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Lipid Accumulation in Synthetic Wastewater-Grown Oleaginous Microorganisms

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LIPID ACCUMULATION IN SYNTHETIC WASTEWATER-GROWN OLEAGINOUS MICROORGANISMS

By

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LIPID ACCUMULATION IN SYNTHETIC WASTEWATER-GROWN

OLEAGINOUS MICROORGANISMS

By

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Wastewater has been shown to contain the necessary nutritive requirements for the growth of microorganisms. The term, oleaginous, has been given to a classification of microorganisms know to produce up to twenty percent of the weight as oil. This study is designed to examine the potential accumulation of lipids within an oleaginous consortium grown on synthetic wastewater. Potential of the fluorescent stain, Nile red, as a lipid detector is also emphasized.

Percentages of extractables greater than thirty-five percent were achieved within the oleaginous consortium using a nitrogen-limited medium. Low pH was found to increase the percentages of extractables. Xylose was shown to be a more optimal carbon source for accumulation than glucose. Nile red was shown to bind to intracellular inclusions and may be useful in monitoring lipid accumulation in industrial settings.

DEDICATION

This thesis is dedicated to my grandparents, Mr. and Mrs. Thomas Jackson Ford, Jr. and Mr. and Mrs. Vance Walker Thompson. They taught me humor, love, and that bootstraps are indestructible.

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The completion of this thesis would not have been possible without the help of many. Foremost, I thank Dr. W. Todd French who showed remarkable patience and supported my efforts at every step. Dr. Lewis R. Brown introduced me to the scientific method, and has a contagious spirit of pride and dedication to his work which inspires me still. I thank Dr. Donald Downer for greeting every academic crisis with a smile, a joke, and a fix. Also, I salute the efforts of all of the fellow graduate students that were instrumental in my success. Most notably, Ben Hartenbower, Scott Crymble, Jacqueline Hall, Bart van den Berg, and Andro Mondala provided humor, support, and great ideas.

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CHAPTER I

INTRODUCTION

The problems of energy consumption and production have had steadily increasing importance over the past years. The present state of energy concerns is wide-ranging, having significant effects on politics, environment, land use, and economics. Reliance on petroleum has reached peak levels and the expanding economies and industrialization of large-population countries such as China and India will further exacerbate current troubles. Adding further magnitude to the scenario, most studies predict world production of oil to peak at some time from 2010-2020 (Hirsch *et al.*, 2005).

Many rising technologies have been proposed to combat current energy concerns. To alleviate the high greenhouse gas emissions and land destruction caused by the burning of coal, researchers have proposed wind, solar, geothermal, and nuclear solutions to the rising demands for electricity. However, the provision of fuels is perhaps the greatest concern. World oil consumption is currently expected to increase from 86.1 million barrels per day in 2007 to 103.9 million barrels per day in 2030 (Dept. of Energy, 2010). When these data are paired with the aforementioned peak oil analysis, the true breadth of the current energy crisis can be clearly understood. With barrels of oil currently rising to record prices, the future economic effects of our demand of petroleum is bleak. Many solutions are under investigation to decrease the dependency on fossil fuels; including bio-oils, bio-ethanol, bio-butanol, biodiesel, solar power, wind power, and geothermal power. Biodiesel is of particular interest for this study.

Biodiesel is a popular solution to the provision of future fuel demands. There are currently many different resources that are being evaluated for their potential as a source of biodiesel. These sources include soybeans, rapeseeds, sunflower seeds, flax, palm oil, and animal fats (Sanford *et al.*, 2009). However, this rush for a new source of fuel has also caused some food crops to be used in the production of fuels. This has resulted in the price increase of many food crops (NPR, 2008). This event has made clear the need for a potential oil source that is not tied to food production. Another issue which effects and will continue to have effects on the potential sustainability of U.S. biodiesel production is the continued decline in farm land. Land designated as farmland in the U.S. decreased by twelve percent from 1982 to 2003 (USDA NRCS, 2007). This presents the further qualification that optimal biodiesel sources should have no value as food and also have a limited acreage requirement. These specifications are difficult to meet with most of the currently studied biodiesel sources.

One source that meets both of these requirements is oleaginous microorganisms. These are microorganisms, primarily yeasts, which have been shown to have the ability to accumulate up to twenty percent of their weight as oil (Ratledge, 1997). The industrial use of these oleaginous microorganisms could offer a cheaper domestic solution to increasing fuel production and satisfying rising demands. One of the limiting resources in many industrial scale reactions is the management of water. This is of particular interest when evaluating the prospects of oil production from oleaginous microorganisms because large volumes of water will be needed to provide a scale of production that can make this a viable solution. A potential solution to this problem is the use of wastewater treatment facilities. This is a viable option in that this solution would utilize existing infrastructure and water resources.

The objective of this study is to examine the potential of oleaginous microorganisms for the production of billions of gallons of biodiesel feedstock. The use of wastewater treatment facilities as biorefineries will also be examined. A particular emphasis will be placed on the use of a consortium of oleaginous microorganisms to facilitate production of a biodiesel-like product. The potential of certain microorganisms to accumulate intracellular inclusions of oil is well-known and widely studied. In order to prevent problems of culture loss and contamination, a consortium of these microorganisms will be examined. Oil accumulation yields will be optimized by using different growth conditions and carbon sources. Glucose and xylose will be examined as potential sources of carbon. Methods of determining lipid accumulation, including fluorescence of the lipophilic stain Nile red, will also be examined. Furthermore, imaging techniques such as confocal laser scanning microscopy and scanning electron microscopy will be used to visualize oil accumulation within cell cultures.

CHAPTER II

LITERATURE REVIEW

Oleaginous Microorganisms

The term oleaginous microorganism refers to any microorganism that has the capability of accumulating up to 20 percent of its weight in lipids (Ratledge *et al.*, 1977). Yeasts are single-celled eukaryotes, and their unique structure and metabolic capabilities have led them to be the most widely studied and commonly labeled oleaginous microorganisms (Ratledge, 2002). The oleaginous microorganism *Rhodotorula glutinis* (formerly *R. gracilis*) was previously examined as having the ability to accumulate over thirty percent of its weight in lipid (Easterling *et al.*, 2009). Similarly, Davies has shown that the oleaginous yeast, *Cryptococcus curvatus*, has the ability to accumulate greater than 60% cell dry weight in lipids utilizing cheese whey as a carbon source (1988). *Lipomyces starkeyi* has been described as accumulating over sixty percent of its weight in lipids when cultivated under conditions of high carbon to nitrogen ratios (Angerbauer *et. al.*, 2008). Rupcic *et al.* showed that even 1% methanol could be utilized to accumulate around 5% lipid in the oleaginous yeast *Candida lipolytica* (1996).

Cultivation of Oleaginous Microorganisms on Varied Carbon Sources

Many different carbon sources have been shown to provide the necessary components of growth and lipid accumulation within oleaginous yeasts. While glucose is, of course, the most commonly researched source of carbon for lipid biosynthesis, the expense has fueled interest in several other cheaper sources. For instance, *Trichosporon*

fermentas has been shown to accumulate over 35% lipid when grown utilizing cane molasses containing primarily sucrose along with other carbohydrates (Zhu *et al.*, 2008). Sewage sludge has also been examined as a cheap and robust source of carbon for the production of biodiesel via oleaginous yeasts (Angerbauer *et al.*, 2008). Rupcic *et al.* showed that hydrocarbons such as 1% methanol could be utilized to accumulate around 5% lipid in the oleaginous yeast *Candida lipolytica* (1996).

Wheat residue hydrolysates have been examined for their potential as a source of xylose and glucose for the growth and accumulation of lipid in the oleaginous yeast *Cryptococcus curvatus* (Greenwalt *et al.*, 2000). Starches have also shown to be converted to greater than 30% lipid yields (Papanikolaou *et al.*, 2007). The tubers of the Jerusalem artichoke were found to be utilized by *Rhodosporidium toruloides* to produce a lipid content of up to 40% (Zhao *et al.*, 2010). The Jerusalem artichoke is a non-grain plant material which increases the potential for its use as a substrate for bio oil production. However the processing required to achieve usable Jerusalem artichoke extracts adds a level of time and expense that could eliminate this feedstock from being a practical solution. Derivatives of animal fats have also been shown to provide the necessary substrates for lipid production in oleaginous yeasts. Papanikolaou *et al.* used industrial fat composed of stearin to produce intracellular lipid in *Yarrowia lipolytica* (2002). While the use of animal fats could be useful to supplement feedstocks for use in large-scale bio oil production, these substrates are not available in the quantities required to be considered a long term solution.

Several fruit substrates and waste products have been examined as potential feedstocks for bio oil production. Banana juice supplemented with some salts and growth factors was shown to be a viable lipid production medium for *Candida curvata* (Vega *et*

al., 1987). Orange peel has also been proposed as a viable substrate for lipid production in the oleaginous yeast *Cunninghamella echinulata* (Gema *et al.*, 2002). Zhao *et al.* found that lipid yields of greater than 60% could be achieved by utilizing a medium containing a 2:1 (wt/wt) concentration of glucose and xylose with *L. starkeyi* (2008). Easterling *et al.* examined xylose, glucose, and glycerol separately and in combination and described yields over 30% in *R. glutinis* (2009).

Nile Red Fluorescence

Nile red is a highly fluorescent dye that can be used to stain intracellular lipid inclusions for detection and imaging purposes. Nile red has been used to stain and image fat and fatty acids within tissue samples (Smith, 1908). Ramoino used Nile red in conjunction with Confocal Laser Scanning Microscopy to examine and visualize lipid deposits within the ciliate *Paramecium primaurelia* under varying conditions of environmental stress. By using filters set at 488-nm excitation and 530-nm emission it was determined that intracellular lipid deposit numbers were greatest in the initial logarithmic phase (Ramoino *et al.*, 1996). Nile red has also been used for the detection of lipids in single-celled prokaryotes and eukaryotes. The accumulation of polyhydroxyalkanoic acids within the bacterial species *Azotobacter vinelandii* has been examined by the addition of Nile red directly to a growth medium and subsequent exposure to ultraviolet light (Spiekermann *et al.*, 1999).

Kimura correlated Nile red fluorescence with actual lipid yields obtained from gas chromatographic analysis of fatty acids from dried cells of the oleaginous species *L. starkeyi* and *C. curvatus*. In testing different excitation and emission spectra, it was found that an excitation wavelength of 488-nm and emission of 565-585-nm were

optimal for detection of lipid fluorescence. This study found that Nile red could be a useful method for rapid estimation of intracellular lipid contents (Kimura *et al.*, 2004). The use of Nile red to estimate intracellular lipids could be useful in efficient determination of optimal times to extract lipids from batch cells to achieve high lipid yields. Although Nile red staining has been shown to be an indicator for intracellular lipids, no literature could be identified that attempted to estimate real time lipid accumulations in an oleaginous consortium via Nile red staining.

Lipid Extraction and Quantification

The most utilized methods for the extraction of lipids from samples follow the model put forth by Bligh and Dyer (1959). These researchers utilized a chemical extraction process to obtain the lipids from fish tissue samples. The Bligh and Dyer method uses chloroform and methanol, along with water, to obtain a percentage of lipids gravimetrically. There have been other models proposed for this purpose, including a calorimetric method that was first designed and utilized to extract lipids from mosquitoes (Van Handel, 1985). Although found to be less expensive and laborious, this calorimetric method has been compared to the previous method utilized by Bligh and Dyer and it was found to have minimal differences in lipid yield percentage (Inouye and Lotufu, 2006). The labor and expense associated with both Bligh and Dyer and calorimetric lipid analysis reinforce the potential advantages of Nile red fluorescence as a potential estimator of lipid accumulation.

Metabolic Biosynthesis of Intracellular Lipid

Much work has been done with the intent of better understanding the mechanics of lipid accumulation within the unique species of oleaginous microorganisms. Of

approximately 600 different species of yeasts, only around 30 have been confirmed to have the biochemical properties required to be classified as oleaginous (Rattray, 1988). Perhaps the most crucial environmental occurrence that promotes lipid accumulation in the oleaginous yeasts is the presence of excess carbon concurrent with the absence of some other limiting growth factor such as nitrogen. Limiting nitrogen under conditions of surplus carbon is the most commonly studied and effective method of examining intracellular lipid accumulation (Ratledge, 2004). Although there is still much to be investigated, there are some known components of the biochemistry involved in lipid accumulation. Several studies have detailed the role of malic enzyme in this process. The inhibition of malic enzyme by sesamol was shown to severely alter the accumulation of intracellular lipids in the filamentous fungus *Mucor circinelloides* (Wynn *et al.*, 1997). Malic enzyme appears to play a significant role in the provision of NADPH which is necessary for lipid biosynthesis. The over expression of malic enzyme by genetically engineered *M. circinelloides* mutants has been shown to increase lipid accumulation by 20% (Zhang *et al.*, 2007). Similar results were found in a study using three strains of *Aspergillus nidulans* that contained wild-type, repressed, and over expressed malic enzyme activity (Wynn and Ratledge, 1997).

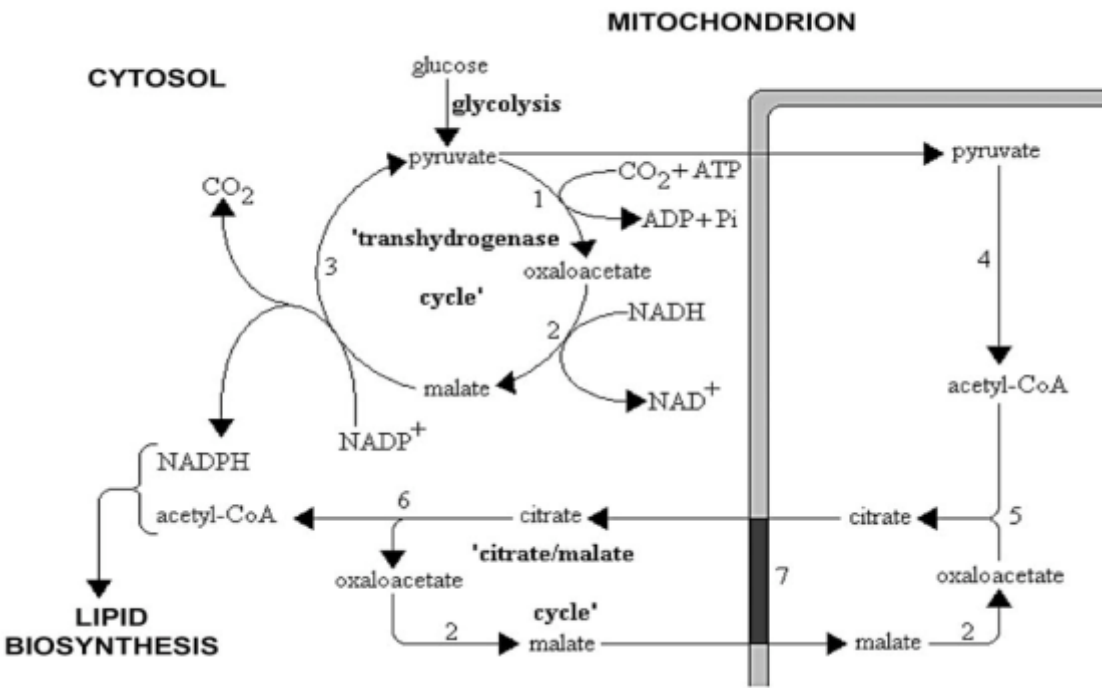

Figure 1 Lipogenesis

Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase;
3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase;
6, ATP:citrate lyase; 7, citrate/malate translocase.
Source: Wynn *et al.*, 2001

Biochemical Distinctions of Oleaginous Microorganisms

While malic enzyme is present in both oleaginous and non-oleaginous microorganisms, it has been proposed that the level of expression within species could be the determining factor for the ability to accumulate intracellular lipid (Zhang *et al.*, 2007). Another significant difference between the oleaginous and non-oleaginous yeasts has been observed. Citric acid is an important metabolite necessary for fatty acid synthesis in all cells. Citric acid is a product of the tricarboxylic acid (TCA) cycle. In the oleaginous yeasts the activity of isocitrate dehydrogenase is regulated by the presence of excess adenosine monophosphate (AMP). This positive feedback loop does not appear

to be present in the non-oleaginous yeasts. The presence of AMP within the cell is regulated by AMP deaminase (Ratlege, 2004).

The activity of AMP deaminase has been shown to increase dramatically at the first signs of depletion of the nitrogen source (Wynn *et al.*, 2001). AMP deaminase up-regulation causes a decreased level of AMP within the mitochondrion and results in excess citrate accumulating. The citrate is moved into the cytosol where it is eventually cleaved into the other primary requirement of lipid biosynthesis, acetyl-CoA. This reaction is facilitated by the enzyme, ATP:citrate lyase. There are other complexities that remain to be elucidated in the biosynthesis of lipid accumulation within the cell, but the synthesis of the two major precursors, NADPH and acetyl-CoA, has been described (Ratledge, 2004).

Glucose or Xylose as Carbon Source for Lipid Accumulation

Glucose and xylose are metabolized differently within both bacteria and eukaryotes. For the purpose of lipid accumulation, the six-carbon glucose is primarily metabolized via glycolysis. This metabolism provides the aforementioned precursors, NADPH and acetyl-CoA, required for lipid biosynthesis. The five-carbon sugar, xylose, is metabolized via the pentose phosphate pathway. This pathway also provides the cell with NADPH and acetyl-CoA (Jeffries, 2006).

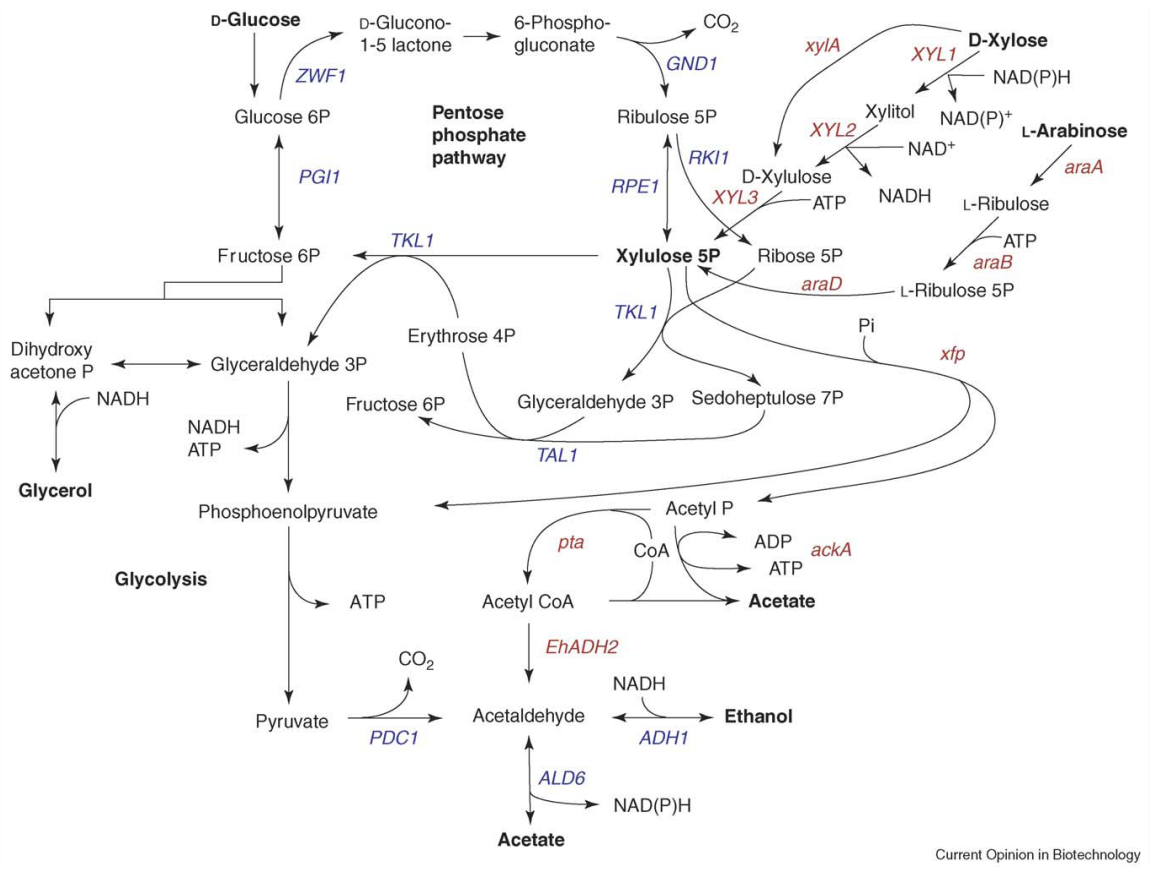

Figure 2 Pentose Phosphate Pathway

Yeast genes in italic caps; bacterial in lower case italics.
Source: Jeffries, 2006

Xylose is of particular importance due to the high yields obtained through acid hydrolysis of biomass. Xylose is the primary hemicellulosic sugar of hardwoods and agricultural residues representing up to 25% of the dry weight biomass (Ladisch *et al.* 1983). Tsao *et al.* previously described xylose yields of up to 33 pounds per 100 pounds of corncobs hydrolysed (1982). Hydrolysis of hemicelluloses from hardwoods has shown yields of up to 25g of xylose per 100g of wood (Maloney *et al.*, 1986). Many of the studies of xylose fermentation have focused on the production of ethanol (Schneider *et al.* 1981). The interest of xylose within this study is to examine the low-cost and

readily available sugar's use as a potential carbon source for lipid biosynthesis using an oleaginous consortium of microorganisms.

CHAPTER III

MATERIALS AND METHODS

Oleaginous Consortium Description and Maintenance

An oleaginous consortium containing the yeasts C. curvatis, *R. glutinis*, and *L. starkeyi* were maintained in a batch 4L flask with automated stirring. The medium used in this batch technique was a synthetic wastewater medium described by LaPara *et al.* (2003). This synthetic wastewater medium contained 5.55 g gelatin (Fisher Scientific, hereinafter referred to as FS), 2.59 g starch (FS), 0.37 g yeast extract (FS), 0.37 g casamino acids (FS), 1.85 g ammonium sulfate (FS), 0.925 g sodium phosphate (FS), 1.11 g potassium phosphate (FS), .0022 g calcium chloride (FS), and 3.7 mL SL7 trace mineral solution per 1L of reverse osmosis water.

Sterilization was achieved with a Steris SG-120 Scientific Gravity Sterlizer (Mentor, Ohio) autoclave. At weekly intervals, 1.5L of the culture was removed and replaced with fresh medium. Although the optimal growth temperature of the consortium yeasts is 30^0 C, the stock was kept at room temperature to slow growth and conserve media. The primary carbon source within this medium was starch. Aseptic techniques were used to maintain consortium consistency. Using this stock culture as the inoculation source, the experiment was divided into two stages.

Growth Stage

The growth phase of the experiment was accomplished by using 1500mL of the synthetic wastewater medium with 3g of Starch. The medium was prepared, autoclaved,

and placed into a 3L beveled Erlenmeyer flask. The flask was then inoculated by spinning down 1200mL of the stock oleaginous consortium, using a ThermoScientific Sorvall ST40 Centrifuge (Waltham, Massachusetts), and adding the cell pellets directly to the medium. This was then placed on a New Brunswick Innova® I26 rotary incubator (Edison, New Jersey) maintained at 30^0 C for 168h. Observations were taken at 24h intervals to ensure proper aeration/stirring was taking place. Optical density was recorded directly after inoculation and again after the 168h incubation period using the Genesys Spectronic 20 Spectrophotometer (ThermoFisher). The target OD_{595nm} for this growth phase was 1.5-2.0. The primary goals of this initial growth stage were to achieve a high cell concentration and to have consistent and quantitative starting inoculae for all lipid accumulation experiments. After the desired optical density was achieved, a portion was spun down and resuspended in reverse osmosis water. This solution was used as the inoculate for the lipid accumulation stage.

Lipid Accumulation Stage (Large flask)

The lipid accumulation medium used for all experiments was a nutritionally sparse medium containing no added nitrogen. The components of this medium were 1g/L KH2PO4 (FS), 1g/L Na2HPO4•12 H2O (FS), 0.8g/L MgSO4 •7H2O (FS), 10mL/1L Trace Mineral solution (FS), and 2.0mL/1L FeSO4 solution (FS). The pH was adjusted to 6.0. The desired concentration of the carbon source (xylose or dextrose) was dissolved, under heat in reverse osmosis water, and then filter sterilized. After autoclaving, the carbon source was added to the medium aseptically. The final volume of medium within each 1L flask was 300mL. The flasks were then inoculated with 100mL of spun down oleaginous consortium from the growth stage. At this point the flasks were

placed on the shaker/incubator at 30^0 C. Three flasks were designated for each lipid accumulation variable (experiment performed in triplicate). Optical Density (405-nm), Nile red fluorescence (488-nm emission, 560-nm excitation) using the Molecular Devices, Inc. Spectramax M5 Microplate Reader (Sunnyvale, California), pH using the ThermoScientific Orion 4 Star (Waltham, Massachusetts) pH meter, cell mass (per 10mL) were recorded at 0h, 48h, 96h, 144h, and 168h. Bligh and Dyer extraction was performed on a sample of the initial inoculum and also on 100mL aliquots at 96h, 144h, and 168h.

Small Scale Lipid Accumulation Stage (100mL Wheaton Bottles)

During the larger shake-flask experiments cell clumping provided difficulty in achieving representative samples. Smaller scale experiments using sacrificial sampling were performed to address variability due to cell clumping within flasks successfully. For these smaller, bottle experiments, the same nitrogen limited medium as flask experiments were used. A volume of 55mL of medium was used in each Wheaton bottle. This volume was attained by adding 25mL of a 2X buffered or non-buffered nitrogen limiting media solution, and 25mL of a 2X filter sterilized sugar solution. The final 5mL contained a concentrated cell inoculum from the growth stage. A total of 150 Wheaton bottles were prepared, representing triplicates of the ten test groups and five time points. Sacrificial sampling was utilized in the experiments conducted in Wheaton bottles. Different bottles were designated for each time point and the entire contents were harvested and analyzed when the designated time points were reached. As in the shake-flask experiments, bottles for each lipid accumulation variable were maintained in triplicate.

Nile Red and Optical Density Analysis

In order to obtain readings for Nile red fluorescence and optical density, a 1.0 mL aliquot was taken and from this aliquot 300µL was transferred into a well on a 96-well plate for an optical density (405-nm) reading. In order to obtain the Nile red fluorescence reading, 7µL of a 5µg/ml Nile red solution in acetone was added to the 1.0mL aliquot, shaken gently, and then 300µL was transferred to a well on the 96-well plate. The 96-well plate was then placed into the Spectramax Microplate Reader. Optical density was quantified at the 405-nm wavelength and the emission wavelength of 488-nm and excitation wavelength of 560-nm was used to calculate Nile red fluorescence. These settings were based on previous work by Kimura *et al.*(2004).

Experimental Variables

Both glucose and xylose were examined for their potential use as carbon sources for lipid accumulation. Each carbon source was tested at both 40g/L and 60g/L concentrations. These sugars and concentrations were also examined under controlled pH conditions. The results of pH control and uncontrolled pH were compared in order to determine the optimal conditions for lipid accumulation. For the controlled pH experiments, both 0.5 M phosphate buffer and a citrate-phosphate buffer containing 24.3mL of 0.1 M citric acid (FS) and 25.7mL of 0.2 M NaHPO4 (FS) per 100mL water (Manual of Methods for General Bacteriology) were used. The target buffering pH was 5.0.

Analysis of Sugar Concentration

Sugar concentrations within the samples were examined to determine carbon utilization and examine growth characteristics within the experiments. The samples were

analyzed using an Agilent 1100 HPLC (Santa Clara, California) equipped with a Softa 200 Evaporative Light Scattering Detector (Westminster, CO). The injection volume was 2 microliters. The method utilized a Restek Pinnacle II Amino column (150 X 4.6mm) with 5 micron packing, and the solvent system was 78 % Acetonitrile/ 22 % Water (Optima Grade from Fisher). Calibration data were generated for both xylose and glucose and fit to a Power Law model with an R-Square of 0.99575 and 0.99677 for xylose and glucose, respectively.

Bligh and Dyer Extraction of Lipids

Extraction of intracellular lipids was accomplished by using an adaptation of the methods described by Bligh and Dyer (1959). The starting sample for this extraction was a cell pellet that had been freeze-dried overnight using a Labconco FreeZone 6L Console Freeze Dry System (Kansas City, Missouri). To this cell pellet 5mL of sterile reverse osmosis water was added. A monophasic mixture was achieved by adding 12.5mL of methanol (FS) and 6.25mL of chloroform (FS). This mixture was then mixed vigorously, by hand, for ten minutes. Next, a biphasic mixture was attained by adding an additional 6.25mL of water and 6.25mL of chloroform. This mixture was then placed in a centrifuge for 10 minutes at 3000rpm. Post-centrifugation, three phases were observable. The aqueous layer was at the top, followed by a cell suspension, and a bottom chloroform layer. The aqueous layer was discarded. The bottom chloroform layer was carefully removed using a Pasteur pipette and passed over a glass wool column and collected into a tared amber vial. This extraction process was repeated twice by adding additional volumes of 12.5mL methanol and 6.25mL chloroform. At the end of the extraction, approximately 19mL of chloroform solution was contained in each tared amber vial.

The vial containing the chloroform solution was then placed in a Caliper LifeSciences TurboVap LV (Hopkinton, Massachusetts) at 50^{0}C with a nitrogen flow rate of 10-15psi until no liquid remained. The vial was then weighed and Bligh and Dyer extracts were calculated by subtracting the weight of the tared vial from the weight of the same vial containing the extracts. A percentage of extractables within each cell pellet was then calculated by dividing the mass of the Bligh and Dyer extracts by the starting cell mass of the pellet and then multiplying by 100.

Production of Fatty Acid Methyl Esters for Analysis

The extracts from the Bligh and Dyer procedures contained acylglycerols, free fatty acids, and phospholipids. Analysis of the extracts required conversion to fatty acid methyl esters (FAME's). This was accomplished by adding 2mL of 2%(v/v) H2SO4 in methanol. These samples were then incubated at 60^{0}C for 2 hours in capped amber vials. After the vials cooled to room temperature, the reactions were quenched by adding 5mL of 3% NaHCO3 and 5% NaCl in water. Next, 2mL of toluene was added and the vials were vortexed vigorously. Another 2mL of toluene was then added and vortexed. The toluene used also contained a tetradecane (C14) internal standard and 100µg/mL butylated hydroxyl toluene (BHT). The top layer consisting of toluene and fatty acid methyl esters was then removed using a Pasteur pipette and placed in an autosampler vial.

Confocal Laser Scanning Microscopy

Nile red was used at a concentration of 5µg Nile red to 1 ml acetone (Ramoino *et al.*, 1996). Samples were taken from the oleaginous consortium grown under nitrogen-limiting conditions for 96 hours. A 1ml sample was applied directly to a precleaned slide

and allowed to air dry. The sample was then heat-fixed and lightly washed with a 0.1M phosphate buffer. The slide was then covered with a 5µg/ml solution of Nile red in acetone for fifteen minutes at room temperature. The slide was then dried with blotting paper. Imaging of intracellular lipid inclusions was accomplished via Confocal Laser Scanning Microscopy. Fluorescent images were acquired using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, Inc) with an Inverted Zeiss Axiovert 200 M Light microscope and a plan apochromat 100 X/1.4 NA objective lens. A FITC/TRITC (Fluorascein/Rhodamine/Transmission) filter set was used in Single and Multi Track channel mode imaging. Excitation wavelengths of 488nm/543nm and Long Pass (LP) Emission wavelengths of 505nm (Green) and 560nm (Red) were acquired at 512x512 or 1024x1024 pixel format for imaging purposes (MSU EM CENTER).

Scanning Electron Microscopy

Samples from the oleaginous consortium grown for 120h were taken and freeze-dried. The samples were then fixed using a 2.5% glutaraldehyde 0.1M phosphate buffer. The fixed samples were washed in 0.1M phosphate buffer and then stained with 0.2% osmium tetroxide in 0.1 phosphate buffer for three hours. Samples were washed with distilled water and then ethanol. The samples were dried using the Polaron E-300 critical-point dryer (San Diego, California) and sputter coated using the Polaron E5100. Images were obtained using the Zeiss EVO-40XVP (Oberkochen, Germany) environmental scanning electron microscope. Back-scattered detection was used to identify intracellular lipid inclusions.

CHAPTER IV

RESULTS AND DISCUSSION

Confocal Imaging of Nile Red Stained Lipid Inclusions.

Lipid staining within cells of the oleaginous consortium was shown to be effective when using Nile red. Confocal laser scanning microscopy appears to be a useful method for visualization of Nile red-stained intracellular lipid inclusions.

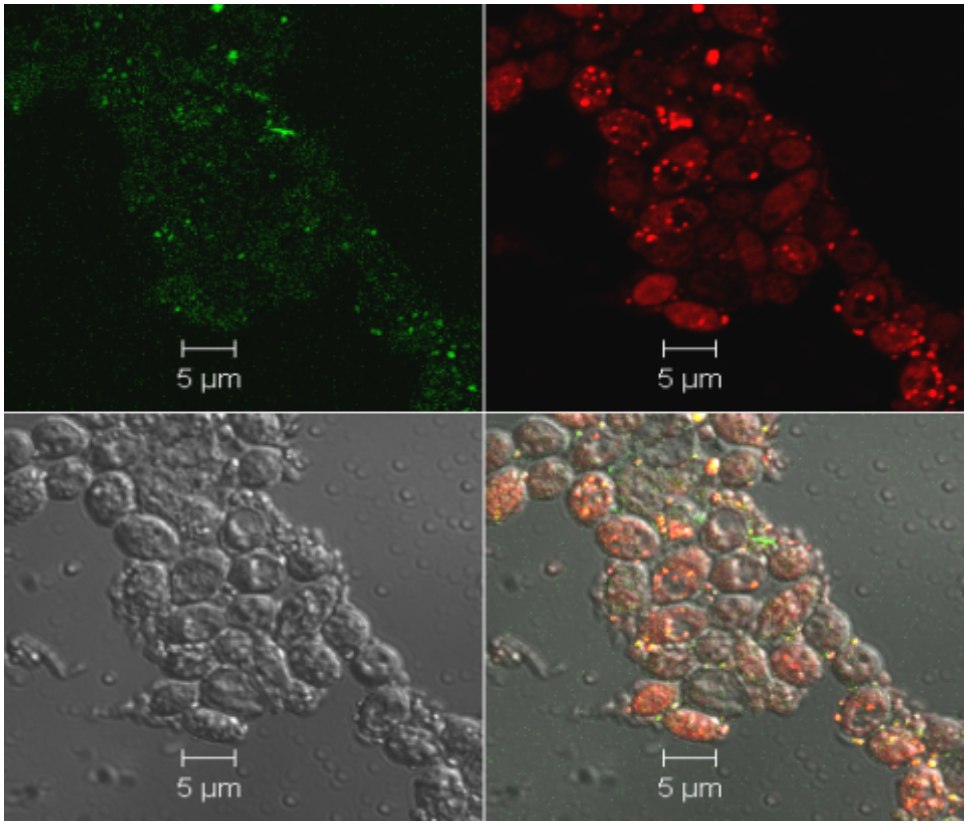

Figure 3 Scanning Confocal Laser Microscopy Image of Lipid Inclusions

Shown are emission wavelengths of 505-nm (Green), 560-nm (Red), No emission (Gray), and overlaid green and red wavelengths.

As observed in Figure 3, the green emission channel showed some inclusions, while the red channel showed the ability of Nile red to show both the topography and intracellular lipids. The channel without emission (gray) shows the image without the benefits of fluorescence. When visualizing the Nile red-stained lipid inclusions the red wavelength (560-nm) emission setting provided the clearest images.

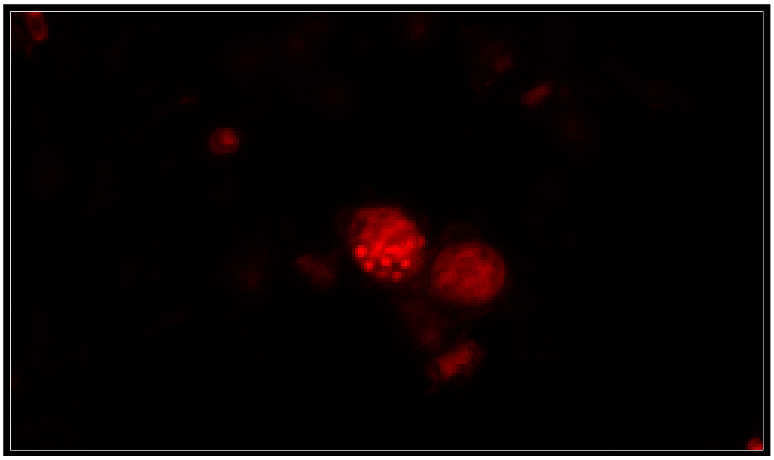

Figure 4 Nile Red-stained Inclusions Within Cells

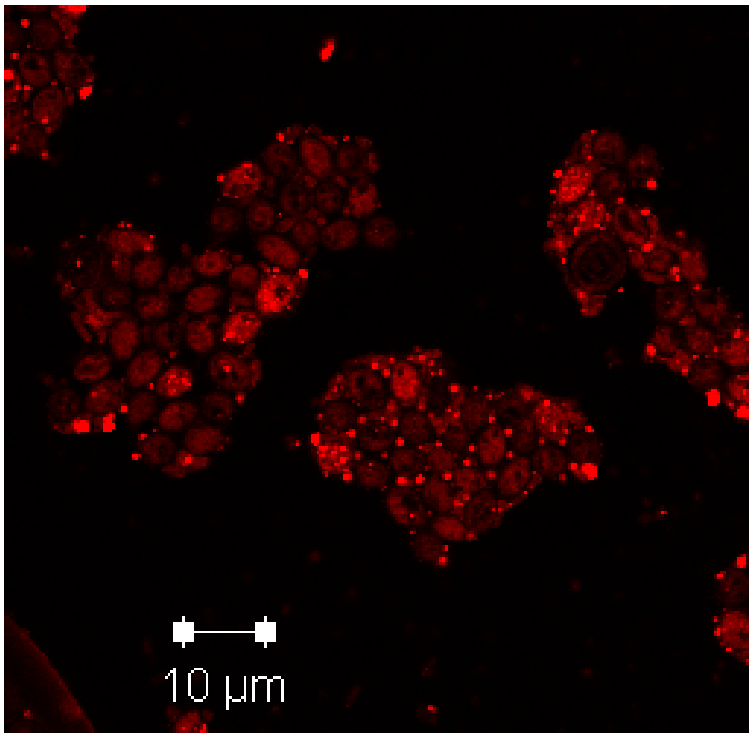

Figure 5 Large Groups of Stained Cells with Lipid Inclusions

Use of Nile red fluorescence and the red emission filter showed intracellular lipid inclusions, but Figures 4-5 show that inclusions within groupings of cells were variable. While useful as an imaging technique, the staining and sample preparation times involved in this procedure would not be efficient in industrial settings. Although scanning confocal laser microscopy is not a feasible method of approximating or detecting the presence of lipid inclusions in a real time industrial setting, the method proved useful in showing Nile red affinity for lipid inclusions. The method also appeared to show that Nile red was able to enter into the cells of the oleaginous consortium and produce a fluorescent response when using excitation wavelengths of 488-nm/543-nm and Emission wavelengths of 505-nm and 560-nm. This showed that Nile red could potentially be useful for providing a real-time estimation of lipid accumulation within cells in an active experimental setting.

Scanning Electron Microscopy

The use of Scanning Electron Microscopy (SEM) allowed for the visualization of three dimensional oleaginous cells with lipid inclusions. Although this technique is impractical for analyzing lipid accumulation in real-time, it was useful for visualizing the dramatic degree to which individual cells within the consortium could accumulate lipids.

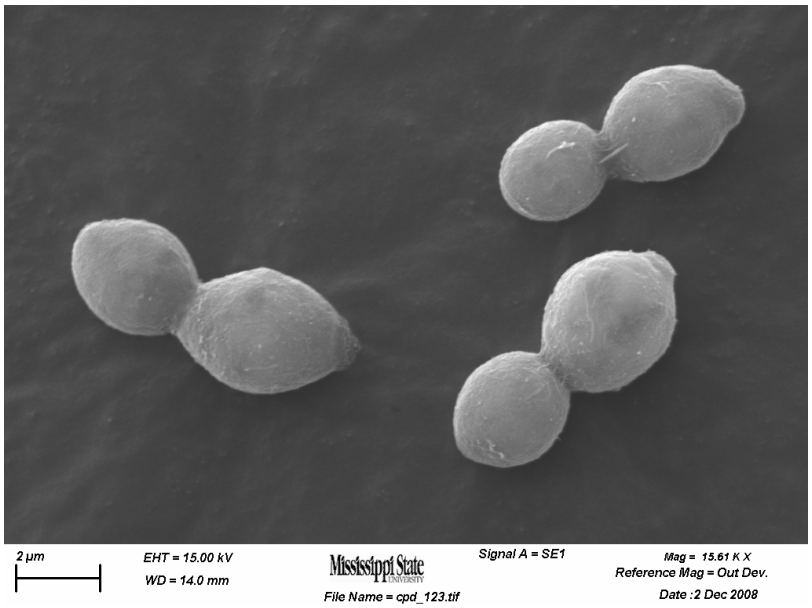

Figure 6 Electron Micrograph of Group of Budding Yeast

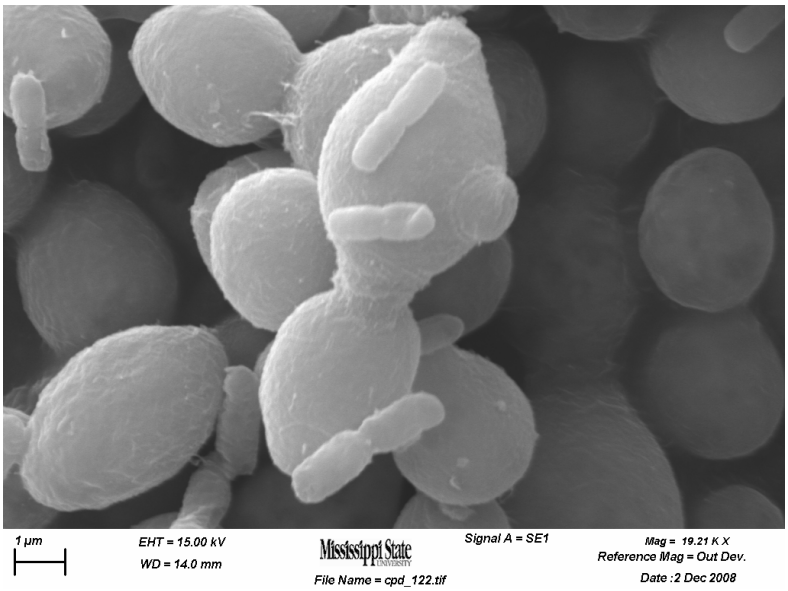

Figure 7 Electron Micrograph Showing a Heterogeneous Consortium Cluster Containing Both Yeast and Bacteria

The use of SEM also provided a definitive, though inefficient, way of ensuring that the consortium was a heterogeneous mixture of different species of oleaginous microorganisms.

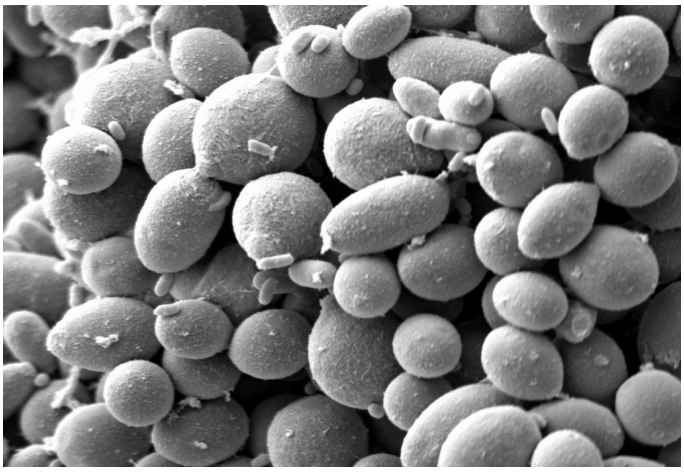

Figure 8 Large Group of Cells From the Oleaginous Consortium

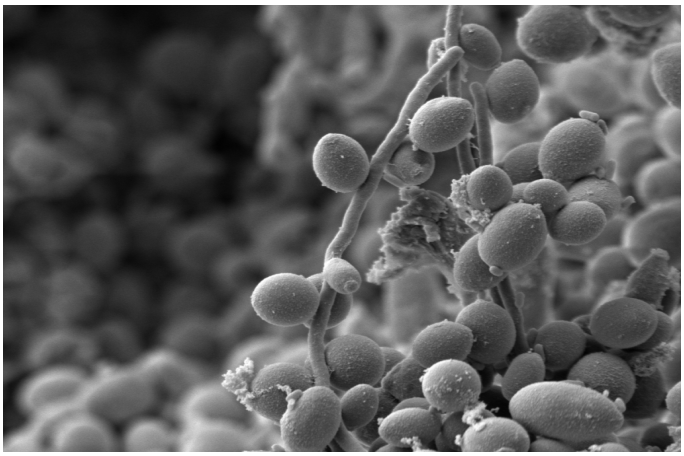

Figure 9 Large Group of Budding Yeasts and Filamentous Yeasts

Figures 6-9 show the degree to which SEM can be used to examine the topography and the heterogeneous characteristics of the oleaginous consortium. Backscatter electron imaging was used to provide contrasted images. Although the use of backscatter imaging diminished the topographical features of the cells, it was useful in showing some structures within the cells. Figure 10 shows a standard image of a cluster of oleaginous cells. Figure 11 shows the same region using backscatter detection. Inclusions within the cells are clearly visible in Figure 11.

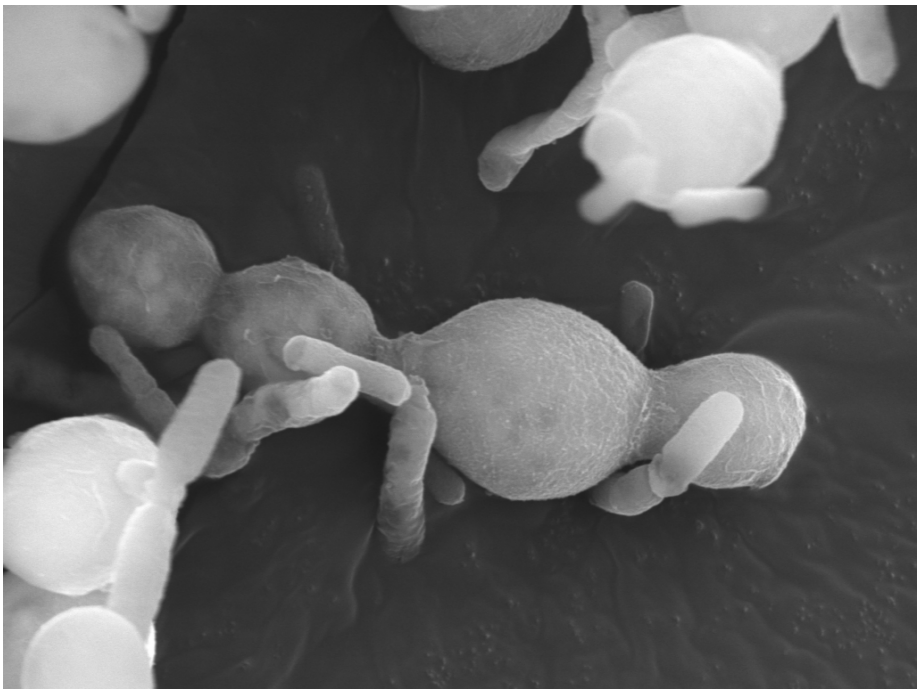

Figure 10 Electron Micrograph of a Cluster of Budding Cells

Figure 11 Use of Back-Scattered Detection to Visualize Inclusions via SEM

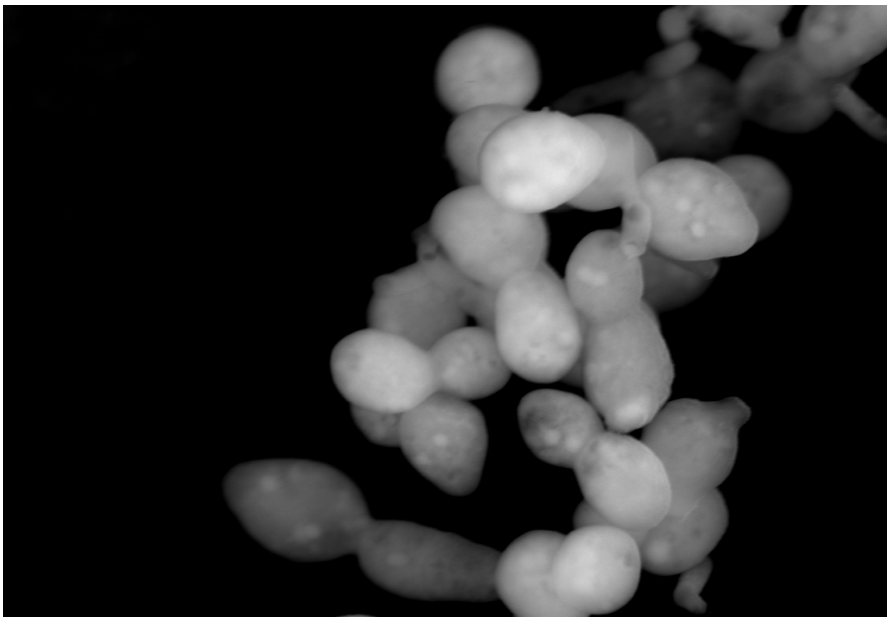

Figure 12 Electron Micrograph of Inclusions Within Cell Cluster

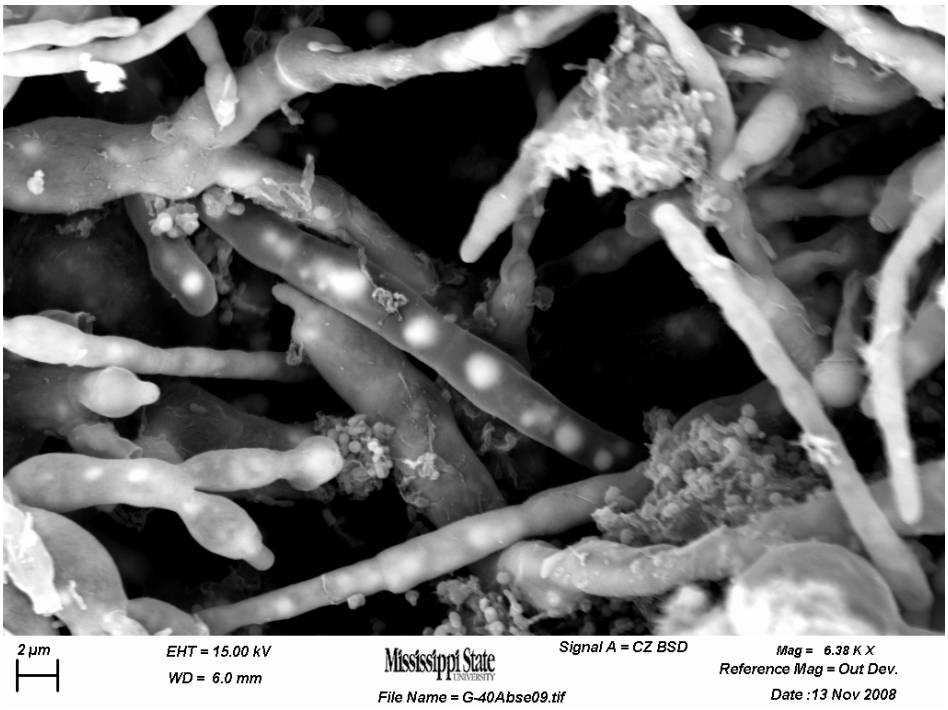

Figure 13 Electron Micrograph Showing Inclusions Within Filamentous Fungi

Figures 12 and 13 show the most conclusive uses of back-scattered detection to visualize intracellular inclusions. In Figure 12, inclusions are observed within a cluster of budding cells. Figure 13 shows similar inclusions within filamentous hyphae.

Shake-Flask One Liter Experiments for Lipid Accumulation

This experiment utilized 300mL of lipid accumulation medium within 1L flasks. Glucose and xylose were examined as carbon sources at concentrations of 40g/L and 60g/L. Buffered conditions were also examined with test groups being maintained at pH of 5 by addition of 0.5 M phosphate buffer.

At intervals of 0h, 48h, 96h, 144h, and 168h Optical Density (405nm), Nile red fluorescence (488nm emission, 560nm excitation), dissolved oxygen, pH, cell mass (per 10mL) were recorded. All test groups were in triplicates.

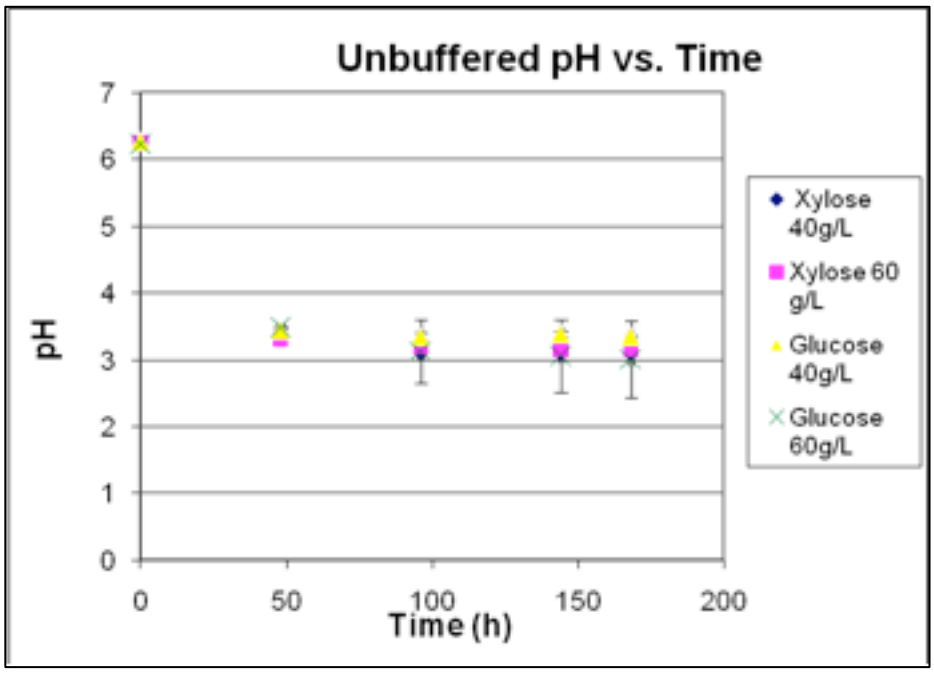

Figure 14 Effects of an Oleaginous Consortium on pH in Flasks Containing Buffered Medium with Different Concentrations of Xylose or Glucose

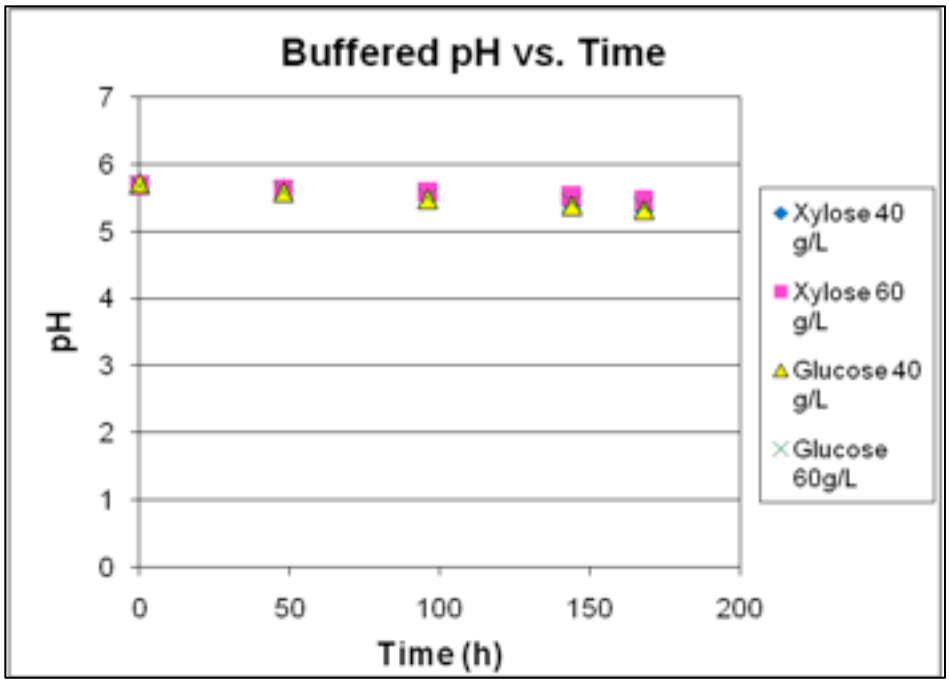

Figure 15 Effects of an Oleaginous Consortium on pH in Flasks Containing Buffered Medium with Different Concentrations of Xylose or Glucose

Figures 14 and 15 show the rapid decline of pH within the flasks and the ability of the 0.5 M phosphate buffer to maintain the pH above 5. In both the buffered and unbuffered conditions the pH was analogous among the different test conditions of sugar/concentration. Figures 16 and 17 suggest that the optical density remained constant in the unbuffered flasks, while optical density steadily increased within the buffered flasks. There is a high degree of variability in the optical density readings of the final two time points within the unbuffered flasks. This variability can be explained by the cell clumping that was observed in both buffer conditions within all test groups. Clumping was more severe within the unbuffered flasks.

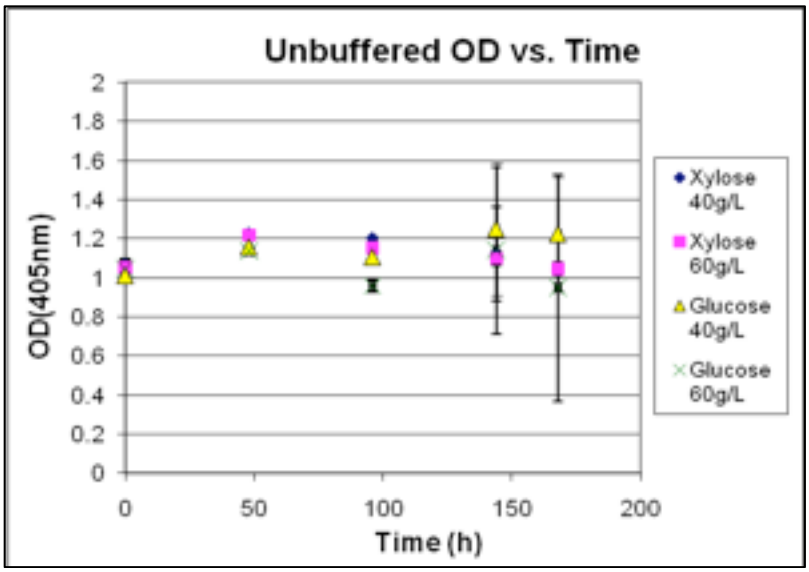

Figure 16 Effects of an Oleaginous Consortium on Optical Density (405-nm) in Flasks Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose

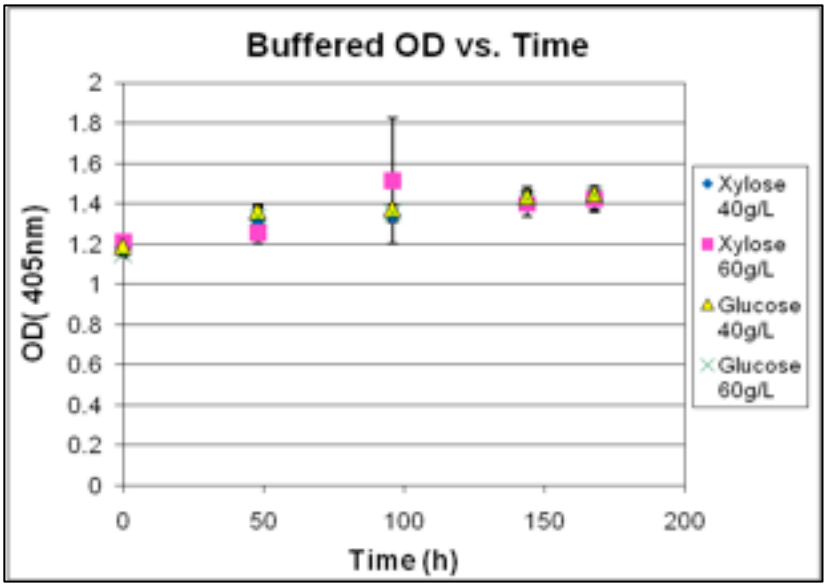

Figure 17 Effects of an Oleaginous Consortium on Optical Density (405-nm) in Flasks Containing Buffered Medium with Different Concentrations of Xylose or Glucose

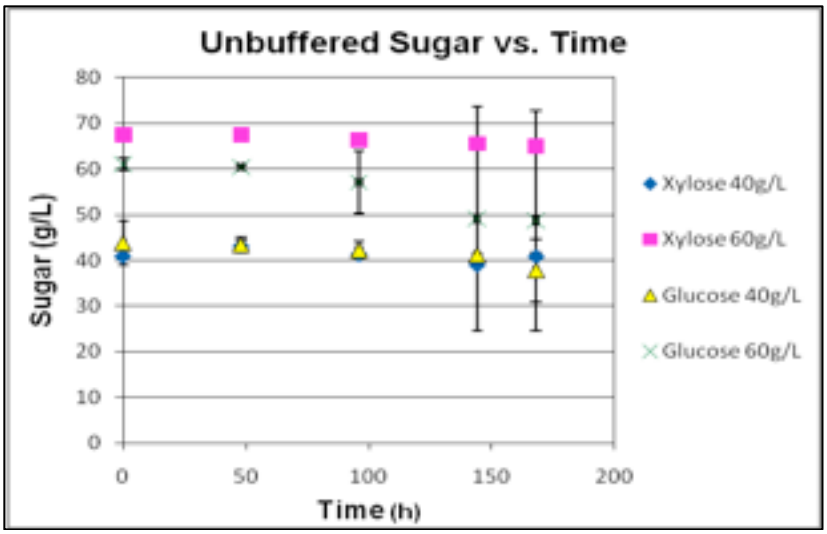

Figure 18 Effects of an Oleaginous Consortium on Sugar Concentrations (g/L) in Flasks Containing Unbuffered Medium with Different concentrations of Xylose or Glucose

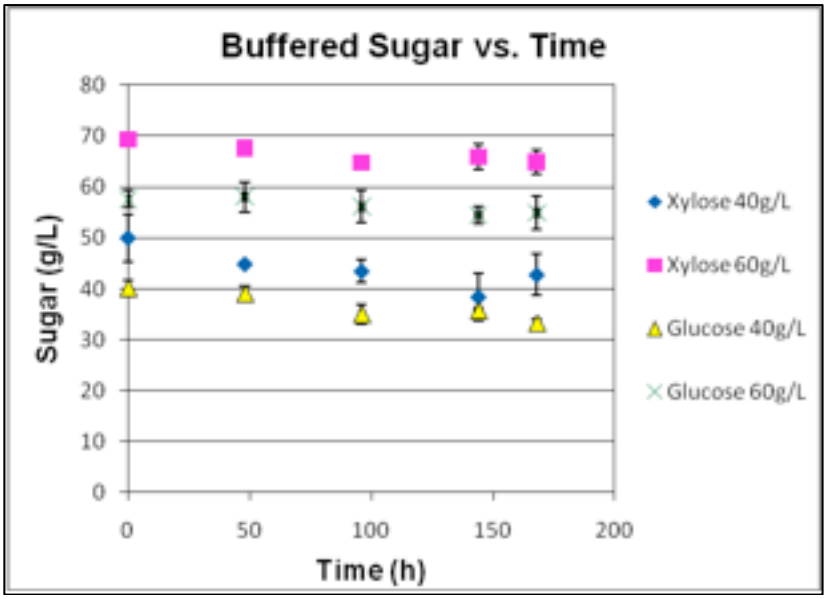

Figure 19 Effects of an Oleaginous Consortium on Sugar Concentrations (g/L) in Flasks Containing Buffered Medium with Different Concentrations of Xylose or Glucose

The concentrations of sugar (g/L) within the flasks are shown in Figures 18 and 19. Sugar concentrations showed a large degree of variability within flasks of the same

test groups. This variability was more severe in the unbuffered flasks. One possible explanation is that the use of a consortium of microorganisms makes it difficult to produce identical conditions within test groupings. While variability was also present in the recording of cell mass (Figures 20 and 21), it is clear that the cells within the buffered flasks continued to grow throughout the length of the experiment and the cell numbers within the unbuffered flasks were constant. These data are reinforced by the optical density readings previously mentioned.

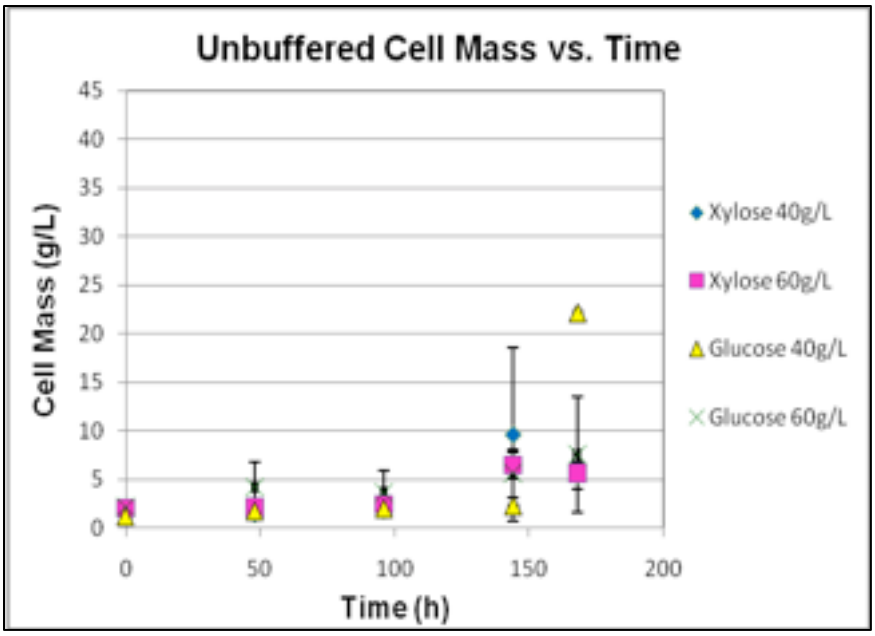

Figure 20 Effects of an Oleaginous Consortium on Cell Mass (g/L) in Flasks Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose

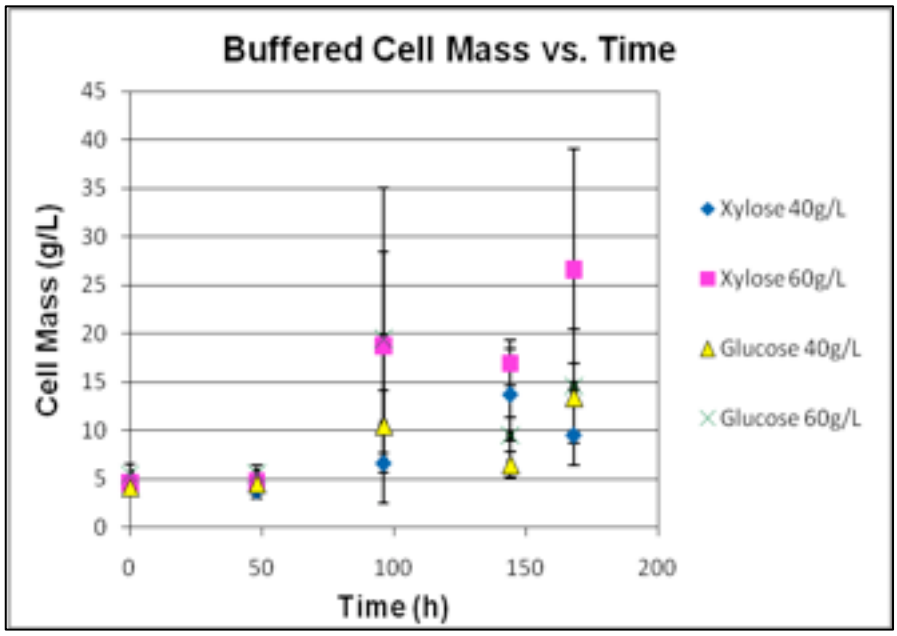

Figure 21 Effects of an Oleaginous Consortium on Cell mass (g/L) in Flasks Containing Buffered Medium with Different Concentrations of Xylose or Glucose

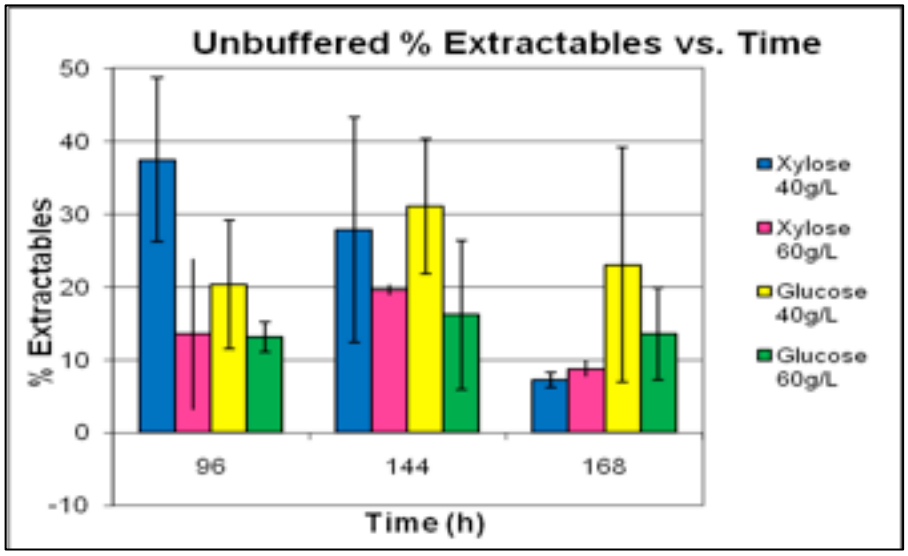

Figure 22 The Observed % Extractables Accumulated Within the Oleaginous Consortium Cultivated in Flasks Containing Unbuffered Medium and Varied Carbon Sources

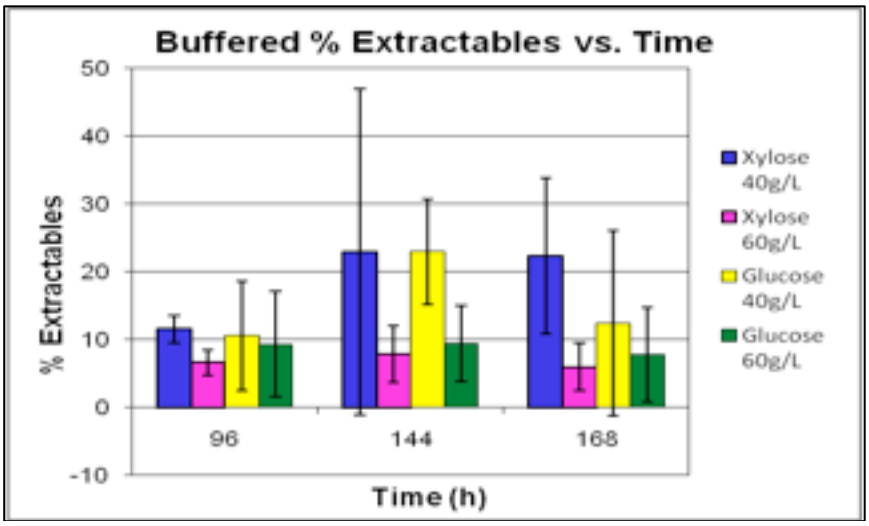

Figure 23 The Observed % Extractables Accumulated Within the Oleaginous Consortium Cultivated in Flasks Containing Buffered Medium and Varied Carbon Sources

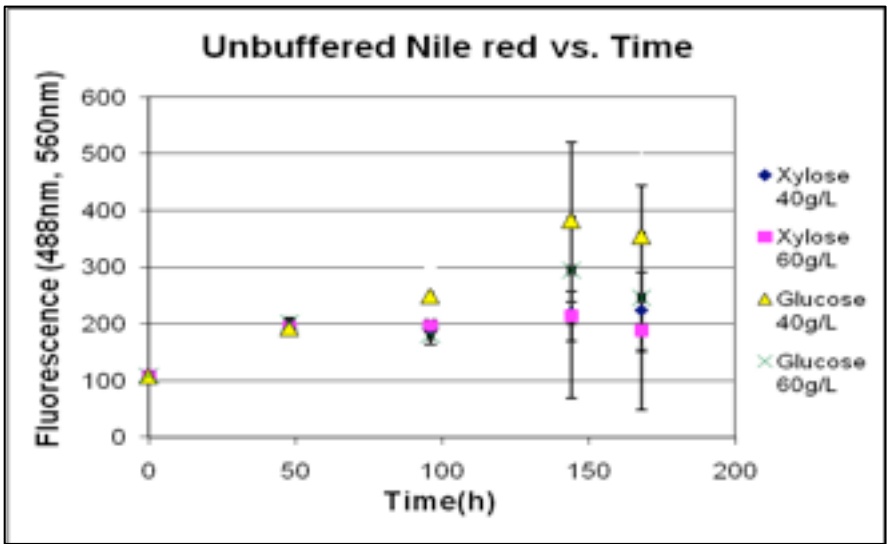

Figure 24 Fluorescence (405nm, 560nm) Measurements of Nile Red Treated Consortium from Unbuffered Medium with Different Concentrations of Xylose or Glucose

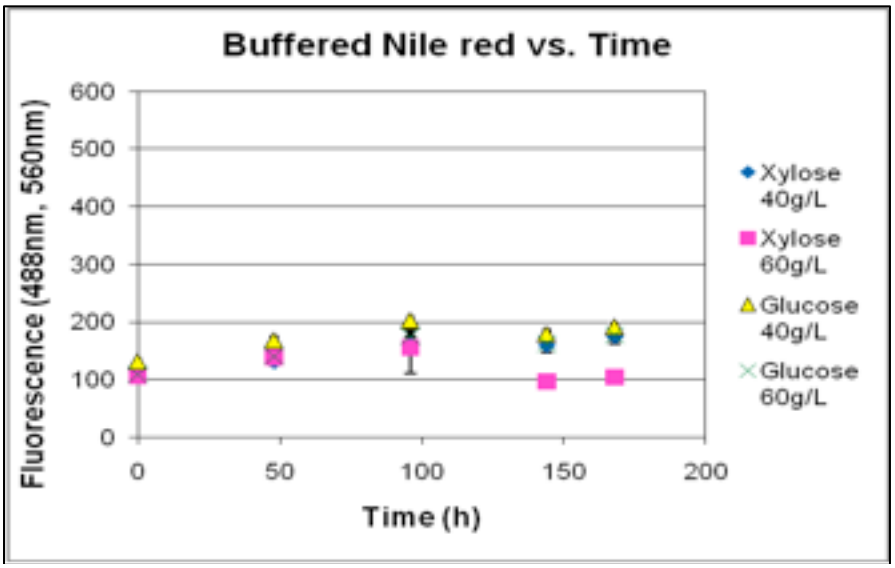

Figure 25 Fluorescence (405nm, 560nm) Measurements of Nile Red Treated Consortium from Buffered Medium with Different Concentrations of Xylose or Glucose

The test groups without pH control appeared to accumulate the largest amount of Bligh/Dyer extractables (Figures 22 and 23) and also the highest Nile red fluorescence. Fluorescence is shown in Figures 24 and 25. The highest fluorescence readings were observed at 144h in the unbuffered flasks which corresponds to the highest actual extractable percentage. The large-scale flask experiments appear to show some correlation with Nile red fluorescence and actual Bligh/Dyer extractables. There was a large degree of variability within test groups of the flask experiment. This variability was most evident in the measurements of optical density, cell mass, and Nile red fluorescence. Many factors could contribute to this degree of deviation. A large amount of cell clumping occurred within the flasks. This made it difficult to obtain uniform samples from each flask. In order to address the variable of cell clumping, experiments were performed utilizing a smaller scale and sacrificial sampling.

Small Scale 100mL Bottle Experiment (Phosphate Buffer)

In order to reduce the variability within the test groups, a small scale experiment utilizing sacrificial sampling was performed. For this experiment 150 Wheaton bottles were prepared using the previously described nitrogen-limited medium and techniques. Each of the ten test groups were examined in triplicate. There were also designated time points appointed to each bottle. Controls containing no sugar were also added to the experiment but were only tested for three time points (0h, 96h, 192h). At the end of each time point, five total, the entire contents of the bottle was sampled and examined. The pH controlled bottles were maintained at ph 5.0 using a 0.5 M phosphate buffer.

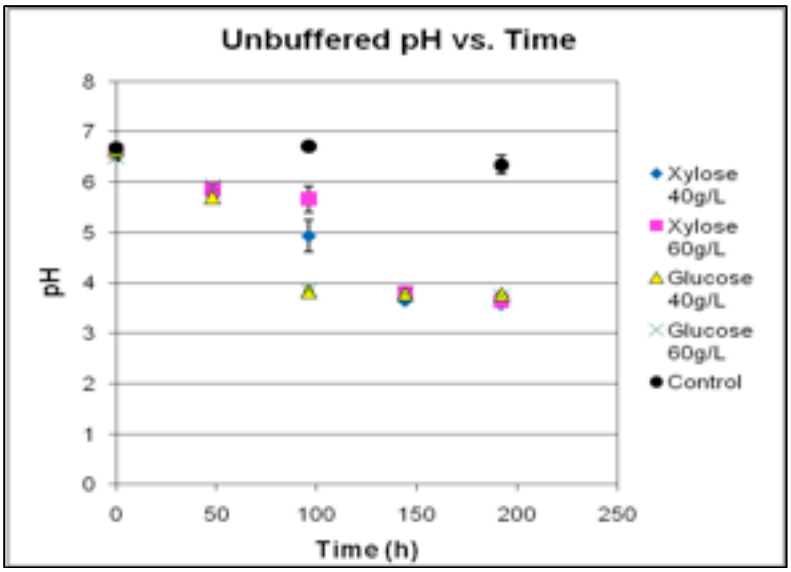

Figure 26 Effects of an Oleaginous Consortium on pH in 100mL Bottles Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose

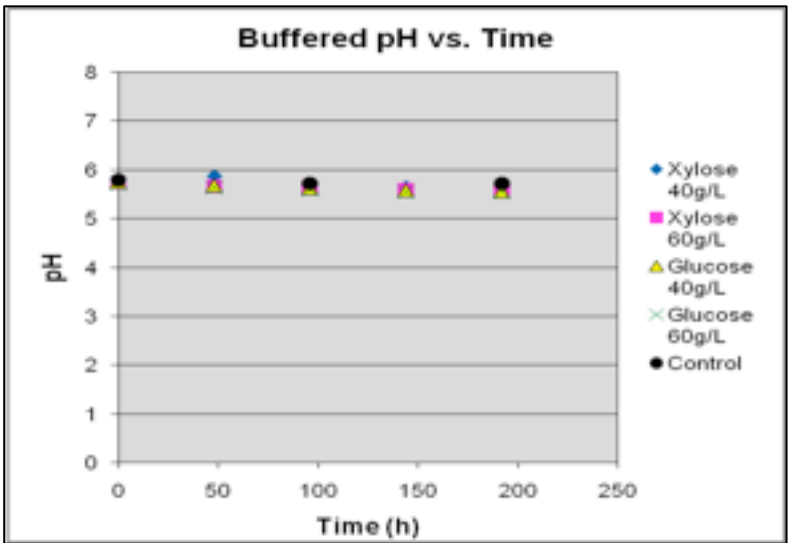

Figure 27 Effects of an Oleaginous Consortium on pH in 100mL Bottles Containing Buffered Medium with Different Concentrations of Xylose or Glucose

Figures 26 and 27 detail the observations of pH for the 100mL bottle experiments in buffered and unbuffered nitrogen-limited media, respectively. As seen in Figure 26, the pH dropped swiftly in all test groups while remaining at pH 7 in the control samples. The pH dropped faster in the samples containing glucose, although at 144h all samples were below pH 4. Figure 27 shows that the 0.5M phosphate buffer was effective in maintaining the pH at slightly below 6 throughout the length of the experiment and in all test groups.

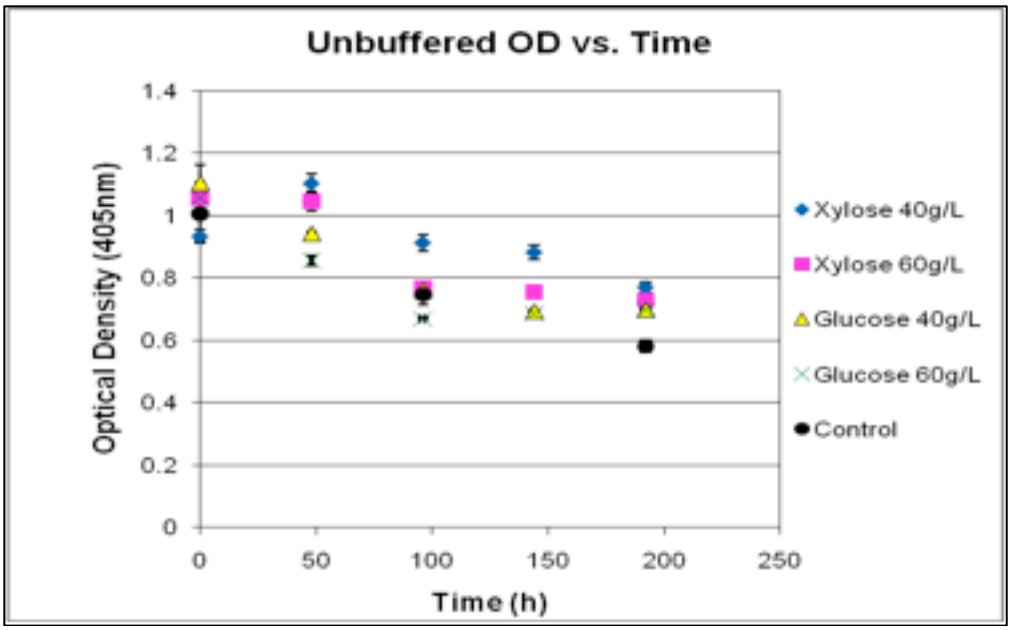

Figure 28 Optical Density (405nm) Versus Time in 100mL Bottles Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose and Oleaginous Consortium

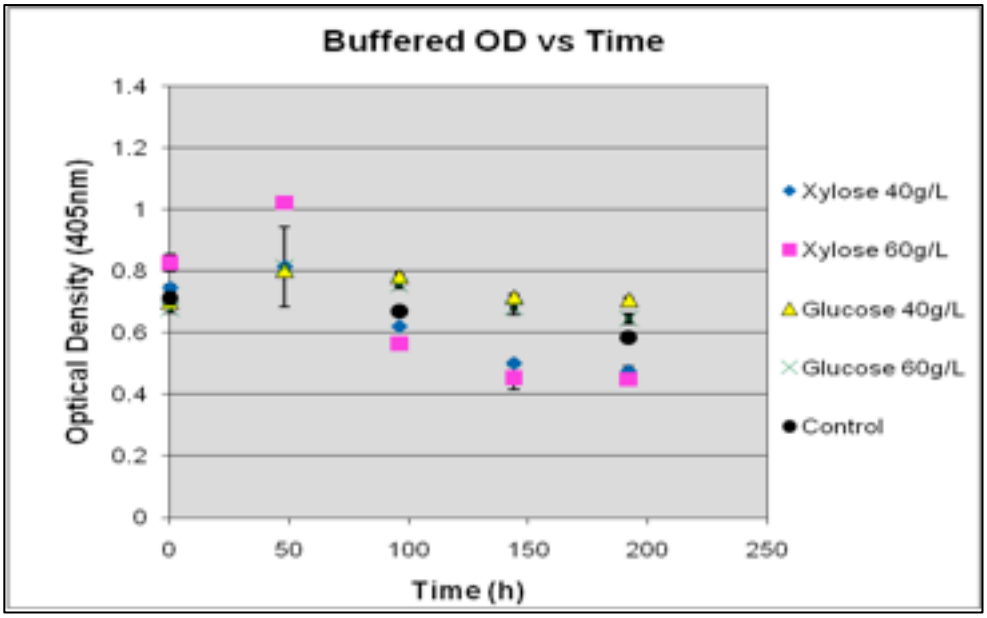

Figure 29 Optical Density (405nm) Versus Time in 100mL Bottles Containing Buffered Medium with Different Concentrations of Xylose or Glucose and Oleaginous Consortium

The optical densities in both the buffered and unbuffered 100mL bottles remained fairly constant showing some decline in the final time points of the experiment. Interestingly, the bottles containing both concentrations of glucose showed lower final optical densities than those containing xylose when no buffer was added (Figure 28). This trend was reversed in the buffered bottles, with both concentrations of xylose producing lower optical densities than the glucose test groups (Figure 29). Under all conditions no growth appeared to be occurring.

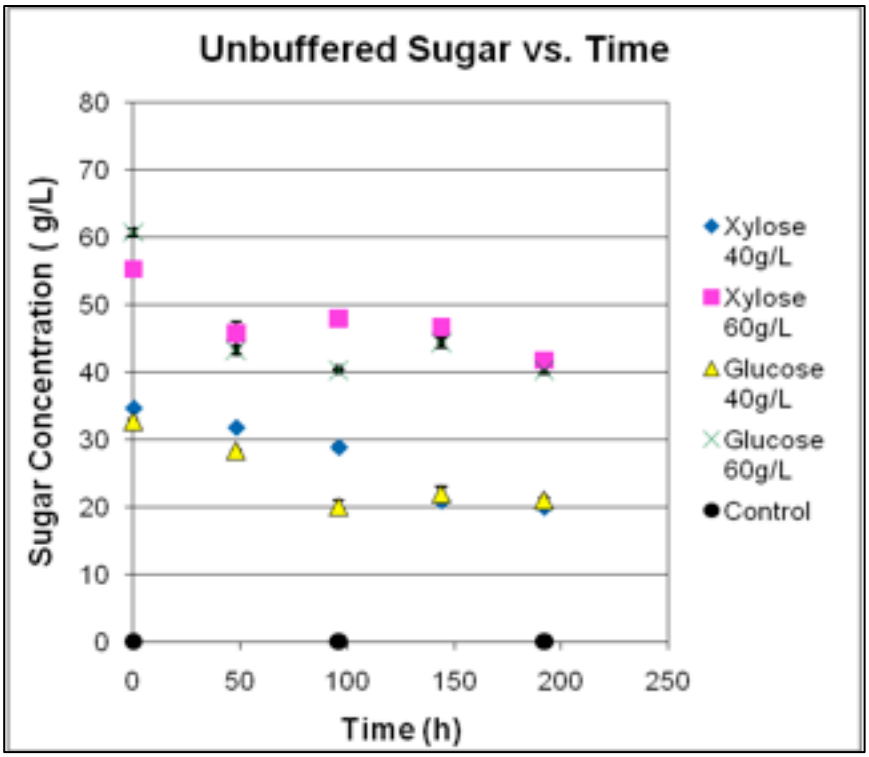

Figure 30 Effects of an Oleaginous Consortium on Sugar Concentrations (g/L) in 100mL Bottles Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose

The concentrations of sugar decreased at a steady rate in both concentrations of xylose and glucose under unbuffered conditions (Figure 30). The sugar decreases were similar among the different sugars at both concentrations with final sugar concentrations

around 40g/L and 20g/L. The sugar concentrations in the buffered 100mL bottles were more variable between the different test groups. As observed in Figure 31, glucose concentrations decreased more rapidly and to lower final concentrations than the comparable samples containing xylose.

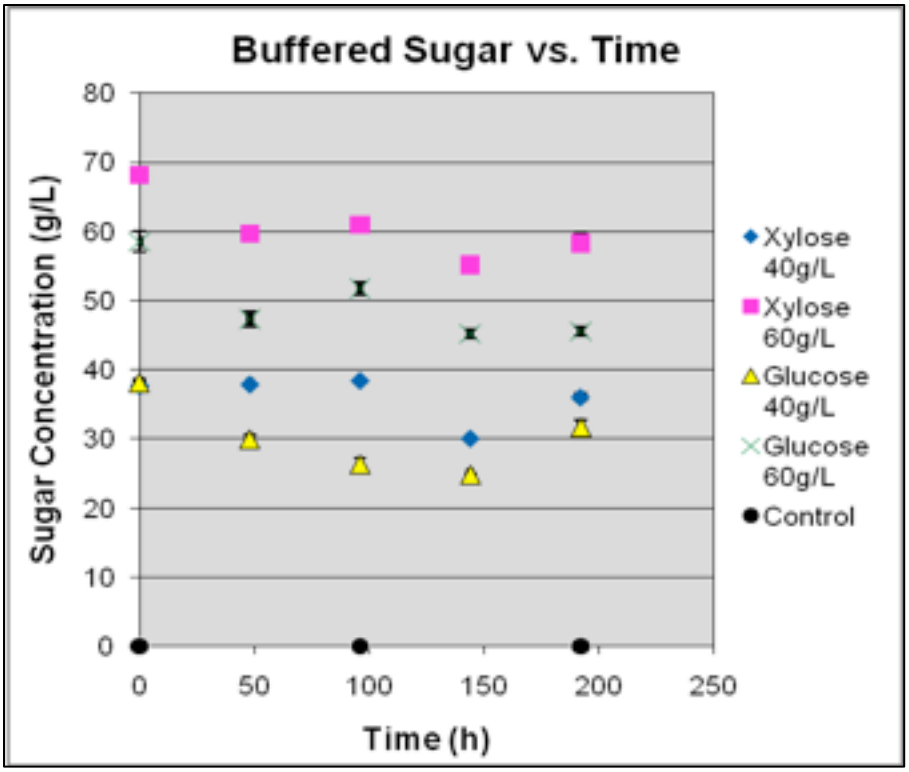

Figure 31 Effects of an Oleaginous Consortium on Sugar Concentration (g/L) in 100mL Bottles Containing Buffered Medium with Different Concentrations of Xylose or Glucose

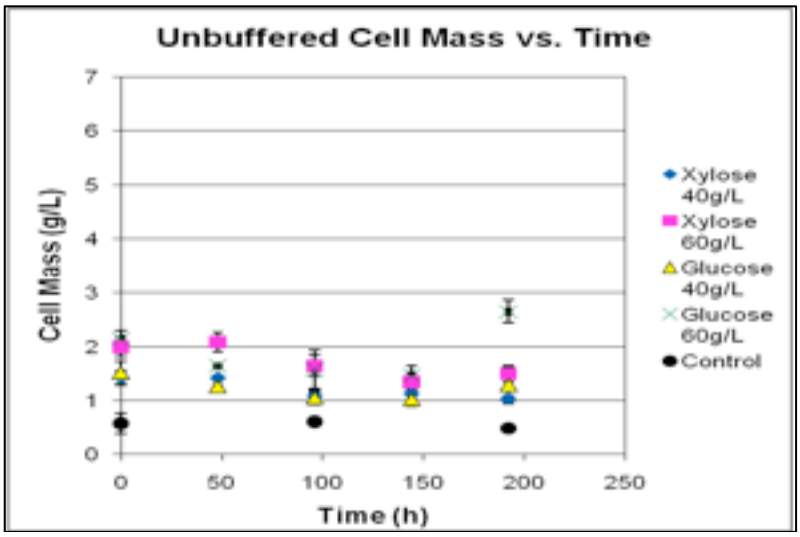

Figure 32 Effects of an Oleaginous Consortium on Cell Mass (g/L) in 100mL Bottles Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose.

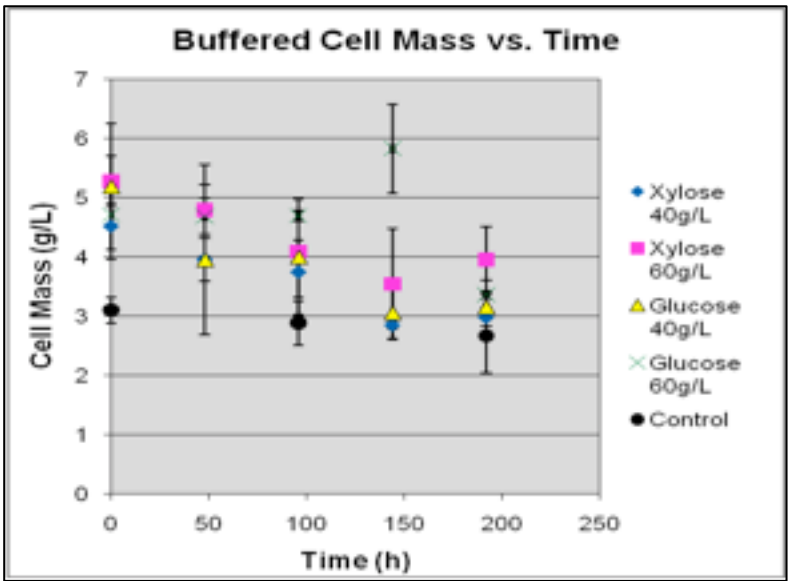

Figure 33 Effects of an Oleaginous Consortium on Cell Mass (g/L) in 100mL Bottles Containing Buffered Medium with Different Concentrations of Xylose or Glucose.

The use of sacrificial sampling and the 100mL bottles appeared to reduce the previously observed problems of cell clumping. In Figure 32 variability is slight between

the different test groups under unbuffered conditions. However, Figure 33b shows a drastic degree of variability within buffered test groups. Also observable is a higher overall initial cell mass as compared to the unbuffered samples. One explanation for this is that the amount of buffer required caused the phosphate to come out of solution and dramatically effect the cell mass readings. This problem was widespread in all samples, including the controls.

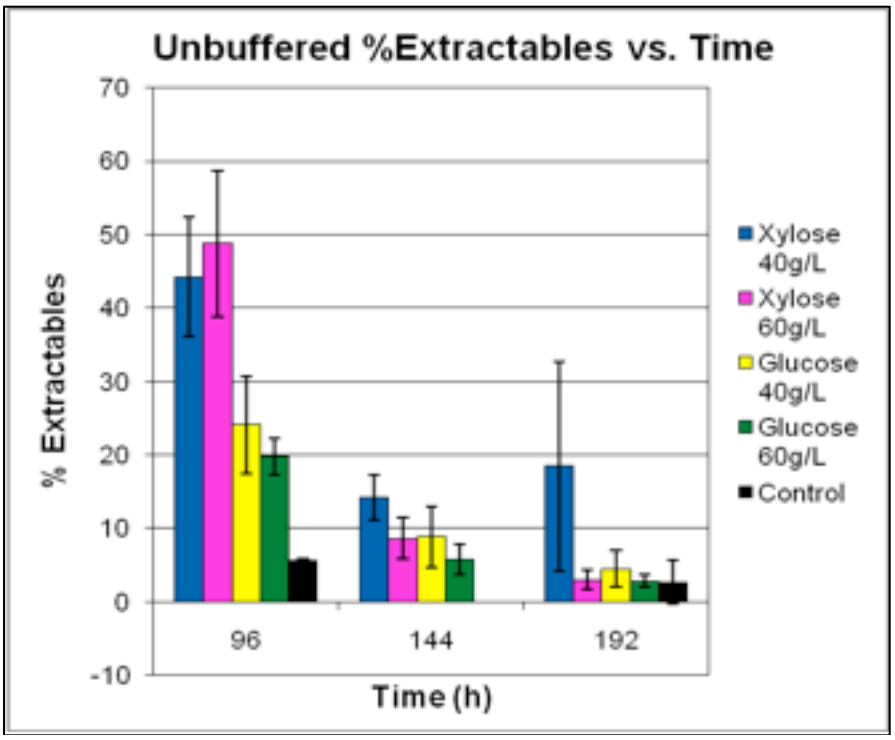

Figure 34 The Observed % Extractables Accumulated within the Oleaginous Consortium Cultivated in 100mL Bottles Containing Unbuffered Medium and Varied Carbon Sources.

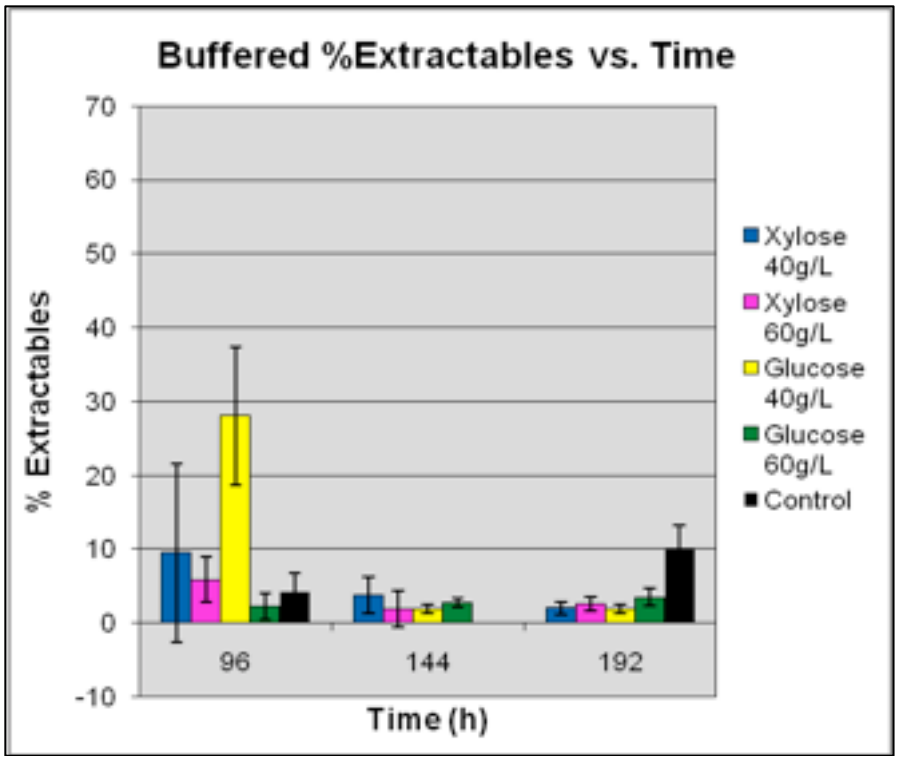

Figure 35 The Observed % Extractables Accumulated within the Oleaginous Consortium Cultivated in 100mL Bottles Containing Buffered Medium and Varied Carbon Sources

The extractables percentages were similar in the 100mL bottle experiment to the data from the flask experiments. The unbuffered samples had higher percentages of extractables than the buffered samples across all test groups (Figures 34 and 35). The highest percentages were achieved during the 96h time point in the unbuffered samples. Again, the samples with xylose achieved the highest extractables percentages.

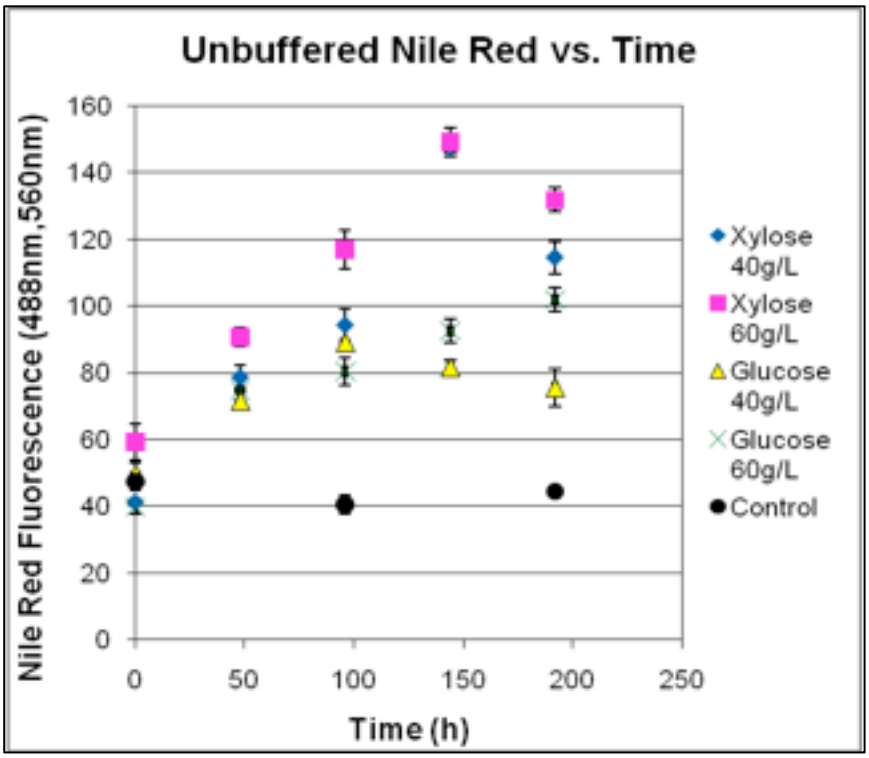

Figure 36 Effects of an Oleaginous Consortium on Fluorescence (488nm, 560nm) in 100mL Bottles Containing Unbuffered Medium with Different Concentrations of Xylose or Glucose

As shown in Figures 36 and 37, Nile red fluorescence variability decreased greatly in the 100mL bottle experiments as compared to the flask experiments. This could be a result of the usage of sacrificial sampling. Overall fluorescence readings were higher in the unbuffered samples. Again, the samples containing xylose produced the highest fluorescence readings.

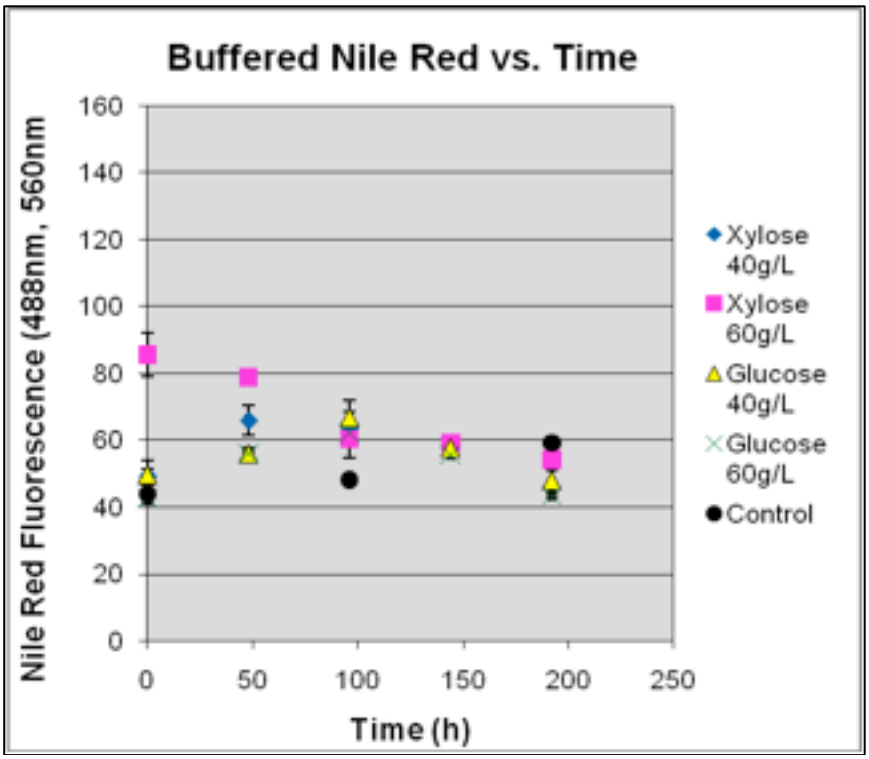

Figure 37 Effects of an Oleaginous Consortium on Fluorescence (488nm, 560nm) in 100mL Bottles Containing Buffered Medium with Different Concentrations of Xylose or Glucose

While some of the problems of variability within the large scale experiments seemed to be effectively resolved using the smaller scale, new difficulties were observed. The 0.5M phosphate buffer was effective in maintaining the pH at five, but the amount of phosphate required for each medium was high enough that it appeared to come out of solution and dramatically affect the values of cell mass. At such a high concentration the phosphate could have also significantly affected the physiological conditions within the oleaginous cells. Such high concentrations of phosphate added an unwanted variable to an already complex experiment. For this reason, a new buffer was needed. Despite these problems, the use of sacrificial sampling seemed to reduce overall variability in the other measured values. This experiment showed that the unbuffered conditions were optimal for lipid accumulation via glucose or xylose. The unbuffered cells with xylose as a

carbon source were able to accumulate the highest percentage of extractables at over forty-five percent for both concentrations. The larger concentrations of carbon source (60g/L) did not seem to be effective in increasing yields of extractables. The small scale experiment reduced the variability observed with Nile red fluorescence. Although the highest measured Nile red fluorescence measurements did not correspond with the highest actual extractable yields in every circumstance, the sample groups with the highest actual extractable yields also had the highest fluorescence. Furthermore the control groups, which yielded the least percentage of extractables, also gave the lowest fluorescence readings.

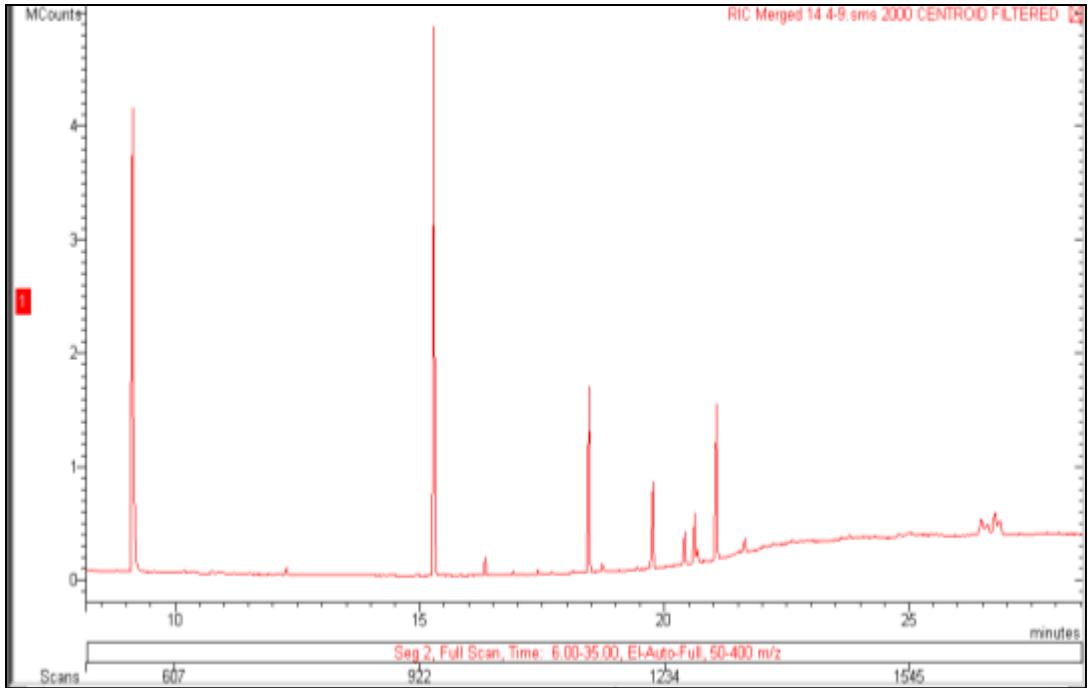

Figure 38 Small Scale (Phosphate buffer) Fatty-Acid Methyl Ester Analysis

1. Dichlorobenzene (int. std.) 2. BHT (stabilizer) 3. Methyl Ester Pentadecanoic Ester ($C_{16}H_{32}O_2$)
4. 2-hexyl-, methyl ester Cyclopropaneoctanoic acid ($C_{18}H_{34}O_2$) 5. Methyl Ester Ocatadecanoic
Acid ($C_{19}H_{38}O_2$) 6. (Z)- Methyl ester 9-octadecenoic acid ($C_{19}H_{36}O_2$)
7. Methyl ester 8,11-octadecadienoic acid ($C_{19}H_{34}O_2$)

Analysis of fatty-acid methyl esters via gas chromatography-mass spectrometry is shown in Figure 38. The largest peaks corresponding to FAMES occurred at 18 to 22 minutes and corresponded to sixteen-carbon, eighteen-carbon, and nineteen-carbon compounds. The highest FAME peak corresponded to a sixteen-carbon compound identified as methyl ester pentadecanoic ester ($C_{16}H_{32}O_2$).

Small Scale 100mL Bottle Experiment (Citrate-Phosphate Buffer)

To address the problems associated with the use of phosphate as a buffer solution, the small scale experiment was repeated using a citrate-phosphate buffer. Additionally,

based on results of previous experiments, only the sugar concentration of 40g/L was tested. All other experimental conditions were conserved.

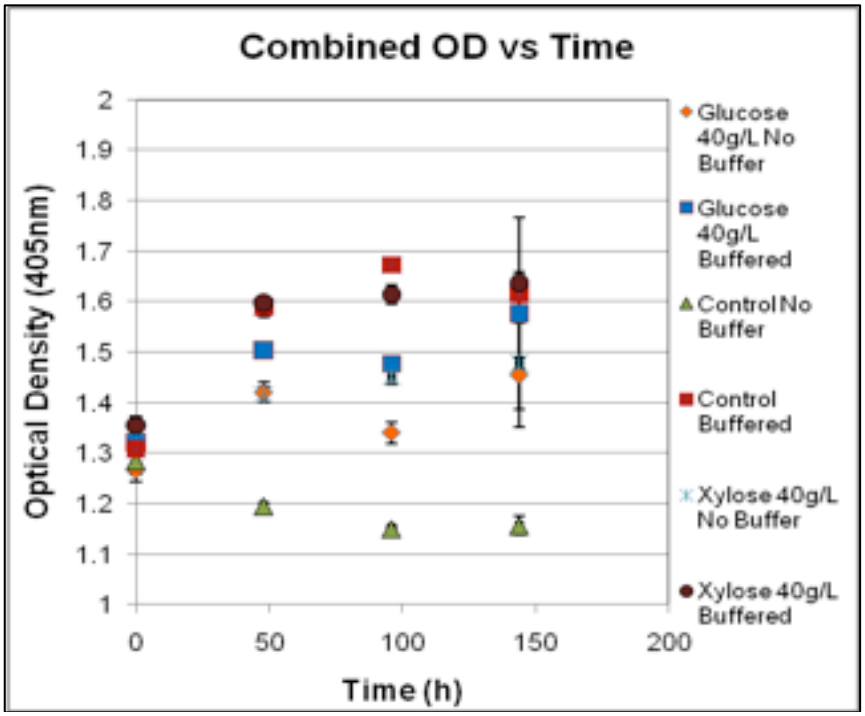

Figure 39 Effects of the Oleaginous Consortium on Optical Density 405nm) in 100mL Bottles Containing Either Citrate-Phosphate Buffer or No Buffer with Lone Sugars or No Added Sugar

Optical density values showed some increase in this experiment as compared to previous flask and bottle experiments (Figure 39). In previous experiments the optical densities declined steadily throughout the experiment so these results were unexpected. The oleaginous consortium was maintained in nitrogen-limited medium in all experiments to prevent the use of the carbon sources for growth and drive the mechanism of lipid accumulation. However, increases in optical density were not sufficient to cause concern about the overall validity of this experiment.

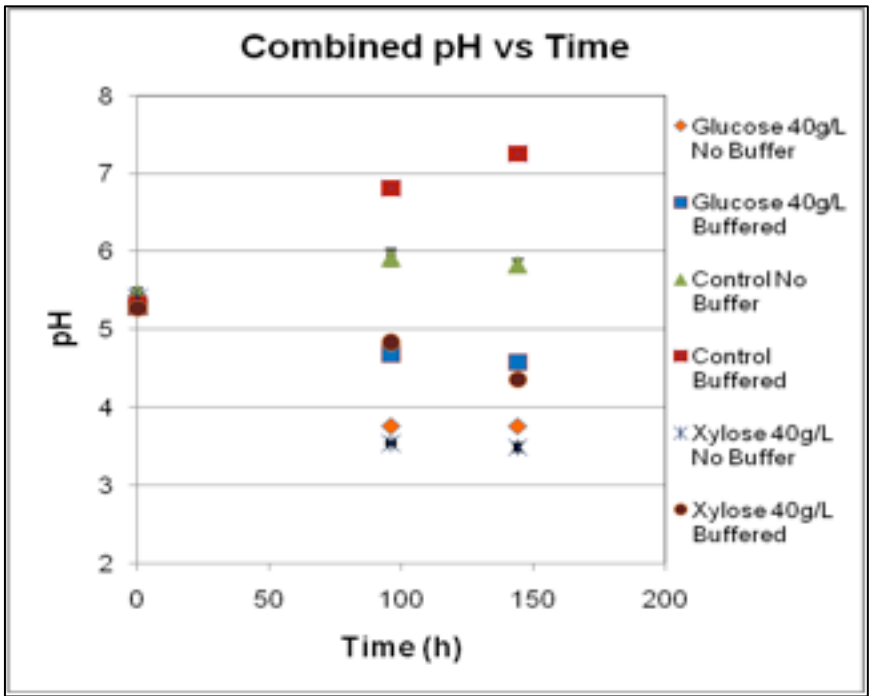

Figure 40 Effects of the Oleaginous Consortium on pH in 100mL Bottles Containing Either Citrate-Phosphate Buffer or No Buffer with Lone Sugars or No Added Sugar

In Figure 40, the data show that the citrate-phosphate buffer was useful in maintaining the buffered medium above pH 4. The unbuffered samples quickly became acidic and reached final pH levels below 4. The citrate-phosphate buffer did not achieve the buffering results of the previous 0.5 M phosphate buffer used (Figure 27). Although the buffered samples did show a decrease in pH, the decrease was less than observed in the unbuffered samples.

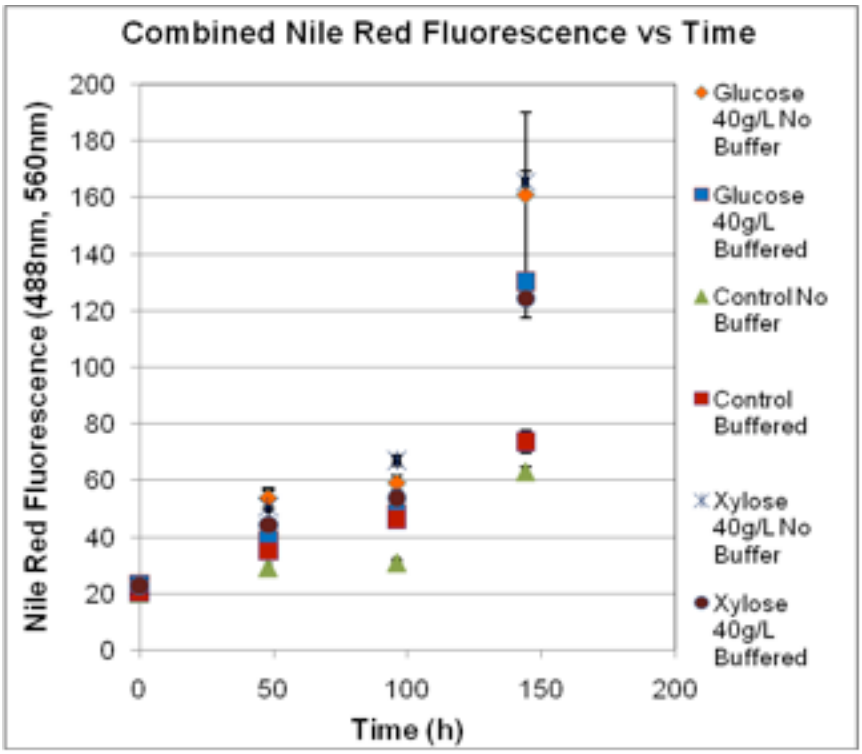

Figure 41 Effects of the Oleaginous Consortium on Fluorescence (488nm, 560nm) in 100mL Bottles Containing Either Citrate-Phosphate Buffer or No Buffer with Lone Sugars or No Added Sugar

In this experiment the fluorescence values increased progressively. The highest fluorescence readings were achieved at the 144h time point (Figure 41). Again, the highest fluorescence readings occurred within the unbuffered samples. At 144h, the unbuffered glucose and xylose test groups showed no statistical difference in fluorescence.

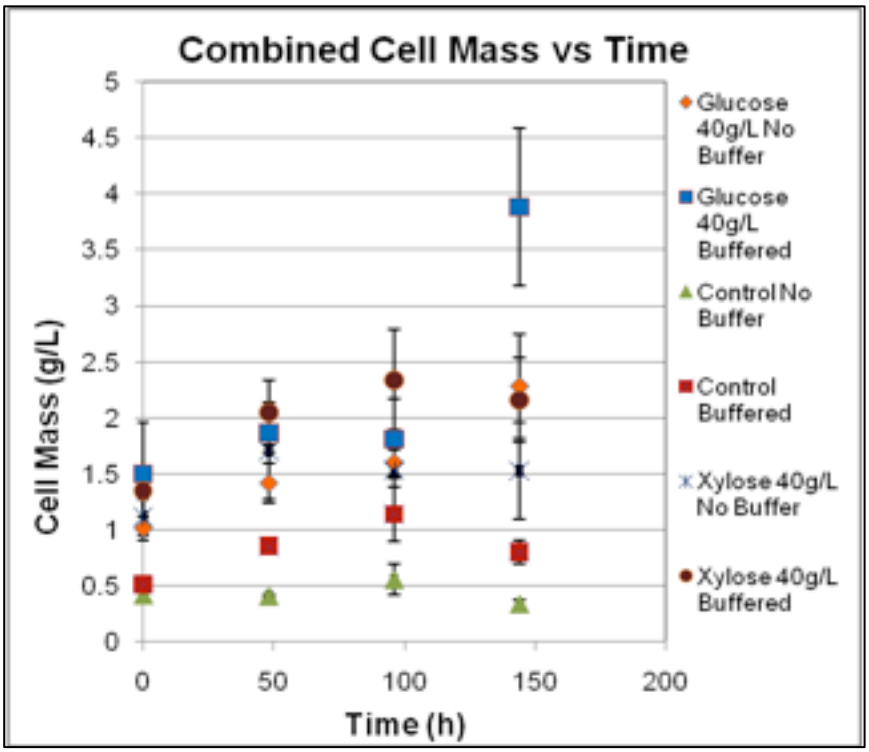

Figure 42 Effects of the Oleaginous Consortium on Cell Mass (g/L) in 100mL Bottles Containing Either Citrate-Phosphate Buffer or No Buffer with Lone Sugars or No Added Sugar

As observed in the optical density readings (Figure 39), there was some growth among cultures within this experiment. The cell mass observations (Figure 42) are variable but reinforce the optical density data. Some growth appeared to occur in the buffered samples. This suggests that the consortium was utilizing the citrate within the buffer as a carbon source.

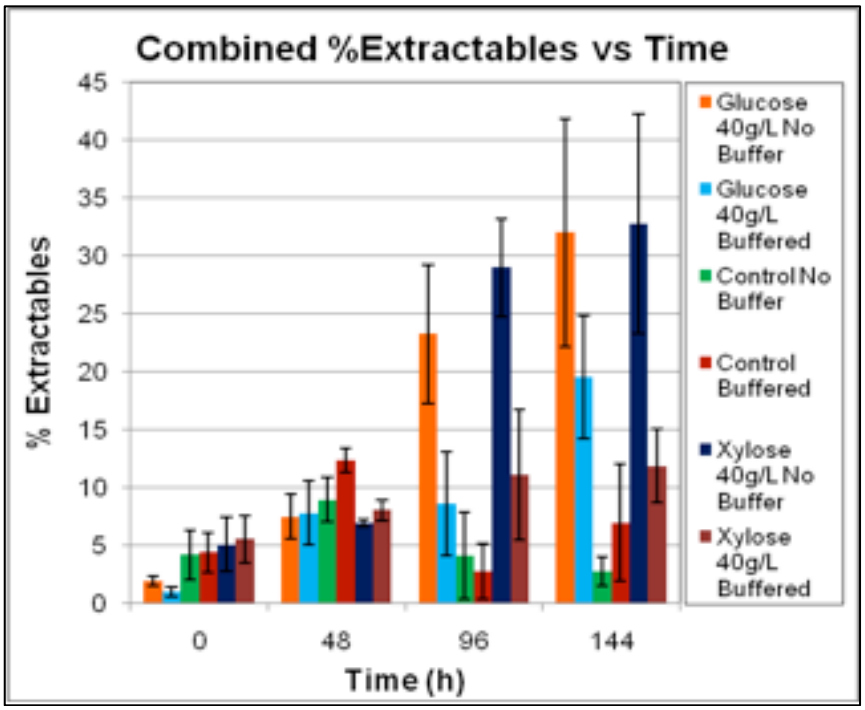

Figure 43 Quantitation of % Extractables Accumulated within the Oleaginous Consortium when Cultivated in 100mL Bottles with Varied Citrate-Phosphate Buffer and Sugar Conditions

The citrate-phosphate buffer appeared to remain in solution, and thus, did not distort the cell mass quantitations. Overall, the experiment with the citrate-phosphate buffer reinforced the observations from other experiments. Highest extractables percentages came from xylose at 40g/L (Figure 43). Also, the non-buffered samples accumulated the highest percentage of extractables with both xylose and glucose. The samples containing buffer achieved higher extractables percentages than previously observed in the experiment using the 0.5M phosphate buffer. This could be explained by the consortium utilizing citrate. Also, the previously stated difference in the buffering capacity of the citrate-phosphate buffer could have an effect. The bottles that contained the citrate-phosphate buffer reached a lower pH than the buffered samples from the 0.5M phosphate experiment, and thus were able to achieve higher yields of extractables.

Again, Nile red appears to be a good indicator of lipid accumulation within cells. In this experiment, Nile red fluorescence correlated well with actual extractables percentages.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

In the worldwide push for discovery of new methods of energy production many solutions will most likely be combined in order to decrease demand on fossil fuels. This study has shown the potential for an oleaginous consortium of microorganisms to accumulate high percentages, over forty percent in some cases, of extractables with fatty-acid methyl ester properties. The study has also evaluated the use of new techniques to detect lipid accumulation within cells.

Both Nile red fluorescence and Scanning Electron Microscopy were proven to be useful methods of envisaging actual lipid inclusions within cells. Nile red fluorescence also shows potential as an indicator of lipid accumulation within cells of an actively cultivated consortium. This potential could prove useful for industrial scale lipid production using wastewater treatment facilities and oleaginous microorganisms. The solvents used for lipid extraction are expensive and the methods are protractive. The use of Nile red fluorescence as a detector of lipid accumulation within batches could reduce cost and also allow for real-time indications of intracellular lipids. Although the data for Nile red fluorescence is variable in terms of showing close percentages of lipid accumulation, the method delineates presence versus absence with great accuracy.

This study has also elucidated the conditions of optimal extractables accumulation. It is apparent that xylose is preferable to glucose when the goal is lipid accumulation. A concentration of 40 g/L was also more optimal than the higher 60 g/L

ratio of carbon source. Furthermore, it appears that a lack of pH control induces optimal conditions for lipid accumulation.

Should ongoing experiments be initiated using a consortium of oleaginous microorganisms, the author suggests increasing the scale. Although large scale flask experiments showed a high degree of variability, the author feels that by utilizing bioreactors variability can be decreased significantly. Experiments should be conducted in reactors to better monitor culture conditions and examine the potential industrial scale use. Finally, the author would recommend examining new techniques of extracting intracellular lipids due to the cost and time constraints of present methods.

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