumination was employed when algal cultures stopped growing at a low incident photon flux. Overall, better biomass concentrations were observed when growth started at low light intensities and progressed higher in comparison to cultures illuminated at higher initial levels. Kinetic parameters related to growth and nitrogen consumption

were measured and were compared between the different phototrophic growth regimes tested.

Results from this study showed that the sequential change in light intensity or PFD during algal growth studied in this research has shown to improve algal growth up to 2-fold. There are multiple factors that affect the growth rate and final cell concentration in this process, which include starting incident light intensity, the difference in light intensity levels or jump in light levels, and the number of light levels used over the favorable range of illumination for a specific algal strain. This study also showed that the utilization of supplied light not only depends on intensity of incident light but also on the culture density. The results from the study suggest that simple periodic increase in light intensity can effectively increase the overall performance of photobioreactors.

Chapter 5: Maximizing Algal Growth in Anaerobic Digester Effluent Using Light and Nitrogen Supplementation

As discussed in Chapter 4, phototrophic growth of algae can be inhibited by unavailability of light at high culture densities. Light penetration into media such as anaerobic digester effluent is inherently poor because particulates and colored organics can scatter and absorb light [13]. Also, algae growing phototrophically utilize CO_2 from bicarbonate (HCO_3^-) ions dissolved in the growth medium. The release of hydroxyl ions (OH^-) as a result of this process increases the pH of the medium [14]. Since ammonia volatilization is significant at high pH (> 8) [15], photrotrophic algae growth in the absence of pH control could become N-limited if ammonia is the only available source of N. Therefore, determining the effects of replenishment of light during high culture densities in closed reactors and supplementing ammonia-nitrogen lost due to volatilization in open pond systems will improve biodiesel production through algal cultivation on

natural substrates.

In the study described in Chapter 5, effect of light supplementation was tested in cultures of *Scenedesmus dimorphous*, *Chlorella vulgaris*, and LLAI by growing them in the effluent of an anaerobic digester in externally illuminated stirred tank reactors. Also, the effect of nitrogen supplementation was examined by growing algal strains in three forms of anaerobic digester effluent i.e. ammonia-N free effluent, ammonia-N supplemented effluent, and artificial anaerobic digester effluent comprised of inorganic constituents and micro-nutrients present in the real effluent.

It was observed in this study that a step increase in illumination resulted in resumption of growth when the cultures stopped growing at a low incident photon flux. Also, application of light supplementation enabled the enhanced uptake of inorganic nutrients and prolonged the phototrophic activity in those algal strains which were unable to effectively utilize native organic nutrients for heterotrophic growth in light limited conditions. The study with nitrogen supplementation revealed that the presence of inorganic-N was essential for algal growth in IBR effluent, even in the presence of nitrogen from organic substrates. Also, it has been found that the ability of an alga to utilize native organic substrates in addition to inorganic nutrients in the effluent could assist in increasing the biomass yield in the culture.

Chapter 6: Identification of an Isolated Algae Strain and Intracellular TAG and CN Ratio at Different Light Intensities Using Anaerobic Digester Effluent as Substrate

Algae growth on a natural media like animal waste treating anaerobic digester effluent can be supported by N and P available from both organic and inorganic compounds [9, 16]. The effluent borne compounds can also supply other macro nutrients

as well as micro-nutrients and vitamins necessary for algal growth [17, 18]. The effluent also contains a range of other compounds such as volatile fatty acids (VFA), carbohydrates, and other forms of organic carbon that can be utilized for the heterotrophic growth of algae [19, 20]. However, despite the effluent containing a variety of substrates that can be utilized by algae, cost-effective production of biodiesel depends on identifying the algal strain capable of utilizing native substrates efficiently in the presence of media borne microorganisms and optimizing the growth conditions to maximize intracellular lipid production [21].

The study described in Chapter 6 involved identification of the strain isolated from the Logan Lagoon Wastewater Reclamation Facility (LLAI) using morphological properties and 23S rRNA genomic sequence of the alga. Also, growth characteristics of LLAI were determined by comparing the biomass production and nutrient uptake patterns at different light intensities, while growth conditions inducing high biodiesel production were determined by comparing intracellular TAG accumulation and corresponding CN ratio at five irradiance levels.

Results from the study showed that characteristically LLAI was found to have great similarity with mixotrophic strains of the genus *Chlorella*. While light micrographs of LLAI showed close resemblance with *Chlorella* spp. in morphology, analysis of 23S rRNA gene sequence of LLAI suggested a 98% match with *C. vulgaris*. The nutrient uptake pattern of LLAI revealed the ability of alga to perform phototrophic as well as heterotrophic growth in the effluent, which enabled it to utilize both inorganic and organic native substrates. Growth of LLAI at different irradiance levels showed that both biomass yield and lipid production can be enhanced by increasing the intensity of light in the

culture.

These results also suggest that the algal strain (LLAI) isolated from a natural environment is well suited for cultivation on inexpensive substrates such as anaerobic digester effluent. Development of strains that can adapt to such media, grow well in the presence of other native microorganisms and efficiently utilize available substrates can significantly reduce the costs associated with algae cultivation for biofuels.

CHAPTER 2

SPECIATION OF DAIRY WASTE TREATING ANAEROBIC DIGESTER EFFLUENTS AND AVAILABILITY OF NUTRIENTS FOR BIOLOGICAL UTILIZATION AND CHEMICAL RECOVERY PROCESSES

Abstract In this study, effluent from an Induced Bed Reactor (IBR) anaerobic digester treating dairy manure was characterized to determine the speciation and availability of nutrients (N and P). The study involved performing mass balances on the major elements in solid and liquid phases and determining the relative distribution of organic and inorganic C, N, and P. The chemical equilibrium model, Visual MINTEQ 2.53 (VM), was then used to describe the speciation of the nutrients (especially PO_4^{-3}) in IBR effluents and predict P dissolution upon dilution of the medium. Experimentally determined PO_4^{-3} solubilization data accurately matched model estimates. These results confirmed the VM prediction that Ca^{+2} and Mg^{+2} were responsible for most of the PO_4^{-3} interactions in digested dairy waste. In addition, Ca^{+2} and Mg^{+2} also interacted with CO_3^{-2} and the model calculations showed that relative magnitude of PO_4^{-3} or CO_3^{-2} precipitates was largely governed by the pH and alkalinity of the effluent. Thus, with as low as five input parameters - concentrations of Ca^{+2}, Mg^{+2}, and PO_4^{-3} along with pH and total alkalinity, the solubility of inorganic P in chemically complex anaerobic digester effluents treating dairy wastes could be predicted.

Introduction

Anaerobic digesters are commonly used manure treatment systems that effectively reduce volatile particulate content, mitigate pathogens and odor, and provide containment of the greenhouse gas, methane, as biogas [1]. In addition to pollution control, this method of treating farm wastes also allows waste products to be converted to value-added products such as biofuels and biofertilizers [2]. Although anaerobic digestion breaks down a large fraction of the carbon contained in suspended and volatile particulates [3] into simpler compounds such as short chain fatty acids, carbon dioxide and methane, the nutrient content of the manure is not altered significantly [4, 5]. As a result, anaerobically digested dairy wastes retain the high concentrations of nutrients (primarily N and P) originally present in the animal waste and could be of concern since leachates or runoff (if effluents are land applied) from these effluents may cause environmental pollution [6].

The biological uptake of nutrients from digested animal waste is dictated by their chemical forms (such as ionic, precipitated, co-precipitated, and adsorbed) in the effluent [9, 22-25]. Further, the type and abundance of specific nutrient species depend on interactions with other chemical components of the effluent [26, 27]. Finally, speciation of nutrients also depends on the extent of manure digestion as well as digester type [2, 28]. The Induced Bed Reactor (IBR) is a commercial anaerobic digestion system that improves upon traditional plug-flow designs to facilitate better retention of microorganisms thereby achieving more rapid digestion (retention time of 5-7 days) in comparison to other traditional systems that generally have hydraulic retention times of 20-30 days [29]. Nutrient availability and speciation in IBR digested animal manure has not been previously described.

Composition analysis of anaerobic digester effluents have been performed in past studies that have also determined the concentrations of various inorganic species [3, 30, 31]. However, a comprehensive mass balance of the organic and inorganic nutrient forms in the effluent liquid and particulate phases has not been reported. Such information facilitates understanding the roles of major species in precipitation, co-precipitation, and adsorption. Also, chemical speciation in native anaerobic digester effluents treating dairy manure has been predicted in the past using chemical/geochemical models such as Mineql+ [32, 33] and Visual MINTEQ 2.23 [30]. However, such model predictions have not been quantitatively validated, although spectroscopic studies have qualitatively confirmed the presence of some predicted mineral phases [26].

In this study, comprehensive mass balance of various chemical species in the effluent of an IBR digester treating dairy manure was first performed. Visual MINTEQ (VM) 2.53 was initially used with ionic strength, pH, alkalinity, and concentrations of all major cations and anions as input parameters to model the chemical speciation in the IBR effluent. However, we found that knowledge of pH, alkalinity, and concentration of Ca^{+2}, Mg^{+2}, and PO_4^{-3} was sufficient to predict the fractionation of ortho-P between soluble and precipitate forms using VM. Inorganic N (NH_4^+) was measured (also predicted by VM) to be in a dissolved form and did not influence PO_4^{-3} speciation. With only five input parameters, VM was then used to predict PO_4^{-3} solubilization upon dilution of the effluent with water. Phosphate dissolution was also experimentally determined and good correlation was observed with model estimates thereby validating the theoretical speciation results. This ability to predict the behavior of nutrients under changing

environmental conditions is useful to develop strategies for removal or utilization of nutrients.

Materials and Methods

Sample Source and Collection

Samples for this study were obtained from the effluent of a dairy waste-treating IBR at Wadeland Dairy, Ogden, UT. Periodic samples were collected between May 2007 and April 2008 and immediately stored at 4°C. Most of the analyses were performed within a week after sample collection, although some portion of the sample was also stored at -20°C for future analysis, if required.

Analytical Methods

Total elemental analysis on samples was performed by digesting samples using nitric acid with peroxide [34] followed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Concentrations of P, K, S, Ca, Na, Mg, Si, Fe, Al, Cu and trace elements (As, B, Ba, Cd, Co, Cr, Mn, Mo, Ni, Pb, Se, Sr, and Zn) were determined by this method. Total-C and total-N content was determined using the Dumas method of combustion [35] followed by analysis through a LECO TruSpec CN analyzer (St Joseph, MI). Besides analysis of whole effluent samples, elemental composition (including C and N) of the liquid and solid portions of the effluent slurry was also determined. The liquid portion of the effluent was isolated by passing samples through a 0.45µm filter. Solids were obtained by centrifuging the sample at 4000g for 45 min. To facilitate mass balances, total solids were determined on the native effluent as well as on its liquid and particulate portions [36].

Electrical conductivity and pH were measured using Mettler Toledo (Columbus, OH) Inlab Versatile pro pH probe and Inlab 730 conductivity probe, respectively. Alkalinity measurements were performed via titration [37]. Alkalinity of undiluted effluent samples was determined first. Since titration of solid precipitates can be slow and incomplete, alkalinity measurements were further performed on serially-diluted (using DI water) effluent samples. After each dilution, the measured alkalinity was multiplied by the dilution factor to calculate alkalinity of the undiluted effluent. Sequential dilutions and alkalinity measurements were continued until calculated alkalinity values did not change upon further dilution. This final value was considered as the total alkalinity of the native effluent and represented the overall alkalinity caused by all dissolved and precipitated ions present in the effluent. Alkalinity in the liquid fraction of the effluent slurry was also measured to determine contribution of the dissolved ions only.

Since C, N, and P can be present in both organic and inorganic forms, samples were analyzed for inorganic C (CO_3^{-2}-C), inorganic-N (NH_3-N) and inorganic-P (ortho-P) in both the native effluent slurry and in the liquid fraction. Organic-C was first determined using heated persulfate oxidation method [38]; inorganic C was calculated by subtracting organic-C content in the sample from total-C (concentration. NH_3-N and ortho-P were measured using the salicylate method [39] and the ascorbic acid method [40], respectively.

Chemical Speciation of IBR Effluent

Visual MINTEQ 2.53 (VM) was used to model the major interactions associated with P speciation in the IBR effluent. VM is the Windows version of MINTEQA2 ver. 4, which was originally developed by US EPA for calculating metal speciation, solution equilibria etc. for natural waters. MINTEQ applies the fundamental principles of thermodynamics to

solve geochemical equilibria from a set of mass balance equations, one for each component. MINTEQ is composed of four submodels. The speciation submodel computes the activities of complexed and uncomplexed cationic and anionic species, neutral ion pairs, and the activities of cationic and anionic redox species. These activities are then fed to the solubility submodel, which calculates ion activity products for solids and minerals. The results from these calculations are used by the mass transfer submodel to calculate the mass of solid that precipitates or dissolves. In the fourth or adsorption submodel MINTEQ models adsorption onto solid surfaces via several mechanisms: an activity Langmuir isotherm, an activity Freundlich isotherm, an ion exchange model, a constant capacitance surface complexation model, and a triple-layer surface complexation model. The calculations completed by each submodel are dependent on the thermodynamic data stored in the MINTEQ database [41].

In this study, the VM program was primarily used to predict the interactions between cations and anions in the IBR effluent and determine concentrations of precipitated and dissolved species. To model speciation, average concentrations of the ionic species along with pH and alkalinity were input into the program. It was assumed that total alkalinity is caused by the inorganic-C in the effluent that is present as either CO_3^{-2} or as HCO_3^{-1}. This is a reasonable assumption since the inorganic-C concentrations were high and the pH was near-neutral.

Results and Discussion

Experimental Determination of Chemical Species

Of 24 elements tested, only 10 were found to be present in significant (> 0.1 mM) concentrations and are reported in this study. Table 2-1 shows the concentration of these elements in the effluent and influent of the IBR for five samples collected over a year-long period. A slight difference between the average concentrations of nutrients in the influent and effluent of the IBR (about 13% N and 20% P) was likely due to volatilization of gases (stripping of NH_3 facilitated by the pH close to 8.0) or retention of particulates (precipitates of P and other cations) by the digester. N and P are possible contaminants and their biological uptake depends on the availability of these nutrients [42] that is, in turn, determined by their chemical speciation [27, 43].

The distribution of major elements in the dissolved phase and particulate phase of the IBR effluent is shown in Table 2-2. It can be seen that C, P, Ca, Mg, Fe, Cu, Zn, and Mn were preferably present as solids whereas Na and K were mostly soluble. N was present in significant amounts in both the solid and liquid phases. This distribution of inorganic elements in particulate phase suggested that the majority of the mineral precipitates in the effluent were likely comprised of Ca, Mg, Fe, Cu, Zn, and Mn. However, due to their relatively low concentrations, Cu, Zn, and Mn precipitates were likely less abundant than those of Ca, Mg, and Fe. Also, Fe preferentially precipitates with anions of sulfur (HS^- or S^{-2}) in anaerobic environments [44] and is therefore unlikely to have significant interaction with PO_4^{-3}. Although S is found in high concentrations in wastes like dairy or swine manure [5], it was not included in our study since it preferably forms stable and relatively insoluble precipitates with metals like Fe, Cu, and Zn in reducing environments

of anaerobic digesters [45, 46]. Hence it is unlikely that S influences speciation of N and P.

C, N, and P can be present in either organic or inorganic forms in both the dissolved and solid phases. Soluble forms are typically considered more bioavailable, although insoluble nutrients can also be slowly utilized. Nutrients in solid mineral phases can also dissolve by addition of water or change in pH. Table 2-3 shows the concentrations of organic and inorganic C, N, and P in the native effluent slurry along with their distributions in the liquid and particulate fractions. It can be seen that organic-C (90.2%), organic-N (59.4%) and organic-P (49%) constituted a major fraction of total C, N and P in the effluent. It can also be calculated from Table 2-3 that most of inorganic-C (83.4 %) and inorganic-N (88.4 %) was in the liquid fraction of the effluent. However, most of the inorganic-P (94.5 %) was in the effluent solids, likely precipitated with cationic species in the effluent. Although most of the inorganic-C was soluble, due to their high concentrations, anions of C i.e. HCO_3^- and CO_3^{-2} could compete with PO_4^{-3} for precipitation with cations.

From the above discussion it can be concluded that ions of Ca, Mg, inorganic-C and inorganic-P are the major precipitate-forming constituents of the effluent. Also, it can be seen that monovalent cation forming elements such as Na, K, and inorganic-N were largely soluble and therefore contributed to the electrical conductivity (or ionic strength) of the effluent.

In addition to concentrations of various inorganic species, pH, electrical conductivity, and total alkalinity are important for estimating the precipitation and chemical speciation of nutrients in the effluent [30, 31, 33, 47]. These three parameters measured in the whole

IBR effluent and in its liquid fraction are shown in Table 2-4. The total alkalinity values of the whole effluent and of the liquid fraction were observed to be similar. Since CO_3^{-2}/HCO_3^- ions (due to much higher relative concentration of inorganic-C and pH ~ 8, from Table 2-3 and 2-4) are the major contributors to the alkalinity of the effluent, these results suggest that only a small fraction of CO_3^{-2}/HCO_3^- precipitated with cations (likely Ca^{+2} and Mg^{+2}) in the effluent. Electrical conductivity of about 16.0 dS/m is indicative of high concentrations of dissolved ions.

Modeling the Major Ionic Speciation in IBR Effluent Using VM Program

VM results indicate that the major precipitates in the IBR effluent consisted of Ca^{+2}, Mg^{+2}, CO_3^{-2} and PO_4^{-3}. Fig. 2-1 shows the fraction of these species present in precipitate form as determined through experiments and chemical equilibrium modeling. It can be seen that VM predictions compared accurately with experimental measurements of anions (CO_3^{-2} and PO_4^{-3}) but the model somewhat over-predicted precipitation of Ca^{+2} and Mg^{+2}. It is possible that Ca and Mg were partially complexed with organic compounds such that a fraction of these species was unavailable for interaction with inorganic anions in the effluent. It has been reported [48] that organic species such as pectin, lignin, and organic acids have a tendency to form complexes with cations like Ca^{+2} and Mg^{+2} in a solution of high ionic strength. Also, organic acids formed by acidogenesis but not converted to methane or carbon dioxide remain in the effluent and could promote formation of complexes with Ca and Mg. Thus, while organic species likely interacted with the cations, their effect on PO_4^{-3} speciation and precipitation was not significant.

VM predicted several possible precipitates in the effluent. Inorganic-P was predicted to interact primarily with Ca and Mg to form hydroxyapatite ($Ca_5(PO_4)_3OH$), tricalcium

phosphate ($Ca_3(PO_4)_2$), monetite ($CaHPO_4$), brushite ($dCaHPO_4.2H_2O$), newbryite ($MgHPO_4.3H_2O$), and bobierrite ($Mg_3(PO_4)_2.H_2O$). Ca and Mg were also predicted to interacted with CO_3^{-2} to form calcite ($CaCO_3$), magnesite ($MgCO_3$), dolomite ($CaMg(CO_3)_2$), and huntite ($CaMg_3(CO_3)_4$). Of all the minerals, three finite particulates (species present as precipitates at equilibrium) were predicted- hydroxyapatite, dolomite, and calcite.

To extend the applicability of the speciation model and further validate its performance, VM was used to predict PO_4^{-3} solubilization after dilution of the effluent with water. One representative IBR effluent sample was appropriately diluted and used in this study. The total concentrations of Ca^{+2}, Mg^{+2} and PO_4^{-3} as well as the alkalinity and pH of this sample were measured and are shown in the first row (dilution factor = 1) of Table 2-5. Concentrations for the diluted samples were calculated by dividing with the appropriate dilution factors (values in rows 2-6 of Table 2-5). Alkalinity and pH were measured in each diluted sample. Using only these five input parameters, soluble PO_4^{-3} concentrations at each dilution were estimated using VM. Soluble PO_4^{-3}concentrations were also determined experimentally after aliquots of the effluent sample were appropriately diluted. Each dilution was made in triplicate. 2-2 shows that measured and predicted values of the fraction of PO_4^{-3} present as dissolved species at various dilutions correspond closely. Thus, despite the numerous organic and inorganic species in the effluent of an anaerobic digester treating dairy waste, only a few decisively affect the P speciation.

Fig. 2-2 shows that the fraction of soluble PO_4^{-3} increases upon dilution since some precipitated minerals dissolve upon addition of water. This dissolution of precipitates also

affects parameters such as alkalinity (as alkalinity depends on the concentration of CO_3^{-2}/HCO_3^- and PO_4^{-3} ions in the dissolved phase of the effluent) and ionic strength of the medium, which in turn affect the speciation of other elements in the effluent besides phosphorous. Thus, although linearly correlated at low dilutions, PO_4^{-3} solubilization asymptotically approaches maximum values at higher dilutions. Due to simultaneous dilution of the medium and dissolution of precipitates, the decrease in TSS upon dilution is also non-linear (Fig. 2-2).

VM also predicted changes in the relative distribution of major precipitates on dilution. Fig. 2-3 shows relative percentages of these precipitates- dolomite, hydroxyapatite, and calcite in the IBR effluent at different dilutions. Among these precipitates present in the native effluent (undiluted), dolomite is most abundant followed by hydroxyapatite and calcite. After dilution, the fraction of calcite precipitates increased while the relative amounts of dolomite and hydroxyapatite became lower. Due to the high alkalinity of the effluent, lowering phosphate concentrations at large dilutions likely changed the equilibrium conditions such that precipitation of CO_3^{-2} with Ca^{+2} became more favorable. These observations underscore the influence of alkalinity on PO_4^{-3} speciation.

Due to limited phosphorous rock reserves [49] and increasing global demands for nitrogenous fertilizer [50], there is a greater emphasis on recovering nutrients [43]. To meaningfully use nutrients from sources like digested dairy waste, it is important to understand the nature and behavior of nutrients in these systems. The experimental and modeling results reported in this study can be used to predict nutrient concentrations when these waste streams are utilized. For example, algal growth for production of biodiesel or

biofertilizer [9, 51] requires appropriate NH_4^+ and PO_4^{-3} concentrations and digested dairy waste effluents can serve as a source of nutrients for this process. Similarly, producing bioplastics from digested wastes is possible through luxury uptake and release of phosphorus if appropriate ratios and/or concentrations of organics and ortho-P are maintained [52]. Production of struvite, a slow release fertilizer, can also be achieved from digested dairy manure through adjusting $NH_4^+/Mg^{+2}/PO_4^{-3}$ ratios [43, 53]. Thus, predicting available nutrients and their speciation is needed in all these applications that seek to better utilize animal wastes.

Conclusions

Our study showed that nearly 95% of ortho-P present in digested dairy waste from the IBR reactor was insoluble and was likely precipitated with Ca^{+2} as hydroxyapatite. Most of the NH_3-N was soluble. Also, inorganic C (as CO_3^{-2} and HCO_3^{-1}), constituted about 12% of the total-C and was responsible for the high alkalinity of the effluent. Further, our results show that speciation of nutrients is primarily dependent on concentrations of Ca^{+2}, Mg^{+2} and alkalinity along with pH and ionic strength of the digested animal waste. Thus, phosphate speciation and availability can be accurately predicted through chemical equilibrium modeling with as low as 5 input parameters.

Table 2-1 Concentrations of major elements in the influent and effluent of IBR. Reported values are average measurements from five samples taken over a period of one year. Values in parentheses indicate one standard deviation

Element	**Influent (pH = 7.8±0.1)) (mg/L) Avg. (Std dev)**	**Effluent (pH = 7.9±0.2) (mg/L) Avg. (Std dev)**
N	3560.0 (*850.0*)	3100.0 (*820.0*)
P	604.0 (*180.0*)	484.0 (*140.0*)
K	2538.0 (*995.0*)	2210.0 (*844.0*)
Ca	2054.0 (*463.0*)	1546.5 (*597.0)*
Mg	897.0 (*164.0*)	642.0 (*168.0*)
Na	778.0 (*206.0*)	653.6 (*197.0*)
Fe	276.0 (*132.0*)	182.0 (*116.0*)
Cu	42.0 (*15.9*)	26.8 (*19.6*)
Zn	14.8 (*3.3*)	14.0 (*5.6*)
Mn	17.2 (*10.1*)	12.9 (*8.9*)

Table 2-2 Mass balance calculations of major elements present in the effluents of IBR. Reported values are average measurements from five samples taken over a period of one year. Values in parentheses indicate one standard deviation

Element	**Concentration in Whole Effluent (W) (mg/Kg) pH = 7.9±0.2**	**Concentration in Dissolved Phase (L) (mg/Kg) pH = 8.1±0.1**	**Concentration in Particulate Phase (S) (mg/kg)**	**Difference** $\left\lvert 100 * \frac{W-(L+S)}{W} \right\rvert$ **(%)**
C	17043.0 (*1600.0*)	3350.0 (*500.0*)	13030.0 (*402.0*)	3.9
N	2695.0 (*713.0*)	1460.0 (*370.0*)	990.0 (*139.0*)	9.1
P	420.0 (*122.0*)	16.1 (*3.3*)	428.0 (*27.0*)	5.7
K	1922.0 (*734.0*)	2002.0 (*449.0*)	0.0 (*0.0*)	4.1
Ca	1345.0 (*519.0*)	84.8 (*15.0*)	1352 (*106.0*)	6.8
Mg	558.0 (*146.0*)	111.0 (*54.0*)	417.0 (*7.0*)	5.4
Na	568.0 (*171.0*)	592.0 (*157.0*)	0.0 (*0.0*)	4.2
Fe	158.0 (*101.0*)	5.4 (*1.4*)	157.0 (*32.3*)	2.8
Cu	23.3 (*17.0*)	1.3 (*0.1*)	23.7 (*4.5*)	7.3
Zn	12.2 (*4.9*)	0.3 (*0.0*)	12.8 (*2.0*)	7.9
Mn	11.2 (*7.7*)	0.3 (*0.2*)	11.3 (*5.6*)	3.8

Table 2-3 Distribution of C, N and P in organic and inorganic forms in the IBR effluent. Reported values are average measurements from five samples. Values in parentheses indicate one standard deviation

Element	Whole effluent					Dissolved phase			
	Total (mg/L)	**Inorganic (mg/L)**	**Inorganic fraction (%)**	**Organic fraction (%)**		**Total (mg/L)**	**Inorganic (mg/L)**	**Inorganic fraction (%)**	**Organic fraction (%)**
C[1]	19600 (*1840*)	2170 (*390*)	9.8 (*1.6*)	90.2 *(3.6)*		3350 (*500*)	1810 (*104.9*)	54.0 (*4.1*)	46.0 (*3.8*)
N[2]	3100 (*820*)	1380 (*301*)	40.6 (*0.6*)	59.4 *(0.7)*		1460 (*370.0*)	1220 (*295*)	84.3 (*3.2*)	16.4 *(1.7)*
P[3]	483.6 (*140.3*)	241.8 (*64.3*)	51.0 (*1.6*)	49.0 *(1.8)*		16.1 (*3.3*)	13.2 (*3.2*)	82.0 (*2.1*)	18.0 (2.3)

[1]Inorganic C = CO_3-C
[2]Inorganic N = NH_3-N
[3]Inorganic P = PO_4-P

Table 2-4 Other nutrient speciation-relevant characteristics of IBR effluents. Reported values are average measurements from five samples. Values in parentheses indicate one standard deviation

Measured parameter	**Whole Effluent**	**Dissolved phase**
pH	7.9 (*0.2*)	8.1 (*0.1*)
Electrical Conductivity (dS/m)	16.4 (*1.4*)	16.0 (*1.0*)
Total Alkalinity mg/L $CaCO_3$	12500 (*800*)	11000 (*600*)

Table 2-5 Inputs for VM to predict solubilization of ortho-P after dilution with water. Total alkalinity and pH were measured with samples after each dilution. Concentrations of ions were measured only in the undiluted sample and were estimated (using the dilution factor) for diluted samples. Each dilution was made in triplicate and the average values are reported. Values in parentheses indicate one standard deviation

Dilution factor	**Total Alkalinity (mg/L $CaCO_3$)**	**pH (std dev)**	**Conc. of ions (mM)**		
			Ca^{+2}	**PO_4^{-3}**	**Mg^{+2}**
1	9500 *(300)*	7.95 *(0.10)*	37.9 *(4.90)*	6.83 *(1.13)*	26.16 *(3.00)*
2	5200 *(180)*	7.90 *(0.10)*	18.95	3.42	13.08
5	2100 *(100)*	7.83 *(0.09)*	7.58	1.37	5.23
10	1060 *(60)*	7.80 *(0.08)*	3.79	0.68	2.62
20	540 *(20)*	7.77*(0.10)*	1.89	0.34	1.31
40	230 *(10)*	7.76 *(0.9)*	0.95	0.17	0.66

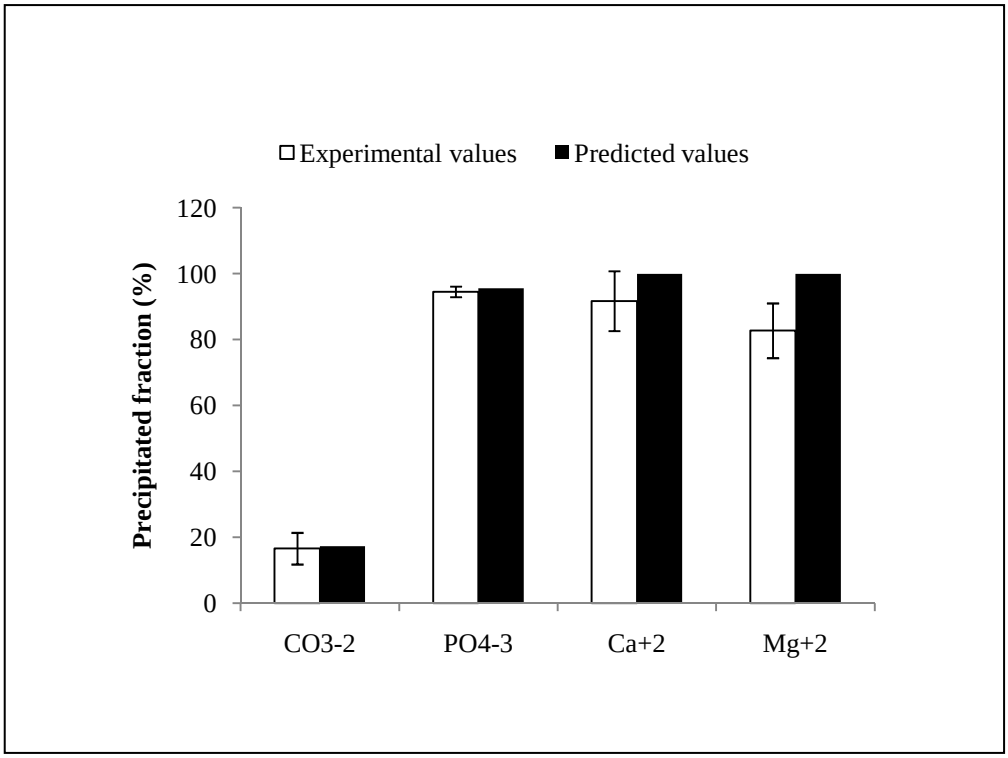


Fig. 2-1 Comparison between VM-predicted and experimentally determined precipitation of major ionic species in the IBR effluent

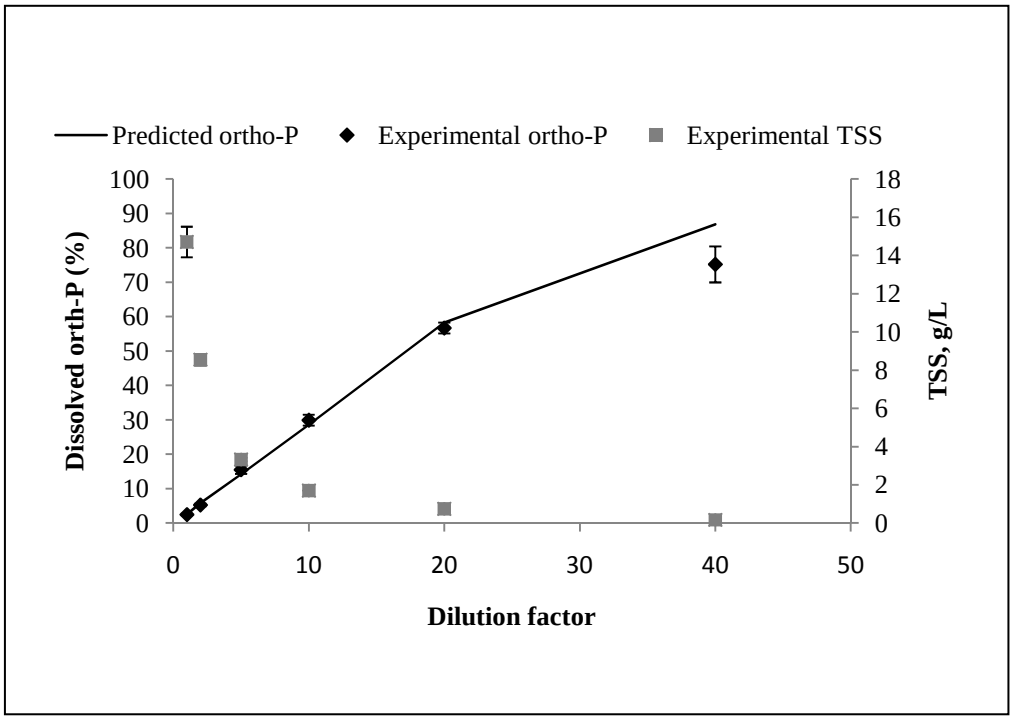


Fig. 2-2 Changes in TSS and dissolved ortho-P on diluting the IBR effluent with water. Symbols indicate average experimental values and the solid line represents VM prediction of solubilized ortho-P. Error bars represent one standard deviation from mean values

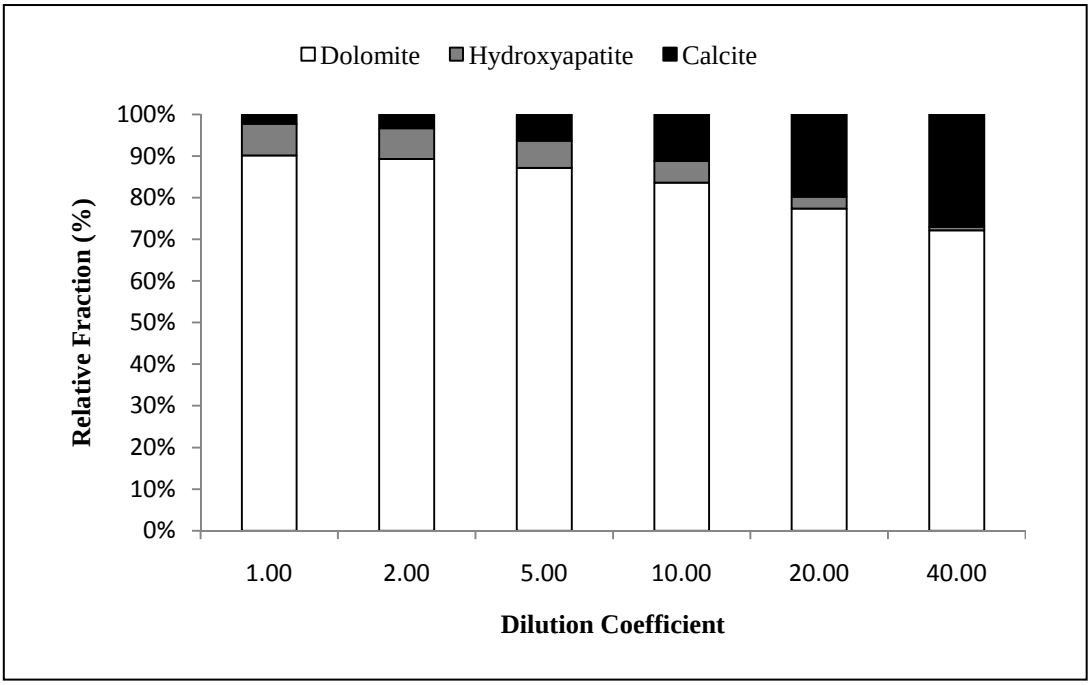


Fig. 2-3 Relative fraction of major precipitates in the effluent of IBR at different dilutions as predicted by chemical speciation modeling

CHAPTER 3

ADAPTATION OF MICROALGAE TO ANAEROBIC DIGESTER EFFLUENT FOR ENHANCED UTILIZATION OF ORGANIC NUTRIENTS AND HIGH BIOMASS PRODUCTION

Abstract Anaerobic digester effluents can serve as inexpensive source of nutrients for the production of biodiesel through growth of microalgae. In this study effluent of an Induced Bed Reactor (IBR) anaerobic digester treating dairy waste was analyzed for availability of nutrients for biological uptake and light conditions available for phototrophic growth in the effluent. Three algae strains - *Scenedesmus dimorphous*, *Chlorella vulgaris* and an alga isolated from the wastewater treatment lagoons (LLAI) in Logan, UT, were grown in the effluent for multiple generations to facilitate their adaptability to the effluent environment. Time course data showed that there was a marked improvement in growth rates (up to 4.8 fold) and biomass production (up to 8.7 fold) in the algal cultures during adaptation study. Growth of adapted algal strains in the effluent suggested that besides utilizing inorganic nutrients, algal strains also utilized significant fractions of organic N (up to 45% of total N) and P (up to 50% of total P), and heterotrophic-C (up to 27% of total C utilized) . The pattern of uptake of organic substrates revealed that algal cultures tend to switch from phototrophic to heterotrophic growth when light penetration within the reactor decreased due to increased culture density. Finally, the fact that the highest uptake of organic substrates was in LLAI culture (which also produced maximum biomass) suggested that since it originated from a natural habitat this algal strain had better adaptability to the effluent environmental conditions than the two type strains. This

adaptation allowed LLAI to utilize native substrates in the effluent and sustain the growth for longer period of time.

Introduction

Anaerobic digesters are useful farm waste management systems, effluents from which contain high concentration of nutrients, N and P [5]. The nutrients in the effluent of an anaerobic digester are generally considered environmental contaminants if not contained or utilized [4]. While nutrient management on farms could become mandatory in the future [6], a separate nutrient removal system would further increase the operational cost of these digesters. However, this additional cost can be countered by integrating anaerobic digester systems with a process such as algal cultivation, where the nutrients in the effluent of digester are utilized by microalgae for the production of biomass that can be processed into biodiesel [54]. The effluent of anaerobic digester effluent is one such growth medium that can supply inexpensive substrate for algal growth. N and P available from both organic and inorganic compounds can supply the macro nutrients [9, 16], whereas a variety of other substances such as micro-nutrients and vitamins can also be utilized during algal growth [17, 18]. The effluent also contains a range of other compounds such as volatile fatty acids, VFA, carbohydrates, and other forms of organic C that can be utilized for the heterotrophic growth of algae [19, 20].

Although uptake of inorganic nutrients (NH_3-N and ortho-P) by algae grown in raw and anaerobically digested dairy waste has been studied in great detail in past studies [9, 25, 55], utilization of native organic compounds (especially org-N and org-C) by algae has not been much emphasized [56, 57]. Since anaerobically digested dairy wastes contain a significant fraction of organic species [48, 58], the potential of utilization of organic

nutrients by algae needs to be researched in greater detail. As algae grown on natural media like domestic wastewater or animal farm effluents are able to perform both heterotrophic and phototrophic growth [59], availability of organic nutrients to support heterotrophic growth could increase overall biomass yield from the culture. Also since uptake of organic nutrients has been related to the stress response of algal cells in light limited conditions [60], algae strains utilizing the organic nutrients could potentially be high lipid storing organisms.

In this study effluent of the Induced Bed Reactor (IBR), a dairy waste treating anaerobic digester was used to determine the nutrient composition and potential of sustained algal cultivation in it. Samples from the IBR were first analyzed to determine the major elements present and their relative distribution in dissolved and particulate phases of the effluent. Thereafter native C, N, and P were examined for their fractionation in organic and inorganic (ionic) forms in the effluent. These analyses were used to determine the bioavailability of substrates essential for algal cultivation in the effluent of anaerobic digesters. Besides this, effluent samples were also analyzed for the penetration of light at different levels of dilution, and the results were used to determine the pretreatments necessary to support phototrophic algal growth in the effluent.

Three algae strains *Scenedesmus dimorphous*, *Chlorella vulgaris* and Logan lagoons algal isolate (LLAI), an alga isolated from the wastewater treating lagoons in Logan, UT, were grown in IBR effluent to investigate the suitability of effluent medium to support algal growth. These algal strains were first grown for multiple generations to give them an opportunity to adapt to the effluent environment. Thereafter, biomass production and the amounts of different forms of nutrient utilized at various stages of growth by the adapted

algal strains grown in sterile and unsterile growth conditions in the effluent were compared. Finally, results were used to determine the growth characteristics and nutrient uptake patterns of three algal strains at various stages of growth in the effluent.

Materials and Methods

Effluent Source, Growth Media Preparation and Characterization of effluent

Anaerobic digester effluent samples for this study were obtained from a dairy waste treating Induced Bed Reactor (IBR) at the Wadeland Dairy, Ogden, UT. Effluent samples from the IBR location were collected and pretreated before stocking. The pretreatment involved fourfold dilution of the samples with DI water followed by centrifugation at 500xG for five minutes. The pretreated stocked samples were further diluted five times before using them for algal growth. Sterile growth experiments were performed with five-fold diluted stock samples autoclaved for 20 minutes at 120°C.

Artificial effluent recipe was based on the concentration of major cations and anions present in the effluent of IBR. The artificial IBR effluent was comprised of 4.2mg/L of $CaCl_2$, 5.2mg/L of $MgCl_2.6H_2O$, 4.7mg/L of NH_4Cl, 1.5mg/L of $K_2HPO_4.3H_2O$, 22.5mg/L of $NaHCO_3$, 15mg/L of $Fe(NH_4)_3(C_6H_5O_7)_2$, and 1ml/L of trace element solution. Trace element solution was prepared using 600mg/L of H_3BO_3, 250mg/L of $MnCl_2.4H_2O$, 20mg/L of $ZnCl_2$, $CuCl_2.2H_2O$, 15mg/L of Na $MoO_4.2H_2O$, $CoCl_2.6H_2O$, 10mg/L of $NiCl_2.6H_2O$, and 10mg/L of KBr. The artificial effluent so prepared was diluted ten-folds, adjusted to pH 7.0 and autoclaved before being used for algal growth experiments.

For characterization, total elemental analysis on samples was performed by digesting

samples using nitric acid with peroxide [34] followed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Concentrations of P, K, S, Ca, Na, Mg, Si, Fe, Al, Cu, and trace elements (As, B, Ba, Cd, Co, Cr, Mn, Mo, Ni, Pb, Se, Sr, and Zn) were determined by this method. C and N content was determined using the Dumas method of combustion [35] followed by analysis through a LECO TruSpec CN analyzer (St Joseph, MI). Besides analysis of whole effluent samples, elemental composition (including C and N) of the liquid and solid portions of the effluent slurry was also determined. The liquid portion of the effluent was isolated by passing samples through a 0.45µm filter. Solids were obtained by centrifuging the sample at 4000g for 45 min. To facilitate mass balances, total solids were determined on the native effluent as well as on its liquid and particulate portions [36].

Since C, N and P can be present in both organic and inorganic forms, samples were analyzed for inorganic C (CO_3^{-2}-C), inorganic-N (NH_3-N) and inorganic-P (ortho-P) in both the native effluent slurry and in the liquid fraction. Organic-C was first determined using heated persulfate oxidation method [38]; inorganic C was calculated by subtracting organic-C content in the sample from total-C concentration. NH_3-N and ortho-P were measured using the salicylate method [39] and the ascorbic acid method [40], respectively.

Algal Strains, Growth and Analyses

Three algal strains studied in this research were 1) Algal isolate from Wastewater Reclamation Facility, Logan, UT, 2) *Scenedesmus dimorphous* (Utex # 417), and 3) *Chlorella vulgaris* (Utex # 265). To ascertain the adaptability of these algal cultures to the effluent's chemical and physical environment, algae sample from the lagoons and UTEX slants were first grown in artificial IBR effluent and then transferred to sterile agar plates

prepared with diluted and pretreated IBR effluent. The strains grown on plates were transferred to sterilized liquid IBR effluent and this process was repeated multiple times to ensure adaptability of alga to the effluent environment.

The adapted algal strains were cultured in triplicates with a uninnoculated sterile control in 1L CORNING spinner flasks (S/N- 3561). The algal cultures were stirred at constant speed of 125 rpm using ISOTEMP 1110049S stir plates and were illuminated from two opposite sides using four 40W fluorescent tubes (Ecolux Sunshine 40). Irradiance of light was measured at the center of the flasks at a height of 3.5inch from the bottom of the flask using WALZ US-SQS/L light sensor and LI-COR LI- 250A light meter. A 12-hour light-dark cycle was used and level of Irradiance was kept constant at 485μmoles/m^2/sec during the entire length of research study. pH of the cultures in all the experiments was kept between 6.9 – 7.0 using CO_2 sparging. The pH of the cultures was checked daily using Inlab Versatile pro pH probe (Mettler Toledo, Columbus, OH) and CO_2 supply was adjusted if the pH was found to be out of the above range.

Algal growth was monitored using total suspended solids (TSS) as representative of growth and was measured using Method 2540D for examination of water and wastewater [61]. Also, daily samples were filtered through 0.45μ syringe filters (Nalgene 190-2545) and analyzed for dissolved total nitrogen (dTN) and ammonia nitrogen (dNH_3-N), dissolved total phosphorous (dTP) and ortho phosphorus (dOP), and dissolved chemical oxygen demand (dCOD). All the analyses were performed according to standard methods for the examination of waters and wastewaters [62]. Dissolved organic N and P were determined by subtracting dNH_3-N from dTN and dOP from dTP, respectively.

Results and Discussion

Biodiesel production from algae grown on inexpensive and locally available sources like effluent of an anaerobic digester treating dairy waste could be highly cost effective as algae in these processes utilize native substrate for growth. However biomass production and intracellular lipid accumulation in such a process could be limited by factors not applicable to controlled algal growth in synthetic media. Some of these factors include bioavailability of native nutrients in the effluent, effect of effluent borne microorganism, and the adaptability of algal strain to the effluent environment [7-10]. These process specific factors render characterization of the effluent for bioavailability of nutrients and determining the ability of an alga to grow in distinct effluent environment as the most important steps towards determining the suitability of an effluent for algal cultivation.

Characteristics of Effluent Affecting the Algal Growth

In this study, nutrients in the IBR effluent was characterized to determine the concentration of major elements in dissolved and particulate phases of the effluent, and relative distribution of C, N, and P in organic and inorganic forms in the effluent. The distribution of major elements in dissolved and particulate phases of the IBR effluent has been shown in Fig. 3-1 (A). It can be seen that a significant portion of most of the elements was present in particulate phase. The presence of these elements (especially C, P, Ca, and Mg) in the particulate phase was likely due to the precipitation of these elements that was assisted by alkaline pH (between 7.8 – 8.0) of the effluent [23, 27].

High amounts of precipitation in the effluent not only limit the availability of these elements for biological uptake but also impede the penetration of light in the effluent that could adversely affect the phototrophic growth of algae [26, 63]. Hence pretreatment of

the effluent was performed to counter the effects of particulates in the effluent. Preliminary dilution (four-fold) of effluent samples was done before removal of coarse solids through centrifugation so to release the precipitates adsorbed or caught in between coarse solids. The second dilution (five-fold dilution of stock samples) was done to increase penetration of light through the effluent as light impeding effect of particulates was diminished due to the dissolution of precipitates on increasing the dilution.

The concentration and distribution of C, N, and P in the IBR effluent in organic and inorganic forms is shown in Fig. 3-1 (B). It can be seen that besides the inorganic form, high percentages of C (85%), N (50%), and P (40%) were also present in organic form in the effluent. As organic fractions of these nutrients are significant, the utilization or removal of this form of nutrients becomes equally important as the inorganic form. Also, algae cultivated on natural substrates like animal waste effluents or wastewater have shown to perform mixotrophic (both phototrophic and heterotrophic) growth and utilize organic substrates along with inorganic nutrients [64]. Hence availability of organic nutrients in growth medium could facilitate higher biomass production in mixotrophic algae due to prolonged availability of usable substrates.

Besides, the availability of nutrients necessary for algal growth, phototrophic algal strains also need optimum level of light for growth. Algal growth on waste from dairy farms or anaerobically digested dairy waste has been seen as a measure of nutrient removal in the past studies [9, 25, 65, 66]. Although these studies showed that phototrophic algal growth is supported by dairy waste effluent, the experiments performed in most of these studies were on treated and standardized effluent. Therefore, to estimate the potential of phototrophic growth in the effluent it is important to determine the

availability of light in its natural form.

Also, treatments such as removal of particulates or dilution affect the light penetration through the effluent [67]. Therefore, identifying the changes in light availability at various stages can help to devise pretreatment steps to ascertain optimal light conditions in the effluent. Availability of light in the effluent for phototrophic growth depends on penetration of light through it. The penetration of light through the effluent depends on two factors: 1) scattering of light because of the suspended solids and 2) absorbance of light due to the color of the effluent because of the presence of colored organic compounds [13]. The combined and individual effects of these factors on the penetration of light through the effluent together with an estimate of decline in their effects due to an increase in dilution have been studied here.

Fig. 3-2 represents the light penetration through the effluent samples at different levels of dilution. The combined effect on light penetration due to scattering of light by suspended solids and light absorption by the color of effluent is shown in part [A], whereas effect on light penetration only due to absorption of light by the effluent color is shown in part [B] of the figure.

While there was no significant penetration of light observed at 10x or lower levels of dilution in whole samples [A], samples with suspended solids removed [B] had at least 12% of light penetrated through them at dilution 10x or higher. It is clear on comparing data from [A] and [B] that the color of the effluent itself has a big effect on penetration of light through the effluent. There was almost no penetration of light through the effluent samples with 0 to 5x dilution even after removal of the suspended solids. Also, it was observed that percent of penetrated light increased steeply at irradiance level 700

µmoles/m^2/s and above both in [A] and [B]. This suggests that color of the effluent (which is common variable in both cases) has stronger effect on light penetration than presence of suspended solids in the medium. This is also to be noted that even at the highest level of dilution (40x) in [B], the percent of light penetrated through was only about 52% of that of clear water, and rest is lost solely because of absorption of light by color of the effluent.

The above discussion supports the need of diluting the effluent to a certain level in order to support any phototrophic growth. The other advantage of diluting the effluent is the increase in concentration of dissolved ortho-P in the effluent with an increase in dilution. Fig. 3-3 shows the relationship between ortho-P dissolution and decrease in TSS (on diluting the effluent). The increase in dissolved ortho-P has been shown by multiplying the concentration of dissolved ortho-P at a particular dilution by the dilution factor. The data show a strong correlation between ortho-P dissolution and TSS removal. This correlation is also consistent with previous observations and prediction on release of ortho-P from the precipitates in the effluent on increasing the dilution [9]. Thus it can be understood that diluting the effluent increases both penetration of light and dissolution of ortho-P but as the dilution increases the overall concentration of nutrients in the effluent decreases. Hence the level of dilution of the effluent for phototrophic utilization of nutrients must be decided in concert with other characteristics of the effluent such as total elemental composition, dissolution of precipitates, and relative ratio of nutrients.

Adaptability of Algal Strains to the Effluent Environment

Since algal strains selected for this study came from different sources, the primary growth environment and media composition for these strains (sewer wastewater for LLAI and standardized culture media for *S. dimorphous* and *C. vulgaris*) were different from the

effluent of IBR in physical and chemical characteristics. Therefore, these algal strains needed to adapt to the complex and unique environment of the IBR effluent, through a process known as physiological acclimation [68]. It has been shown that changes in habitat of microalgae induce adaptive mutations in algal cells that help in evolutionary acclimation to the growth environment [69]. While the ability of an algal strain to adapt to a natural growth medium depends on various factors, the most important being the availability of nutrients required for growth, physico-chemical conditions suitable for algal growth, and competition from native microorganisms for substrate [70, 71].

This part of the study was performed to determine the adaptability of algal strains to sterile effluent medium, while the effect of native microorganisms on algal growth will be discussed in the following section. During this adaptability study the algal strains were first grown on agar plates made out of IBR effluent and then grown multiple times in the liquid effluent to ensure their adaptability to the effluent environment. Table 3-1 shows log-phase growth rate and biomass production in cultures of algal strains in different generations of growth during adaptability study. The increase in these parameters over different generations was used to identify the acclimation of algal strains to the effluent. An algal strain was considered adapted to the effluent environment when no significant increase in log-phase growth rate and biomass production was observed in subsequent generations of growth.

Data shown in Table 3-1 indicate that both biomass production and growth rate increased from the previous stages suggesting the enhanced adaptability of the algal strains due to physiological acclimation. It can be seen that growth rates of log phase and biomass production increased significantly during the adaptability study in all the algal

cultures. However, the increase in biomass production was higher in LLAI culture (8.7 times) than algal cultures of type strains of *S. dimorphous* (5.1 times) and *C. vulgaris* (4.2 times). Also, while final growth rates of type strains were superior to LLAI, overall increase in growth rate of LLAI was greater than type strains during physiological acclimation of algae to the effluent. This increase was found to be 4.8 folds in LLAI compared to 3.6 and 3.8 folds in *S. dimorphou*s and *C. vulgaris*, respectively.

Higher increase in biomass production and growth rate in LLAI during physiological acclimation suggests better adaptation of this alga to the new growth medium (IBR effluent) than the type strains. This superior adaptation of LLAI was possibly due to its original habitat being a natural medium as opposed to the standardized growth medium of type strains. As it has been found in the past studies that algal strains isolated from natural habitats generally have flexibility in their systems that allows them to undergo physiological adaptation or genetic mutation strains in stressed environments [72]. This flexibility in the system of isolated algae strains enables them to adapt to a new growth environment better than pure strains, which probably have much hardened system resistive to any physiological or genetic changes [73].

Comparison between Sterile and Unsterile Growth

The effluent of an anaerobic digester treating dairy waste contains a number of indigenous microorganisms [74]. For a successful survival of a foreign species of microorganisms, it should be able to dominate the microbial consortium and outcompete native microorganisms for utilization of resources in the effluent. The dominance of a foreign species could be achieved by providing the environmental conditions that are species specific and at the same time unfavorable or neutral to the native organisms. In a

growth medium like anaerobic digester effluent where the majority of native microorganisms are either obligate or facultative anaerobes [75], an aerobic process could provide such environmental conditions. Algal culturing is one such process as it is generally carried out either with continuous mixing or sparging of gases (CO_2 or compressed air), which induces aerobic conditions in the growth medium detrimental to most of the anaerobic microorganisms [76].

In this part of the study the effects of native microorganisms in the IBR effluent on growth characteristics of the algal strains was determined. Algal strains were simultaneously grown in the IBR effluent in sterile and unsterile growth conditions, and biomass production and amounts of different forms of nutrient utilized at various stages of growth were compared. Fig. 3-4 shows the comparison between growth curves and dissolved-COD removal from the three algal strains grown in sterile and unsterile environment in the IBR effluent. It can be seen that the log-phase growth rates were reduced in all three algal cultures in unsterile growth conditions, culture densities also decreased in the strains of *S. dimorphous* (24.5%) and *C. vulgaris* (11.5%) whereas an increase (35.7%) in culture density was observed in LLAI. The increased culture density in LLAI was possible due to its slower and longer growth in unsterile conditions than *S. dimorphous* and *C. vulgaris*. This prolonged growth allowed LLAI to better adapt to the effluent environment than the other two strains.

The effect of native microorganisms can also be estimated on the basis of utilization of substrate by the algal cultures. From Fig. 3-4, removal of dissolved chemical oxygen demand (dCOD) indicates a rise in uptake of heterotrophic-C in all three algal cultures in unsterile growth conditions. However, TSS data indicate an increase in algal biomass

production only in LLAI culture. It can also be calculated that the increase in the ratio of dCOD removal to algal biomass produced was smaller in LLAI (from 150 to 180 mg of dCOD removed per gram of biomass) than in two type strains (from 66 to 145 mg of dCOD removed per gram of biomass in *S. dimorphous* and from 49 to 93mg of dCOD removed per gram of biomass in *C. vulgaris*).These results suggest that while dCOD removal in LLAI was mostly due to heterotrophic algal growth, significant microbial activity of native microorganisms could be the cause of increased dCOD removal in the cultures of two type strains in unsterile growth conditions.

Similar results were obtained from the analyses of nutrient (N and P) removal by algal cultures grown in the IBR effluent. Fig. 3-5 shows the removal of inorganic and organic forms of N and P by three algal cultures in sterile and unsterile growth conditions. It can be seen that there was an increase in nitrogen removal (with a small decrease in phosphorous removal) in *S. dimorphous* and *C. vulgaris* cultures despite a decrease in algal biomass production in unsterile conditions. An increase in both N and P proportional to the production of algal biomass was observed in LLAI culture when sterile and unsterile growths were compared. This further supports the argument that while the presence of native microorganisms did not affect the growth and nutrient utilization in LLAI culture, it significantly hampered the production of algal biomass and changed the nutrient uptake patterns in *S. dimorphous* and *C. vulgaris* cultures.

Utilization of Organic Substrates by Algal Strains

Unlike the growth of an alga in a defined and strain specific medium, growth in a natural medium such as effluent of an anaerobic digester treating dairy waste depends on the ability of the strain to utilize available forms of nutrients in complex environmental

conditions. In a growth medium with limited availability of light for phototrophic growth (Fig. 3-2) and abundance of organic substrates (Fig. 3-1) to support heterotrophic growth, an algal strain capable of performing both phototrophic and heterotrophic growth can be expected to thrive. The two type strains studied in this research, *S. dimorphous* and *C. vulgaris* have been reported to be found in various wastewater treatment systems around the world and shown to perform mixotrophic growth in various research studies [17, 59, 77]. While the third strain LLAI, being originated from natural medium (wastewater lagoon) could be expected to perform mixotrophic growth in suitable conditions [78].

As suggested by the uptake of organic substrates i.e. organic-C (estimated from dCOD removal), and organic N and P shown in Figs. 3-4 and 3-5, it can be concluded that all the three algal strains performed heterotrophic growth along with phototrophic growth in both sterile and unsterile growth conditions. However, higher amounts of algal biomass were found to be produced in the cultures showing greater uptake of organic nutrients. In other words algal strains performing higher percentage of heterotrophic growth (LLAI) produced more algal biomass than the other strains, which underwent a greater percentage of phototrophic growth.

This relationship between uptake of organic substrates and biomass production can be explained through the trends of nutrient uptake in algal cultures. It can be noticed in the trend curves (especially those of LLAI) shown in Fig. 3-6, there is an increase in uptake of organic substrate after an initial phase. This increase in the uptake of organic nutrient suggests the tendency of algal cells to enhance heterotrophic growth when light penetration in the effluent diminished due to high culture density. The ability of algal cells to switch between phototrophic and heterotrophic growth has been studied in the past, and

it has been found that during light limited conditions some strains of algae perform heterotrophic growth to survive [79, 80].

Therefore, based on the results discussed above, it can be understood that an algal strain capable of enhancing heterotrophy in light limited conditions can sustain growth for a longer period of time. As discussed in previous sections that out of the three algal strains studied in this research, LLAI was best adapted to the IBR effluent. This superior adaptability of LLAI enabled it to sustain heterotrophic growth for a longer period of time in light limited conditions than the other two strains, hence higher algal biomass production was seen in LLAI culture.

Conclusions

Characterization of the effluent of an IBR anaerobic digester done in this study revealed that nearly half of N and P, and ninety-percent of C in the effluent were present in organic form. It was also shown that major fractions of elements such as C, P, Ca, and Mg were present in particulate phase in the effluent. Further experiments suggested due to high amounts of particulates and limited availability of light in the effluent, pretreatment involving removal of coarse solids and dilution of the effluent should be done before utilizing it for algal growth. The results showed that pretreatment of the effluent not only improved the availability of light for phototrophic algal growth but also increased the concentration of ortho-P in the effluent, due to dissolution of precipitates on increasing the dilution.

Since the algal strains selected for this study came from different sources, these algal strains were given the opportunity to adapt to the complex and unique environment of the IBR effluent. A marked improvement in biomass production and growth rates was seen in

all three cultures during the adaptability study. However, the adaptation of Logan lagoons algal isolate (LLAI) to the effluent environment was much superior to that of the other two strains. Results showed that biomass production and growth-rate of log-phase increased by 8.7-fold and 4.8-fold, respectively, in LLAI culture as compared to 5.1- and 3.6-fold, respectively, in *S. dimorphous* and 4.3 and 3.8-fold respectively in *C. vulgaris* cultures.

The experiments performed to determine the effect of presence of native microorganisms in the effluent on growth and nutrient utilization by the algal strains showed that all the three strains were able to grow in unsterile growth conditions. On the other hand, comparative study between algal cultures grown in sterile and unsterile effluent revealed that culture densities in *S. dimorphous* and *C. vulgaris* decreased (24.5 and 11.5 percent, respectively) in the presence of native microorganisms, whereas it increased (35.7 percent) due to extended growth of LLAI in unsterile conditions. Results also suggested that while the removal of organic nutrients (C, N, and P) in LLAI culture was mostly due to heterotrophic algal growth, significant microbial activity of native organisms contributed to the removal of organic substrate in cultures of two type strains in unsterile growth conditions.

It was seen in this study that LLAI has produced higher biomass production and utilized more nutrients (organic and inorganic) than the type strains *S. dimorphous* and *C. vulgaris* both in sterile and unsterile growth conditions. This could be attributed to the ability of LLAI strain to adapt to the effluent environment which enabled it to utilize the available form of nutrients. It has been suggested that LLAI since originated from a natural habitat had better adaptability to the new environmental conditions than the two type strains that allowed it to grow for longer period of times in the effluent.

It was also found that besides utilizing inorganic nutrients, the algal strains also utilized significant fractions of organic N (up to 45% of total N) and P (up to 50% of total P), and heterotrophic-C (up to 27% of total-C utilized). The pattern of uptake of organic substrates revealed that algal cultures tended to switch from phototrophic to heterotrophic growth when light penetration within the effluent was reduced due to increased culture density. Results showed LLAI culture grew the longest, removed the highest percentage of organic nutrients and attained highest culture density among the three cultures. It has been suggested in this study that the algal strain that has the ability to incorporate heterotrophic growth with phototrophic growth when light penetration in the effluent diminished due to high culture density has better chances of survival in a growth medium such as anaerobic digester effluent.

Table 3-1 Biomass production and growth rate of log phase in different algal cultures grown on IBR effluent in different generations of adaptation study

Algal Culture	Generation of culture	Biomass production, mg/L	Growth rate of log-phase mg-dry-cells/L/day
Logan Lagoon Algal Isolate	**1st**	140	25
	2nd	280	30
	3rd	770	40
	4th	980	95
	5th	1220	120
S. dimorphous	**1st**	170	50
	2nd	310	120
	3rd	870	180
C. vulgaris	**1st**	120	50
	2nd	280	130
	3rd	510	190

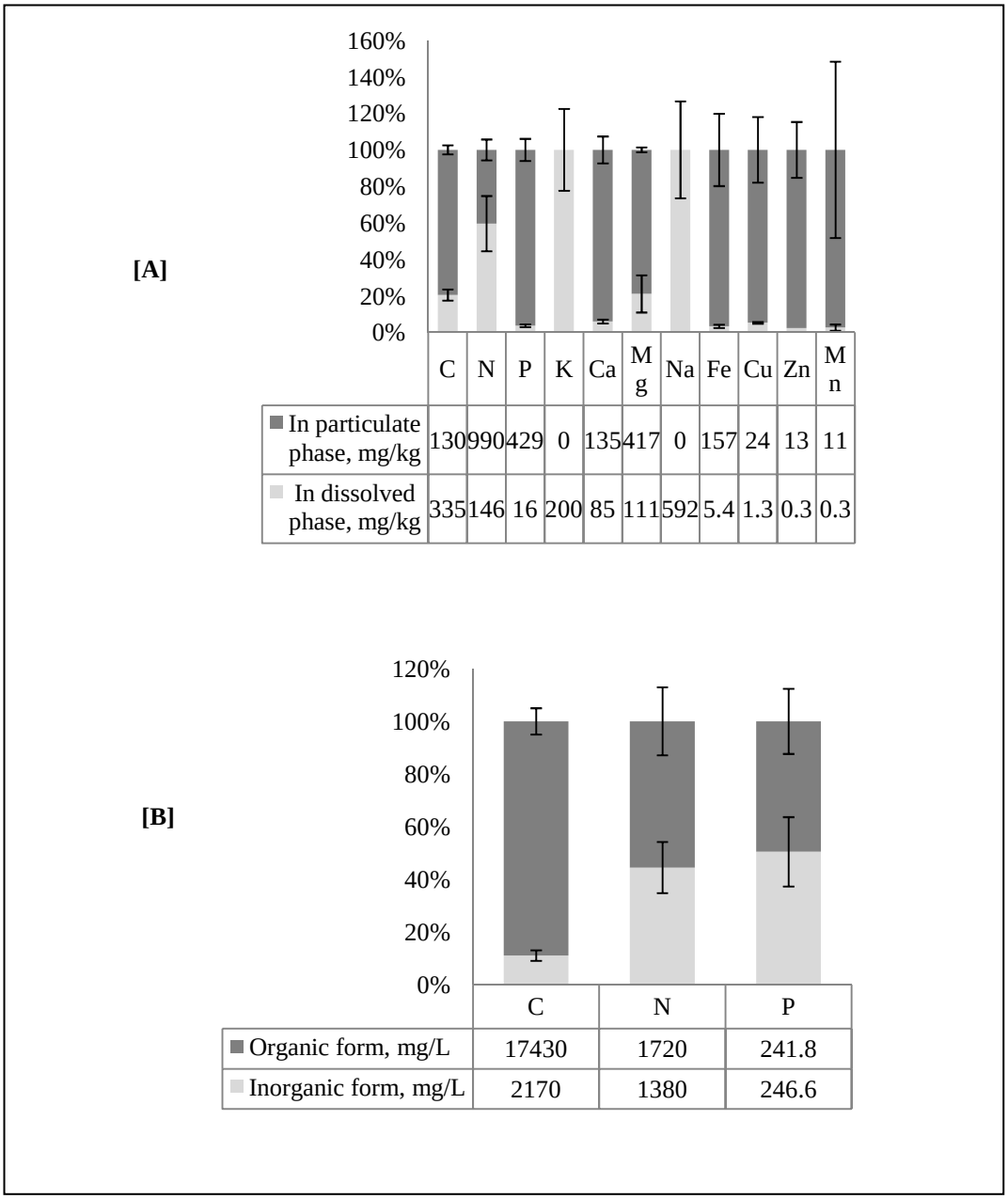


Fig. 3-1 Distribution of different elements in dissolved and particulate phases of the whole (untreated) IBR effluent [A] and distribution of C, N, and P in organic and inorganic forms in the whole (untreated) IBR effluent [B]

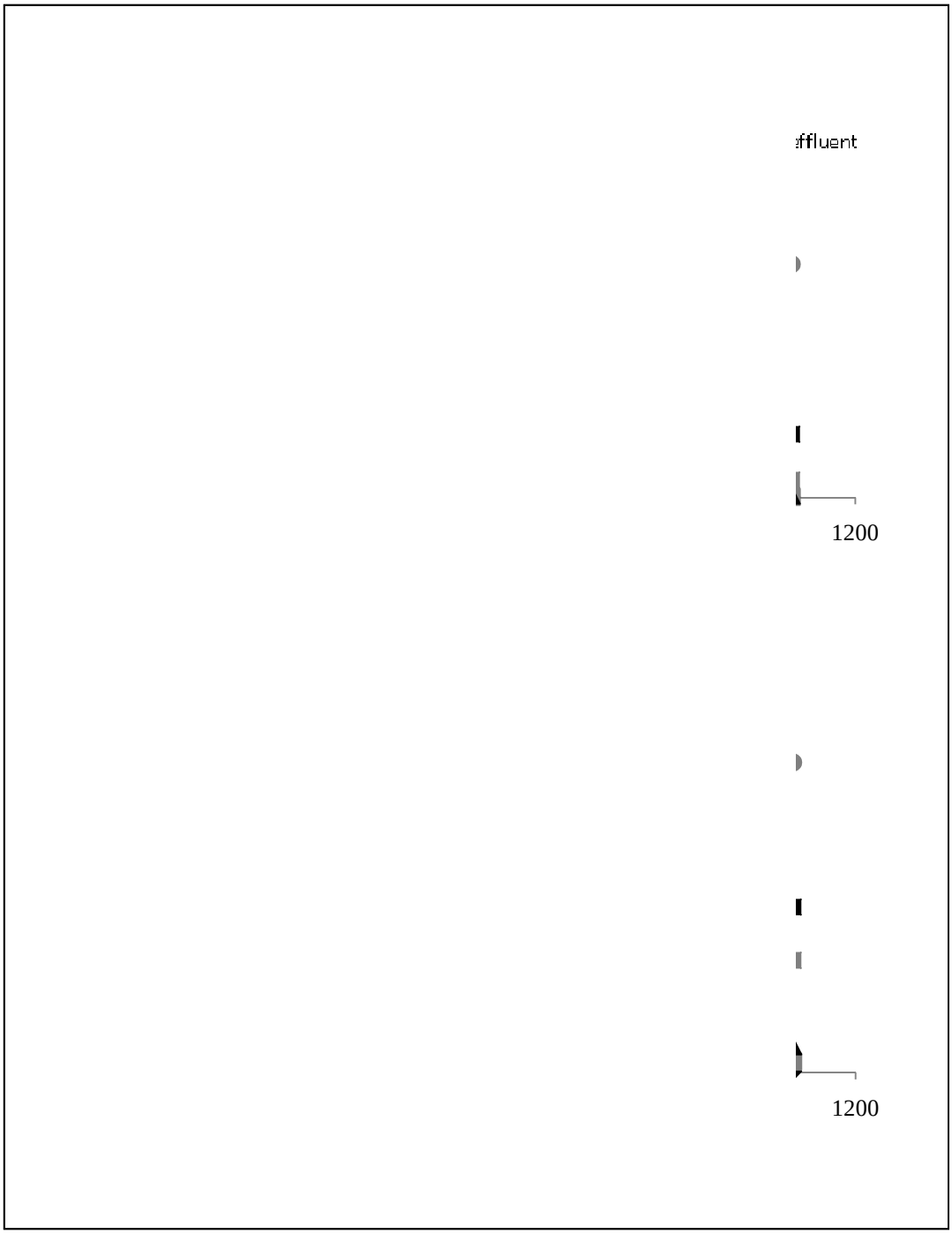


Fig. 3-2 Penetration of light through the IBR effluent at different levels of dilution: [A] Whole effluent and [B] Effluent with suspended solids removed

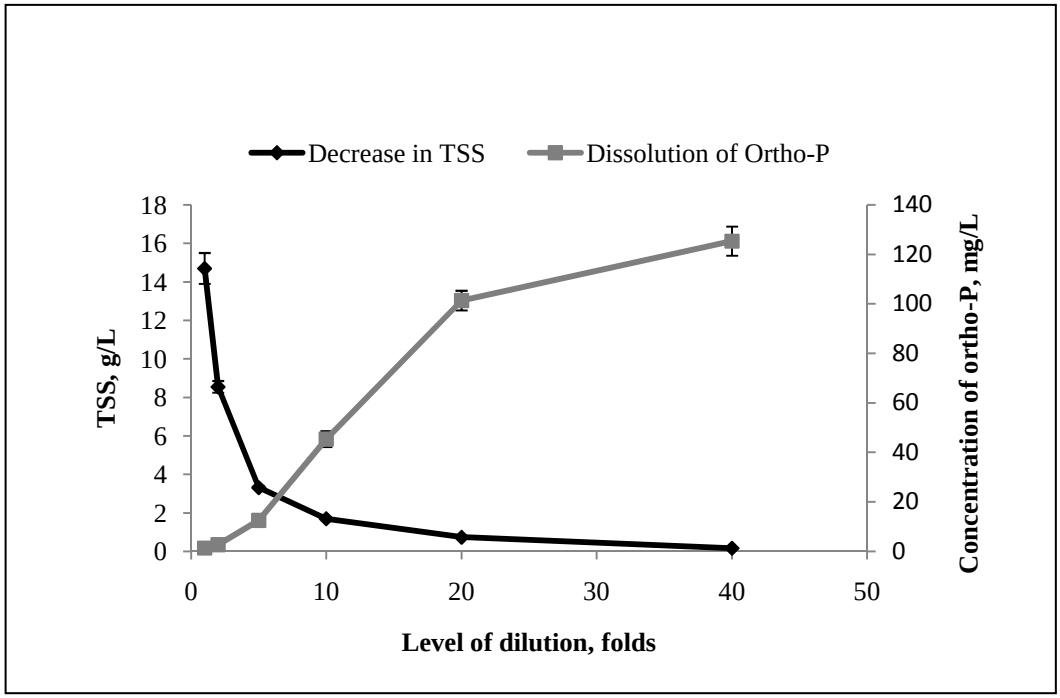


Fig. 3-3 Decrease in TSS concentration and dissolution of ortho-P on increasing the dilution of IBR effluent

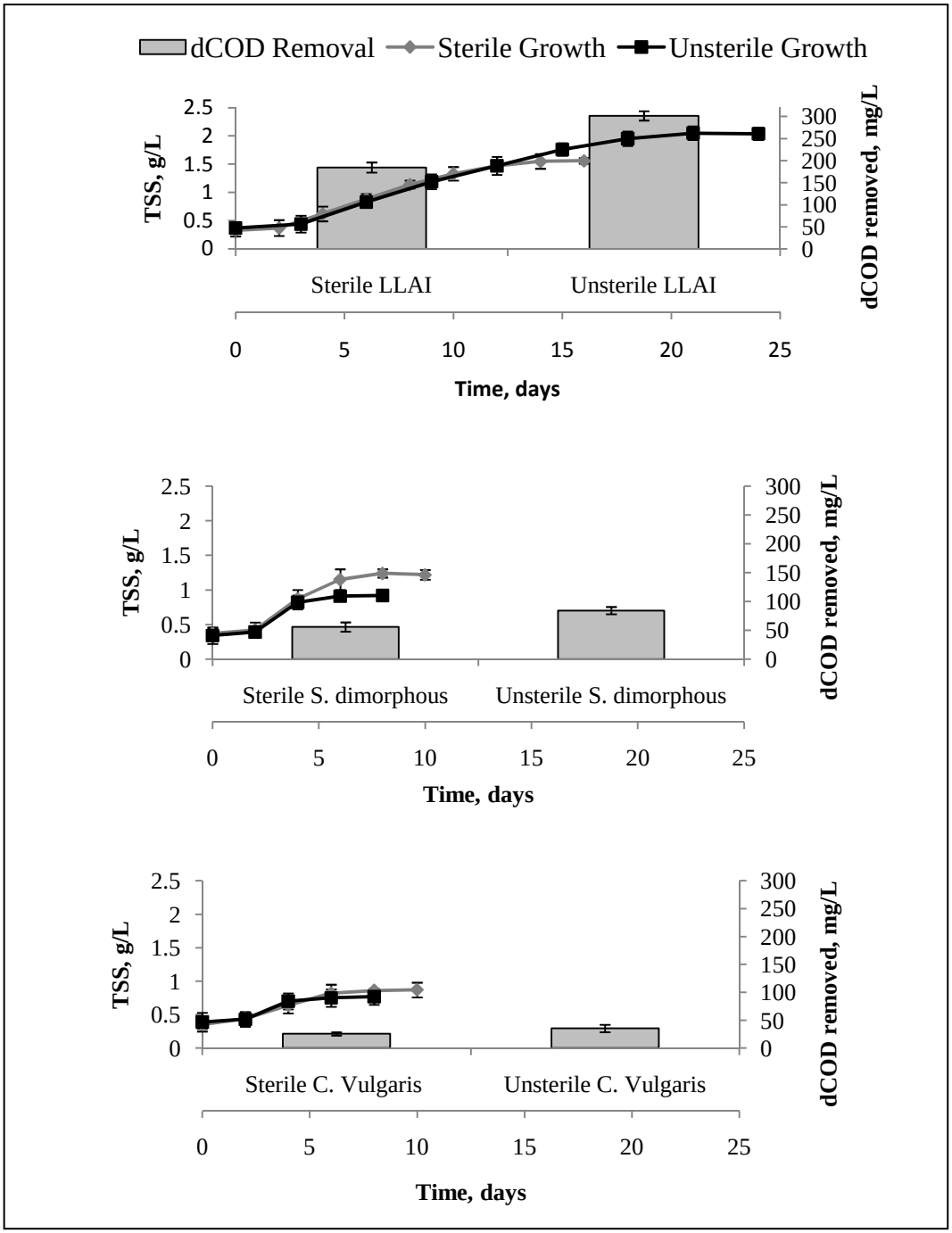


Fig. 3-4 Growth curves and dCOD removal from three algal strains grown in sterile and unsterile environment in the IBR effluent

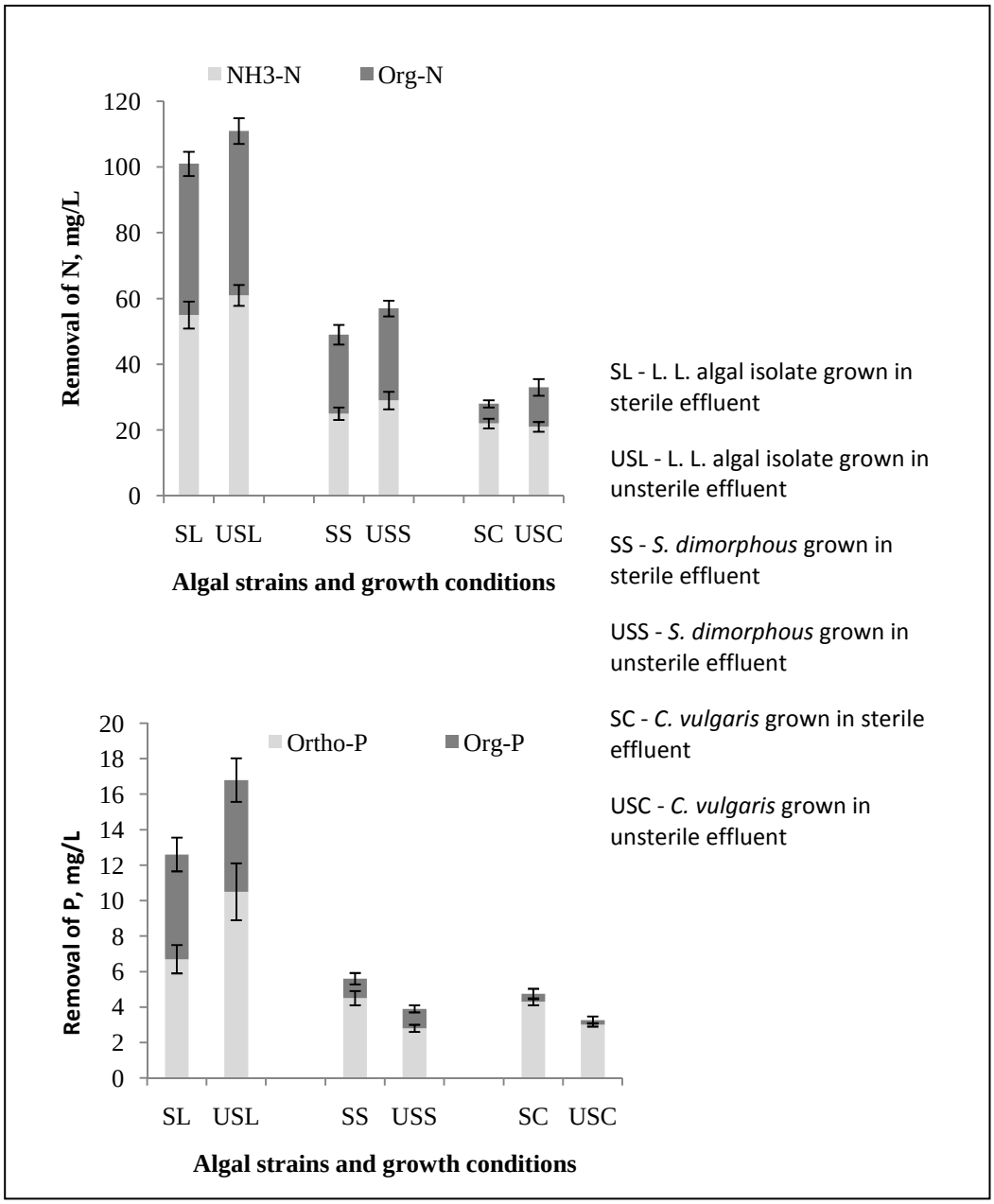


Fig. 3-5 N and P removal by the algal strains grown in sterile and unsterile IBR effluent

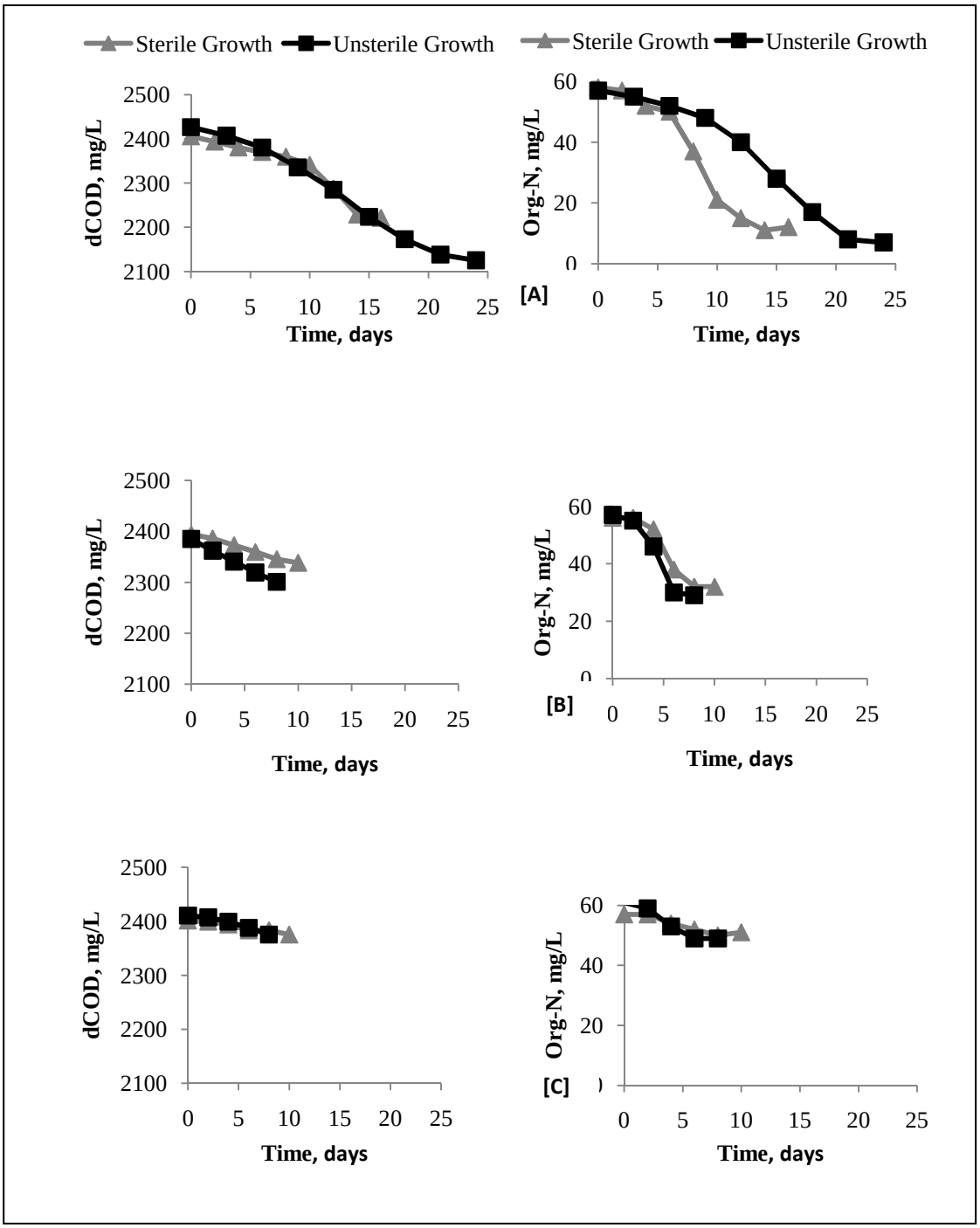


Fig. 3-6 dCOD and Organic-N removal by algal cultures during growth in sterile and unsterile IBR effluent. [A] Logan Lagoon Algal Isolate, [B] *S. dimorphous*, [C] *C. vulgaris*

CHAPTER 4

MAXIMIZING ALGAL GROWTH IN BATCH REACTORS USING SEQUENTIAL CHANGE IN LIGHT INTENSITY[1]

Abstract Algal growth requires optimal irradiance. In photobioreactors, optimal light requirements change during the growth cycle. At low culture densities, a high incident light intensity can cause photoinhibition, and in dense algal cultures, light penetration may be limited. Insufficient light supply in concentrated algae suspensions can create zones of dissimilar photon flux density inside the reactor, which can cause suboptimal algal growth. However, growth of dense cultures can also be impaired due to photoinhibition if cells are exposed to excessively high light intensities. In order to simultaneously maintain optimal growth and photon use efficiency, strategies for light supply must be based on cell concentrations in the culture. In this study, a lipid-producing microalgal strain, *Neochloris oleoabundans*, was grown in batch photobioreactors. Growth rates and biomass concentrations of cultures exposed to constant light were measured and compared with the growth kinetic parameters of cultures grown using sequentially increasing light intensities based on increasing culture densities during batch growth. Our results show that reactors operated under conditions of sequential increase in irradiance levels yield up to a 2-fold higher biomass concentration when compared with reactors grown under constant light without negatively impacting growth rates. In addition, this tailored light supply resulted in less overall photon use per unit mass of generated cells.

[1]S. Wahal and S. Viamajala, 2010"Maximizing algal growth in batch reactors using sequential change in light intensity." Applied Biochemistry and Biotechnology 161(1-8): 511 - 522

Introduction

Microalgae can serve as feedstocks for a variety of products including biodiesel [51, 81], biohydrogen [82], pigments, polyunsaturated fatty acid [83, 84], and for environmental purposes such as capture of CO_2 from flue gases [85, 86] and remediation of environmental pollutants [87]. There are two primary options for mass production of algae—open systems such as raceway ponds and closed photobioreactors. Outdoor culture systems, while cheaper, have been shown to have lower long-term productivity due to limited options for light and carbon management as well as susceptibility to contamination [84, 88]. On the other hand, while initial capital investments for photobioreactors are higher than those for open ponds, better contaminant management and improved utilization of photosynthetically active radiation as well as carbon can lead to superior overall productivities [89].

Since phototrophic growth requires inputs of light and inorganic carbon (most often derived from dissolved carbon dioxide), algae culturing can potentially be limited by inadequate availability of either of these components to growing cells. When culture densities increase during growth, cellular absorption of light as well as other optically relevant effects such as scattering and shading results in a significant decrease in overall light penetration into culture vessels [63, 90]. This creates zones of dissimilar photon flux density (PFD) inside the reactor with light availability mostly restricted to regions near the illuminated surfaces. Consequently several reactor designs, including flat-plate and tubular-type, seek to maximize the ratio of illuminated surface to volume [91]. Mixing significantly improves photon distribution to the growing cultures in these reactors by assisting the periodic transport of cells into the lighted regions of the photobioreactors [92-

94]. Higher growth yields are generally observed in well mixed reactors [95, 96]. Other designs integrate optics with growth reactors using sunlight collection and distribution devices [97, 98]. Such optical designs seek to deliver light to growing reactors based on photosynthetic needs and thereby improve efficiency of photon use as well as areal productivity [97].

When culture densities are low during early stages of logarithmic growth, light effectively penetrates through the entire culture medium, and a low light flux is adequate. In dense suspensions, higher intensity light is required to penetrate deeper and be more available to growing cultures. Thus, supply of light can be tailored to the requirements of algal cultures as they progress through their growth cycle to enhance light use while achieving high cell concentrations. Previous studies have shown that light control in photobioreactors can serve to improve growth rates [99, 100] as well as final biomass concentrations [100, 101]. However, these studies utilize sophisticated control methods that include frequent changes in light intensities. In large-scale systems processing several million gallons of algal cultures for biofuel production, such precise controls are likely to be difficult to implement, and perhaps unnecessary. Simpler methods that involve only periodic alteration of growth parameters are more practical. Based on this supposition, we hypothesized that phototrophic growth could be sustained to reach high algal culture densities by a “fed-batch” approach to light supply. In such a system, increase in incident intensities would serve to replenish light within the bioreactor when photon limitations became evident from a decline in growth rate.

In this study, we tested our hypothesis using a lipid-producing microalgal strain *Neochloris oleoabundans* [11, 12] grown in externally illuminated stirred tank reactors.

We observed that after the cultures stopped growing at a low incident photon flux, a step increase in illumination resulted in resumption of growth. Overall, better biomass concentrations were observed when growth started at low light intensities and progressed higher in comparison to cultures illuminated at higher initial levels. Kinetic parameters related to growth and nitrogen consumption were measured and are compared between the different phototrophic growth regimes tested.

Materials and Methods

Organism, Media and Growth Conditions

N. oleoabundans (UTEX # 1185) cultures were grown in a modified Bristol media that contained the following: $NaNO_3$ (9 mM), KH_2PO_4 (1.4 mM), $MgSO_4.7H_2O$ (0.3 mM), $CaCl_2.2H_2O$ (0.17 mM), NaCl (0.43 mM), and ferric ammonium citrate (15 mg/L). Media pH was adjusted to 7.0 prior to autoclaving (121°C, 20 min). The 100-mL cultures were first grown in 250-mL Erlenmeyer flasks on an illuminated shaker table (120 rpm), and log phase cultures were transferred to 6-L Cytostir® reactors (Kimble/Kontes, Vineland, NJ, USA) (working volume=5 L) that were used to perform growth studies reported here. All experiments were performed at room temperature (20°C).

Experimental Setup

To determine the effect of different light intensities on algal biomass accumulation under constant illumination and the range of light intensities supporting maximal algal growth, *N. oleoabundans* cultures were grown at six different levels of irradiance. The irradiance was provided by fluorescent light tubes placed around the bioreactors, and different light levels were attained by changing the distance between fluorescent tubes and

the bioreactors. A schematic of the experimental setup that depicts an end-on view of the arrangement is shown in Fig. 4-1. For the “sequential light change experiments,” the reactors were illuminated by a bank of 12 (six on each side of bioreactor) Ecolux Sunshine 40 W fluorescent tubes (GE Lighting, Cleveland, OH, USA) set on a frame such that the lights were 3 in. away from the vessel walls. The length of the frame was 40 in. and using this setup, light could be supplied to three reactors when placed adjacent to each other (3 in. apart). For our experiments, three levels of light intensity were created by switching on four, eight, or 12 (two, four, or six each on both sides) light tubes. The location of the light tubes that remained illuminated at each intensity level and the corresponding average photon flux at the center of the reactor is depicted in the table inset in Fig. 4-1. Average light intensity was determined as the mean value of the irradiance measured in five directions (N–S–E–Wand upward) at the center of the base of empty reactor placed in light enclosure. Light measurements were performed using a light meter (model LI-250A, Li-Cor Biosciences, Lincoln, NE, USA) equipped with a quantum sensor (model LI-190SA, Li-Cor Biosciences, Lincoln, NE, USA). The average light intensities with four, eight, or 12 fluorescent tubes switched-on were calculated to be 91.2, 177.8, and 273.1μmol $m^{-2}s^{-1}$, respectively.

The reactors for the experiments were placed on stir plates that were used to rotate the built-in paddle impellers. An air–CO_2 mixture was sparged through each reactor, and flow rates were monitored using gas flow meters. The entire reactor setup containing media and sparge system was autoclaved prior to inoculation. After the start of the experiments, gas flow rates were manually adjusted using in-line valves to maintain circum-neutral pH. This method of pH adjustment has been successfully used in previous studies [102], and in

our experiments, the overall gas flow changes required to maintain near-neutral pH were only 10% higher than the initial set point. Besides pH control, gas sparging supplemented inorganic carbon to the algal cultures and also provided a means for axial mixing in the reactor in addition to the radial fluid motion provided by the reactor paddles. Throughout the experiments, the cultures were exposed to a light–dark cycle of 12 h that was maintained using a timer connected to the light circuit.

Experimental Design

Initial experiments were performed at six PFDs—70.8, 91.2, 130.4, 177.8, 220.0, and 273.1μmol $m^{-2}s^{-1}$ to determine the effect of different light intensities on algal biomass accumulation under constant illumination. Thereafter, effects of sequential increase in incident light intensities on growth were studied using three light levels and two illumination schemes. All the three light levels were used sequentially in scheme I, whereas in scheme II, only levels 2 and 3 were employed in sequential order. In illumination scheme I, growth was started at a light intensity equal to 91.2μmol $m^{-2}s^{-1}$ (scheme I, level 1) and sequentially increased to 177.8μmol $m^{-2}s^{-1}$ (scheme I, level 2) and finally up to 273.1μmol $m^{-2}s^{-1}$ (scheme I, level 3). In the illumination scheme II, light intensities of 177.8 (scheme II, level 2) and 273.1μmol $m^{-2}s^{-1}$ (scheme II, level 3) were sequentially employed. During both illumination schemes, changes to higher light levels were made when growth was observed to stop at the lower intensity.

Analytical Methods

During growth studies, 15 mL of sample was periodically withdrawn from each reactor and analyzed for optical density, total suspended solids (TSS), and nitrate. For all samples, optical density was measured as absorbance at 685 nm using a Spectronic

Genesys 5 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). At higher culture densities, TSS measurements were also made using procedures outlined in the Standard Methods recommended by the American Public Health Association (Method 2540 D) [61]. Due to poor method sensitivity at low culture densities, TSS was measured only when at least partial growth was achieved. Correlations between optical density and TSS were developed and TSS concentrations in dilute cultures were determined from the calibration based on absorbance data. Nitrate (NO_3–N) was measured by the Cadmium reduction (Method 4500 –NO_3^{-1}) [103] using Hach nitrate analysis kits (Hach Company, Loveland, CO, USA).

Calculations

Total moles of incident photons during algal growth were calculated based on incident light flux as

$$N_{SL} = A_{eff}(I_{in}\Delta t) \tag{1}$$

where NSL represents the total incident photons during growth at L_{th} level under S_{th} illumination scheme of the experiment. I_{in} is the average incident light flux or PFD (μmol $m^{-2}s^{-1}$) at that level, and Δt represents the total duration during which light irradiance was available to the algal culture at that light level in the given scheme of experiment. A_{eff} is the effective surface area of the bioreactor (m^2) that is calculated as the projected area of liquid culture exposed normal to the incident light and given by

$$A_{eff} = \pi DH \tag{2}$$

where H is the liquid depth in the reactor (=7 in.), and D is the internal diameter of the

reactor (= 7 in.).

For example, total incident photons during growth at level 2 in scheme II of the experiment can be calculated using I_{in}=177.8μmol $m^{-2}s^{-1}$, Δt=13d at 12 hr/d=561,600s, and A_{eff} = π×7×7 in.^{2}=154 in.2=0.1 m^2.

$$\text{Hence; } N_{22} = 9.98 \times 10^6\ \mu\text{mol}$$

Rates of growth $\Delta X/\Delta t$ were calculated as the slope of the linear portion of the growth curve, whereas biomass yields per gram of NO_3–N consumption, $Y_{X/N}$, were calculated as the ratio of biomass accumulated during a given time period in an illumination scheme and NO_3–N consumed in that duration.

Results and Discussion

Photosaturation Studies with *N. oleoabundans*

In photobioreactors, available irradiance depends on the geometry of the reactor in addition to incident light intensity [104]. Therefore initial studies were performed to determine the incident light flux that would result in favorable algae growth in our reactor set-up. These tests were performed using constant irradiance in the range 70–273.1 μmol $m^{-2}s^{-1}$. Final biomass concentrations obtained at the end of these experiments were measured.

The results from these tests are shown in Fig. 4-2. Biomass concentrations were observed to first increase with incident PFD but after a certain level of illumination, a decrease in the final amount of biomass produced was observed, suggesting a behavior consistent with photosynthetic saturation. At low light intensity, the photosynthetic processing capabilities are not adequately utilized resulting in low bioproductivity. Higher illumination results in photosynthetic saturation where all the photons are fully utilized by

the phototrophic organism, and at still higher intensities, the available photons are not fully utilized and instead get dissipated [105]. Eventually, as irradiance level increases, photosynthesis is inhibited [106]. Most commonly, photosynthesis saturation curves are reported in terms of microalgal growth rates [63, 99, 107]. However, since biomass concentrations in photobioreactors, such as stirred vessels, are also proportional to incident light intensities, yield based data can also describe the photosaturation effects [108]. In such systems, under conditions of moderate mixing, phototrophic growth rates have been reported to be proportional to biomass concentrations [108].

Based on Fig. 4-2, it appears that photosaturation occurs at approximately 180μmol $m^{-2}s^{-1}$ and is consistent with other observations that report flux tolerances in the range 200–400μmol m−2s−1 for microalgal cultures grown in photobioreactors [92]. To avoid inhibition of low density starting cultures, initial light intensities for subsequent tests were chosen to be 91.2 and 177.8μmol m^{-2} s^{-1}.

Algal Growth Studies with Sequential Increase in Light Intensities

Growth data for the two sequential illumination tests are shown in Fig. 4-3. Nitrate concentrations monitored in these experiments to obtain nutrient-specific stoichiometric yield coefficients are also included. These data show the progressive accumulation of biomass over time and the corresponding nutrient consumption. It can be observed that the nitrogen consumption over the entire experimental duration was less than 50% of what was initially added. Also, less than half the phosphorus initially added was consumed (data not shown), suggesting that the growth was not nutrient limited. However, at each illumination level, growth stopped after a certain biomass concentration was achieved. Increase in light intensity led to a resumption of growth but the cultures reached stationary

phase again without complete depletion of nutrients. This phenomenon is likely due to inadequate availability of light to sustain growth at higher cell concentrations due to the balance between energy absorbed by the photosynthetic mechanism and that dissipated by respiration as hypothesized by previous research [109-111].

Overall biomass accumulation was enhanced when light intensities were sequentially increased under both illumination schemes (Fig. 4-3). When light flux closer to the photosynthetic saturation limit was initially used (illumination scheme II with initial PFD of 177.8μmol $m^{-2}s^{-1}$), the growth rates and yields were higher, as expected (Table 4-1). Also, when suboptimal photon flux was used initially (illumination scheme I with initial PFD of 91.2μmol $m^{-2}s^{-1}$), biomass yield based on nitrate consumption ($Y_{X/N}$) was lower. These results are corroborated by other previous research, which shows that algal cells show higher specific uptake of nitrogen when grown under low light and slower growth conditions in comparison to growth under higher light intensities that facilitate faster growth [112].

Figures 4-4 and 4-5 show the comparison between algal growth under illumination schemes I and II at lower (scheme I level 1 and scheme II level 2) and higher (scheme I level 2 and scheme II level 3) light levels, respectively. It can be seen that when the light intensity was increased to a higher level under both illumination schemes, growth rate trends were similar to those observed during lower level tests. Although illumination scheme II used light concentrations that were observed to be photoinhibitory during single light intensity experiments (Fig. 4-2), it appears that the inhibitory effect was mitigated due to exposure of the dense growing culture to this light intensity. Since the penetration of light is less in dense cultures, cells were likely exposed to the higher intensities for

smaller periods as they moved in and out of the illuminated region due to mixing. This limited exposure likely prevented the inhibitory effects that would otherwise be observed if cultures were exposed to high light intensities initially when fewer cells are present in the liquid medium [92]. Although cultures grew faster under illumination scheme II that had overall higher light intensities, the final biomass concentrations (after exposure to light level 3) were higher during growth under illumination scheme I. It can be observed from Figs. 4-4 and 4-5 that under illumination scheme I, growth was prolonged, although slower, such that final culture densities were higher. One possible explanation for this behavior could be that under illumination scheme I, light intensities doubled between lower (level 1) and higher (level 2) levels, whereas they increased by a factor of only 1.5 under scheme II. The bigger jump in light intensity in scheme I possibly allowed better penetration of light through the algal culture, which resulted in phototrophic activity for a longer duration.

During growth studies under illumination scheme I, a third level of light intensity (273.1μmol $m^{-2}s^{-1}$) was applied when growth stopped after level 2. The cultures appeared to continue to grow as a result of this switching for first 2 days but showed no enhancement in cell concentrations after the third day. During this period (2 days), about 0.11 g/L of biomass was accumulated, yielding a growth rate of 0.055 g cells $L^{-1}day^{-1}$- the highest observed among all the levels in illumination scheme I. These unexpected high growth rates could be attributed to “flashing effect” where cells were likely exposed to very high intensities for short periods due to the small penetration depth of light in these dense cultures. It has been reported in previous studies that flashing or intermittent exposure of high intensity light increases the efficiency of photosynthesis by algal cells

[92, 113], and it is possible that the fortuitous combination of mixing speeds, light intensity, and culture density resulted in this phenomenon in our tests.

Comparison of Culture Performance between Illumination Conditions Tested

The concentrations of biomass accumulated at the end of every level of growth during both illumination sequences are shown in Table 4-1. We can consider level 1 tests in each illumination sequence experiment as the constant-light control since uniform illumination was supplied during this portion of growth. On comparing biomass accumulated at the end of first level with biomass obtained from the entire growth study under the individual illumination sequence (including all levels), we can get the net effect of sequential change in light intensity on overall algal biomass accumulation. Through simple calculations, we can see that during scheme I, the overall biomass accumulation ($\Delta X_1 + \Delta X_2 + \Delta X_3$ = 1.02 g cells/L) increases by 1.92-fold as compared to level 1 growth (ΔX_1=0.53 gcells/L). Similarly, a 1.38-fold increase is observed during scheme II experiments ($\Delta X_2 + \Delta X_3$ = 0.84 compared to ΔX_2 = 0.61). Another appraisal of the system performance is through measure of productivity under the two illumination schemes tested; 1.02 g cells/L were obtained over 22 days (neglecting the 2 days of no growth) when illumination scheme I was employed such that the productivity under this scheme was 0.046 gcells $L^{-1}day^{-1}$. Productivity with illumination scheme II can similarly be calculated to be 0.0525 gcells L^{-1} day^{-1} showing that although final biomass concentrations were lower during growth on higher intensity lights, overall productivities stayed higher.

One final measure of system performance is through evaluation of efficiency of photon energy use during the two illumination schemes. Table 4-2 shows total incident photons on the liquid culture during different levels of algal growth. Using these data, it

can be calculated that total moles of photons supplied under illumination scheme I (N_{11} + N_{12} + N_{13} = 10.55 mol) is only 0.66 times of photons supplied under illumination scheme II (N_{22} + N_{23} = 15.88 mol). However, the final biomass accumulation at the end of scheme I (ΔX_1 + ΔX_2 + ΔX_3 = 1.02 g cells/L) is 1.21 times of biomass accumulated at the end of scheme II (ΔX_2 + ΔX_3 = 0.84). This suggests that it is more energy efficient to start culture growth with lower intensity light to maximize utilization of incident radiation.

Conclusions

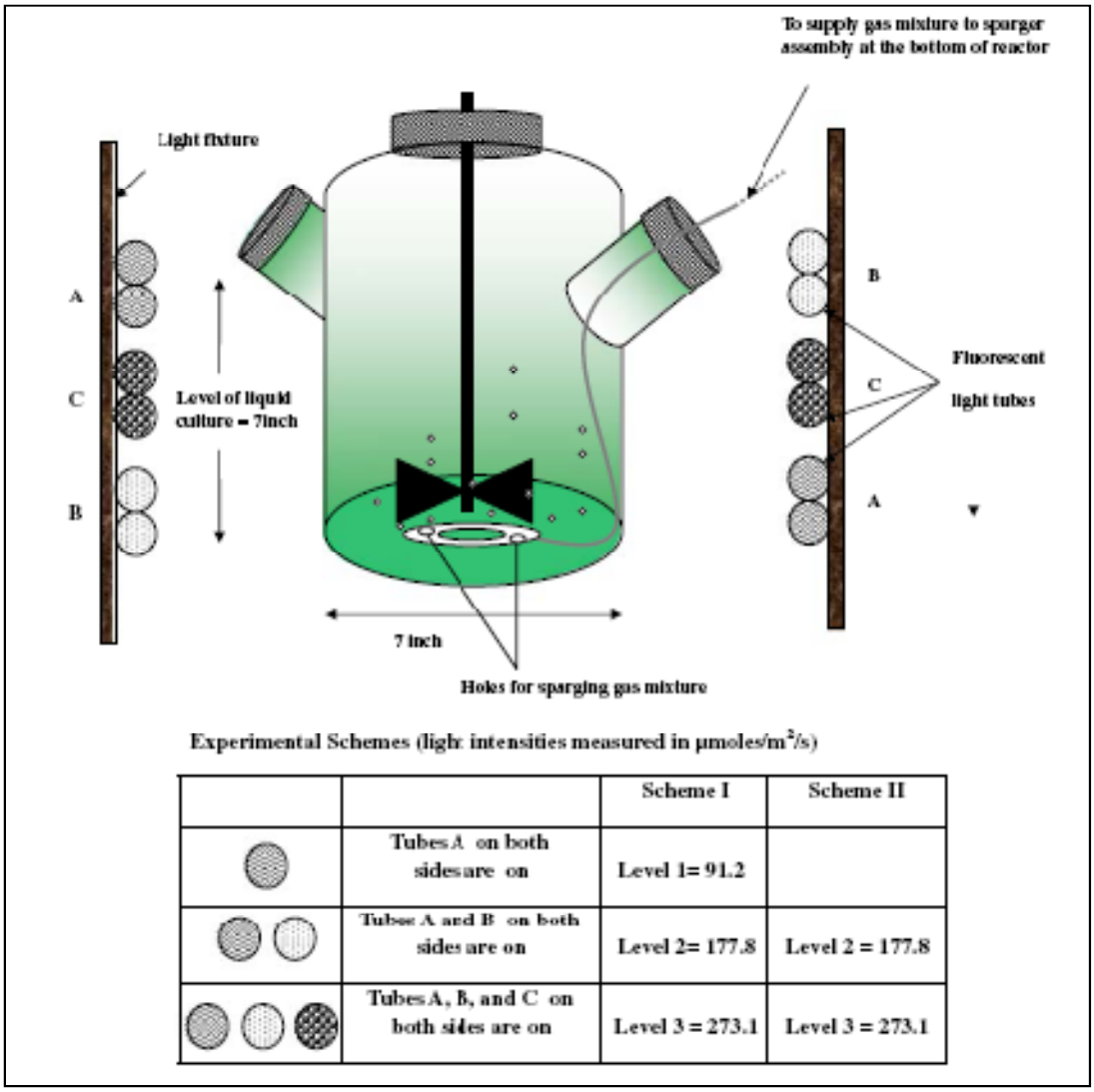
The sequential change in light intensity or PFD during algal growth studied in this research has shown to improve algal growth up to 2-fold. There are multiple factors that affect the growth rate and final cell concentration in this process, which include starting incident light intensity, the difference in light intensity levels or jump in light levels, and the number of light levels used over the favorable range of illumination for a specific algal strain. This study also showed that the utilization of supplied light not only depends on intensity of incident light but also on the culture density. Our study has also shown that simple periodic increase in light intensity can effectively increase the overall performance of photobioreactors.

Table 4-1 Yields and NO_3-N consumption at the end of different levels of growth in schemes I and II

ILLUMIN-ATION SCHEME	LEVEL 1			LEVEL 2			LEVEL 3		
	ΔX_1, g cells/L Avg. (std dev)	$\Delta X/\Delta t$, g cells/L/day Avg. (std dev)	$Y_{X/N}$, g cells/g N Avg. (std dev)	ΔX_2, g cells/L Avg. (std dev)	$\Delta X/\Delta t$, g cells/L/day Avg. (std dev)	$Y_{X/-N}$, g cells/g N Avg. (std dev)	ΔX_3, g cells/L Avg. (std dev)	$\Delta X/\Delta t$, g cells/L/day Avg. (std dev)	$Y_{X/N}$, g cells/g N Avg. (std dev)
I	0.53 (0.006)	0.050 (0.0005)	13.57 (0.98)	0.38 (0.010)	0.047 (0.002)	12.74 (1.04)	0.11 (0.015)	0.055 (0.003)	11.5 (0.71)
II				0.61 (0.013)	0.075 (0.001)	16.68 (1.23)	0.23 (0.011)	0.056 (0.002)	12.40 (0.92)

Table 4-2 Total moles of incident photons, N_{SL} supplied to the culture at different stages of the experiment

ILLUMINATION SCHEME	LEVEL 1	LEVEL 2	LEVEL 3
I	N_{11} = 5.12 x 10^6 µmoles	N_{12} = 3.07 x 10^6 µmoles	N_{13} = 2.36 x 10^6 µmoles
II		N_{22} = 9.98 x 10^6 µmoles	N_{23} = 5.90 x 10^6 µmoles



		Scheme I	Scheme II
	Tubes A on both sides are on	Level 1= 91.2	
	Tubes A and B on both sides are on	Level 2= 177.8	Level 2 = 177.8
	Tubes A, B, and C on both sides are on	Level 3 = 273.1	Level 3 = 273.1

Fig. 4-1 Schematic diagram of the reactor and illustration of light levels

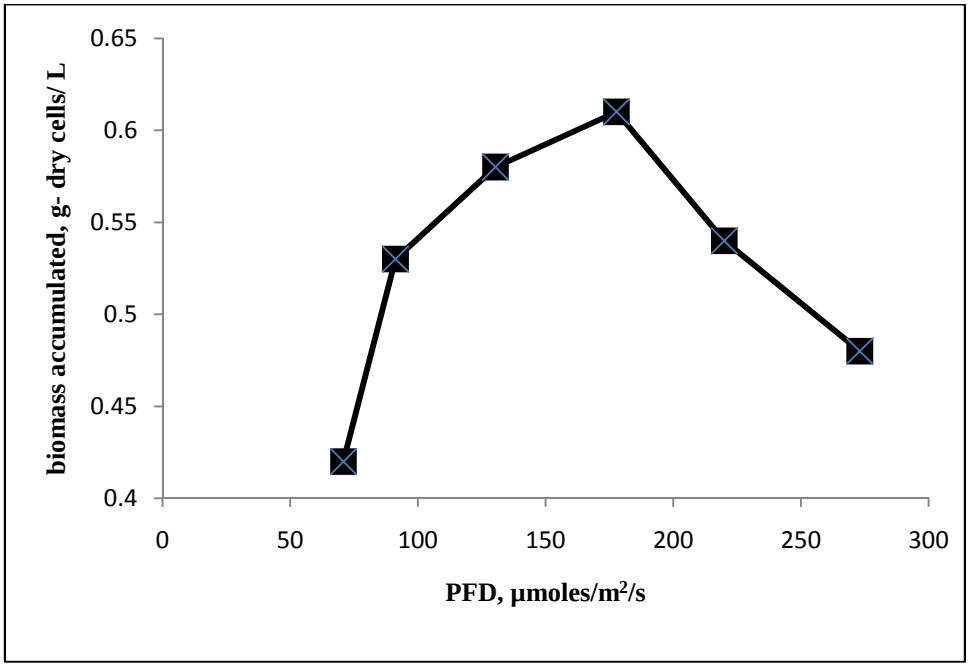


Fig. 4-2 Biomass accumulation at different incident light intensities with constant light intensity throughout the growth

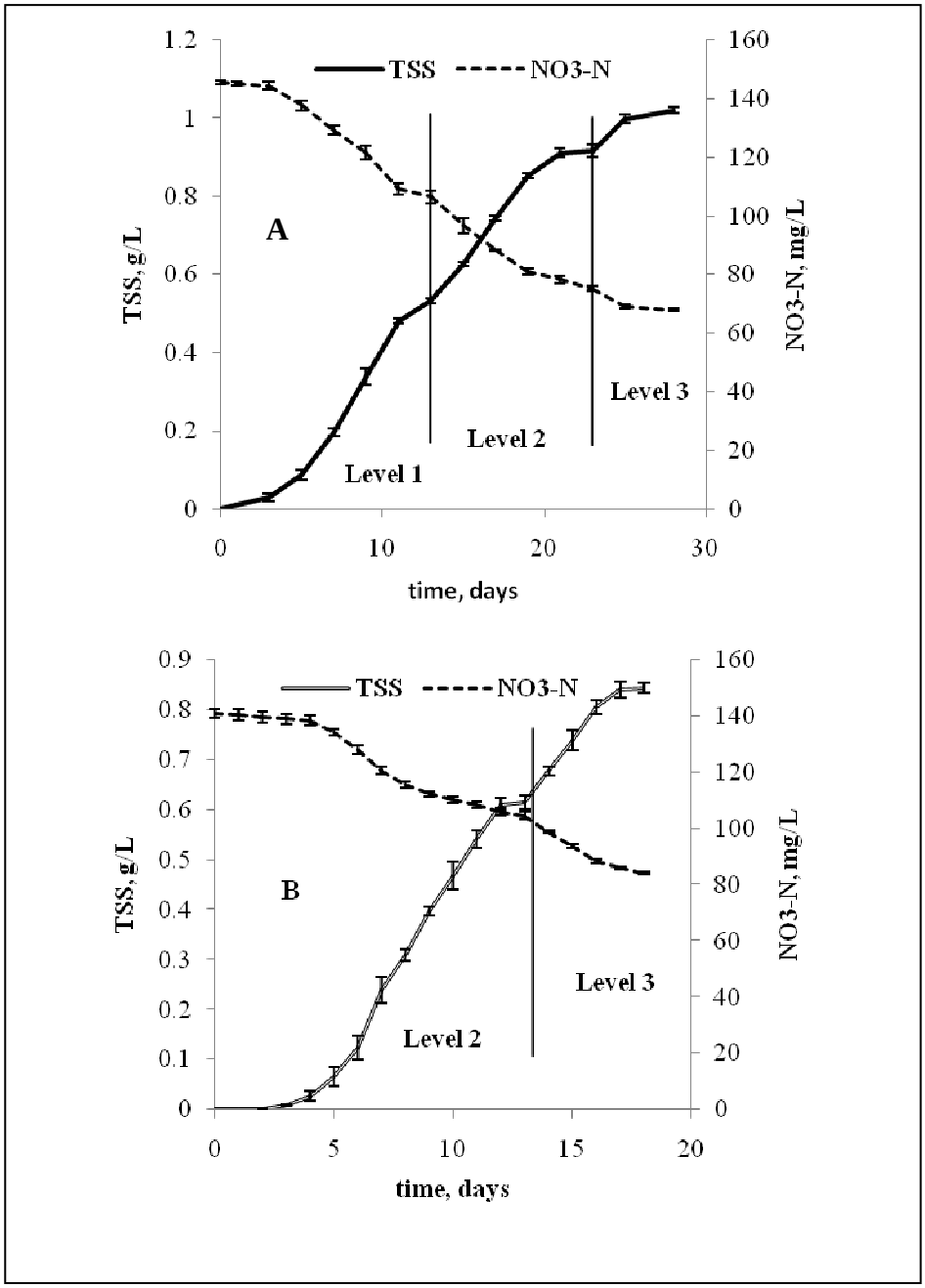


Fig. 4-3 Algal growth and NO_3-N uptake during studies under (A) illumination scheme I (sequentially illuminated with light intensities of 91.2, 177.8, 273.1 µmoles/m^2/s) and (B) illumination scheme II (sequentially illuminated with light intensities of 177.8 and 273.1 µmoles/m^2/s)

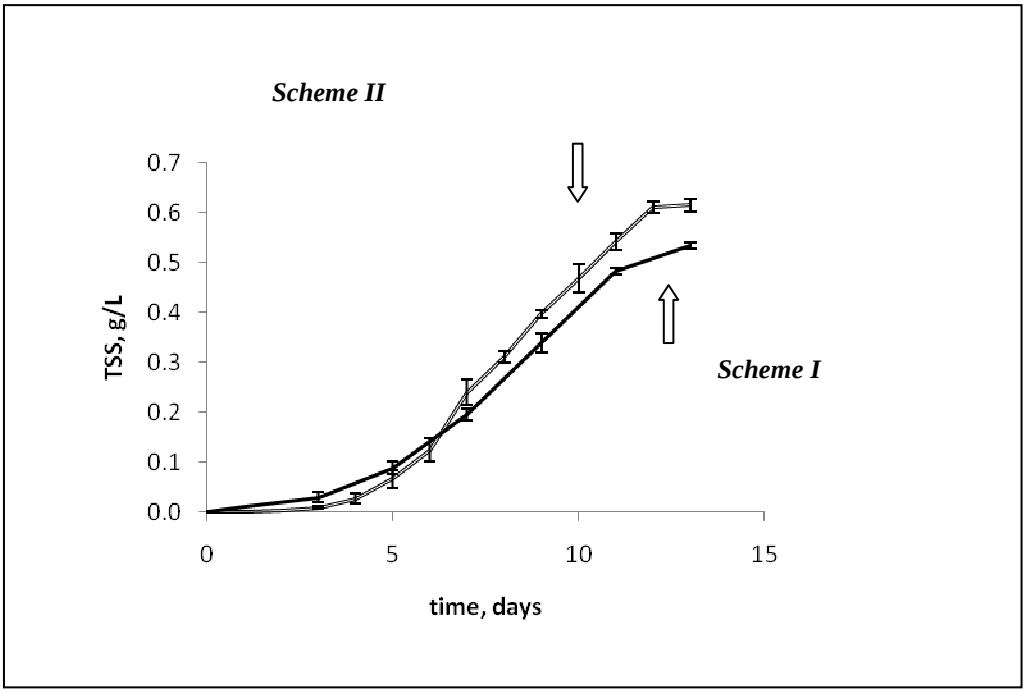


Fig. 4-4 Comparison between lower (first) light level growths under illumination schemes I and II. Photon flux densities during this stage of growth were 91.2 and 177.8 µmoles/m^2/s, respectively, for the two illumination schemes

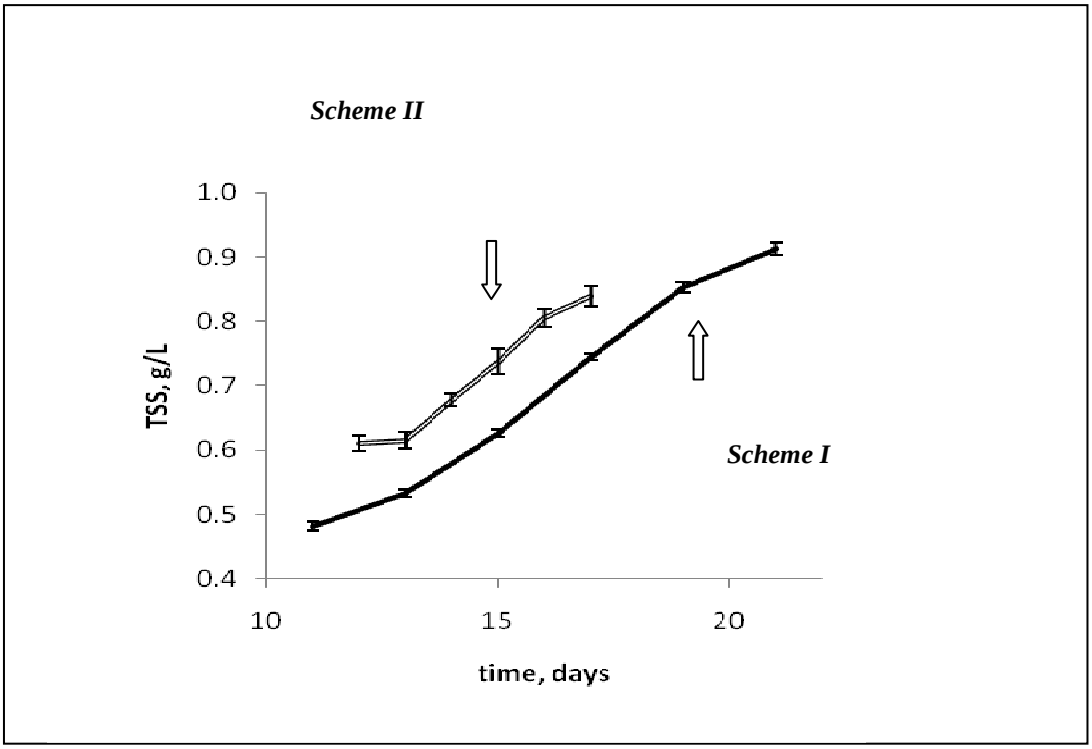


Fig. 4-5 Comparison between higher (second) light level growths under illumination schemes I and II. Photon flux densities during this stage of growth were 177.8 and 273.1 µmoles/m^2/s, respectively, for the two illumination schemes

CHAPTER 5

MAXIMIZING ALGAL GROWTH IN ANAEROBIC DIGESTER EFFLUENT USING LIGHT AND NITROGEN SUPPLEMENTATION

Abstract Naturally available nutrient rich sources such as animal waste treating anaerobic digester effluent can be used for biodiesel production through cultivation of microalgae. However algal growth in these effluents can be inhibited by unavailability of light due to increased culture density that reduces light penetration in the bioreactor. In this study a step increase in incident irradiance has been used to supplement the light lost in the bioreactor. It was shown that this strategy not only sustained the phototrophic growth inhibited by light limitation but also increased biomass production up to 2.7 fold in the algal cultures. Besides this application of light supplementation enabled the enhanced uptake of inorganic nutrients and prolonged the phototrophic activity in those algal strains unable to utilize native organic nutrients for heterotrophic growth to survive in light limited conditions. Also since algal growth can be inhibited by loss of ammonia-N in an uncontrolled pH environment such as open ponds. Therefore, in order to determine the effect of ammonia-N on algal growth in anaerobic effluent environment, algal strains were grown in ammonia-stripped and ammonia-supplemented effluent. Experiments showed that none of the algal strains studied could grow in absence of ammonia-N in the effluent. On the other hand, supplementation of ammonia not only facilitated the growth in algal cultures; it also assisted the utilization native substrates in the effluent. This utilization of native substrates resulted in an improvement of up to 2.8 times in biomass production in the algal cultures.

Introduction

Cultivation of microalgae for the production of biodiesel requires an inexpensive substrate to subside the high production costs. Naturally available substrates such as effluents of an anaerobic digester treating animal waste are inexpensive nutrient sources that have shown to support algal growth [66]. However, due to much lower algal biomass yields than most of industrial media [114], biodiesel production through cultivation of microalgae on these effluents is unlikely to be applied at the commercial scale. It has been found that reduced penetration of light due to increasing culture density in the effluent reduces phototrophic algal growth in a closed reactor [63, 115], and loss of N due to volatilization of ammonia adversely affect both the heterotrophic and phototrophic growths in open pond systems [116]. Therefore, determining the effects of replenishment of light during high culture densities in closed reactors and supplementation of ammonia-nitrogen lost due to volatilization in open pond systems will improve the estimates of biodiesel production through algal cultivation on natural substrates.

Since phototrophic growth requires optimum input of light, algal culturing in bioreactors can potentially be limited by inadequate irradiance during growth. When culture densities increase during growth, cellular absorption of light at the extremities of the reactor creates shading or loss of light at the inside of the reactor. This shading effect is enhanced in a growth medium like animal waste treating anaerobic digester effluent where absorption and scattering of light due to color and suspensions within the effluent respectively significantly reduced penetration of light in the culture [63, 115]. Hence due to these optically relevant effects biomass production by algae grown in anaerobic digester effluent in a bioreactor is significantly reduced. However, in dense algal cultures, higher

intensity light can penetrate deeper and more light could be available to growing cultures.

Thus, supply of light can be tailored to the requirements of algal cultures as they progress through their growth cycle to enhance light use while achieving high cell concentration [117]. In this study, effect of light supplementation has been tested in the cultures of *Scenedesmus dimorphous*, *Chlorella vulgaris* and an alga isolated from wastewater treating lagoons by growing them in the effluent of an anaerobic digester in externally illuminated stirred tank reactors. It was observed in all the three algal cultures that after the cultures stopped growing at a low incident photon flux, a step increase in illumination resulted in resumption of growth. Also, application of light supplementation enabled the enhanced uptake of inorganic nutrients and prolonged the phototrophic activity in those algal strains which were unable to effectively utilize native organic nutrients for heterotrophic growth to sustain in light limited conditions.

Since inorganic-N for algal growth in an anaerobic digester effluent almost entirely comes from the dissolved ammonia, hence both phototrophic and heterotrophic growths are dependent on the concentration of NH_3-N in the effluent [74]. Algal growth in an uncontrolled pH environment such as open ponds, suffers the loss of nitrogen due to volatilization of ammonia that can adversely affect the biomass yield [118, 119]. Although algae growing in a natural medium such as anaerobic digester effluent could utilize both organic and inorganic forms of nitrogen, algal growth exclusively on organic-N is not generally reported in the literature [18, 56]. Therefore, it is important to understand the effect of ammonia-N on growth of algae in unique anaerobic effluent environment, and how the presence of ammonia-N with native organic-N affects the biomass production in the algal culture.

In this part of the study algal strains were grown in three forms of anaerobic digester effluent i.e. ammonia-N free effluent, ammonia-N supplemented effluent, and artificial anaerobic digester effluent comprised of inorganic constituents and micronutrients present in the effluent. The study revealed that presence of inorganic-N was essential for algal growth in IBR effluent, even in the presence of nitrogen from organic substrates. Also, it has been found that the ability of an alga to utilize native organic substrates in addition to inorganic nutrients in the effluent could assist in increasing the biomass yield in the culture.

Materials and Methods

Effluent Source and Preparation of Growth Media

Anaerobic digester effluent samples for this study were obtained from an Induced Bed Reactor (IBR) treating dairy waste at the Wadeland Dairy, Ogden, UT. Effluent samples from the IBR location were collected and pretreated before stocking. The pretreatment involved fourfold dilution of the samples with DI water followed by centrifugation at 500xG for five minutes. The pretreated stocked samples were further diluted five times before use for algal growth.

Ammonia-N free IBR effluent was prepared by volatilizing ammonia off the pretreated effluent by raising the pH to 11.5; the rise in pH was induced by adding 5N NaOH solution to the effluent. pH of the effluent was readjusted to 7.0 before using it for algal growth. Ammonia-N supplemented effluent samples were prepared by adding 100ml of untreated IBR effluent per liter of ammonia-N free IBR effluent, which brought the ammonia-N concentration of effluent samples up to 100 mg/L. Artificial effluent was prepared on the basis of concentration of major cations and anions present in the effluent

of IBR. The artificial IBR effluent was comprised of 4.2mg/L of $CaCl_2$, 5.2mg/L of $MgCl_2.6H_2O$, 4.7mg/L of NH_4Cl, 1.5mg/L of $K_2HPO_4.3H_2O$, 22.5mg/L of $NaHCO_3$, 15mg/L of $Fe(NH_4)_3(C_6H_5O_7)_2$. Also, 10ml of real IBR effluent was added per liter of artificial effluent to provide the native micro-nutrients. The artificial effluent so prepared was diluted ten-folds, adjusted to pH 7.0 and autoclaved before being used for algal cultivation.

Algal Strains, Growth and Analyses

Three algal strains studied in this research were 1) Algal isolate from Wastewater Reclamation Facility, Logan, UT, 2) *Scenedesmus dimorphous* (Utex # 417), and 3) *Chlorella vulgaris* (Utex # 265). To ascertain the adaptability of these algal cultures to the effluent's chemical and physical environment, algae sample from the lagoons and UTEX slants were first grown in artificial IBR effluent and then transferred to sterile agar plates prepared with diluted and pretreated IBR effluent. The strains grown on plates were transferred to sterilized liquid IBR effluent and this process was repeated multiple times to ensure adaptability of alga to the effluent environment.

The adapted algal strains were cultured in triplicates (unless specified otherwise) with a uninnoculated sterile control in 1L CORNING spinner flasks (S/N- 3561). The cultures were stirred at constant speed of 125 rpm using ISOTEMP 1110049S stir plates and were illuminated from two opposite sides using 40W fluorescent tubes (Ecolux Sunshine 40). Irradiance of light was measured at the center of the flasks at a height of 3.5'' from the bottom of the flask using WALZ US-SQS/L light sensor and LI-COR LI- 250A light meter. Throughout the experiments, the cultures were exposed to a light–dark cycle of 12 h that was maintained using a timer connected to the light circuit. pH of the cultures in all

the experiments was kept between 6.9 – 7.0 using CO_2 sparging. The pH of the cultures was checked daily using Inlab Versatile pro pH probe (Mettler Toledo, Columbus, OH) and CO_2 supply was adjusted if the pH was found to be out of the above range.

Algal growth was monitored using total suspended solids (TSS) as representative of growth and was measured using Standard Method 2540D for examination of water and wastewater [61]. Also, daily samples were filtered through 0.45μ syringe filters (Nalgene 190-2545) and analyzed for dissolved total nitrogen (dTN) and ammonia nitrogen (dNH_3-N), dissolved total phosphorous (dTP) and ortho phosphorus (dOP), and dissolved chemical oxygen demand (dCOD). All the analyses were performed according to standard methods for the examination of waters and wastewater [62]. Dissolved organic N and P were determined by subtracting dNH_3-N from dTN and dOP from dTP respectively.

Results and Discussion

Algal Growth with Light Supplementation

Optimal light requirements for phototrophic algal growth change as culture density increases in a photobioreactor. When culture density is low during early stages of logarithmic growth, light effectively penetrates through the entire culture medium and a low light flux is adequate. In a dense suspension, cellular absorption of light as well as other optically relevant effects such as scattering and shading result in a significant decrease in overall light penetration into the bioreactor [63, 115]. This creates zones of dissimilar photon flux density inside the reactor with light availability mostly restricted to regions near the illuminated surfaces. Therefore, algal culturing can potentially be limited by inadequate supply of light to the growing cells inside the reactor. Also, light penetration in a medium like effluent of an anaerobic digester treating animal waste is also

be hampered by scattering and absorption due to the suspensions and organic compounds respectively present in the effluent [13]. Hence availability of light for algae growing in these media types could become limited at a much lower culture density than in clear medium [120].In this study a step increase in irradiance level or photon flux intensity has been used to supplement the irradiance required to sustain phototrophic activity when culture growth appeared to come to an end due to light limitation. Three algal strains (Logan lagoons algal isolate) LLAI, *S. dimorphous* and *C. vulgaris* were grown in the effluent of IBR at 485µmoles/m^2/s (*Level I*), light irradiance was switched to 745µmoles/m^2/s (*Level II*) when the logarithmic growth ceased to continue in the culture. Fig. 5-1 shows growth curves of algal cultures grown in the effluent with supplementation of light, arrows shown in the figure represent the points where irradiance levels were switched during the growth. It can be seen that after a culture stopped growing at lower incident photo flux, the step increase in illumination resulted in resumption of growth in cultures of all the three algal strains. This resumption of growth due to light supplementation resulted in a significant increase in overall culture densities, as indicated by higher TSS content in algal cultures at the end of growth at *Level II* than *Level I*.

Also, it can be noticed in Fig. 5-1 that the growth rate of biomass production ($\Delta X/\Delta t$) is lower in *Level II* than *Level I* in all the three algal cultures, which was likely due to a relatively small difference (1.53 fold) in the levels of photon flux intensities chosen for the study. As it has been found in previous studies that logarithmic growth rate after light supplementation depends on the difference between the levels of irradiance used for light supplementation [117]. This means that higher difference in photon flux intensities between the two levels might have induced faster logarithmic growth in the second stage

of the experiment. However, it has also been reported that extremely high light intensity could be detrimental for algal growth as it causes photo-bleaching of the cells [106]. This photo-inhibitory effect of irradiance depends on the actual availability of photons in the culture and time of exposure of algal cells to the light [113]. Therefore, the difference in levels of irradiance should be decided on the basis of optically relevant characteristics of the growth media and reactor properties controlling the distribution of photons in the culture.

The increase in biomass production from three algal cultures as a result of application of light supplementation can be estimated using the results from Table 5-1, which shows biomass production and dCOD removal by the algal cultures grown with and without light supplementation in the IBR effluent. It can be seen that there was an increase of 1.6, 2.5, and 2.7 fold in biomass production in cultures of LLAI, *S. dimorphous*, and *C. vulgaris* respectively when grown with light supplementation. Also as apparent by lower dCOD removal (suggestive of lower heterotrophic growth) in algal cultures grown with light supplementation, it can be understood that this strategy encouraged higher phototrotrophic activity in the cultures.

Fig. 5-3 shows comparison between the organic and inorganic nutrient (N and P) removal by three algal cultures grown with and without light supplementation. The results show that enhanced biomass production in algal cultures due to the application of light supplementation resulted in an increased uptake of inorganic nutrients (ammonia-N and ortho-P). It can be seen that the fraction of ammonia-N in total nitrogen removed by algal culture increased from 55% to 68% in LLAI, 61% to 70% in *S. dimorphous* and 64% to 74% in *C. vulgaris*. The corresponding increase in ortho-P content of total phosphorous

removed in cultures of LLAI, *S. dimorphous* and *C. vulgaris* were 0.5, 1.0 and 2.0 percent respectively. This increased utilization of inorganic nutrients was likely due to enhanced phototrophic activity in the algal cells, which was possible as a result of prolonged availability of light in light-supplemented algal cultures.

In normal growth conditions (with no light supplementation) in anaerobic digester effluent, availability of light is likely restricted to regions near the illuminated surfaces at high culture densities [91, 115]. Algal strains capable of performing mixotrophic (both phototrophic and heterotrophic) growth tend to switch to heterotrophic growth during these light limited conditions [79, 80]. The results in this study showed that a step change in irradiance level at high culture density enables deeper penetration of light inside the reactor, which prolonged the phototrophic growth and inorganic nutrient uptake in the algal cultures. Also, application of light supplementation induced higher biomass productions in cultures of *C. vulgaris* and *S. dimorphous*, which unlike LLAI were not able to effectively utilize organic nutrients (or switch to heterotrophic growth) to sustain growth during light inhibition in normal growth conditions.

Algal Growth with Nitrogen Supplementation

Algae growing phototrophically utilize carbon dioxide dissolved in the growth medium, this raises the pH of the medium due to the production of hydroxyl ions (OH^-) as a result of this process [14]. As loss of ammonia due to volatilization significantly increases at high (> 8) pH values [15], therefore, photrotrophic algae growing in the absence of pH control could become nitrogen starved if dissolved ammonia is the only available source of nitrogen. However, since a natural medium such as wastewater or effluent of an anaerobic digester treating animal waste contains significant amount of

organic nitrogen, algal strains adapted to be growing in it might be able to grow solely on organic-N in the absence of inorganic-N [18-20, 121]. To test the growth of algal strains exclusively on organic-N, the three algal strains i.e., Logan lagoons algal isolate, *Scenedesmus dimorphous* , and *Chlorella vulgaris* were grown in the IBR effluent in absence of any ammonia-N. It was found that none of the three algal strains was able to utilize native organic substrates for growth in the effluent in absence of inorganic-N (data not shown). On the other hand, all the three strains showed significant growth when the effluent was supplemented with ammonia-N. Growths of these strains were compared with those grown in artificial effluent, and results are shown in Table 5-2. As shown by these results, two type strains grew better in artificial effluent with higher utilization of inorganic nutrients (NH_3-N and ortho-P) than in ammonia-N supplemented real effluent whereas LLAI showed better growth and higher utilization of inorganic-N and P in real effluent. Biomass production in LLAI was 2.8 fold higher in real effluent supplemented with ammonia-N than artificial effluent whereas production of algal biomass was 1.1 and 1.5 times higher in artificial effluent than the real in case of algal strains of *S. dimorphous* and *C. vulgaris* respectively. Results in Table 5-2 also show that the uptake of inorganic nutrients per gram of biomass production increased with the rise in algal growth in artificial effluents in the strains of *S. dimorphous* and *C. vulgaris*. Whereas, utilization of NH_3-N and ortho-P per gram of biomass decreased in LLAI culture in the real effluent despite higher production of algal biomass compared to the artificial effluent.

This difference in growth and nutrient uptake pattern of LLAI as compared to the other two strains can be explained on the basis of nutrient forms utilized by algae when grown in the IBR effluent. Fig. 5-3 shows the amounts of organic and inorganic nutrients

utilized by algal strains when grown in IBR effluent supplemented with ammonia-N. It can be seen that a significant portion of nitrogen (48%) and phosphorous (41%) utilized by LLAI came from the native organic nutrients present in the effluent, whereas uptake of organic nutrients was negligible in *S. dimorphous* and *C. vulgaris*. On the other hand dCOD removal, (which is suggestive of heterotrophic growth in the effluent) was found to be 344.5, 58.0, and 21.8 mg/L in cultures of LLAI, *S. dimorphous*, and *C. vulgaris* respectively. Both these nutrient (Nand P) and dCOD removal data suggests that enhanced growth of LLAI in effluent supplemented with ammonia-N could be due to higher adaptability of LLAI than type strains *S. dimorphous* and *C. vulgaris* that enabled it to utilize native organic substrates available in the effluent [78].

The study showed that none of the algal strains researched could grow or utilize organic nutrients in absence of inorganic nitrogen in the effluent. However, the supplementation of ammonia-N not only facilitated the growth in all the three algal cultures, but alga LLAI was also able to exploit native substrates to enhance the growth in the effluent. Besides this, maximum biomass production and nutrient utilization in this part of the study was found in LLAI culture when grown in ammonia-N supplemented effluent. Also, it has been shown that growth yields from alga LLAI (supposedly more adapted to the effluent environment than the other two strains) was higher than those of pure strains grown in sterile artificial media. This means that chemical composition of a natural growth media like effluent of an anaerobic digester treating animal waste not only supports the algal growth but native substrates present in it could be utilized by well-adapted algal strain to maximize biomass production.

Conclusions

It has been shown in this study that supplementation of light can be used to sustain phototrophic activity when algal growth is inhibited by unavailability of light at high culture densities in an optically unsupportive medium such as anaerobic digester effluent. Cultures using step change in light intensity during growth were shown to produce algal biomass multiple times higher than the ones without light supplementation. It was found that the strains of *S. dimorphous* and *C. vulgaris* unlike Logan lagoons algal isolate (LLAI) were not able to utilize native substrates to undergo heterotrophic growth in normal growth condition without light supplementation. However, the application of light supplementation enabled these strains to perform enhanced uptake of inorganic nutrients and prolonged phototrophic activity, which resulted in much higher biomass yields in cultures of these strains than normal.

The study performed to determine the effect of dissolved ammonia on algae in unique growth environment of anaerobic digester environment showed that none of the strains researched could grow in the absence of ammonia-N. On the other hand, supplementation of ammonia not only facilitated algal growth but also assisted the utilization of native substrates along with the added ammonia-N in the cultures. It was found that well-adapted algal strains like LLAI could also utilize native substrates in the presence of ammonia-N to maximize their growth and productivity in the effluent.

Since low biomass yields in cultures can adversely affect the economics of a process utilizing algal cultivation, therefore techniques ensuing in an improvement in productivity of an algal culture are needed. The strategies of light and nitrogen supplementation discussed in this research were shown to enhance both nutrient utilization and biomass

production in the algal cultures grown in effluent an anaerobic digester treating animal waste. The results of this study not only add to the understanding of algae in complex media types such as anaerobic digester effluent but also help towards finding ways of maximizing algal growth in naturally occurring nutrient sources for the production of biodiesel.

Table 5-1 Biomass production and dCOD removal in the algal cultures grown with and without light supplementation in the IBR effluent

Algal strain and growth conditions	**Biomass, g/L Avg. (std dev)**	**dCOD removed, mg/L Avg. (std dev)**
Logan lagoons algal isolate without light supplementation	1.68 (0.10)	111.0 (10.4)
Logan lagoons algal isolate with light supplementation	2.71 (0.49)	238.9 (11.5)
S. dimorphous without light supplementation	0.56 (0.07)	84.2 (6.4)
S. dimorphous with light supplementation	1.38 (0.26)	69.4 (8.8)
C. vulgaris without light supplementation	0.36 (0.13)	35.5 (6.6)
C. vulgaris with light supplementation	0.99 (0.14)	42.3 (7.1)

Table 5-2 Biomass production and nutrient uptake by the three algal cultures grown in real and artificial IBR effluent

Algal strain and growth conditions	**Biomass, g/L Avg. (std dev)**	**NH_3-N, mg/L Avg. (std dev)**	**Ortho-P, mg/L Avg. (std dev)**
Logan lagoons algal isolate in real effluent	2.18 (0.34)	87.7 (3.5)	12.2 (1.9)
Logan lagoons algal isolate in artificial effluent	0.78 (0.14)	53.8 (3.9)	6.9 (2.5)
S. dimorphous in real effluent	1.14 (0.16)	52.5 (6.6)	5.1 (0.8)
S. dimorphous in artificial effluent	1.23 (0.13)	79.6 (4.2)	9.7 (1.4)
C. vulgaris in real effluent	0.72 (0.11)	37.7 (3.1)	4.5 (0.6)
C. vulgaris in artificial effluent	1.12 (0.07)	72.6 (1.9)	6.9 (0.8)

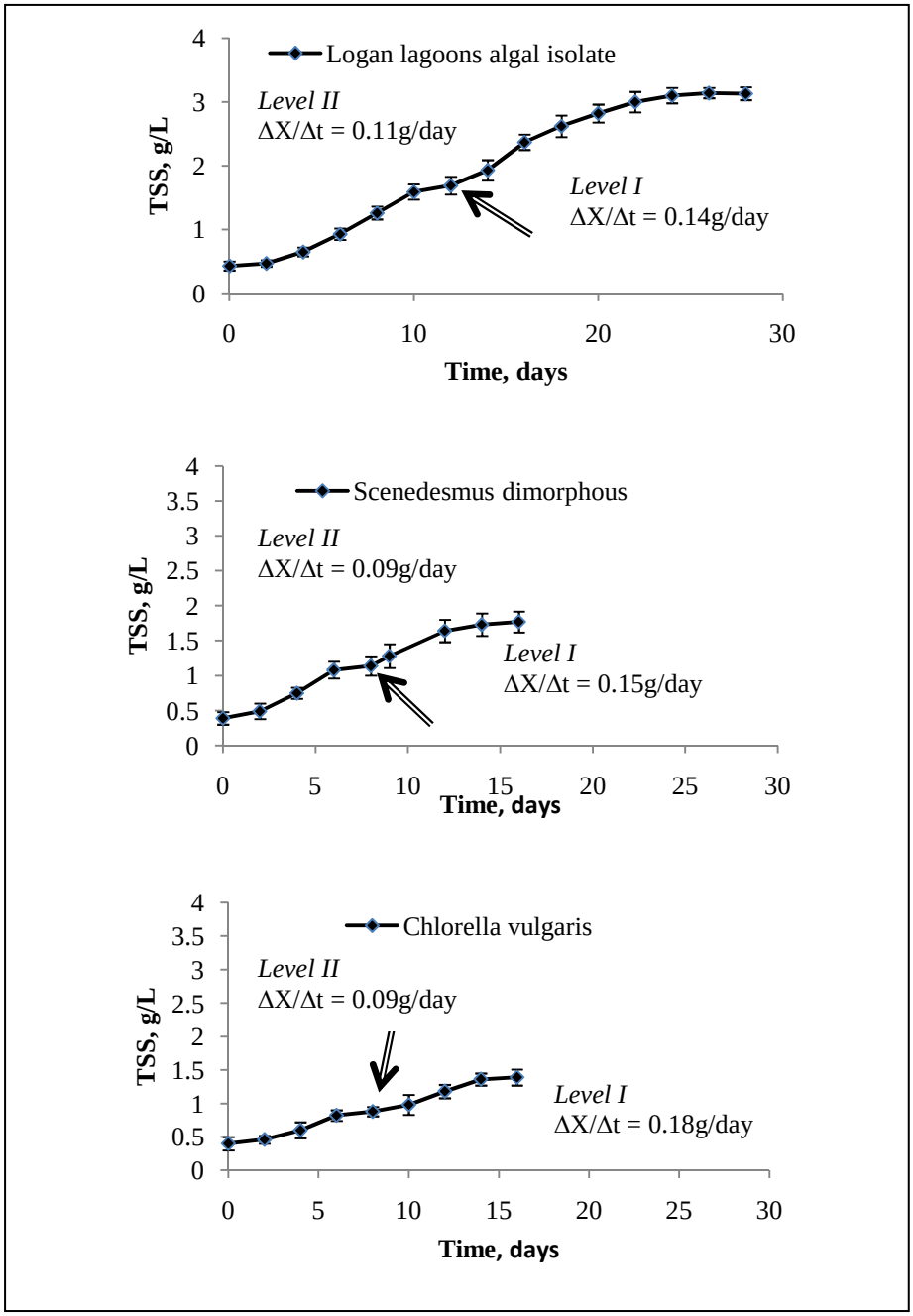


Fig. 5-1 Growth curves of algal cultures grown in IBR effluent with light supplementation

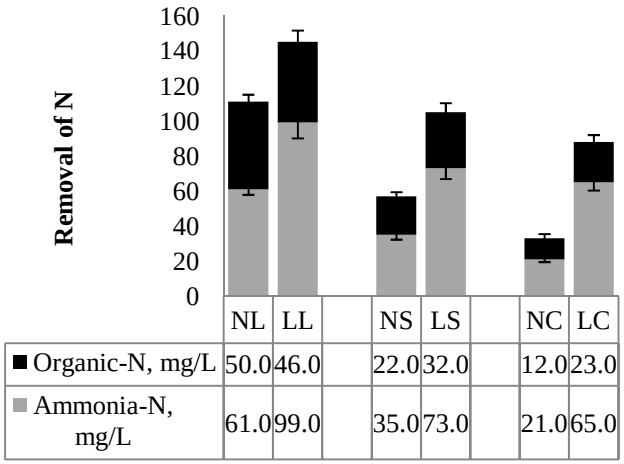


Fig. 5-2 Comparison between the nutrient removal by three algal cultures grown with and without light supplementation

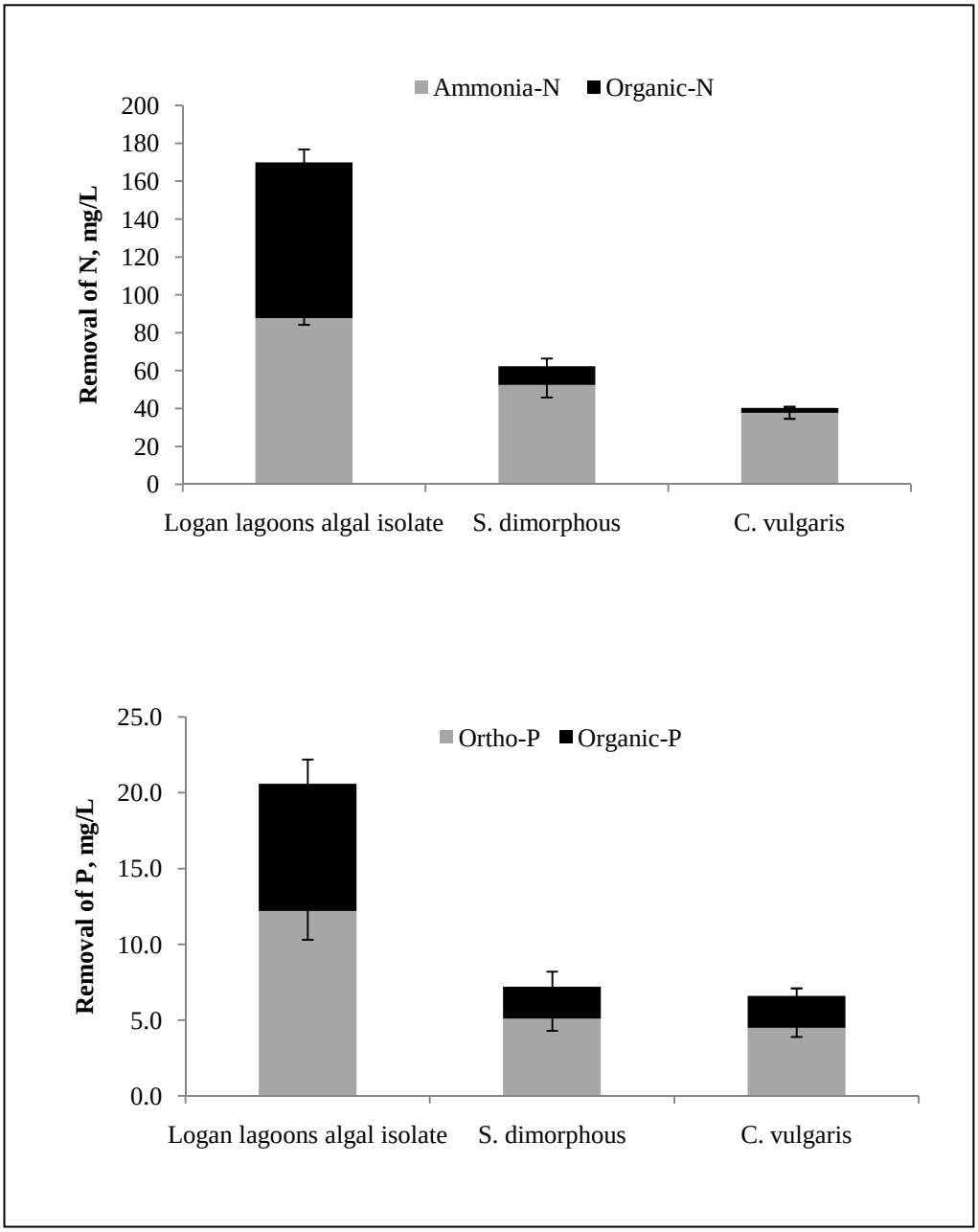


Fig. 5-3 N and P removal by algal cultures grown in ammonia-N supplemented IBR effluent

CHAPTER 6

IDENTIFICATION OF AN ISOLATED ALGAE STRAIN AND INTRACELLULAR TAG AND CN RATIO AT DIFFERENT LIGHT INTENSITIES USING ANAEROBIC DIGESTER EFFLUENT AS SUBSTRATE

Abstract In this study an algal strain from a natural assemblage of microorganisms was isolated by cultivating it on the effluent of an anaerobic digester. The isolated strain, Logan lagoons algal isolate (LLAI) was identified using gene sequencing and morphological characteristics. While the morphology of LLAI cells as seen under 1000x and 400x magnification resembled Chlorella family, 23S rRNA gene sequence of the strain showed close phylogenic relationship (98%) with *C. vulgaris*. Growth profiles of LLAI in anaerobic digester effluent at five (255, 485, 745, 885, and 1100µmolesm^{-2}s^{-1}) irradiance levels revealed higher uptake of organic nutrients at low light intensities, which suggested that algal cells performed greater heterotrophic growth to survive in light limited conditions. Also, biomass production in LLAI cultures was found to increase with the rise in level of irradiance. Time-course study on triacylglyceride (TAG) production in LLAI cells showed that accumulation of TAGs in algal cells started in exponential growth and continued to increase until it peaked during the stationary phase of growth. Intracellular TAG production in LLAI showed dependence on intensity of light and was found to increase consistently with an increase in irradiance level. Also it was found while there was no significant change in intracellular carbon content at different light intensities, nitrogen content of LLAI cells increased significantly from low to high levels of irradiance.

Introduction

The global demand for sustainable and renewable source of energy is presently as high than ever before [122], and research efforts are being raised towards finding renewable energy processes that are simple, inexpensive and have wide application [123]. Cultivating microalgae for production of biodiesel is one such process that is relatively simple and can be applied to different environmental conditions and geographical locations [124]. Even though significant advancement in identification of key variables and optimization of process for algal biodiesel production have been seen in past few years [123, 125], yet, high production cost compared to petroleum based fuels can potentially hamper the acceptability of algal biodiesel at a commercial scale [126]. There are various factors that can adversely affect the economic feasibility of algal biodiesel at different scales of production [124]. However, sustaining optimum supply of nutrients and maintaining sterile growth conditions in the cultures can be a highly cost intensive process especially for large scale production of algal biodiesel [114, 126].

One of the approaches for reducing the cost so incurred is integrating algae cultivation with a preexisting large scale process such that the combined process has better sustainability and economic feasibility. Cultivation of algae in the effluent of an anaerobic digester treating animal waste is such a combination where two large scale individual processes (biodiesel production and anaerobic digestion) can be integrated to increase the economic viability of each other. High concentrations of nutrients (N and P) in the effluent is an environmental concern, therefore, if not managed the presence of these nutrients can adversely affect the acceptability of anaerobic digestion technology on large confined animal farm if discharge from anaerobic digesters are more stringently regulated in the

future [6]. On the other hand due to the presence of concentrations of nutrients, effluent from an anaerobic digester can be used as an inexpensive source of nutrients for cultivation of algae, which can significantly reduce the production cost of algal biodiesel [66].

Algae growth on a natural media like effluent of an anaerobic digester treating animal waste can be supported by N and P available from both organic and inorganic compounds [9, 16]. The effluent borne compounds can also supply other macro nutrients as well as micro-nutrients and vitamins necessary for algal growth [17, 18]. The effluent also contains a range of other compounds such as volatile fatty acids (VFA), carbohydrates, and other forms of organic C that can be utilized for the heterotrophic growth of algae [19, 20]. However, despite the effluent containing a variety of substrates that can be utilized by algae, cost-effective production of biodiesel depends on identifying the algal strain capable of utilizing native substrates efficiently in the presence of media borne microorganisms and optimizing the growth conditions to maximize intracellular lipid production [21].

In this study an algal strain from a mixed population of microorganism in a waste water treatment facility was isolated and cultured in the effluent of an anaerobic digester treating dairy waste. Identification of the isolated strain was performed using morphological properties and 23S rRNA genomic sequence. Growth characteristics of the algal strain were determined by comparing the biomass production and nutrient uptake patterns at different light intensities, while growth conditions inducing high biodiesel production were determined by comparing intracellular triacylglycerides (TAG) accumulation and corresponding CN ratio at five irradiance levels.

Materials and Methods

Growth Media, Algal Strain and Experimental Setup

Anaerobic digester effluent samples for this study were obtained from an Induced Bed Reactor (IBR) treating dairy waste at the Wadeland Dairy, Ogden, UT. Effluent samples from the IBR were collected and pretreated before stocking. The pretreatment involved fourfold dilution of the samples with DI water followed by centrifugation at 500xG for five minutes. The pretreated stocked samples were further diluted five times before use for algal growth.

Algal strain studied in this research was an isolate from Logan Lagoons Wastewater Treatment Facility, Logan, UT. To ascertain the adaptability of the Logan lagoons algal isolate (LLAI) to the effluent's chemical and physical environment, algae sample from the lagoons was first grown on sterile agar plates prepared with diluted and pretreated IBR effluent, and single algal colony transfer on plates were done for multiple generations. The algal strain isolated on agar plates was then grown in liquid sterile IBR effluent and this process was repeated multiple times to ensure adaptability of alga to the effluent environment.

Algal cultures for this study were grown in triplicates (unless specified otherwise) with a uninnoculated sterile control in 1L CORNING spinner flasks (S/N- 3561). The cultures were stirred at constant speed of 125 rpm using ISOTEMP 1110049S stir plates and were illuminated from two opposite sides using 40W fluorescent tubes (Ecolux Sunshine 40). Irradiance of light was measured at the center of the flasks at a height of 3.5'' from the bottom of the flask using WALZ US-SQS/L light sensor and LI-COR LI-250A light meter. Throughout the experiments, the cultures were exposed to a light–dark

cycle of 12 h that was maintained using a timer connected to the light circuit. pH of the cultures in all the experiments was kept between 6.9 – 7.0 using CO_2 sparging, the pH of the cultures was checked daily using Inlab Versatile pro pH probe, respectively (Mettler Toledo, Columbus, OH) and CO_2 supply was adjusted if the pH was found to be out of the above range. For dark culturing of LLAI bioreactors were covered with aluminum foil to stop the light from getting inside the culture.

Identification of Algae Strain

For morphological observation, LLAI cells were observed using a Zeiss Axioskop 40 microscope coupled with Axiocam HR3 camera (Thornwood, NY, USA). The objectives and contrast setting used for 400 and 1000x magnifications were A-plan 40x/0.65 with phase-2 contrast and A-plan 100x/1.25 oil with phase-3 contrast, respectively.

For the analysis of plastid 23S rRNA gene sequence of the strain, DNA from LLAI cells was extracted and purified using Power Soil DNA Kit (MO BIO Laboratories Inc, Carlsbad, CA, USA) according to the manufacturer's guideline. The primer pair p23SrV_f1 (5' GGA CAG AAAGAC CCT ATG AA 3') and p23SrV_r1 (5' TCA GCCTGT TAT CCC TAG AG 3') were used to amplify 23S rRNA gene in the DNA[127]. Genomic DNA extract, 3µL, was mixed with the polymerase chain reaction (PCR) master mixture containing 5µL of 10× PCR buffer (Promega), 4µL of 10mM dNTP mix, 4µL of $MgCl_2$ (1mM), 2µL of each primer (0.4mM), 0.3µL Taq polymerase (Promega), and 30µL of Nanopure H_2O. The PCR program was run on Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) using the following cycling conditions: initial denaturation at 94^0C for 2 min, followed by 35 cycles of 94^0C for 20 s, 55^0C for 30 s, and 65^0C for 30 s, with a final extension of 65^0C for 10 min. The PCR

products so obtained were then sent to Utah State University Biotech Center (Logan, UT, USA) for DNA sequencing. The resulting gene sequence was aligned and compared to the nucleotide sequences of some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov).

Analytical Methods

Algal growth was monitored using total suspended solids (TSS) as representative of growth and was measured using Standard Method 2540D for examination of water and wastewater [61]. Also, daily samples were filtered through 0.45μ syringe filters (Nalgene 190-2545) and analyzed for dissolved total nitrogen (dTN) and ammonia nitrogen (dNH_3-N), dissolved total phosphorous (dTP) and ortho phosphorus (dOP), and dissolved chemical oxygen demand (dCOD). All the analyses were performed according to standard methods for the examination of waters and wastewaters [62]. Dissolved organic N and P were determined by subtracting dNH_3-N from dTN and dOP from dTP respectively. Algal cells from experimental cultures were harvested by centrifugation and lyophilized to be tested for percent triacylglyceride (TAG) and intracellular C, N, and P content. For TAG analyses lyophilized cells were sonicated and lipids were extracted in 1:1:1 (v/v/v) mixture of hexane, chloroform and tetrahydrofuran. Gas chromatography of extracted lipids was carried out on a Shimadzu GC-15A, using 15m long RTX-biodiesel column and FID. Helium was used as the carrier gas with the column flow rate of 2.73ml/min. Identification and quantification of TAGs were done using reference substances and standards. Intracellular C and N contents were determined by analyzing a known weight of algal biomass analyzed through LECO TruSpec CN analyzer (St Joseph, MI).

Results and Discussion

Identification of Strain

The morphological characteristics of Logan lagoons algal isolate (LLAI) grown on the effluent of IBR were determined using light microscopy at 400x and 1000x magnification, and the representative micrographs are shown in Fig. 6-1. LLAI cells gave grass green colored colonies of the size ranging between 0.05-0.2cm with circular shapes (Fig. la). The color and size of the microalgal colonies were found similar to those representative of eubacteria [128]. The observation of LLAI cells at 1000x magnification confirmed the spherical to oval morphologies of 1.5 to 3.0μm diameter (Fig. lb). The closer examination of LLAI cells at 1000x magnification showed the presence of a thin cell wall and a cup or girdle shaped bright green single parietal chloroplast (Fig. 1c). Light micrographs of LLAI cells also suggest the mode of multiplication as autosporulation where autospores were found to be released through the splitting of mother cell (Fig. 1d).

23S rRNA gene sequence of LLAI was analyzed to determine phylogenetic lineage of alga. It has been shown that primer pair developed by Sherwood & Presting (2007) to amplify plastid 23S rRNA gene has remarkable universality in both cyanobacteria and eukaryotic green algae [127]. The PCR product with amplified 23S rRNA region of LLAI plastid genome was sequenced to obtain a gene sequence comprised of 384 nucleotides. On comparing this gene sequence with other nucleotide sequences of known microorganisms, 98% match was found with two entries in GenBank database. *Chlorella vulgaris* chloroplast large subunit ribosomal RNA gene (Accession # L43357.1) and *Chlorella vulgaris* C-27 chloroplast DNA (Accession # AB001684.1) were found to align with LLAI gene sequence including 23S rRNA with a total score of 634 and query

coverage of 94%.

These results suggest that the morphological characteristics of LLAI cells closely match with the cell morphologies of *Chlorella* spp. described in past research studies [128-132]. Also, the portion of LLAI gene including 23S rRNA sequence that was used to compare the nucleotide sequences of other microorganisms, showed strong similarities with two available sequences of *C. vulgaris* in Genbank database [133, 134]. Although it will need a complete genomic sequence to establish whether it is a new strain or a mutant of *C vulgaris*, through the results of this identification study it can be inferred that the alga isolated from wastewater treating lagoons (LLAI) belongs to Chlorella family of algal strains.

Biomass Production and Nutrient Uptake

Light penetration in the effluent of an anaerobic digester treating animal waste can potentially be obstructed due to the presence of particulates and high concentrations of (colored) organic compounds [13]. Hence algae growing in such an optically limited media can suffer growth inhibition due to unavailability of light long before the influx of chemical toxicity in the culture [120]. However, an algal strain adapted to the growth environment could utilize native organic substrates to sustain growth in light limited conditions [8, 60]. Therefore in order to determine the effect of available light intensity, LLAI cells were grown at five irradiance levels i.e. 255, 485, 745, 885, and1100 $\mu moles.m^{-2}.s^{-1}$ in the IBR effluent, and growth profiles, nutrient uptake pattern and biomass production in algal culture were compared.

Biomass production and corresponding removal of dissolved-COD (dCOD), and growth profiles of LLAI cultures at five different irradiance levels are shown in Figs. 6-2

and 6-3 respectively. It can be seen in Fig. 6-2 that a significant removal of dCOD took place in LLAI cultures at each level of irradiance, however, the amounts of dCOD removed was not proportional to biomass produced in the culture. This implies that although LLAI cells performed mixotrophic (both phototrophic and heterotrophic) growth at all the irradiance levels, the relative percentage of phototrophic or heterotrophic growth changed with an alteration in light intensity. Also from Fig. 6-2, it can be seen that biomass production in the algal cultures increased with an increase in light intensity, and largest increase (1.67 to 3.57g/L) was observed when the difference in intensities was the highest (485 to 745µmoles.$m^{-2}s^{-1}$). Furthermore as shown in Fig. 6-3, growth rates in algal cultures increased considerably at 885 and 1100 µmoles$m^{-2}s^{-1}$ whereas significantly lower rates of growth were observed at 255 and 485µmoles.$m^{-2}s^{-1}$. The comparison of dCOD removal in LLAI cultures at different light intensities in Fig. 6-2 shows highest removal at 745µmoles$m^{-2}s^{-1}$while there was a decrease in the removal of dCOD when LLAI cells were grown with either higher or lower irradiance than this level of irradiance.

These behaviors of LLAI cells can be explained by determining suitability of growth conditions favorable for phototrophic and heterotrophic growth at different levels of irradiance. From Fig. 6-2, mean ratio of dCOD removal per gram of algal biomass produced in LLAI culture was found to be 175, 180, 125, 100, and 90 mg of dCOD/g of biomass produced from lowest to highest level of irradiance, respectively. This shows that while higher biomass production in LLAI cultures was observed each time the level of irradiance was increased (Figs. 6-2 and 6-3), relative percentage of heterotrophic growth performed by algae was found to decrease almost conversely. From Fig. 6-3, mean growth rates of algal biomass in LLAI cultures were found to be 45, 80, 190, 210, and

220mg.L^{-1}.$d^{-1}$from lowest to highest level of irradiance respectively. Since phototrophic growth rates in algal cultures are generally found to be higher than heterotrophic rates [114], it can be understood that algal cells preferably performed phototrophic growth at higher levels of irradiance (due to increased availability of light in the culture), which resulted in higher growth rates at these light levels. Since growth inhibition due to limited availability of light arises at higher culture densities when high intensities of light are used [135], LLAI cultures produced greater amounts of algal biomass at elevated levels of irradiance (Fig. 6-2).

From the results discussed above it can be understood that algal growth at lower light intensities (255 and 485µmoles.m^{-2}.s^{-1}) were predominantly heterotrophic and primarily phototrophic growth at higher irradiance levels (885 and 1100µmoles.m^{-2}.s^{-1}). On the other hand growth trend at 745µmoles.m^{-2}.s^{-1} represents a transition between heterotrophic and phototrophic growth. Depending on available light conditions mixotrophic algae can switch between phototrophic and heterotrophic growths [79, 80], which may also affect the type (organic or inorganic) of nutrients (N and P) predominantly removed in the culture [114]. Therefore, the pattern of nutrient uptake could be expected to represent the type of growth preferably performed by LLAI culture at different levels of irradiance.

Table 6-1 shows nutrient removed in LLAI cultures grown in the effluent of IBR at five different levels of irradiance. It can be seen that fractions of organic-nitrogen in total-nitrogen removed were higher at low levels of irradiance (with highest of 38% at 485µmoles.m^{-2}.s^{-1}) whereas relatively lower removal of organic-N (with lowest of 28% at 1100µmoles.m^{-2}.s^{-1}) was observed at higher light levels. Similar trends were found with removal of phosphorous in LLAI cultures with highest; 56 % organic-phosphorous

removed at 255µmoles.m^{-2}.s^{-1} and lowest; 25% organic-phosphorous removed at 885µmoles.m^{-2}.s^{-1}.

These results indicate that light limited conditions during algal growth in the effluent at lower levels (255 and 485µmoles.m^{-2}.s^{-1}) while being inhibitory for phototrophic growth, induced higher uptake of organic substrates in LLAI cultures. This is suggestive of mixotrophic algae (LLAI) enhancing the heterotrophic activity to sustain growth in light limited conditions [79]. Furthermore, when the level of irradiance was increased to 745µmoles.m^{-2}.s^{-1}, availability of light prolonged in the culture and algal cells were able to continue phototrophically for a longer period of time. This is also underscored by lower heterotrophic activity (lower uptake of organic substrates) at 745µmoles.m^{-2}.s^{-1} than lower at light levels. As the level of irradiance increased further (885 and 1100µmoles.m^{-2}.s^{-1}), LLAI cells predominantly performed phototrophic growth. This resulted in higher growth rates and lower uptake of organic substrates in the culture than observed at any lower level of irradiance.

Finally, it was also found that although LLAI performed mixotrophic (phototrophic and heterotrophic) growth in the IBR effluent but it was not able to grow in the absence of light as experiments for dark culturing of LLAI did not yield any algal biomass or show significant utilization of substrates. This suggested LLAI could undergo heterotrophic (or uptake of organic substrates) growth only in conjunction with the phototrophic growth. At low irradiance levels despite the culture being light limited and cells preferably using heterotrophic growth to survive, mixing of the culture facilitated the intermittent exposure of cells to the lighted region towards the periphery of the reactor [93, 94, 106], which likely enabled the cells to retain a degree of phototrophic activity in them. However, with

any increase in culture density the exposure time of these cells [117] decreased and algal growth ceased in the culture. This condition took longer to arise at higher light intensities as there was prolonged availability of light in the algal culture at these irradiance levels. This argument was also supported by rapid flattening of growth profiles of LLAI cultures at higher levels of irradiance. High growth rates at high light intensities hastened the advent of light limited condition of low exposure time for algal cells at high culture densities, which resulted in sudden loss of phototrophic activity in the cells that brought the algal growth to come to an end in the culture.

TAG Accumulation and Intracellular C and N Content

To estimate the biodiesel potential of LLAI cells in the IBR effluent, a time course determination of intracellular triacylglycerides (TAGs) accumulation was performed. Fig. 6-4 shows the typical relationship between algal growth and TAG accumulation in LLAI culture in the IBR effluent. It can be seen in the figure that accumulation of TAGs in algal cells started during exponential phase and continued to increase in stationary growth phase of LLAI culture. The TAG accumulation in LLAI cells peaked during stationary growth and started to decrease nearly two days after any significant increase in biomass production was observed in the culture. The results obtained through this study are consistent with past research studies with other algal species [136, 137]. These studies have shown that smaller fatty acids produced during lag phase or initial exponential growth phase start to incorporate in longer fatty acids and subsequently in TAGs during later stages of exponential algal growth that enhances the intracellular TAGs in algal cells. This incorporation of smaller fatty acids into the TAGs increases upon the transition of growth to stationary phase as algal cells tend to store intracellular carbon in long-chained

fatty acids or TAGs during starvation or other growth limited conditions [137, 138].

Accumulation of fatty acids or lipids inside algal cells is influenced by several factors that includes species type, composition of growth medium, aeration, light intensity, temperature and age of the culture [136]. Effect of light intensity on TAG accumulation and intracellular C and N content in LLAI cells cultured in the effluent of IBR is shown in Fig. 6-5. Results shown in Fig. 6-5 suggest that TAG accumulation in LLAI increased with an increase in the irradiance level. Also, it can be seen in the figure that there was no significant change in intracellular carbon content at different light intensities. However a 50% decrease in intracellular nitrogen content was observed in LLAI cells grown from low to high light intensities. This increase in nitrogen content resulted in a continuous decrease in CN ratio in LLAI cells ranging from 12.2 at 285μmoles.m^{-2}.s^{-1} to 6.6 at 1100μmoles.m^{-2}.s^{-1}.

Difference in intracellular TAG content in LLAI cells under various levels of irradiance was consistent with previous findings that show that the same algal strain grown at different light intensities exhibits marked difference in the intracellular composition of fatty acid [89, 139]. Growth at low irradiance levels induces formation of polar lipids in algal cells [140, 141]. Whereas growth at high levels of irradiance results in an increase in the amount of neutral storage lipids, mainly TAGs with a simultaneous decrease in polar lipids content of the cells [142, 143]. The decrease in intracellular CN ratio in LLAI was likely due to increased phototrophic activity of the cells at high levels of irradiance as suggested by the nutrient uptake patterns in LLAI culture. It has been found that the enhanced phototrophic activity at high light intensities causes an increase in intracellular accumulation of photosynthetic organic compounds such as chlorophylls and

carotenoids [144]. Assimilation of these compounds in algal cells also involves photosynthetic fixation of intracellular nitrogen [145], which results in an increase in intracellular nitrogen content, and hence a decrease in CN ratio of algal cells at high light intensities.

Conclusions

Logan lagoon algal isolate (LLAI) studied in this research had been shown to grow on the effluent on anaerobic digester, IBR in the presence of native microorganisms and minimally controlled environment conditions. Characteristically LLAI was found to have great similarity with mixotrophic strains of genus Chlorella. While light micrographs of LLAI showed close resemblance with *Chlorella* spp. in morphology, analysis of 23S rRNA gene sequence of LLAI suggested a 98% match with *C. vulgaris*. The nutrient uptake pattern of LLAI revealed the ability of alga to perform phototrophic as well as heterotrophic growth in the effluent, which enabled it to utilize both inorganic and organic native substrates. Growth of LLAI at different irradiance levels showed that both biomass yield and lipid production can be enhanced by increasing the intensity of light in the culture.

These results showed that an algal strain (LLAI) isolated from a natural consortium of microorganisms (wastewater treating lagoons) can be cultivated successfully on inexpensive substrates such as anaerobic digester effluent. Since algae grown on the effluent utilize only native substrates in nonsterile environment, identification and development of such a combination of algal strain and growth media can significantly reduce the cost incurred in production of algal biodiesel.

Table 6-1 Nutrient removed by Logan lagoon algal isolate grown under different levels of irradiance in the IBR effluent

Irradiance µmoles/m²/s	**Dis-TN (sd) mg/L**	**Dis-NH_3-N (sd) mg/L**	**Dis-Org-N (sd) mg/L**	**Dis-TP (sd) mg/L**	**Dis-Ortho-P (sd) mg/L**	**Dis-Org-P (sd) mg/L**
255	63.0 (4.7)	39.2 (1.8)	23.8 (1.9)	10.8 (2.0)	6.9 (1.2)	3.9 (1.6)
485	111.3 (5.9)	61.2 (3.2)	50.1 (3.9)	16.8 (1.4)	10.5 (1.6)	6.3 (1.2)
745	213.8 (10.1)	133.5 (4.5)	80.3 (6.0)	27.6 (3.7)	19.9 (2.2)	7.7 (2.4)
885	234.0 (9.0)	158.0 (8.8)	76.0 (5.8)	26.1 (4.9)	20.9 (3.8)	5.2 (2.0)
1100	255.2 (10.8)	182.7 (7.3)	72.5 (7.1)	41.4 (5.8)	27.9 (3.6)	13.5 (3.8)

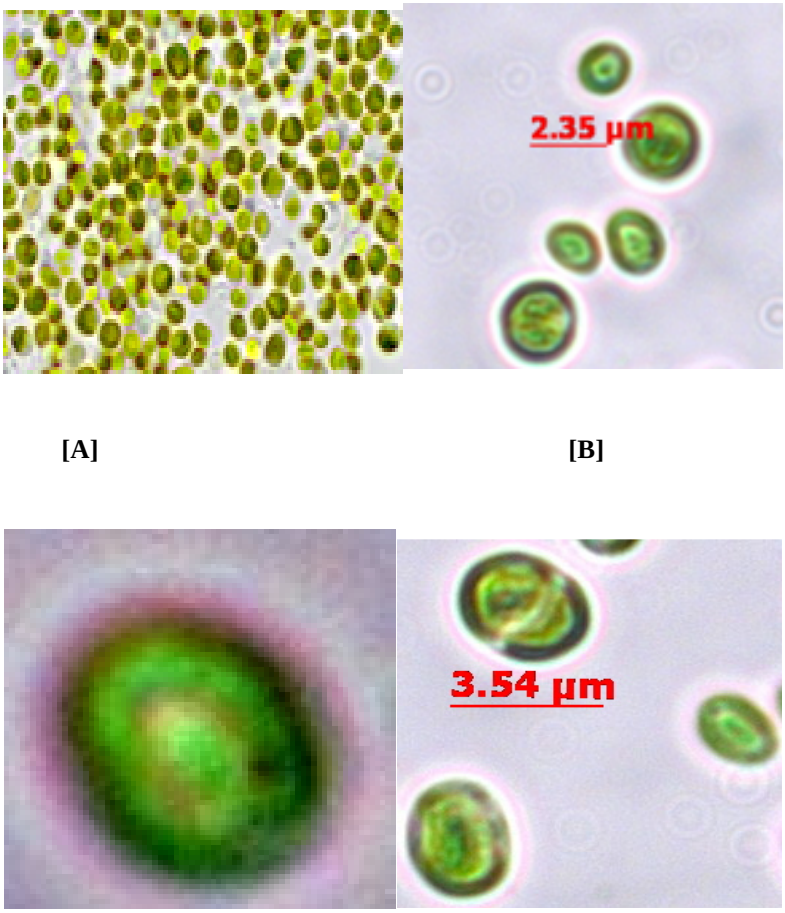


Fig. 6-1 Light micrographs of Logan lagoons isolate algal cells

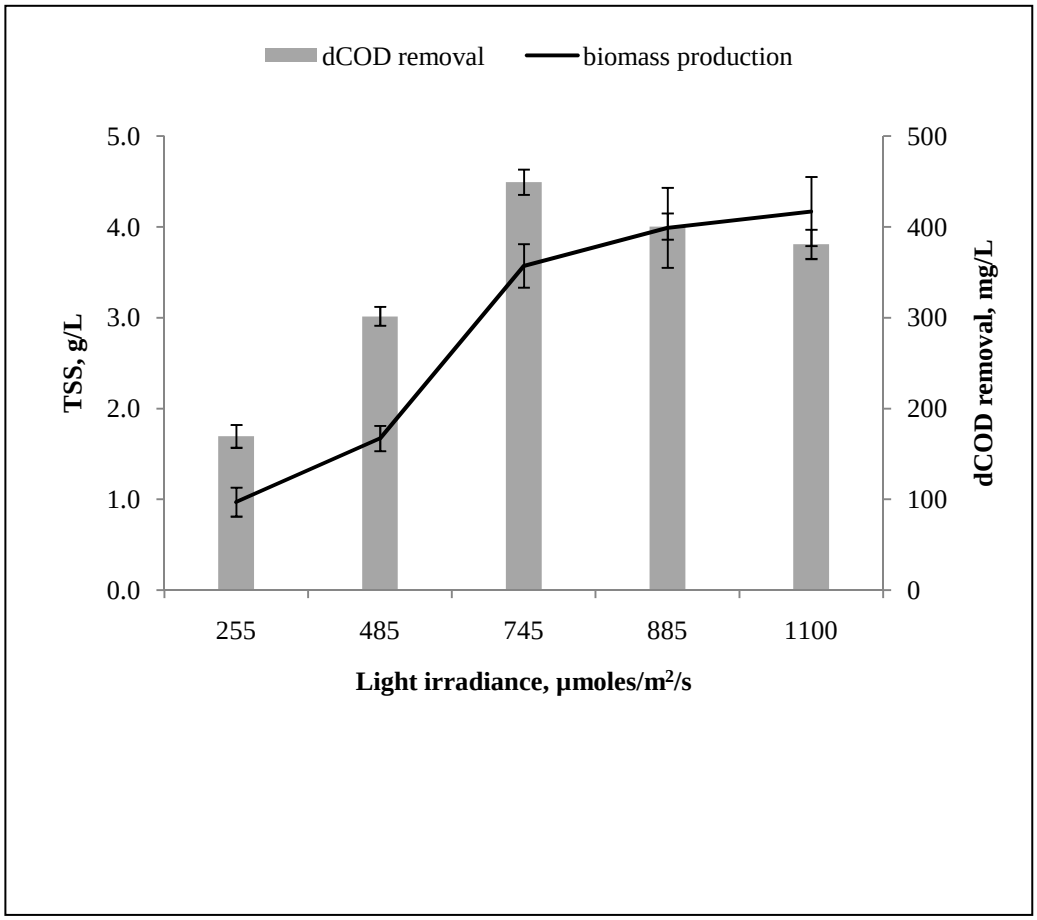


Fig. 6-2 Growth curves of Logan lagoons algal isolate grown under different levels of irradiance in the IBR effluent

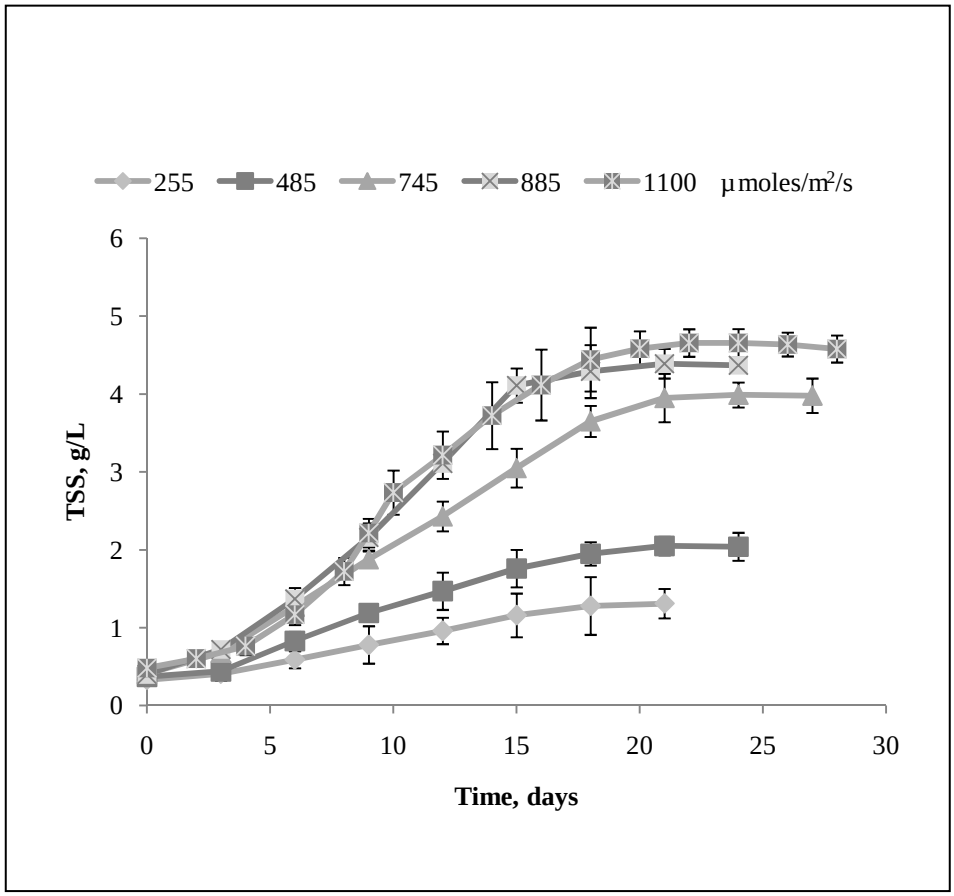


Fig. 6-3 Growth curves of Logan lagoons algal isolate grown under different levels of irradiance in the IBR effluent

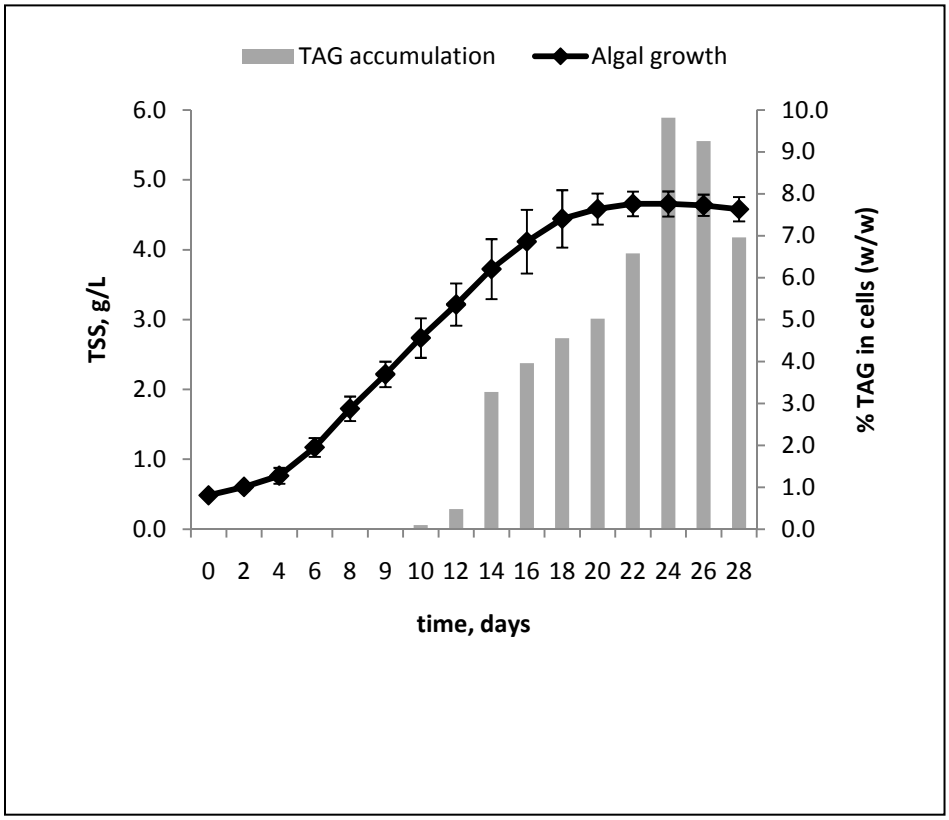


Fig. 6-4 Typical relationship between growth and TAG accumulation in Logan lagoons algal isolate culture in the IBR effluent

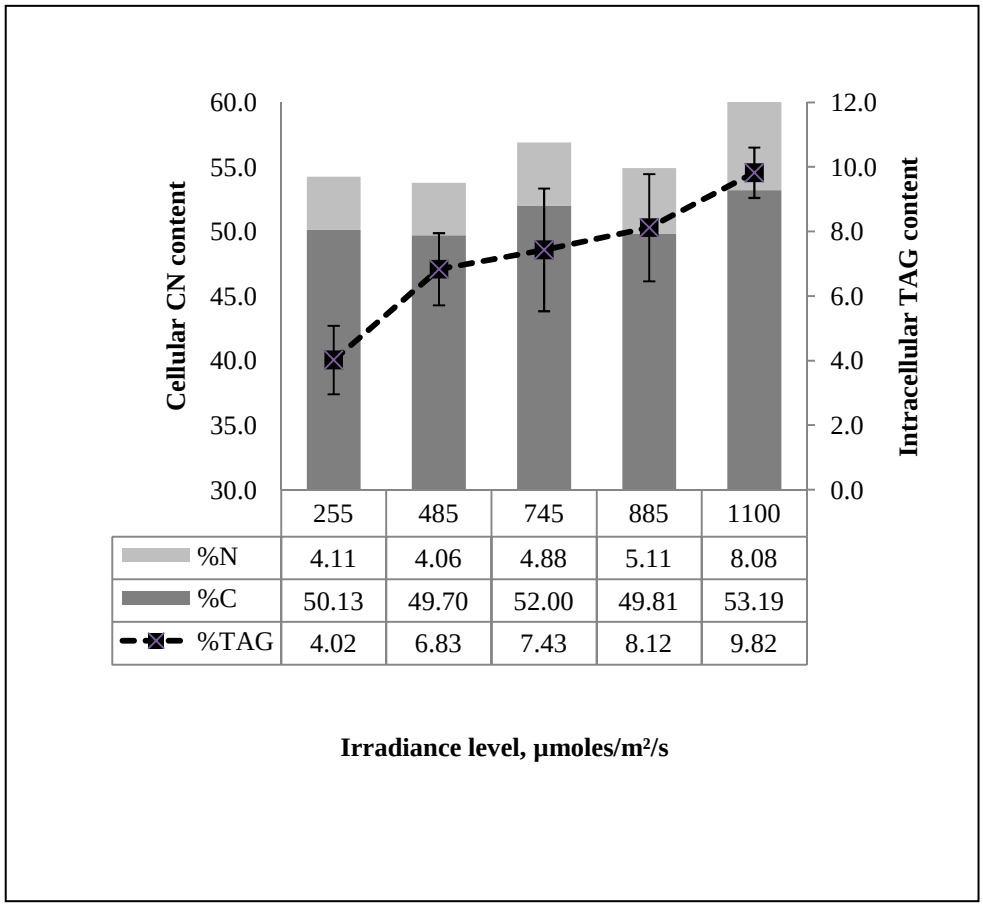


Fig. 6-5 TAG accumulation and intracellular C and P content at different light intensities in LLAI cells grown at different light intensities in IBR effluent

CHAPTER 7

SUMMARY AND FUTURE WORK

Summary of the Research Work

To meaningfully use nutrients from sources like digested dairy waste, it is important to understand the nature and behavior of nutrients in these systems. The current study provides valuable information on characteristics of the effluent of an anaerobic digester treating dairy wastes and a useful theoretical tool to predict the speciation of nutrients in various physico-chemical environments. Pretreatment strategies based on nutrient speciation and light interaction in the effluent employed in this study showed an increase in the bioavailability of nutrients and penetration of light through the effluent. It was shown in this research that native substrates available in organic and inorganic forms can be utilized by algae in the presence of indigenous microorganisms if algal strains are given the opportunity to adapt to the effluent environment.

One of the important findings regarding algal growth reported in this study was the pattern of nutrient uptake by different algal strains in the effluent. It was shown that algal strains adapted to the effluent have the ability to switch from phototrophic growth to heterotrophic growth when light limited conditions arise in the effluent. It has been suggested in this study that the algal strain that has the ability to incorporate heterotrophic growth with phototrophic growth when light penetration in the effluent diminished due to high culture density has better chances of survival in a growth medium such as anaerobic digester effluent.

The strategies of light supplementation and nutrient replenishment proposed in this study proved to maximize the biomass production and nutrient uptake in algal cultures. It

was shown that application of light supplementation enabled the enhanced uptake of inorganic nutrients and prolonged the phototrophic activity in those algal strains unable to utilize native organic nutrients for heterotrophic growth to survive in light limited conditions. On the other hand, supplementation of ammonia not only facilitated the growth in algal cultures; it also assisted the utilization of native substrates in the effluent. This utilization of native substrates resulted in a marked improvement in biomass production in the algal cultures.

Isolation and identification of Logan lagoons algal isolate (LLAI) is another key finding of this research. LLAI showed the best adaptability to the effluent environment and produced highest amounts of algal biomass among the three algal strains studied here. It was suggested that since LLAI originated from a natural habitat this algal strain had better adaptability to the effluent environmental conditions than the two type strains. This adaptation allowed LLAI to utilize native substrates in the effluent and sustain the growth for longer period of time.

The experimental and modeling results reported in this study can be used to predict nutrient concentrations when these waste streams are utilized. The study is specifically important during the current surge of interest in process optimization to reduce the cost of biodiesel production from algae. It has been shown in this study that different strains of algae can be adapted to a natural medium, effluent of an animal waste treating anaerobic, to maximize the utilization of native nutrients present in both organic and inorganic forms. Since algae grown on the effluent utilize only native substrates in nonsterile environment, identification and development of such a combination of algal strain and growth media can significantly reduce the cost incurred in production of algal biodiesel.

Suggestions for Future Work

To extend this study, following hypotheses based on the findings of current research work are suggested to be tested in the future.

Hypothesis 1

The characterization of anaerobic digester effluent in this study has elucidated the presence of C, N, and P in organic forms in significant fractions. Also algae growing on the effluent have shown to utilize organic forms of these nutrients along with inorganic forms especially in low light conditions. Hence identifying the organic compounds preferably utilized by algae in anaerobic digester effluent could improve understanding of algal growth in light limited conditions.

Organic compounds such as volatile fatty acids, carbohydrates and proteins are abundantly found in the effluent of an anaerobic digester treating dairy waste. Utilization of these compounds by algae for heterotrophic-C or organic-N can be examined under various sets of growth conditions supporting either phototrophy or heterotrophy in the algal cells. Also, the study on uptake of effluent borne substrates by algae at different stages of growth can be used to determine the extracellular compounds utilized by algae to assimilate various intracellular compounds.

Hypotheses 2

Lagoons algal isolate researched in this study was shown to produce triacylglycerides (TAGs) when cultured on anaerobic digester effluent. High amounts of lipids including TAGs have been reported to accumulate in algae cells under stressful growth conditions such as nutrients limitation or rise in pH in the culture. Determining such lipid inducing triggers in the growth environment of anaerobic digester effluent could improve TAG

yields from algal cultures grown in the effluent.

Limitation of inorganic-nitrogen in algal cultures has been documented as the trigger for accumulation of intracellular neutral lipids including TAGs. However, algal strains researched in this study utilized both inorganic-nitrogen as well as nitrogen present in organic compounds in the effluent. Therefore, effect of limitation of different forms of nitrogen can be examined for its potential of inducing lipid accumulation in algal cells cultures in anaerobic digester effluent. Besides this, other growth limiting conditions such as rise in the pH of culture or unavailability of light can also be tested as possible lipid inducing triggers in algal cells.

Hypothesis 3

It was shown in this study that biomass accumulation as well as TAG yields from algal cultures in anaerobic digester effluent increased with the rise in level of irradiance. Since this increase showed upward trend till the maximum light intensity (1100µmoles.$m^{-2}s^{-1}$) tested in this research, it can be hypothesized that biomass and TAG yields from algal culture grown in the effluent would continue to increase at irradiance levels even higher than 1100µmoles.$m^{-2}s^{-1}$.

Using the model of algae growing in outdoor bioreactors or raceway ponds, algal strains adapted to the IBR effluent can be grown under sunlight to examine the effect of irradiance at levels higher than ones used in this research. Also, the combination of indoor and outdoor light can be used to increase the irradiance level to sustain the growth inhibited at lower levels of irradiance due to limitation of light.

Hypothesis 4

23S rRNA genomic analysis of Logan lagoons algal isolate (LLAI) performed in this study showed close phylogenetic resemblance of LLAI with *Chlorella vulgaris*. However, in parallel growth comparison, LLAI showed much better biomass production and nutrient removal than pure *C. vulgaris* strain. The complete genomic sequence of LLAI cells would determine whether the isolated strain is genetically different or merely a mutation of *C vulgaris*.

A complete sequencing of genomic DNA of LLAI will be required to establish the exact phylogenic relationship between isolated strain and the known species of algal strains. Genomic analysis performed on LLAI cells from different generations of adaptability study will also help identifying the changes taken place in LLAI cells at the genetic level that enabled them to adapt to the effluent environment.

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APPENDIX

March 28, 2010
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I am preparing my dissertation in the Biological and Irrigation Engineering department at Utah State University. I hope to complete my degree in the spring of 2010.

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CURRICULUM VITAE

SHANTANU WAHAL

EDUCATION

PhD, Biological Engineering, Utah State University, Logan UT, USA *(April 2010)*

MS, Biological Engineering, Utah State University, Logan UT, USA *(Dec 2004)*

BTech, Agricultural Engineering, G B Pant University, Pantnagar, INDIA *(Sep 2001)*

WORK EXPERIENCE

Graduate Research Assistant, USU Biofuels Center, Logan UT, USA *(Jan 2007 – Present)*

- Developed innovative nutrient and irradiance management strategies to increase the productivity in large-scale bioreactors
- Developed modeling techniques to ascertain the bioavailability of nutrients for algal culturing in different types of natural and artificial media
- Evaluated the rate of nutrient uptake by specific strains of algae, and determined nutrient-specific triggers on algal growth for high production of lipids and proteins by algae

Graduate Research Assistant, Biol. Engineering, USU, Logan UT, USA *(Jan 2006 – Dec 2006)*

- Developed analytical techniques for chemical analyses of anaerobic digester effluent and wastewater from pilot-scale bioreactors
- Characterized the effluent for speciation of nutrients and environmental conditions that affect the chemical composition using *Chemical Speciation Models*
- Determined the potential of nutrient removal through three different strategies- chemical precipitation, enhanced biological phosphorus removal, and algal growth

Research Assistant, Nutrition and Food Science, USU, Logan UT, USA *(May 2006 – Aug 2006)*

- Developed biochemical techniques for identification and isolation of milk borne proteins and fatty acids

- Performed lab scale fermentation of different enzymes used for processing of milk and cheese
- Developed analytical techniques for quantification of volatile organic compounds using GC-MS, identification and quantification of compounds in liquid samples using FTIR and HPLC

Graduate/ Teaching Assistant, Biological Engineering, Utah State University, Logan UT *(Sep 2006 – Dec 2006) and (Sep 2007 – Dec 2007)*

- Assisted in teaching, grading, preparation of tests and homework assignments for 6000/5000 level courses
- Worked as lab instructor in Food and Biological Engineering lab, developed and prepared protocols for different lab experiments

Quality Assurance Lab Technician, Gossner Foods, Logan UT, USA *(Jan 2005 – Oct 2005)*

- Performed quality control tests on different on-line food products
- Developed standard operating procedures (SOPs) for various experimental analyses
- Performed biological surveys and conducted calibration of in-plant instrumentation

TRAINING AND CERTIFICATIONS

- Lab Safety and Chemical Hygiene Training, 2009 and 2007
- Certificate Training in Microbial Fermentation: Development and Scale-up, 2007
- BioSys Graduation Certificate: training on MicroFoss 128 microbial detection system, 2005
- 40-hour OSHA 29 CFR 1910.120, 2004

RESEARCH PAPERS (Published and pending)

- **S. Wahal**, S. Viamajala "Characteristics of an isolated algae strain and intracellular lipid production at different light intensities using anaerobic digester effluent as substrate", manuscript in progress to be submitted, May 2010
- **S. Wahal**, S. Viamajala "Maximizing algal growth in anaerobic digester effluent using light and nitrogen supplementation", manuscript completed to be submitted, April 2010
- **S. Wahal**, S. Viamajala, C. L. Hansen, "Speciation of dairy waste treating anaerobic digester effluents and availability of nutrients for biological utilization and chemical recovery processes", **Under review**
- **S. Wahal**, S. Viamajala, C. L. Hansen, "Adaptation of microalgae to anaerobic digester effluent for enhanced utilization of organic nutrients and high culture density", **Under review**

- **S.Wahal**, S. Viamajala, 2010 “Maximizing algal growth in batch reactors using sequential change in light intensity”, ***Applied Biochemistry and Biotechnology***, 161: 511 – 522

RELATED SCHOLARY WORK

Grant proposals

- Recovery of nutrients from digested dairy manure using controlled chemical precipitation, Sep 2008 , submitted to USDA-SBIR

Poster presentations

- 31st Symposium on Biotechnology for Fuels and Chemicals, May 2009, San Francisco, CA "Maximizing algal growth in batch reactors through sequential change in light intensity"
- Institute of Biological Engineering Regional Conference, Oct 2008, Logan, UT, USA “Chemical Speciation in Anaerobic Digester Effluents and Bio-based Strategies for Utilization of Nutrients”
- IBE Mar 2008, Chapel Hill, NC, USA “Assessment of chemical interactions in anaerobic digester effluent and bio-based strategies for nutrient utilization”

Podium presentations

- PNW- ASABE/CSBE Sep 2007, Moscow, ID, USA “Utilization of anaerobic digester effluent for the production of high value bioproducts”

PROFESSIONAL MEMBERSHIPS

- Society of Industrial Microbiology **(SIM)**
- Institute of Biological Engineering **(IBE)**

HONORS AND AWARDS

- Honorable Mention Award, for poster presentation at IBE Conference, USU, USA 2008
- Inclusion in Dean’s List for outstanding scholastic achievement in MS, 2004
- Gold medal for academic excellence in BTECH (undergraduate degree program), 2001