HETEROTROPHIC BIO-OIL PRODUCTION FROM MICROALGAE

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Emre ÇAĞLAR

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ABSTRACT

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The purpose of the thesis is to investigate the parameters affecting heterotrophic production of microalga, *Chlorella minutissima*. The aim is to use crude glycerol, a waste product derived from biodiesel production, as a carbon and energy source for microalgal growth and examine the optimum growth conditions in chemostat mode, as well as the productivity of oil using FTIR based technique. The highest lipid productivity achieved was 1.04 $gl^{-1}h^{-1}$, at the temperature 25^0C, with the dilution rate of 0.25 h^{-1} and using a substrate concentration of 80 gl^{-1} in feeding medium. The lipid, protein and carbohydrate content at this conditions was 14.36%, 47.89% and 8.06%, respectively.

ÖZET

SU YOSUNUNDAN HETEROTROFİK YÖNTEMLE BİYO-YAĞ ÜRETİMİ

Bu çalışmada, *Chlorella minutissima* mikroalginin karbon kaynağı ile heterotrof yöntemle çoğaltılması ve bu süreci etkileyen parametlerin araştırılması amaçlanmıştır. Biyodizel üretiminden atık olarak çıkan ham gliserolün mikroalg çoğaltılmasında enerji ve karbon kaynağı olarak kullanılması, kemostat üretim şeklinde optimum çoğalma koşullarının ve yağ üretim hızlarının FT-IR temelli bir yöntemle araştırılması hedeflenmiştir. Çalışmada en yüksek yağ üretim hızı, 25^{o}C sıcaklıkta, 0.25 h^{-1} seyreltme hızında ve 80 gl^{-1} ham gliserol besleme derişiminde, 1.04 $gl^{-1}h^{-1}$ olarak elde edilmiştir. Bu koşullarda, mikroalgin içeriği, %14.36 yağ, %47.89 protein ve %8.06 karbonhidrat olarak belirlenmiştir.

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CHAPTER 1

INTRODUCTION

One of the most important problems that mankind has been facing is the issue of global warming. Therefore, the efforts for finding alternative energy and fuel sources is greatly increased. One of the successfully applied alternative is called as “biodiesel fuel” which is carbon neutral fuel source since it is produced from oil coming from agricultural crops.

It is known that up to 20% of processed oil in biodiesel production ends up with a waste product; crude glycerol. It is a low value waste product because it contains only 40 – 60% glycerol, and the rest are water, methanol, salts and other impurities. It is an expensive process to purify glycerol to convenient percentages. Therefore, crude glycerol from biodiesel production is a waste product that intensive research has been done for finding alternative ways to process it.

Literature states that bio-fuel production from microalgae could be a promising alternative. Microalgae are single cell plants and some strains are able to accumulate significant amounts of lipid. The limiting factors for microalgae growth rate are the light and nutrient concentration. These limitations prevents this idea from being feasible. It is also known that some strains of microalgae can deplete organic carbon for its nutrient and energy requirements.

Qingyu Wu et al. (2010) applied heterotrophic growth of *Chlorella protothecoides* and it contained lipid content of 55.2%. To increase the biomass and reduce the cost of alga, corn powder hydrolysate instead of glucose was used as organic carbon source in heterotrophic culture medium in fermenters. The result showed that cell density significantly increased under the heterotrophic condition, and the highest cell concentration reached 15.5 g/l. In 5 l fermenter, the cell growth reached 15.5 g/l after 184 h culture, and then microalgal oil was efficiently extracted from the heterotrophic cells. Biodiesel which was obtained from heterotrophic microalgal oil by acidic transesterification was characterized by a high heating value of 41 MJ/kg, a density of 0.864 kg/L, and a viscosity of 5.2×10−4 Pa s (at 40^0C). The results suggest that the new process was a low-cost, feasible, and effective method for the production of

high quality biodiesel from microalgae.

Heterotrophic microalgae can utilize carbon sources such as glucose, ethanol, glycerol, and fructose depending on the microalgal species used. In order to lower the production cost of microalgal oils as biodiesel, cheaper carbon sources should be considered.

Heterotrophic oil production from microalgae greatly exceeds that of vegetable oil crops. However, commercial application of biodiesel production from *C. protothecoides* is restricted due to the high cost, which mostly exists in the fermentation substrate. According to a previous estimate, the cost of glucose accounted for 80% of the total medium cost (Wu et al., 2007).

In order to lower the production cost of microalgal oils as biodiesel, cheaper carbon sources should be considered. For example, Qingyu Wu et al. (2007), investigated sweet sorghum which contains sugars as substrate for fermentation.

Crude glycerol is also used for microalgal fermentation to produce valuable products such as docosahexaenoic acid (DHA, 22:6 n-3) using microalga *Schizochytrium limacinum* and highest DHA yield of 4.91 g/l with 22.1 g/l cell dry weight was obtained (Zhanyou et al., 2007). Another study using the same microalgae were grown on crude glycerol and 35 g/l dry weight concentration with the cellular lipid content of 73.3 % was obtained (Liang et al., 2010).

Cryptococus curvatus, an oleaginous yeast was also grown on crude glycerol derived from yellow grease and cultured in a one stage fed batch mode process wherein crude glycerol and nitrogen source were fed intermittently for 12 days and final biomass of 32.9 g/l and the lipid content of 52% at the end of 12 days were obtained.

Glycerol-rich streams generated in large amounts by the bio-fuel industry, especially during the production of biodiesel, present an excellent opportunity to establish bio-refineries. Once considered a valuable ‘co-product’, crude glycerol is rapidly becoming a ‘waste product’ with a disposal cost attributed to it. Proposed research is based on using crude glycerol as the carbon source for microalgal fermentation. Consequently, the main motivation of this work is to use waste crude glycerol from biodiesel production as a carbon source for microalgae, and producing oil out of microalgae, and to investigate the feasibility of this process. In this way, crude glycerol can be processed into a valuable product, while microalgal oil is produced as an alternative fuel source.

CHAPTER 2

LITERATURE REVIEW

2.1. Photoautotrophic Production

Under natural growth conditions photoautotrophic algae absorb sunlight, and assimilate carbon dioxide from the air and nutrients from the aquatic habitats. Therefore, as far as possible, artificial production should attempt to replicate and enhance the optimum natural growth conditions.

Under natural growth conditions, microalgae assimilate CO_2 from the air. Most microalgae can utilize high levels of CO_2 and it can be fed to medium from different sources such as flue gases from coal power plans and exhaust from brewing industry. However, high levels of sulfur in flue gases can be poisonous to microalgae and therefore it must be removed from gas stream before feeding. This limits direct use of such sources easily as carbon source. Other important limitation is the low solubility of CO_2 in water. Other inorganic nutrients required for algae production include nitrogen, phosphorus and silicon (for diatoms). Although some blue-green algae can fix nitrogen from air, nitrogen fixation is highly energy intensive process for these species of microalgae. Therefore, most of microalgae require nitrogen in soluble from. Phosphorus required in very small amount in microalgae structure but since not all the phosphorus is bioavailable, it must be added in excess amounts to medium.

Currently, photoautotrophic production is the only method which is technically and economically feasible for large-scale production of algae biomass for non-energy production (Borowitzka M., 1997). Two systems that have been deployed are based on open pond and closed photobioreactor technologies (Borowitzka M., 1999). The technical viability of each system is influenced by intrinsic properties of the selected algae strain used, as well as climatic conditions and the costs of land and water (Borowitzka M., 1992).

2.1.1. Open Pond Production Systems

Algae cultivation in open pond production systems has been used since the 1950's (Borowitzka M., 1999). These systems can be categorized into natural waters (lakes, lagoons, and ponds) and artificial ponds or containers.

Raceway ponds are the most commonly used artificial system. They are typically made of a closed loop, oval shaped recirculation channels, generally between 0.2 and 0.5 meter deep, with mixing and circulation required to stabilize algae growth and productivity. Raceway ponds are usually built in concrete, but also ponds with white plastic have also been used. In a continuous production cycle, algae broth and nutrients are introduced in front of the paddle wheel and circulated through the loop to the harvest extraction point. The paddle wheel is in continuous operation to prevent sedimentation. The microalgae's CO_2 requirement is usually satisfied from the surface air, but submerged aerators may be installed to enhance CO_2 absorption (Terry et al., 1985).

Compared to closed photobioreactors, open pond is the cheaper method of large-scale algal biomass production. Open pond production can be done in areas that are not good for agriculture. Open ponds also have lower energy input requirement, and regular maintenance and cleaning are easier and therefore may have the potential to return large net energy production.

In 2008, the unit cost of producing *Dunaliella salina*, one of the commonly cultivated algae strains, in an open pond system was about €2.55 per kilogram of dry biomass, which was considered to be too high to justify production for bio-fuels (Borowitzka M., 1999).

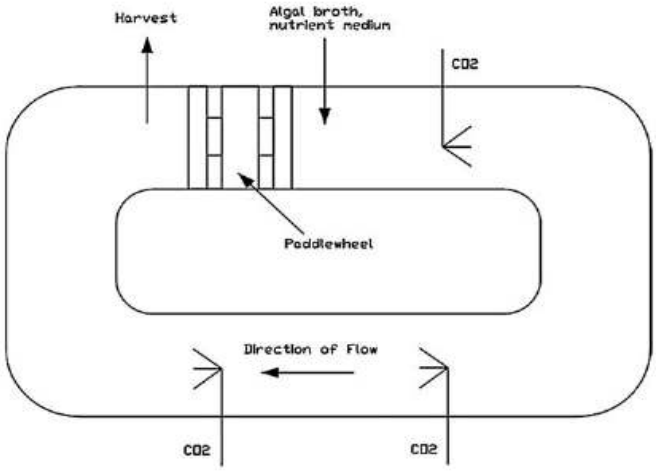

Figure 2.1. Plan view of a raceway pond. Algae broth is introduced after the paddle wheel, and completes a cycle while being mechanically aerated with CO_2. It is harvested before the paddle wheel to start the cycle again (Source: Chisti Y., 2008).

Table 2.1. Advantages and limitations of open ponds and photobioreactors

Production System	**Advantages**	**Limitations**
Raceway pond	Relatively cheap Easy to clean Utilises non-agricultural land Low energy inputs Easy maintenance	Poor biomass productivity Large area of land required Limited to a few strains of algae Poor mixing, light and CO_2 utilisation Cultures are easily contaminated
Tubular photobioreactor	Large illumination surface area Suitable for outdoor cultures Relatively cheap Good biomass productivities	Some degree of wall growth Fouling Requires large land space Gradients of pH, dissolved oxygen and CO2 along the tubes
Flat plate photobioreactor	High biomass productivities Easy to sterilise Low oxygen build-up Readily tempered Good light path Large illumination surface area Suitable for outdoor cultures	Difficult scale-up Difficult temperature control Small degree of hydrodynamic stress Some degree of wall growth
Column photobioreactor	Compact High mass transfer Low energy consumption Good mixing with low shear stress Easy to sterilise Reduced photoinhibition and photo-oxidation	Small illumination area Expensive compared to open ponds Shear stress Sophisticated construction

Open pond systems, require highly selective environments due to inherent threat of contamination and pollution from other algae species and protozoa. Monoculture cultivation is possible by maintenance of extreme culture environment, although only a small number of algae strains are suitable. For example, the species *Chlorella* (adaptable to nutrient-rich media), *D. salina* (adaptable to very high salinity) and *Spirulina* (adaptable to high alkalinity) can be grown in such systems.

An example of large-scale monoculture cultivation is the production of *D. salina* for β-carotene in the extremely halophilic waters of Hutt-Lagoon, Western Australia. However, long production periods for such approaches do not necessarily exclude bacterial and other biological contaminants (Lee et al., 2001). As far as biomass productivity is concerned, open pond systems are less efficient when compared with closed photobioreactors. This is caused by several determining factors; evaporation losses, temperature fluctuation in the growth media, CO_2 deficiencies, inefficient mixing, and light limitation. Temperature fluctuations due to seasonal variations are difficult to control in open ponds. Potential CO_2 deficiencies due to diffusion into the atmosphere may result in reduced biomass productivity due to less efficient utilization of CO_2. Also, poor mixing by inefficient stirring mechanisms, may result in poor mass CO_2 transfer rates causing low biomass productivity (Ugwu et al., 2008). Light limitation due to top layer thickness may also cause reduced biomass productivity. However, enhancing light supply is possible by reducing layer thickness; using thin layer inclined types of culture systems, and improved mixing can minimize impacts to enhance biomass productivity.

High algae biomass production rates are achievable with open pond systems. However, there are still inconsistencies in the production rates reported in literature.. Jimênez et al. extrapolated an annual dry weight biomass production rate of 30 tonnes per hectare using data from a 450 m^2 and 0.30 m deep raceway pond system producing biomass dry weight of 8.2 gm^{-2} per day in Malaga, Spain. Using similar depth of culture, and biomass concentrations of up to 1 gl^{-1}, estimated dry biomass productivity in the range of 10–25 gm^{-2} per day. However, the only open pond system for large-scale production that has achieved such high biomass productivity is the inclined system developed by Setlik et al. (1970) for the production of *Chlorella*. In this system, a biomass concentration of higher than 10 gl^{-1} was achieved, with extrapolated productivity of 25 gm^{-2} per day. Weissman and Tillett (1992) operated an outdoor open pond (0.1 ha) in New Mexico, USA, and attained an average annual dry weight biomass

production rate of 37 tonnes per hectare with a mixed species culture (four species), highest yields were confined to the 7 warmest months of the year.

2.1.2. Closed Photobioreactor Systems

Microalgae production based on closed photobioreactor technology is designed to overcome some of the major problems associated with the described open pond production systems. For example, pollution and contamination risks with open pond systems, prevents their use for the preparation of high-value products for use in the pharmaceutical and cosmetics industry. Also, unlike open pond production, photobioreactors permit culture of single-species of microalgae for prolonged durations with lower risk of contamination.

Closed systems include the tubular, flat plate, and column photobioreactors. These systems are more appropriate for sensitive strains as the closed configuration makes the control of potential contamination easier. Owing to the higher cell mass productivities attained harvesting costs can also be significantly reduced. However, the costs of closed systems are substantially higher than open pond systems (Carvalho et al., 2006). Photobioreactors consist of an array of straight glass or plastic tubes as shown in Figure 2.2. The tubular array captures sunlight and can be aligned horizontally, vertically, inclined or as a helix, and the tubes are generally 0.1 m or less in diameter (Molina et al., 2001). Algae cultures are re-circulated either with a mechanical pump or airlift system, the latter allowing CO_2 and O_2 to be exchanged between the liquid medium and aeration gas as well as providing a mechanism for mixing (Eriksen N., 2008). Agitation and mixing are very important to encourage gas exchange in the tubes.

Table 2.2. Biomass productivity figures for closed photobioreactors

Species	Reactor type	Volume (l)	X_{max} (g l^{-1})	Paerial (gm^{-2} day^{-1})	Pvolume (g l^{-1} day^{-1})	PE (%)	Reference
Porphyridium cruentum	Airlift tubular	200	3	-	1.5	-	Camacho Rubio et al., 1999
Phaeodactylum tricornutum	Airlift tubular	200	-	20	1.2	-	Acien Fernandez et al., 2001
Phaeodactylum tricornutum	Airlift tubular	200	-	32	1.9	2.3	Molina Grima et al., 2001
Chlorella sorokiniana	Inclined tubular	6	1.5	–	1.47	–	Ugwu CU et al., 2002
Arthrospira platensis	Undular row tubular	11	6	47.7	2.7	–	Carlozzi P., et al., 2003
Phaeodactylum tricornutum	Outdoor helical t.	75	–	–	1.4	15	Hall DO et al., 2003
Haematococcus pluvialis	Parallel tubular (AGM)	25	–	13	0.05	–	Olaizola M., 2000
Haematococcus pluvialis	Bubble column	55	1.4	–	0.06	–	Garcia et al., 2003
Haematococcus pluvialis	Airlift tubular	55	7	–	0.41	–	Garcia et al., 2003
Nannochloropsis sp.	Flat plate	440	–	–	0.27	–	Cheng-Wu et al., 2001
Haematococcus pluvialis	Flat plate	25,000	–	10.2	–	–	Huntley et al., 2007
Spirulina platensis	Tubular	5.5	–	–	0.42	8.1	Converti et al., 2006
Arthrospira	Tubular	146	2.37	25.4	1.15	4.7	Carlozzi P., 2003
Chlorella	Flat plate	400	–	22.8	3.8	5.6	Doucha et al., 2005
Chlorella	Flat plate	400	–	19.4	3.2	6.9	Doucha et al., 2005
Tetraselmis	Column	ca. 1,000	1.7	38.2	0.42	9.6	Chini et al., 2006
Chlorococcum	Parabola	70	1.5	14.9	0.09	–	Sato et al., 2006
Chlorococcum	Dome	130	1.5	11.0	0.1	–	Sato et al., 2006

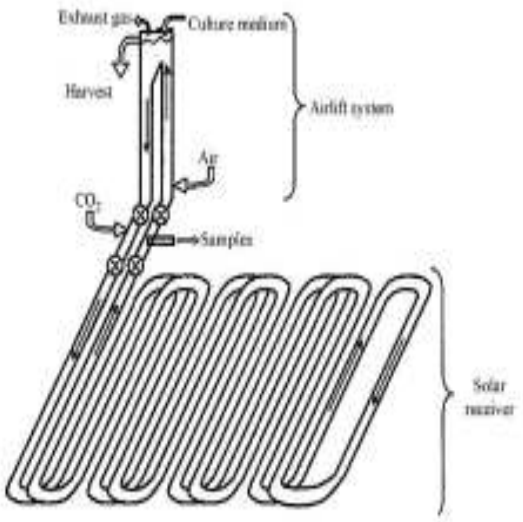

Figure 2.2. Basic design of a horizontal tubular photobioreactor (Source: Becker, 1994)

Some of the earliest forms of closed systems are flat-plate photobioreactors which have received much research attention due to the large surface area exposed to illumination and high densities of photoautotrophic cells (>80 g l^{-1}) observed (Hu et al., 1998). The reactors are made of transparent materials for maximum solar energy capture, and a thin layer of dense culture flows across the flat plate, which allows radiation absorbance in the first few millimetres thickness. Flat-plate photobioreactors are suitable for mass cultures of algae due to low accumulation of dissolved oxygen and the high photosynthetic efficiency achieved when compared to tubular versions (Richmond A., 2000). Tubular photobioreactors have design limitations on length of the tubes, which is dependent on potential O_2 accumulation, CO_2 depletion, and pH variation in the systems (Eriksen N., 2008). Therefore, they cannot be scaled up indefinitely; hence, large-scale production plants are based on integration of multiple reactor units. However, tubular photobioreactors are considered to be more suitable for outdoor mass cultures since they expose a larger surface area to sunlight. The largest closed photobioreactors are tubular, e.g. The 25 m^3 plant at Mera Pharmaceuticals, Hawaii, and the 700 m^3 plant in Klotze, Germany.

Column photobioreactors offer the most efficient mixing, the highest volumetric mass transfer rates and the best controllable growth conditions (Eriksen N., 2008). They are low-cost, compact and easy to operate. The vertical columns are aerated from the bottom, and illuminated through transparent walls, or internally (Suh et al., 2003). Their

performance compares favorably with tubular photobioreactors. Closed photobioreactors have received major research attention in recent years. The most important advantages of pilot-scale production using closed photobioreactors compared to open raceway ponds are rigorous process control and potentially higher biomass production rates. Therefore, potentially higher production of bio-fuel and co-product production could be possible.

2.1.3. Hybrid Production Systems

The hybrid two-stage cultivation is a method that combines distinct growth stages in photobioreactors and in open ponds. The first stage is in a photobioreactor where controllable conditions minimize contamination from other organisms and favour continuous cell division. The second production stage is aimed at exposing the cells to nutrient stresses, which enhances synthesis of the desired lipid product. This stage is ideally suited to open pond systems, as the environmental stresses that stimulate production can occur naturally through the transfer of the culture from photobioreactors to the open pond.

Huntley and Redalje (2007) used such a two-stage system for the production of both oil and astaxanthin (used in salmon feed) from Haematococcus pluvialis, and achieved an annual average microbial oil production rate >10 ton ha^{-1} per annum with a maximum rate of 24 ton ha^{-1} per annum. They also demonstrated that under similar conditions, rates of up to 76 ton ha^{-1} per annum was feasible using species with higher oil content and photosynthetic efficiency.

2.2. Heterotrophic Production

Heterotrophic production has also been successfully used for algal biomass and metabolites (Miao et al., 2006). In this process microalgae are grown on organic carbon substrates such as glucose in stirred tank bioreactors or fermenters. Algae growth is independent of light energy, which allows for much simpler scale-up possibilities since smaller reactor surface to volume ratio's may be used. These systems provide a high degree of growth control and also lower harvesting costs due to the higher cell densities achieved. The set-up costs are minimal, although the system uses more energy than the

production of photosynthetic microalgae because the process cycle includes the initial production of organic carbon sources via the photosynthesis process.

Table 2.3. Biomass productivity figures for heterotrophic microalgae cultures

Species	Product	Culture	X_{max} (g l^{-1})	Total Lipid (%)	Pvolume (g l^{-1} day^{-1})	Reference
Galdieria sulphuraria	C-phycocyanin	Continuous	83.3	–	50.0	Graverholt et al., 2007
Galdieria sulphuraria	C-phycocyanin	Fed-batch	109	–	17.50	Graverholt et al., 2007
Chlorella protothecoides	Biodiesel	Fed-batch	3.2	57.8	–	Xiong et al., 2008
Chlorella protothecoides	Biodiesel	Fed-batch	16.8	55.2	–	Xiong et al., 2008
Chlorella protothecoides	Biodiesel	Fed-batch	51.2	50.3	–	Xiong et al., 2008
Chlorella	Docosahexaenoic acid	Fed-batch	116.2	–	1.02	Wu et al., 2007
Crypthecodinium cohnii	Docosahexaenoic acid	Fed-batch	109	56	–	Swaaf et al., 2003
Crypthecodiniu cohnii	Docosahexaenoic acid	Fed-batch	83	42	–	Swaaf et al., 2003
Chlorella	N/A	Fed-batch	104.9	–	14.71	Wu et al., 2007
Chlorella protothecoides	Biodiesel	Fed-batch	15.5	46.1	–	Li et al., 2007
Chlorella protothecoides	Biodiesel	Fed-batch	12.8	48.7	–	Li et al., 2007
Chlorella protothecoides	Biodiesel	Fed-batch	14.2	44.3	–	Li et al., 2007

Li et al. (2007) outlined the feasibility for large-scale biodiesel production based on heterotrophic cultivation of *Chlorella protothecoides*. Other studies also suggest higher technical viability of heterotrophic production compared to photoautotrophic methods in either open ponds or closed photobioreactors. Miao and Wu (2006) also studied *C. protothecoides* and found that the lipid content in heterotrophic cells could be as high as 55%, which was 4 times higher than in autotrophic cells at 15% under similar conditions. Hence, they concluded that heterotrophic cultivation could result in higher production of biomass and accumulation of high lipid content in cells.

2.3. Mixotrophic Production

Many algal organisms are capable of using either metabolism process (autotrophic or heterotrophic) for growth, meaning that they are able to photosynthesize as well as metabolize organic materials (Graham et al., 2009). The ability of mixotrophs to process organic substrates means that cell growth is not strictly dependent on photosynthesis, therefore light energy is not an absolutely limiting factor for growth as either light or organic carbon substrates can support the growth (Chen et al., 1996).

Examples of microalgae that displays mixotrophic metabolism processes for growth are the cyanobacteria *Spirulina platensis*, and the green alga *Chlamydomonas reinhardtii*. The photosynthetic metabolism utilizes light for growth while aerobic respiration uses an organic carbon source. Growth is influenced by the media supplement with glucose during the light and dark phases, hence, there is less biomass loss during the dark phase.

Table 2.4. Biomass productivity figures for microalgae mixotrophic cultures

Species	**Organic carbon source**	**μ_{max} (day^{-1})**	**X_{max} ($g\ l^{-1}$)**	**Pvolume ($g\ l^{-1}\ day^{-1}$)**	**Reference**
Spirulina platensis	Glucose	0.62	2.66	–	Chen et al., 1996
Spirulina platensis	Acetate	0.52	1.81	–	Chen et al., 1996
Spirulina Sp.	Glucose	1.32	2.50	–	Andrade et al., 2007
Spirulina platensis	Molases	0.147	2.94	0.32	Andrade et al., 2007

Growth rates of mixotrophic algae compare favorably with cultivation of photoautotrophic algae in closed photobioreactors. The rates are higher than for open pond cultivation but are considerably lower than for heterotrophic production. Chojnacka et al. (2004) compared *Spirulina sp.* growth in photoautotrophic, heterotrophic and mixotrophic cultures. They found that mixotrophic cultures reduced photo-inhibition and improved growth rates over both autotrophic and heterotrophic cultures. Successful production of mixotrophic algae allows the integration of both photosynthetic and heterotrophic components during the growth cycle. This reduces the

impact of biomass loss during dark respiration and decreases the amount of organic substances utilized during growth. These features infer that mixotrophic production can be an important part of the microalgae-to-biofuels process.

CHAPTER 3

OBJECTIVES

The main aim of this study is to investigate the optimum parameters of microalgal fermentation on crude glycerol-rich medium and to determine the lipid, protein and carbohydrate contents of resulting microalgal biomass using FT-IR. The goals of the study are listed below:

- ✓ To get an calibration equation for the Dry Weight (DW) of a microalgae suspension against the UV spectra at 550 nm, so that fast measurements of DW can be possible,
- ✓ To examine the effects of the parameters: Temperature (^{0}C), Substrate Concentration (g/l), Dilution rate (h^{-1}) and Productivity (g biomass/l/h) and the composition of algal biomass in terms of lipid, protein and carbohydrate,
- ✓ To optimize the values of the most effective parameters, using a Box Benkhen design sets of experiments,
- ✓ To determine lipid productivity of such microalgal system using crude glycerol, using FT-IR technique,
- ✓ Compare the lipid productivity values obtained as a result of Box Benkhen design with values reported in the literature and report these data into the literature.

CHAPTER 4

EXPERIMENTAL STUDY

4.1. Materials, Algal Strain and Culture Conditions

Crude glycerol was obtained from a local glycerol refinery. It is pretreated in order to remove residual methanol and acidified to split soaps into FFA's. Therefore, the crude glycerol used in this study was approximately 80% purity with impurities of water, phospholipids, MONG (non glycerol compounds) and ash. Before using the pH of crude glycerol is adjusted to 6.5. The green microalga, *Chlorella minutissima* (UTEX 2341) was provided from the Culture Collection of Algae at the University of Texas (Austin, Texas, USA). This algal strain is maintained in modified Bristol's medium (referred to as CZ-M1), and this medium is used as growth medium in the experiments which consisted of (per litre): 0.75 g $NaNO_3$; 0.175 g KH_2PO_4; 0.075 g K_2HPO_4; 0.075 g $MgSO_4 \cdot 7H_2O$; 0.025 g $CaCl_2 \cdot 2H_2O$; 0.025 g NaCl; 5mg $FeCl_3$; 0.287 mg $ZnSO_4 \cdot 7H_2O$; 0.169 mg $MnSO_4 \cdot H_2O$; 0.061 mg H_3BO_3; 0.0025 mg $CuSO_4 \cdot 5H_2O$; and 0.00124 mg $(NH_4)6Mo_7O_{24} \cdot 7H_2O$.

New Brunswick BioFlo410 Bioreactor/Fermentor is used in this study for the experiments. Complete PID control is available for parameters such as pH, temperature, agitation, air injection, foam, and dissolved oxygen (DO). Heat sterilization is also automatically controlled before inoculation. Agitation speed (rpm) and air flow rate (l/min) is cascaded to (DO) to keep it over 20% saturation. Air flow rate is changed between 1 – 25 l/min, and agitation speed is changed between 50 – 300 rpm. Alarm shut-down is set for max level in case of excess foaming. The culture is grown in the medium supplemented with 10 g/l crude glycerol as inoculum. The pH of the medium is adjusted to 6.5 prior to autoclaving at 121 ^{0}C for 20 min, and it is kept at the pH of 6.8 using PID control of the bioreactor, for optimum pH point determination. An inoculum of 10% (by volume, average cell concentration of 0.5 g/l) is inoculated into the bioreactor. Different substrate concentrations of crude glycerol is used at different dilution rates and different temperatures for chemostat mode runs. For changing dilution rates, flow rate and/or the working volume of the medium is adjusted.

4.2. Methods

4.2.1. Biomass Evaulation

The biomass was evaluated by the dry weight DW (g/l), and its relationship with the optical density (OD) at 550 nm and correlated as following:

$$DW = (1.81*OD_{550} - 0.94)*D \quad (4.1)$$

where D is the dilution number for the suspensions that are too dense to be measured by the spectrometer (Multiskan, Thermo).

4.2.2. Determination of Biochemical Compositions

A broth with biomass concentration of 1.0 mg ml^{-1} was prepared. Two-hundred microlitres of suspension was dropped on the CaF_2 window (32 x 3 mm) for formation of a circle with a diameter of 10 mm. The sample was then dried in the vacuum drying oven at 40 ^0C for 1.0 h. The absorbance of samples were collected on the FT-IR spectrometer (TENSOR 27, Bruker) at a resolution of 4 cm^{-1} with sample and background scan time of 16 scans. OPUS 6.5 was employed to process the FT-IR spectra ranging from 400 to 4000 cm^{-1}. ‘‘Rubberband correction” was chosen to correct the spectra baseline, using 64 baseline points and excluding CO_2 bands. Then the characteristic peak areas of lipids, proteins and carbohydrates were calculated by integration. The amounts of biomolecules and their peak areas were correlated as the followings (Pistorins et al., 2009):

$$A_L = - 2.30 + 78.96 * T_L \quad (4.2)$$

$$A_P = - 0.27 + 12.72 * T_P \quad (4.3)$$

$$A_C = 0.07 + 2.05 * T_C \quad (4.4)$$

where T_L (mg), T_P (mg) and TC (mg) represent the total amounts of lipids, proteins and carbohydrates, and A_L, A_P and A_C are characteristic peak areas of lipids, proteins and carbohydrates, respectively.

Table 4.1. Band assigment for infrared spectroscopy

Wavenumber (cm^{-1})	Assignment	Comments
3000–2800	νC–H of saturated CH	
~ 1740	νC=O of ester groups, primarily from lipids and fatty acids	
~ 1650	νC=O of amides from proteins	amide I band
~ 1540	δN–H of amides from proteins	amide II band
~ 1455	δ_{as} CH_3 and δ_{as} CH_2 of proteins	
~ 1398	δ_s CH_3 and δ_s CH_2 of proteins, and ν_s C–O of COO^- groups	
~ 1250–1230	ν_{as} P=O of phosphodiester groups of nucleic acids and phospholipids	
~ 1200–900	νC–O–C of saccharides	generally obscured by the presence of silicate
~ 1075	νSi–O of silicate frustules	

ν_{as} asymmetric stretch; ν_s symmetric stretch; δ_{as} asymmetric deformation; δ_s symmetric deformation.

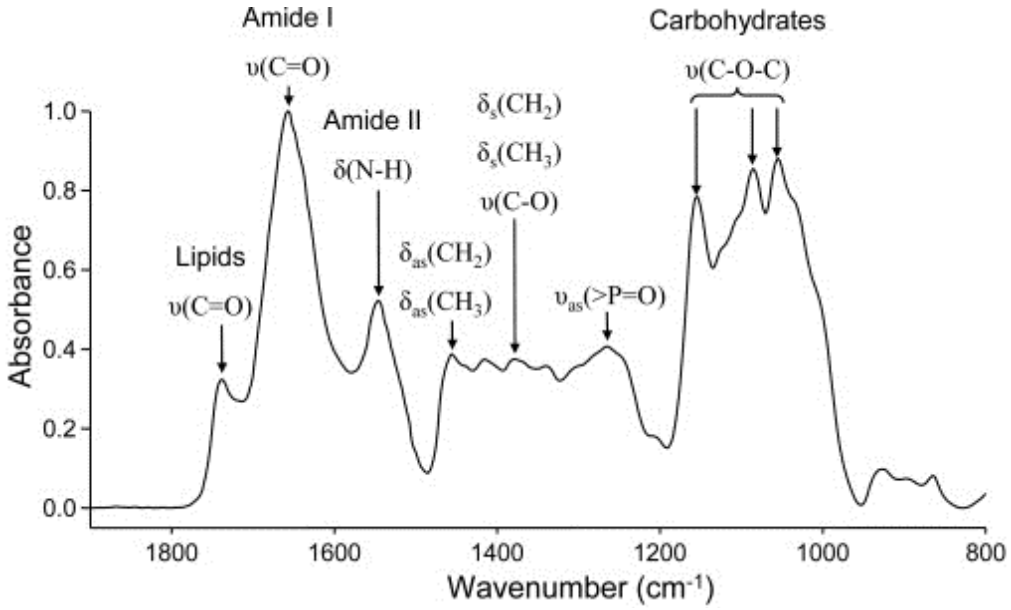

Figure 4.1. Typical spectra for macromolecules

4.2.3. Box-Benkhen Experimental Design

An experimental design is prepared using the software Design Expert Version 8.0.2. There are 3 factors; temperature, dilution rate and substrate concentration (crude glycerol) and 5 responses; dry weight, productivity, lipid, protein and carbohydrate contents. The design data matrix and limits for the factors are shown in Table 4.2.

Table 4.2. Box Benkhen experimental design data matrix

Data Matrix (randomized)

Run	A	B	C
1	0	+	+
2	-	+	0
3	0	0	0
4	+	0	+
5	0	-	+
6	+	+	0
7	0	0	0
8	+	0	-
9	0	+	-
10	-	0	-
11	0	0	0
12	-	0	+
13	0	-	-
14	-	-	0
15	+	-	0

	Low Limit	Midpoint	High Limit
Temperature	25	35	40
Dilution rate	0,05	0,15	0,25
Substrate Conc.	10	80	150

Table 4.3.Box Benkhen Experimental Layout

Std Order	Run #	Factor 1 Temperature (^{0}C)	Factor 2 Dilution rate (h^{-1})	Factor 3 Substrate Concentration (g/l)
8	1	35.00	0.25	150.00
14	2	25.00	0.25	80.00
6	3	35.00	0.15	80.00
12	4	40.00	0.15	150.00
5	5	35.00	0.05	150.00
3	6	40.00	0.25	80.00
1	7	35.00	0.15	80.00
2	8	40.00	0.15	10.00
4	9	35.00	0.25	10.00
15	10	25.00	0.15	10.00
11	11	35.00	0.15	80.00
9	12	25.00	0.15	150.00
13	13	35.00	0.05	10.00
10	14	25.00	0.05	80.00
7	15	40.00	0.05	80.00

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Lipid, Protein and Carbohydrate Detection by FTIR Spectroscopy

FTIR spectra of *Chlorella minutissima* showed nine distinct absorption bands over the wavenumber range 1900 – 800 $cm^{-1.}$. The bands were assigned to specific groups on the basis of biochemical standarts and published studies, as described previously (Stehfest et al., 2005). Table 5.1. shows the integrated band areas.

Table 5.1. Integrated band areas

Designation	Wavenumber (cm^{-1})
Amide I	1705 – 1575
Amide II	1575 – 1480
Carbohydrates	1064 – 880
Lipid	1780 – 1708
Phosphour	1350 – 1190

5.2. Productivity

The highest productivity is obtined in the first run with the value of 7.42 g/l/h (Table 5.2). Surface graphs in Figure 5.1, 5.2 and 5.3 shows the effects of factors on productivity.

Table 5.2. Cell dry weight and productivity responses

Run	Factor 1 Temperature (^{0}C)	Factor 2 Dilution rate (h^{-1})	Factor 3 Substrate Concentration (g/l)	Dry weight (g/l)	Productivity (g/l/h)
1	35.00	0.25	150.00	29.71	7.42
2	25.00	0.25	80.00	28.84	7.21
3	35.00	0.15	80.00	38.70	5.80
4	40.00	0.15	150.00	50.59	7.58
5	35.00	0.05	150.00	61.04	3.05
6	40.00	0.25	80.00	27.10	6.77
7	35.00	0.15	80.00	39.86	5.97
8	40.00	0.15	10.00	2.76	0.41
9	35.00	0.25	10.00	2.38	0.59
10	25.00	0.15	10.00	2.92	0.43
11	35.00	0.15	80.00	35.80	5.37
12	25.00	0.15	150.00	44.50	6.67
13	35.00	0.05	10.00	2.70	0.13
14	25.00	0.05	80.00	40.15	2.01
15	40.00	0.05	80.00	35.51	1.78

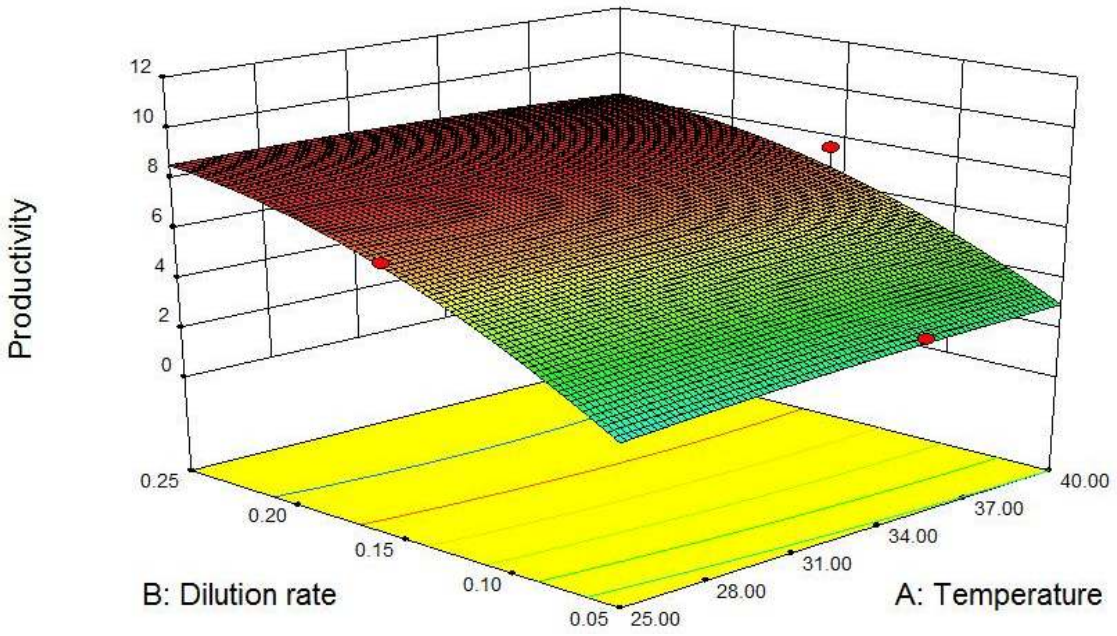

Figure 5.1. Surface graph for productivity (g/l/h) for factors dilution rate and temperature

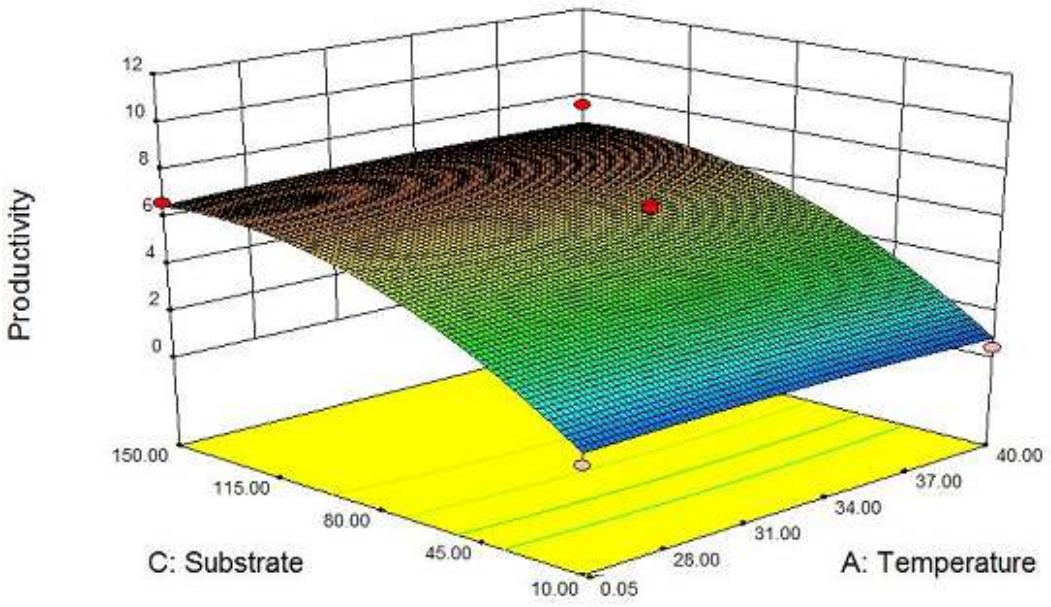

Figure 5.2. Surface graph for productivity (g/l/h) for factors substrate concentration and temperature

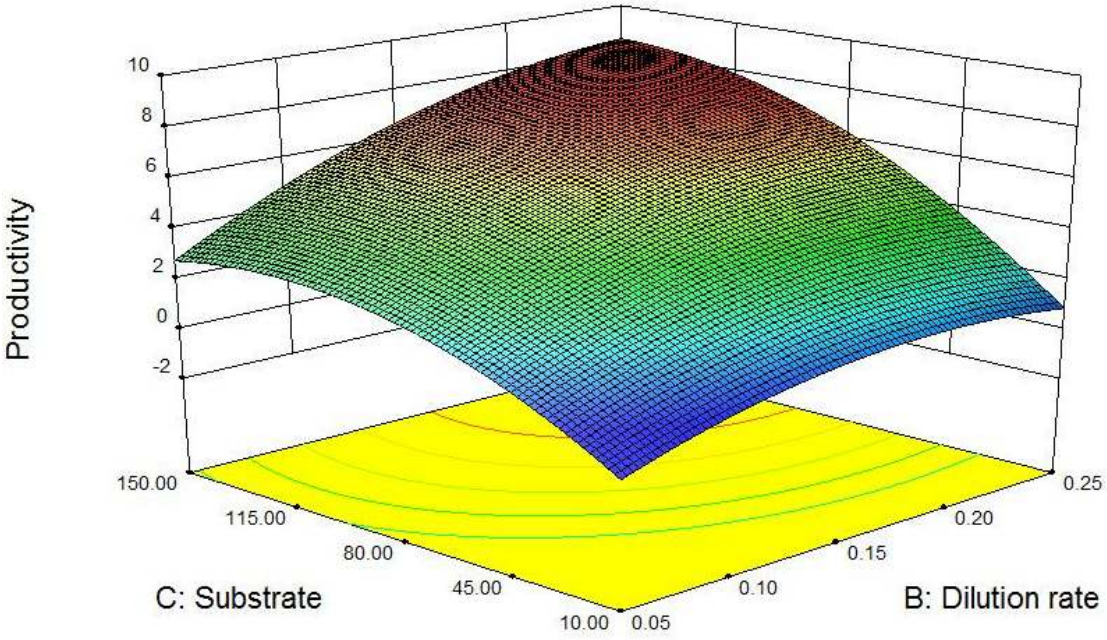

Figure 5.3. Surface graph for productivity (g/l/h) for factors substrate concentration and and dilution rate

5.3. Lipid Content

The highest lipid content was obtained in the second run with the value of 14.35% and with a lipid productivity of 1.04 g/l/h (Table 5.3). Graphs in Figures 5.4, 5.5 and 5.6 shows the change of lipid content in term of different factors.

Table 5.3. Cell dry weight and lipid (%) responses

Run	Factor 1 Temperature (^{0}C)	Factor 2 Dilution rate (h^{-1})	Factor 3 Substrate Concentration (g/l)	Dry weight (g/l)	Lipid (%)
1	35.00	0.25	150.00	29.71	13.90
2	25.00	0.25	80.00	28.84	14.35
3	35.00	0.15	80.00	38.70	10.71
4	40.00	0.15	150.00	50.59	10.19
5	35.00	0.05	150.00	61.04	8.91
6	40.00	0.25	80.00	27.10	13.20
7	35.00	0.15	80.00	39.86	9.77
8	40.00	0.15	10.00	2.76	8.43
9	35.00	0.25	10.00	2.38	8.02
10	25.00	0.15	10.00	2.92	9.70
11	35.00	0.15	80.00	35.80	12.13
12	25.00	0.15	150.00	44.50	12.71
13	35.00	0.05	10.00	2.70	13.48
14	25.00	0.05	80.00	40.15	7.55
15	40.00	0.05	80.00	35.51	9.08

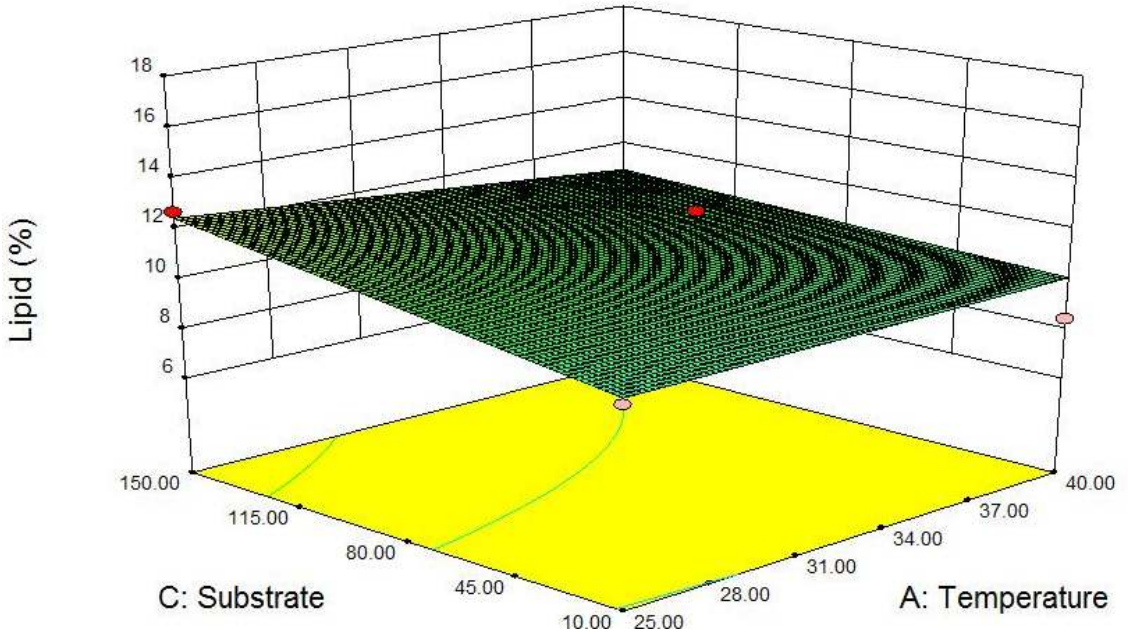

Figure 5.4. Surface graph for Lipid (%) for factors substrate concentration and temperature

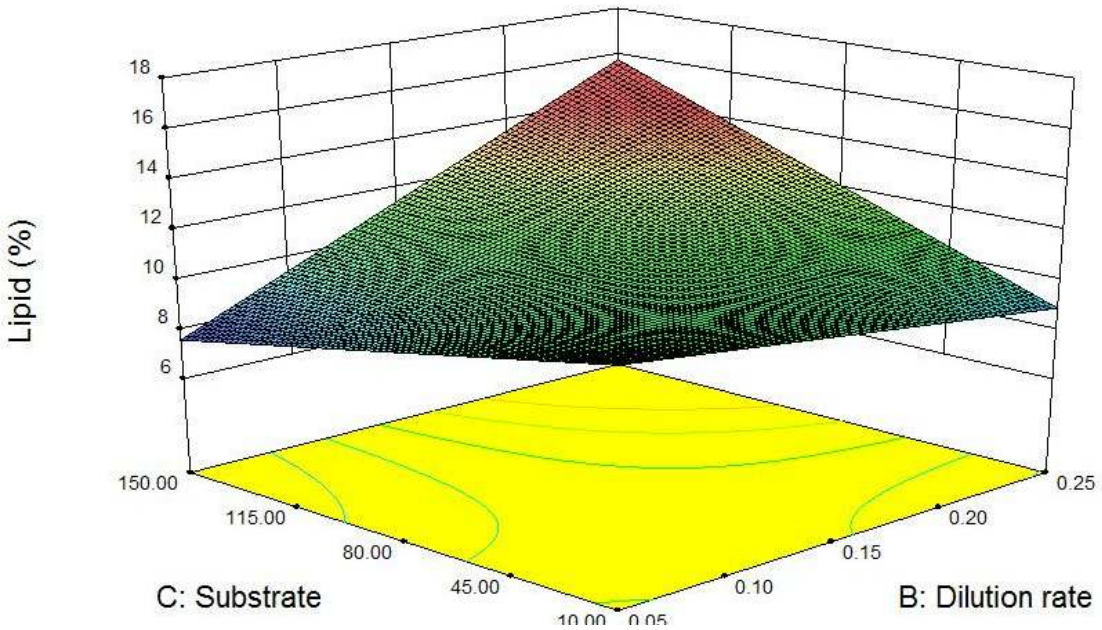

Figure 5.5. Surface graph for Lipid (%) for factors substrate concentration and dilution

rate

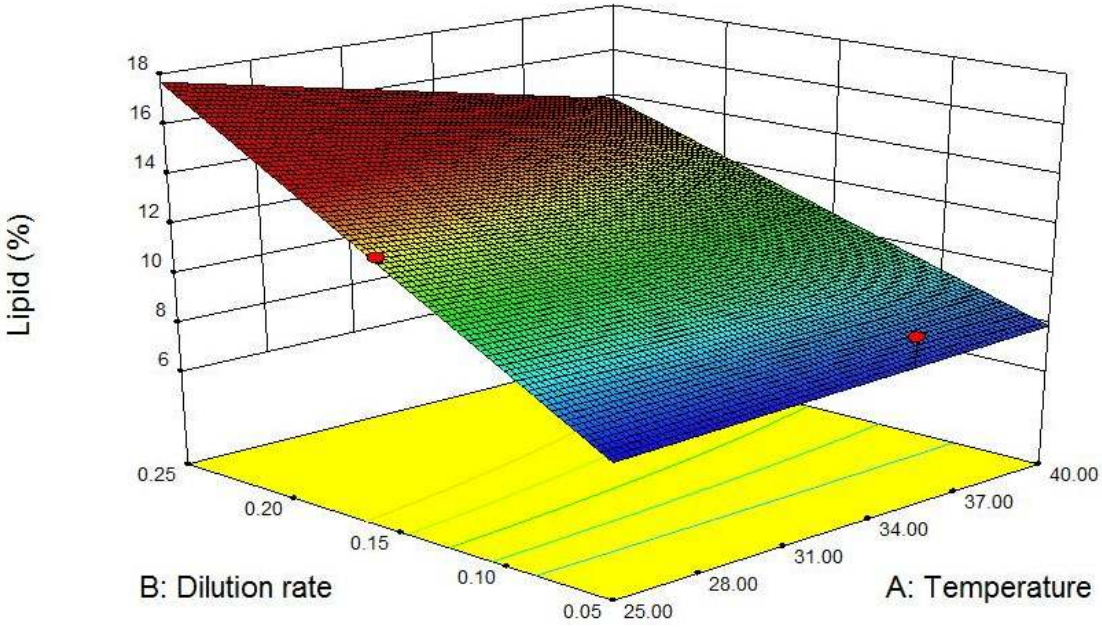

Figure 5.6. Surface graph for Lipid (%) for factors temperature and dilution rates

5.4. Protein and Carbohydrate Content

The highest protein content was observed in the second run while the highest carbohydrate content was observed in the run 14 (Table 5.4).

Table 5.4. Protein and carbohyrate responses

Run	Factor 1 Temperature (^{0}C)	Factor 2 Dilution rate (h^{-1})	Factor 3 Substrate Concentration (g/l)	Protein (%)	Carbohydrate (%)
1	35.00	0.25	150.00	48.40	8.05
2	25.00	0.25	80.00	47.89	8.06
3	35.00	0.15	80.00	35.05	29.45
4	40.00	0.15	150.00	43.45	29.59
5	35.00	0.05	150.00	38.90	32.53
6	40.00	0.25	80.00	36.80	35.33
7	35.00	0.15	80.00	17.95	45.37
8	40.00	0.15	10.00	25.98	55.48
9	35.00	0.25	10.00	14.91	58.18
10	25.00	0.15	10.00	43.89	25.88
11	35.00	0.15	80.00	24.71	40.74
12	25.00	0.15	150.00	46.43	25.87
13	35.00	0.05	10.00	36.65	44.09
14	25.00	0.05	80.00	22.01	50.08
15	40.00	0.05	80.00	39.89	25.66

Although lipid content of microalgae in this study (14%) is lower than reported in the literature (up to 55%), high lipid productivities (1.04 g/l/h) in total was achieved in this study due to the high dry weight concentration and dilution rates. Also, protein content is high compared to literature which can be used as animal feed after oil extraction for fuel purposes. This could be a valuable side product and would significantly affect the economics of such process.

Biodiesel is regarded to be one of the most promising alternatives to fossil fuel because it is renewable and environmental friendly. Currently, biodiesel is produced mainly from soybeans, rapeseed, canola oil, palm oil, animal fat and waste cooking oil. It is important to note that current supply is far less than demand and the price is high. A country such as the United States produced 491 million gallons biodiesel in 2007, far below the annual biodiesel demand. It is not practical to increase biodiesel production by increasing planting area of oil crops in concern of limited land. Another alternative method for biodiesel production is offered by fermentation of high lipid yield

microorganism. Heterotrophic *Chlorella Sp.* has been reported to be a very good candidate for biodiesel production because of its high lipid content and cell density. The high cost of the feedstock glucose is the main obstacle for commercialization. And in the long run, it is not practical to produce biodiesel from the food-based sugar. Crude glycerol is a problematic side product because it contains lots of impurities, and so having low economic value in biodiesel production. So, this study investigated the application of crude glycerol for oil production by heterotrophic *Chlorella minutissima*. In this research, the lipid yield reached up to 1.04 g/l/day. Typically, the lipid productivity by phototrophic microalgae was significantly lower at 17–204 mg/l/day (Mata et al., 2009). As regard to carbon efficiency, there are two advantages in this heterotrophic process. Firstly, the lipid productivity in heterotrophic process is much higher than the phototrophic system. Thus, to produce the same amount of lipid, the heterotrophic process needs less energy for maintenance, irradiation, mixing and collection of microalgae. The save of energy, which is commonly generated by burning fossil fuel, would reduce the emission of CO_2. Secondly, crude glycerol is directly used as a natural carbon source, so the feedstock of the process is obtained by bio-fixation of atmospheric CO_2. From the global perspective, the heterotrophic fermentation has special characteristics, and the improvement of technology would further increase the carbon efficiency and biodiesel productivity.

The work presented here showed the potential of using less expensive crude glycerol from the biodiesel industry to produce oil by microalgal culture. The optimal crude glycerol content for algal oil production was around 150 g/L. Using statistically based experimental designs, it was found that substrate concentration and dilution rate were factors significantly influencing the algal oil production from crude glycerol. The optimal level of these two factors for oil yield were determined as 150 g/l and 0.25 1/h.

Under the optimal culture conditions, oil content in the algae biomass was more than 14%; with a yield of oil 4.14 g/l. Successful application of this process could have a significant impact on the biodiesel industry as it helps to solve the problem of waste glycerol disposal in the biodiesel industry, while simultaneously produces a valuable nutraceutical.

CHAPTER 6

CONCLUSIONS

In this study, the usability of waste crude glycerol from biodiesel processes and some important factors affecting the productivity and chemical composition of microalga, *Chlorella minutissima*, are investigated. From the results it was concluded that such a process is possible with a productivity values up to 7.42 g biomass/l/h and with a lipid content of 14.32 %.

Protein content of the resulting biomass was also in high percentages suggesting that the cake after oil extraction for fuel can be used for animal feed purposes. Processing waste glycerol by feeding it to microalgae in bioreactors can be a promising solution for biodiesel producers that are producing high amounts of waste crude glycerol. Microalgal fermentation is advantageous over photoautotrophic systems in terms of high productivity and easier downstream process handling due to high biomass concentrations achieved in bioreactors. This way, not only the waste glycerol is removed, also it can be converted into bio-oil to be used back in biodiesel industry and also resulting another valuable product as animal feed due to its high protein content.

REFERENCES

Acien Fernandez FG, Fernandez Sevilla JM, Sanchez Perez JA, Molina Grima E, Chisti Y. Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance. Chemical Engineering Science 2001;56(8):2721–32.

Andrade MR, Costa JAV. Mixotrophic cultivation of microalga Spirulina platensis using molasses as organic substrate. Aquaculture 2007;264(1–4): 130–4.

Becker EW. Microalgae. Cambridge: Cambridge University Press; 1994.

Brown LM. Uptake of carbon dioxide from flue gas by microalgae. Energy Conversion and Management 1996;37(6–8):1363–7

Borowitzka M. Microalgae for aquaculture: opportunities and constraints. Journal of Applied Phycology 1997;9(5):393–401.

Borowitzka MA. Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of Biotechnology 1999;70(1–3):313–21.

Borowitzka M. Algal biotechnology products and processes—matching science and economics. Journal of Applied Phycology 1992;4(3):267–79.

Carvalho AP, Meireles LA, Malcata FX. Microalgal reactors: a reviewof enclosed system designs and performances. Biotechnology Progress 2006;22(6):1490–506.

Chen G-Q, Chen F. Growing phototrophic cells without light. Biotechnology Letters 2006;28(9):607–16.

Chiu S-Y, Kao C-Y, Tsai M-T, Ong S-C, Chen C-H, Lin C-S. Lipid accumulation and CO2 utilization of Nanochloropsis oculata in response to CO2 aeration. Bioresource Technology 2009;100(2):833–8.

Çelekli A, Yavuzatmaca M, Bozkurt H. Modeling of biomass production by Spirulina platensis as function of phosphate concentrations and pH regimes. Bioresource Technology 2009;100(14):3625–9.

Chisti Y. Biodiesel frommicroalgae. Biotechnology Advances 2007;25(3):294–306.
Martin-Je´ze´quel V, Hildebrand M, Brzezinski MA. Silicon metabolism in diatoms: implications for growth. Journal of Phycology 2000;36(5):821–40.

Chisti Y. Biodiesel from microalgae beats bioethanol. Trends in Biotechnology 2008;26(3):126–31.

Chini Zittelli G, Rodolfi L, Biondi N, Tredici MR. Productivity and photosynthetic efficiency of outdoor cultures of Tetraselmis suecica in annular columns. Aquaculture 2006;261(3):932–43.

Chen F, Zhang Y, Guo S. Growth and phycocyanin formation of Spirulina platensis in photoheterotrophic culture. Biotechnology Letters 1996;18(5):603–8.

Camacho Rubio F, Acie´n Ferna´ndez FG, Sa´nchez Pe´ rez JA, Garcı´a Camacho F, Molina Grima E. Prediction of dissolved oxygen and carbon dioxide concentration profiles in tubular photobioreactors for microalgal culture. Biotechnology and Bioengineering 1999;62(1):71–86.

Carlozzi P. Dilution of solar radiation through ‘‘culture’’ lamination in photobioreactor rows facing south-north: a way to improve the efficiency of light utilization by cyanobacteria (Arthrospira platensis). Biotechnology and Bioengineering 2003;81(3):305–15.

Cheng-Wu Z, Zmora O, Kopel R, Richmond A. An industrial-size flat plate glass reactor for mass production of Nannochloropsis sp. (Eustigmatophyceae). Aquaculture 2001;195(1–2):35–49.

Converti A, Lodi A, Del Borghi A, Solisio C. Cultivation of Spirulina platensis in a combined airlift-tubular reactor system. Biochemical Engineering Journal 2006;32(1):13–8.

Chojnacka K, Noworyta A. Evaluation of Spirulina sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. Enzyme and Microbial Technology 2004;34(5):461–5.

De Swaaf ME, Sijtsma L, Pronk JC. High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga Crypthecodinium cohnii. Biotechnology and Bioengineering 2003;81(6):666–72.

De Swaaf ME, Pronk JT, Sijtsma L. Fed-batch cultivation of the docosahexaenoic- acid-producing marine alga Crypthecodinium cohnii on ethanol. Applied Microbiology and Biotechnology 2003;61(1):40–3.

Doucha J, Straka F, Lı´vansky´ K. Utilization of flue gas for cultivation of microalgae (Chlorella sp.) in an outdoor open thin-layer photobioreactor. Journal of Applied Phycology 2005;17(5):403–12.

Emma Huertas I, Colman B, Espie GS, Lubian LM. Active transport of CO_2 by three species of marine microalgae. Journal of Phycology 2000;36(2):314–20.

Eriksen N. Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. Applied Microbiology and Biotechnology 2008;80(1):1–14.

Eriksen N. The technology of microalgal culturing. Biotechnology Letters 2008;30(9):1525–36.

Garcia-Malea Lopez MC, Del Rio Sanchez E, Casas Lopez JL, Acie´n Ferna´ndez FG, Fernandez Sevilla JM, Rivas J, et al. Comparative analysis of the outdoor culture of

Haematococcus pluvialis in tubular and bubble column photobioreactors. Journal of Biotechnology 2006;123(3):329–42.

Graverholt O, Eriksen N. Heterotrophic high-cell-density fed-batch and continuous-flow cultures of Galdieria sulphuraria and production of phycocyanin. Applied Microbiology and Biotechnology 2007;77(1):69–75.

Graham LE, Graham JM, Wilcox LW. Algae, 2nd ed., San Francisco: Pearson Education, Inc.; 2009.

Hall DO, Acien Fernandez FG, Canizares Guerrero E, Krishna Rao K, Molina Grima E. Outdoor helical tubular photobioreactors for microalgal production: modeling of fluid-dynamics and mass transfer and assessment of biomass productivity. Biotechnology and Bioengineering 2003;82(1):62–73

Hsieh C-H, Wu W-T. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology 2009;100 (17):3921–6.

Huntley M, Redalje D. CO2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. Mitigation and Adaptation Strategies for Global Change 2007;12(4):573–608.

Hu Q, Kurano N, Kawachi M, Iwasaki I, Miyachi A. Ultrahigh-cell-density culture of amarine alga Chlorococcum littorale in a flat-plate photobioreactor. Applied Microbiology and Biotechnology 1998;46:655–62.

Janssen M, Tramper J, Mur LR, Wijffels RH. Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale-up, and future prospects. Biotechnology and Bioengineering 2003;81(2):193–210.

Jime´nez C, Cossı´o BR, Labella D, Xavier Niell F. The feasibility of industrial production of Spirulina (Arthrospira) in southern Spain. Aquaculture 2003;217(1–4):179–90.

Liang, Y., et al. Converting crude glycerol from yellow grease to lipids through yeast fermentation. Bioresource Technology (2010), doi: 10.1016/j.biortech.2010.04.061

Lee YK. Microalgal mass culture systems and methods: their limitation and potential. Journal of Applied Phycology 2001;13(4):307–15.

Li X, Xu H, Wu Q. Large-scale biodiesel production from microalga Chlorella protothecoides through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 2007;98(4):764–71.

Miao X, Wu Q. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 2006;97(6):841–6.

Muller-Feuga A, Le Gue´des R, Herve´ A, Durand P. Comparison of artificial light photobioreactors and other production systems using Porphyridium cruentum. Journal of Applied Phycology 1998;10(1):83–90.

Moreno J, Vargas MA´ , Rodrı´guez H, Rivas J, Guerrero MG. Outdoor cultivation of a nitrogen-fixing marine cyanobacterium, Anabaena sp. ATCC 33047. Biomolecular Engineering 2003;20(4–6):191–7.

Molina Grima E, Belarbi EH, Acie´n Ferna´ndez FG, Robles Medina A, Chisti Y Tubular photobioreactor design for algal cultures. Journal of Biotechnology 2001;92(2):113–31.

Olaizola M. Commercial production of astaxanthin from Haematococcus pluvialis using 25,000-liter outdoor photobioreactors. Journal of Applied Phycology 2000;12(3):499–506.Hydrodynamic aspects and Arthrospira growth in two outdoor tubular undulating row photobioreactors. Applied Microbiology and Biotechnology 2000;54(1):14–22.

Pistorins, A.M.A., DeGrip, W.J., Egorova-Zachernyuk, T.A., 2009. Monitoring of biomass composition from microbiological sources by means of FT-IR spectroscopy. Biotechnology and Bioengineering 103, 123–129.

Pulz O, Scheinbenbogan K. Photobioreactors: design and performance with respect to light energy input. Advances in Biochemical Engineering/Biotechnology 1998;59:123–52.

Pulz O. Photobioreactors: production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology 2001;57(3):287–93.

Qingyu Wu, Chanfang Gao, Yan Zhai. Application of sqeet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Applied Energy 87 (2010) 756 – 761

Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, et al. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 2008;102(1):100–12.

Richmond A. Microalgal biotechnology at the turn of the millennium: a personal view. Journal of Applied Phycology 2000;12(3–5):441–51.

Sato T, Usui S, Tsuchiya Y, Kondo Y. Invention of outdoor closed type photobioreactor for microalgae. Energy Conversion and Management 2006;47(6):791–9.

Samson R, Leduy A. Multistage continuous cultivation of blue-green alga Spirulina maxima in flat tank photobioreactors. Canadian Journal of Chemical Engineering 1985;63:105–12.

Setlik I, Veladimir S, Malek I. Dual purpose open circulation units for large scale culture of algae in temperate zones. I. Basic design considerations and scheme of a pilot plant. Algologie Studies (Trebon) 1970;1(11).

Suh IS, Lee SB. A light distribution model for an internally radiating photobioreactor. Biotechnology and Bioengineering 2003;82:180–9.

Suh IS, Lee CG. Photobioreactor engineering: design and performance. Biotechnology and Bioprocess Engineering 2003;8(6):313–21.

Tan HH. Algae-to-biodiesel at least five to 10 years away, Available from: http://www.energycurrent.com/index.php?id=3&storyid=14415;2008 [cited 12.01.09].

Terry KL, Raymond LP. System design for the autotrophic production of microalgae. Enzyme and Microbial Technology 1985;7(10):474–87.

Ugwu CU, Ogbonna J, Tanaka H. Improvement of mass transfer characteristics and productivities of inclined tubular photobioreactors by installation of internal static mixers. Applied Microbiology and Biotechnology 2002;58(5):600–7.

Ugwu CU, Aoyagi H, Uchiyama H. Photobioreactors for mass cultivation of algae. Bioresource Technology 2008;99(10):4021–8.

Wang B, Li Y, Wu N, Lan C. CO2 bio-mitigation using microalgae. Applied Microbiology and Biotechnology 2008;79(5):707–18.

Weissman JC, Tillett DM. Design and operation of outdoor microalgae test facility. In: Brown LM, Sprague S, editors. Aquatic species report; NREL/MP- 232-4174. National Renewable Energy Laboratory; 1992. p. 32–57.

Wu Z, Shi X. Optimization for high-density cultivation of heterotrophic Chlorella based on a hybrid neural network model. Letters in Applied Microbiology 2007;44(1):13–8.

Xiong W, Li X, Xiang J, Wu Q. High-density fermentation of microalga Chlorella protothecoides in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 2008;78(1):29–36.

Zhang XW, Zhang YM, Chen F. Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of Spirulina platensis. Process Biochemistry 1999;34(5): 477–81.

APPENDIX A

DRY WEIGHT CORRELATION for 550 nm

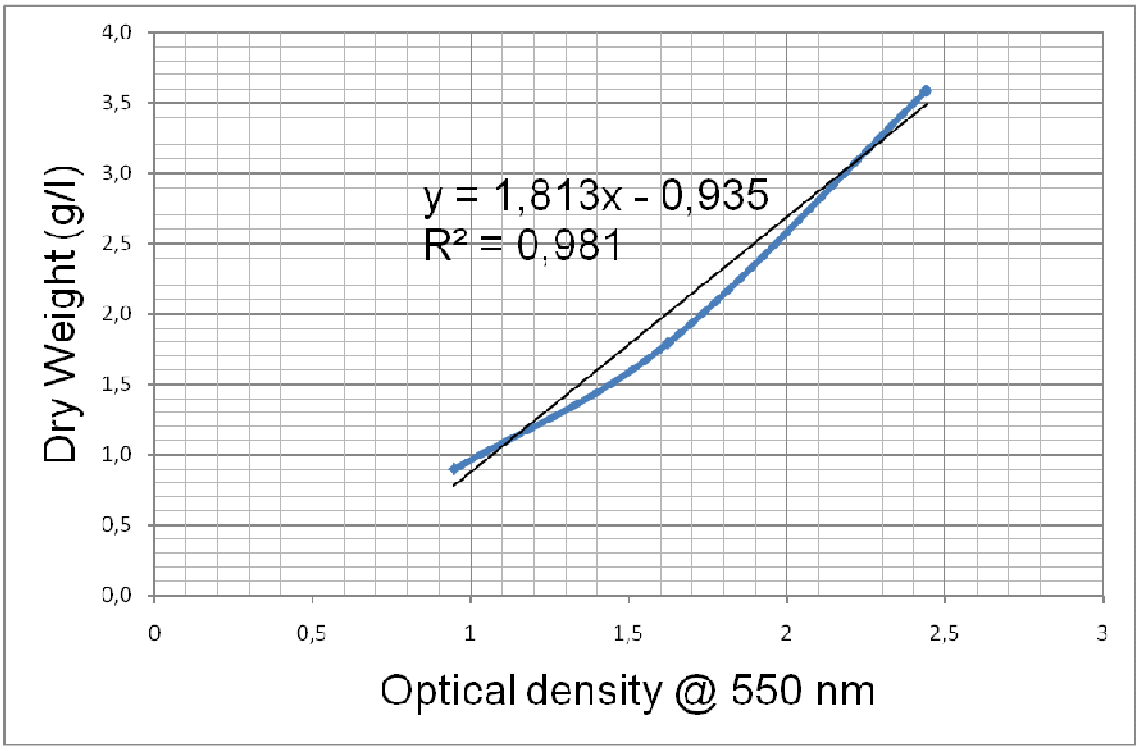

Figure A.1. Correlation curve for Dry weight (g/l) versus optical density at 550 nm

APPENDIX B

FTIR SPECTRAS for SAMPLES

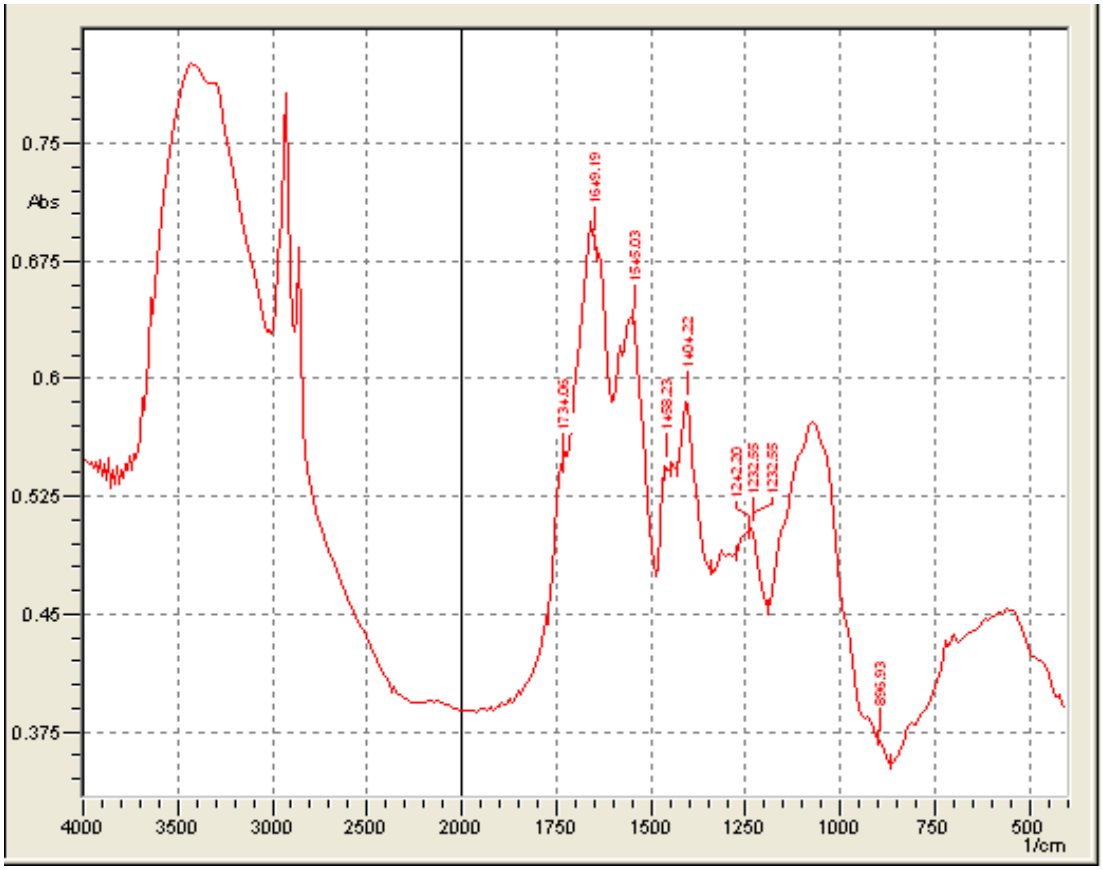

Figure B.1. FTIR Spectra for run #1

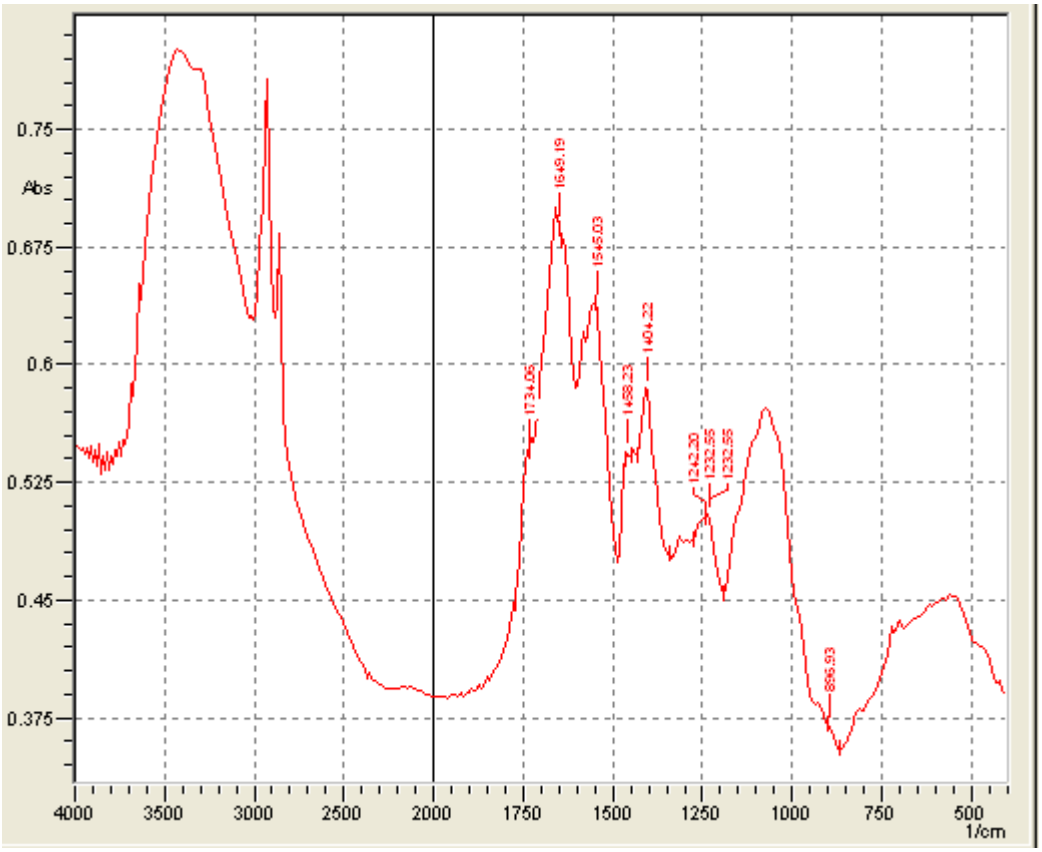

Figure B.2. FTIR Spectra for run #2

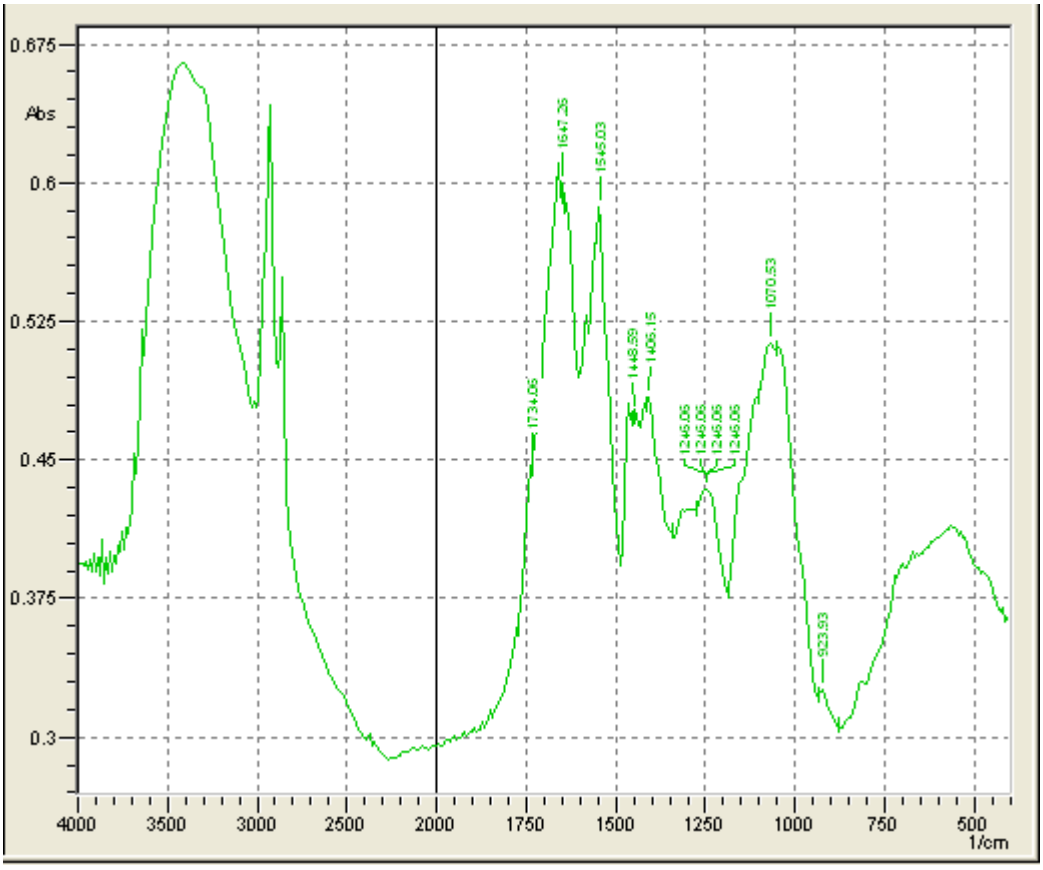

Figure B.3. FTIR Spectra for run #3

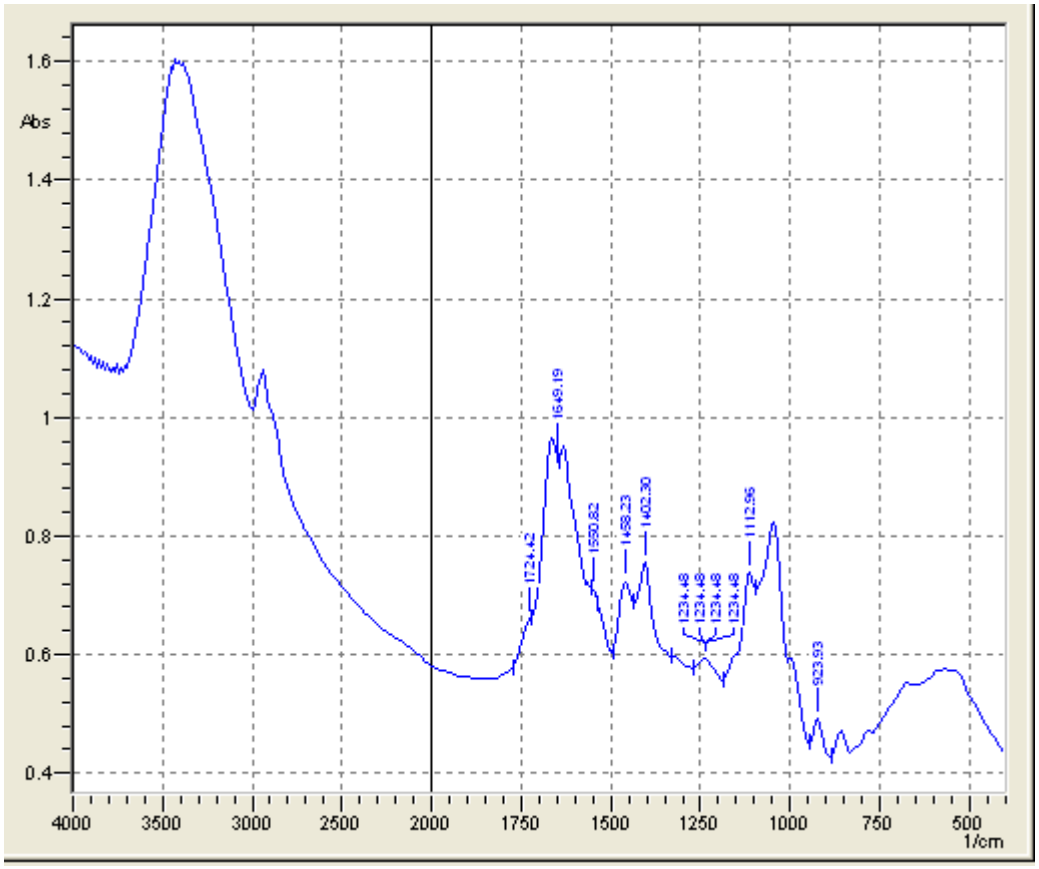

Figure B.4. FTIR Spectra for run #4

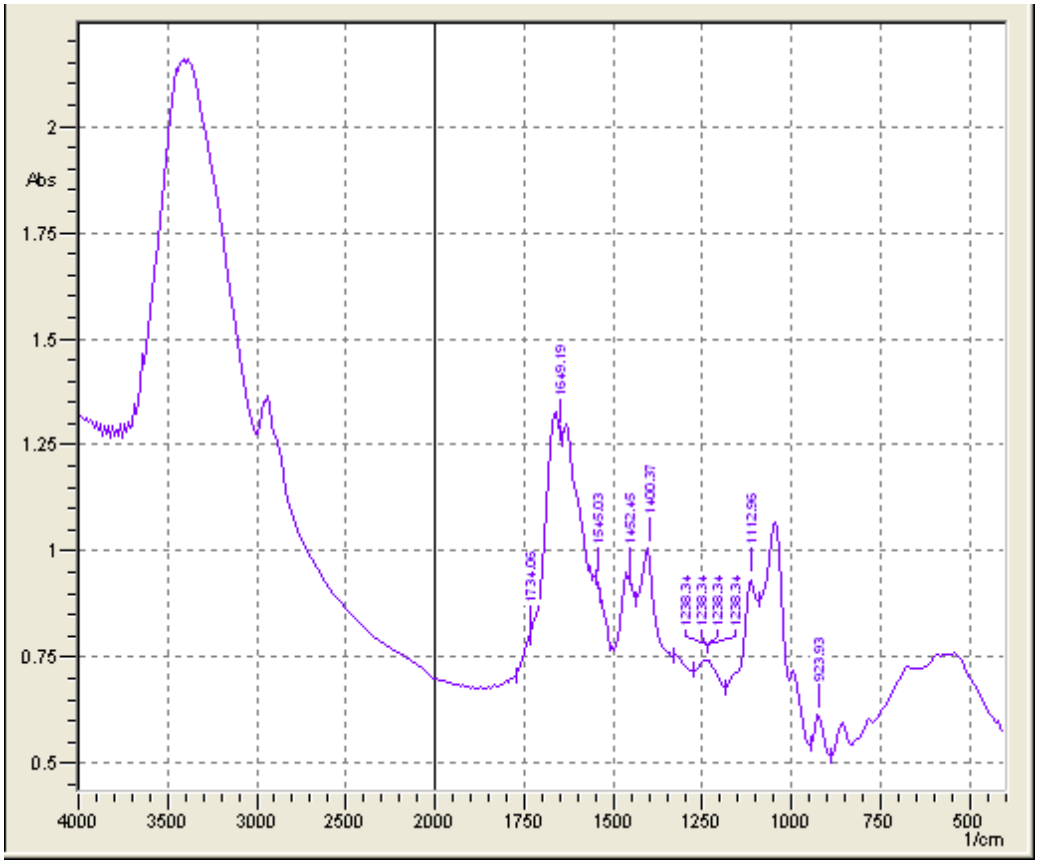

Figure B.5. FTIR Spectra for run #5

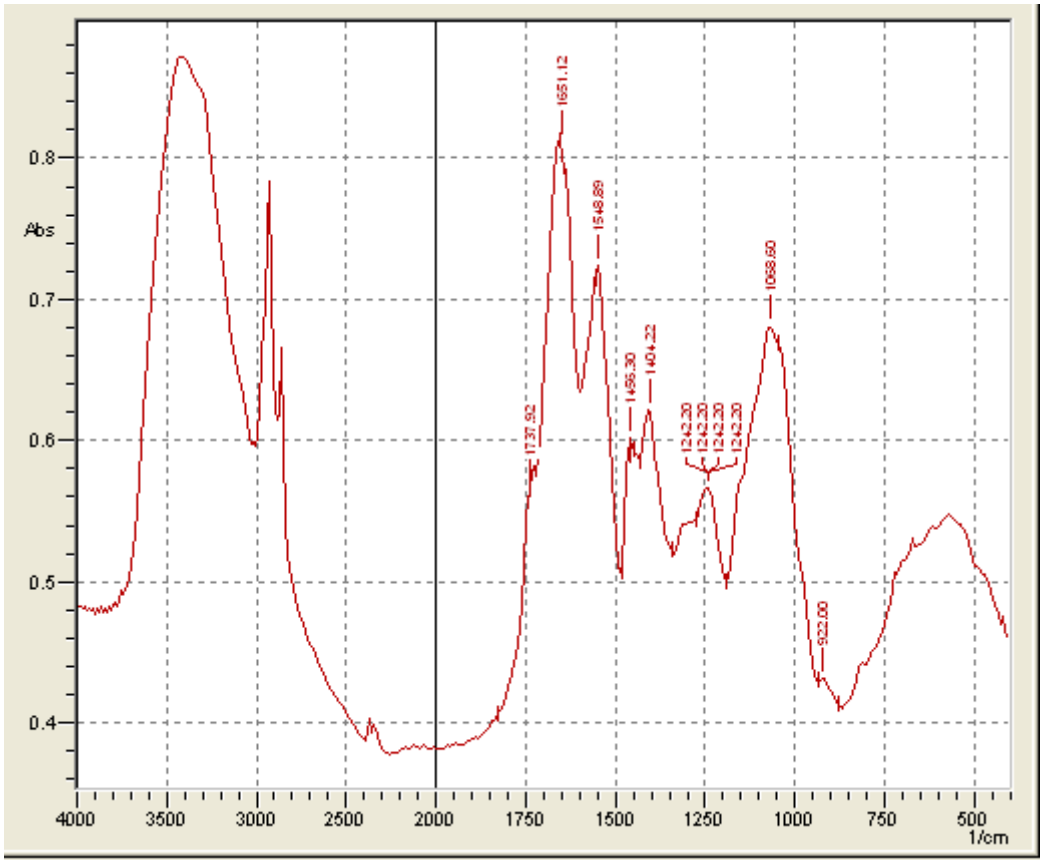

Figure B.6. FTIR Spectra for run #6

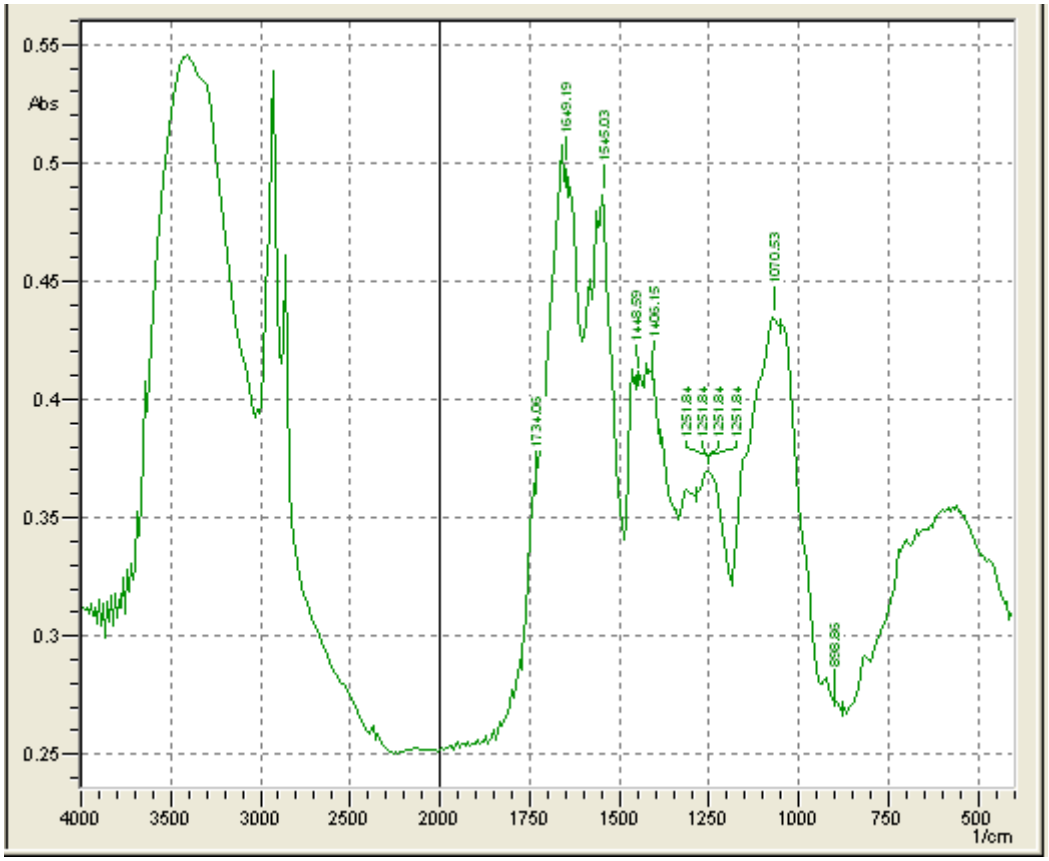

Figure B.7. FTIR Spectra for run #7

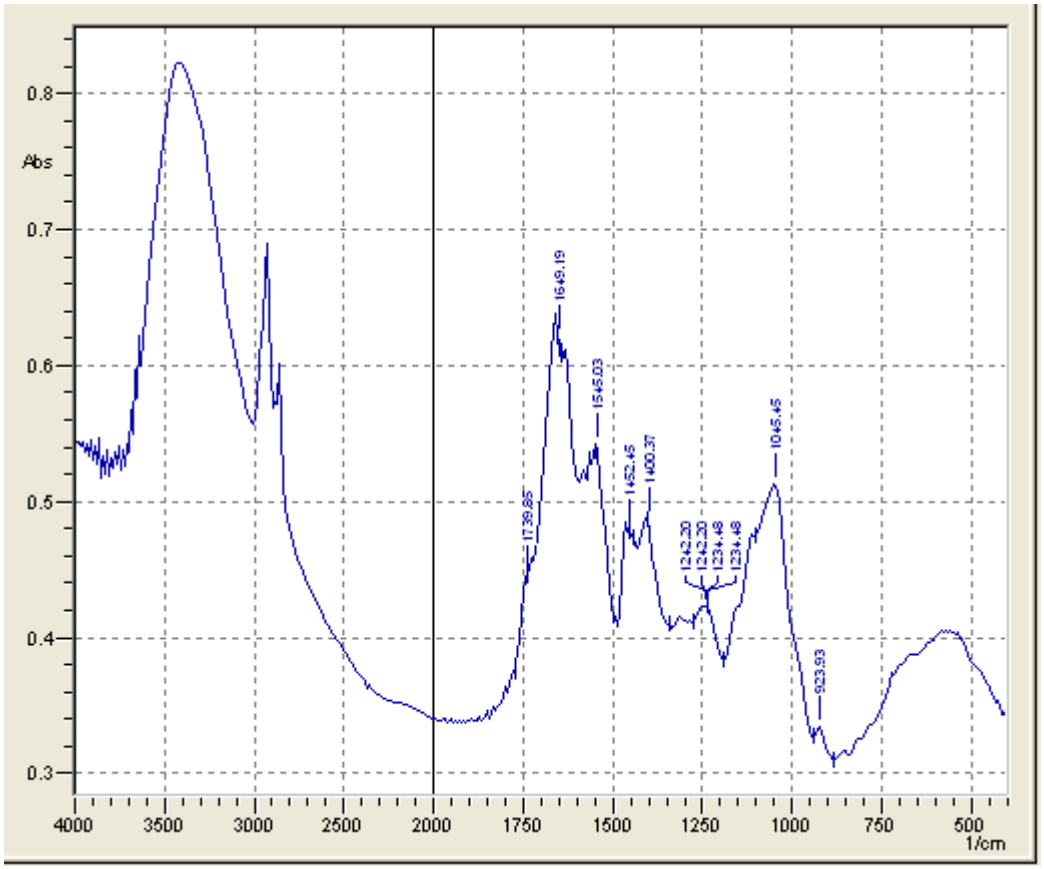

Figure B.8. FTIR Spectra for run #8

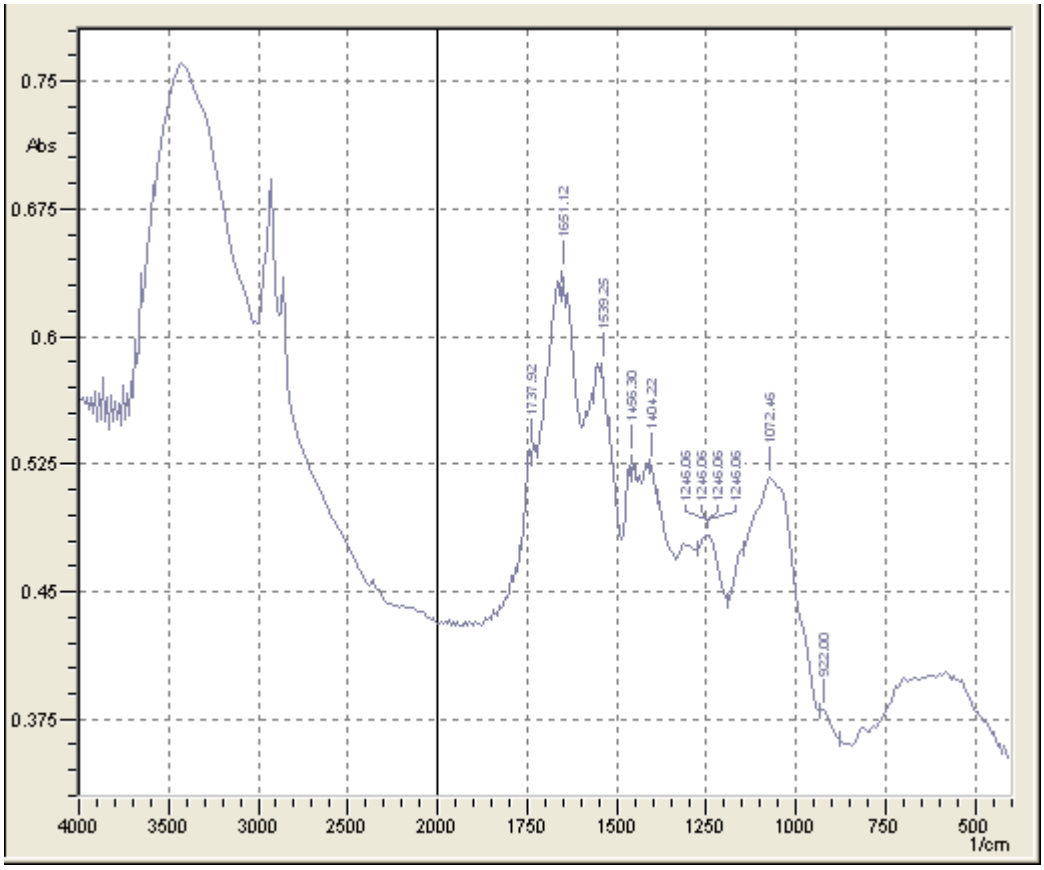

Figure B.9. FTIR Spectra for run #9

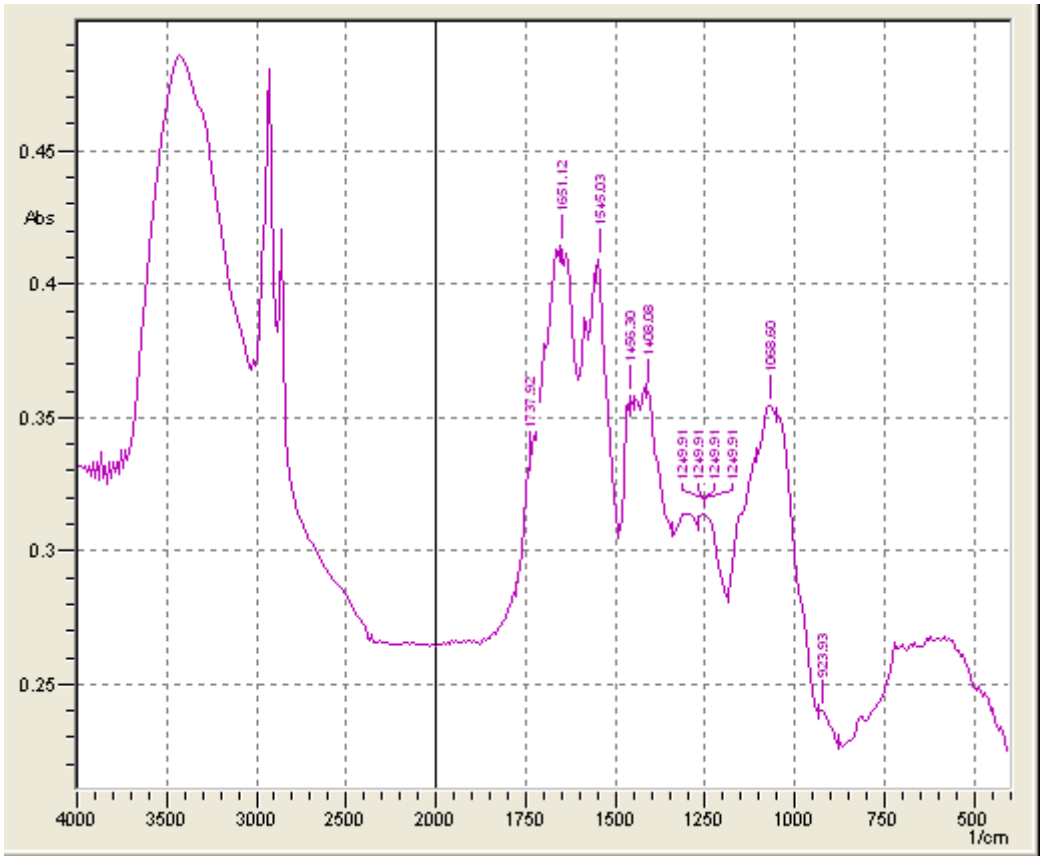

Figure B.10. FTIR Spectra for run #10

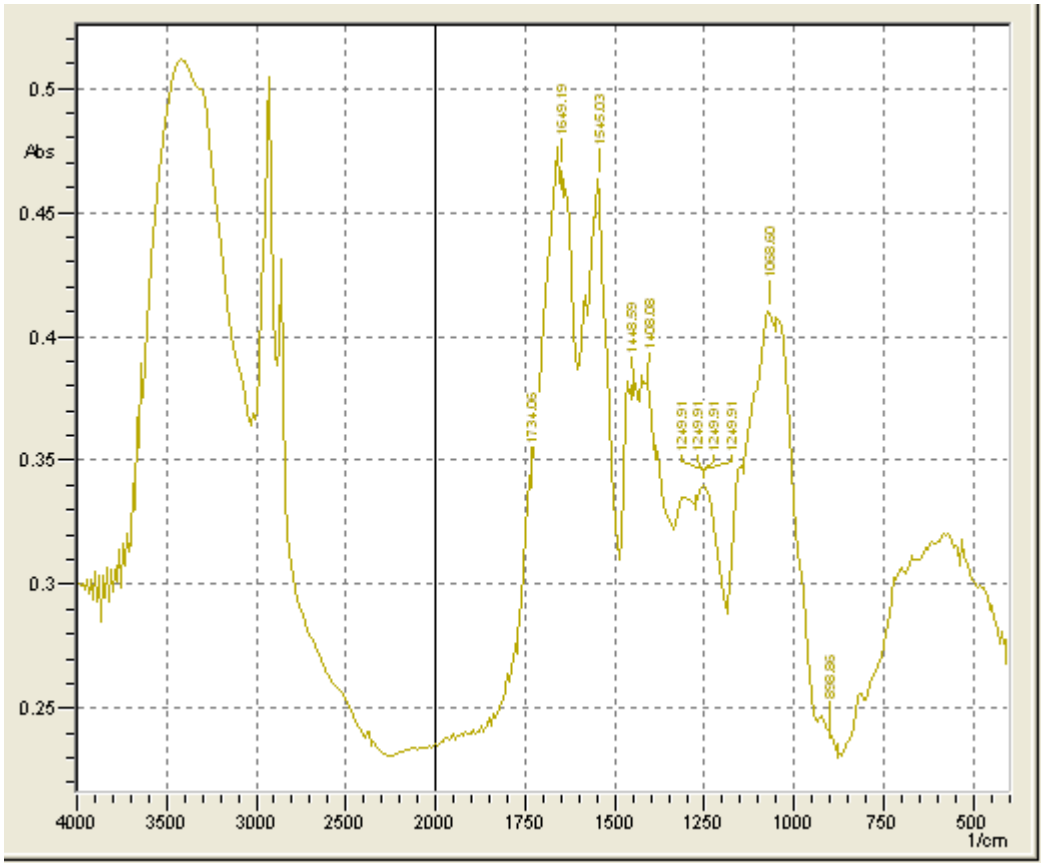

Figure B.11. FTIR Spectra for run #11

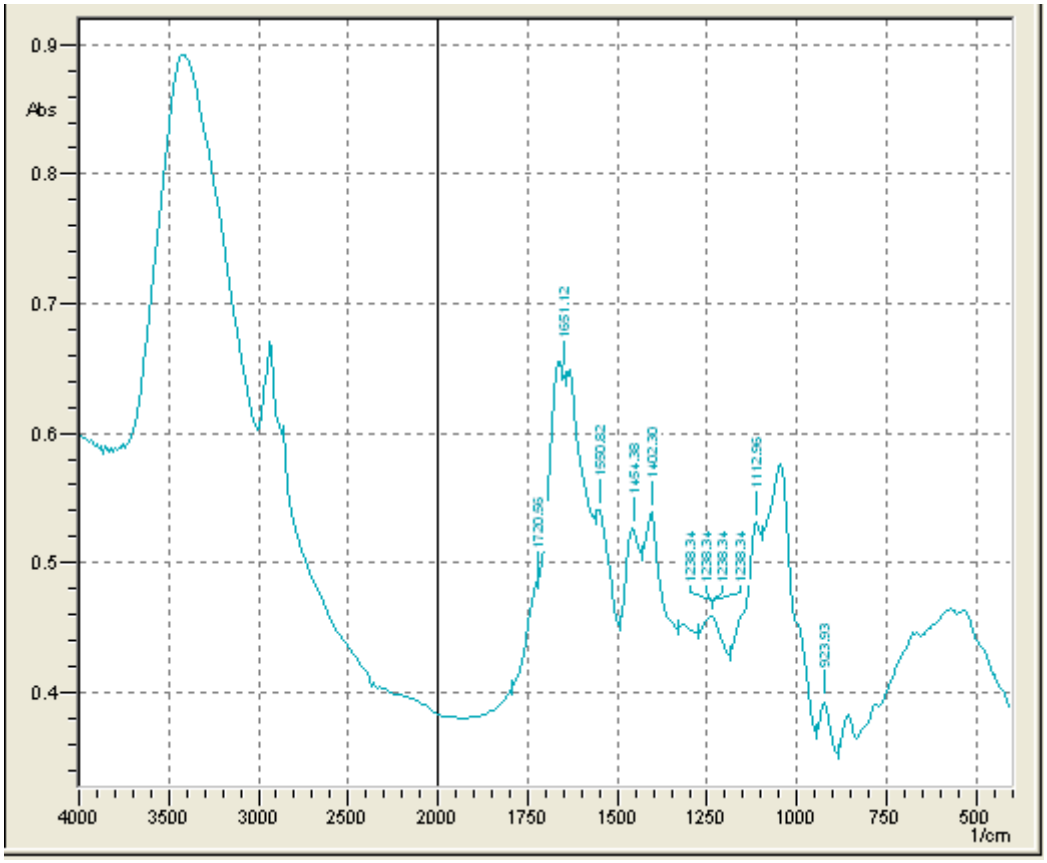

Figure B.12. FTIR Spectra for run #12

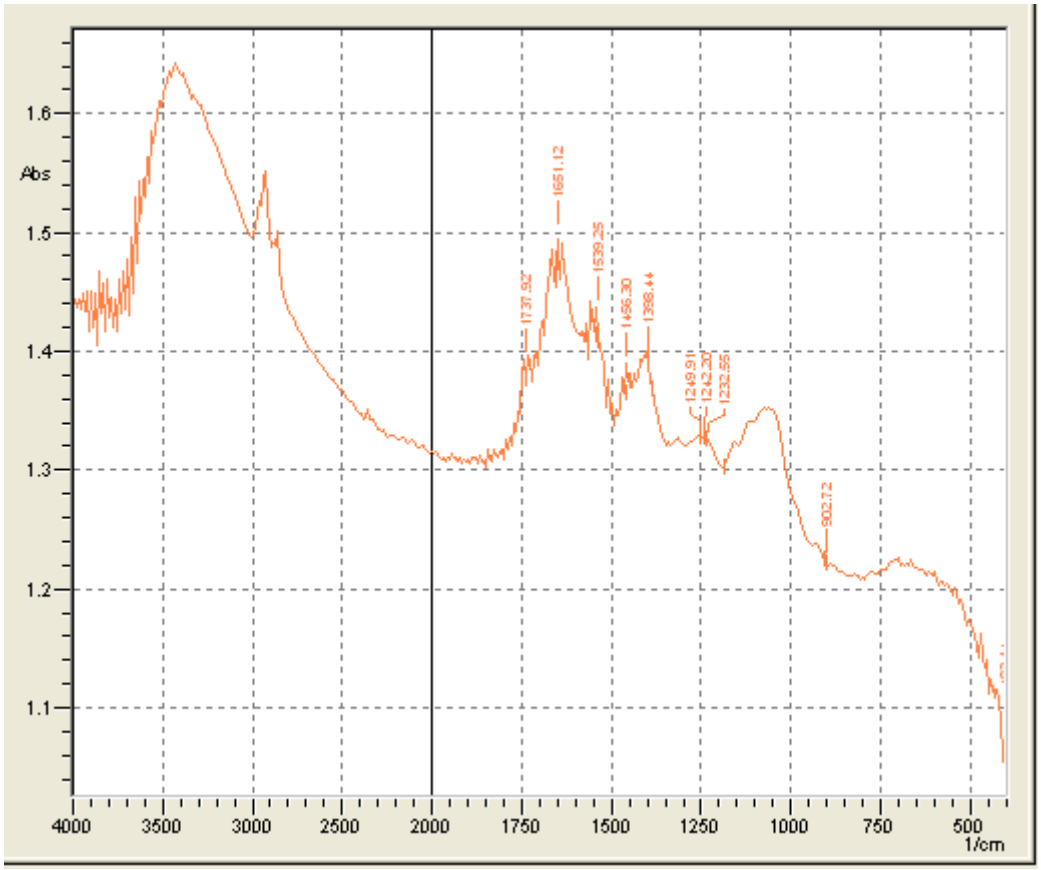

Figure B.13. FTIR Spectra for run #13

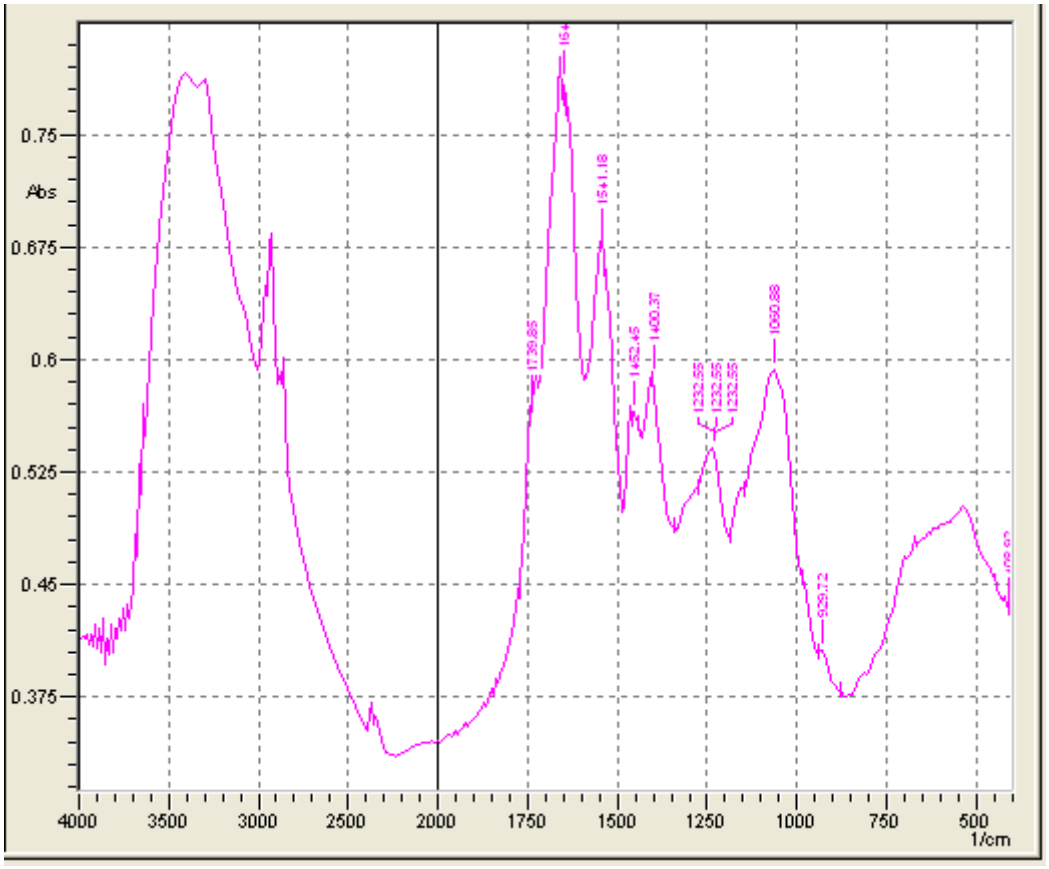

Figure B.14. FTIR Spectra for run #14

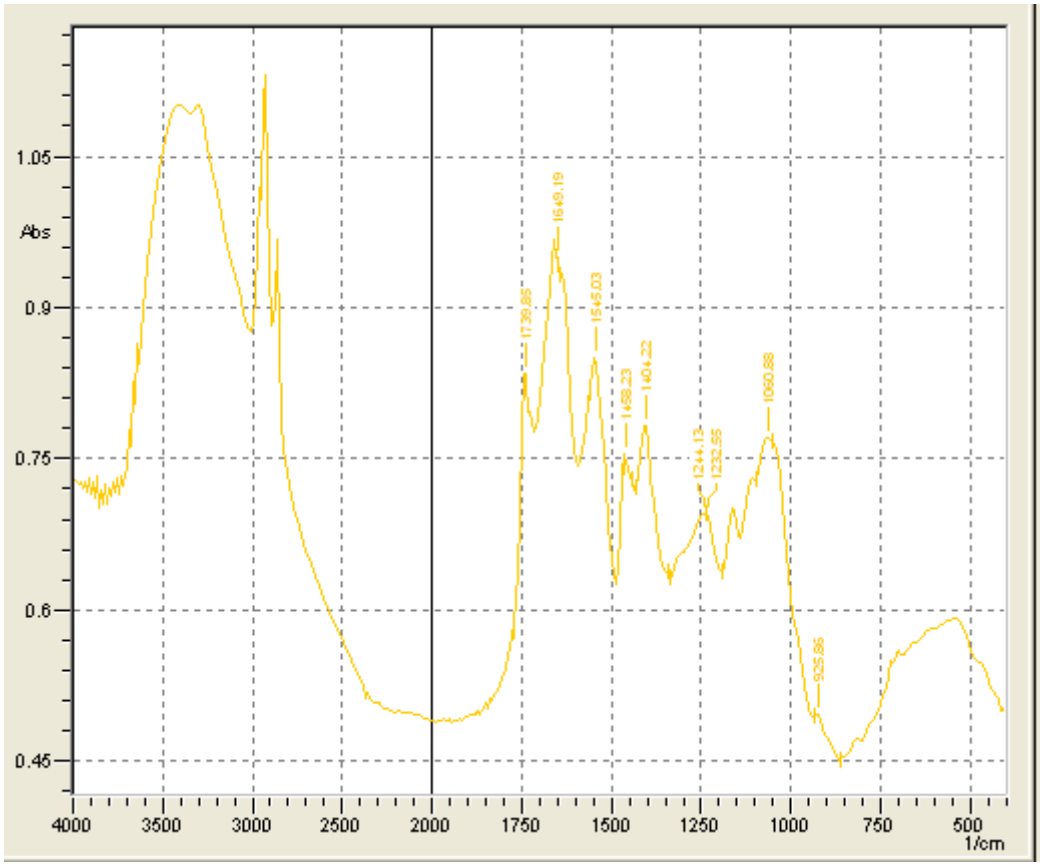

Figure B.15. FTIR Spectra for run #15