Microorganism Utilization for Synthetic Milk Production

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A desired architecture for long duration spaceflight, such as aboard the International Space Station (ISS) or for future missions to Mars, is to provide a supply of fresh food crops for the astronauts. However, some crops can create a high proportion of inedible plant waste. The main goal of this project was to produce the components of milk (sugar, lipid, protein) from inedible plant waste by utilizing microorganisms (fungi, yeast, bacteria). Of particular interest was utilizing the valuable polysaccharide, cellulose, found in plant waste, to naturally fuel- through microorganism cellular metabolism- the creation of sugar (glucose), lipid (milk fat), and protein (casein) to produce a synthetic edible food product. Environmental conditions such as pH, temperature, carbon source, aeration, and choice microorganisms were optimized in the laboratory and the desired end-products, sugars and lipids, were analyzed. *Trichoderma reesei*, a known cellulolytic fungus, was utilized to drive the production of glucose, with the intent that the produced glucose would serve as the carbon source for milk fat production and be a substitute for the milk sugar lactose. Lipid production would be carried out by *Rhodosporidium toruloides*, yeast known to accumulate those lipids that are typically found in milk fat. Results showed that glucose and total lipid content were below what was expected during this phase of experimentation. In addition, individual analysis of six fatty acids revealed that the percentage of each fatty acid was lower than naturally produced bovine milk. Overall, this research indicates that microorganisms could be utilized to breakdown inedible solid waste to produce useable products.

Nomenclature

ARC	=	Ames Research Center
ASE	=	Accelerated solvent extraction
ATCC	=	American Type Culture Collection
BSL	=	Biosafety Level
CDW	=	Cell Dry Weight
FB	=	Fed Batch

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FPU   = Filter Paper Unit
FAME   = Fatty Acid Methyl Ester
GC   = Gas Chromatography
GMO   = Genetically Modified Organism
GO   = Glucose Oxidase Assay
HPLC   = High Performance Liquid Chromatography
ISS   = International Space Station
mg   = milligram
mL   = milliliter
nm   = nanometer
PDA   = Potato Dextrose Agar
RB   = Rhodosporidium Batch Culture
USDA   = United States Department of Agriculture
YPD   = Yeast Peptone Dextrose Agar

I.   Introduction

The overarching vision for this project was to explore the use of biological agents- bacteria, fungi, and yeast- as synthetic food processors to produce the consumable animal product, milk. The main components of bovine milk include casein (milk protein, ~3.2%), lactose (milk sugar, ~4.8%), and butterfat (milk fat, ~3.9%). This project focuses on the production and analysis of milk sugar and milk fat. However, instead of trying to produce lactose, the monosaccharide glucose was pursued. Glucose would serve as a suitable fuel source for a variety of microorganisms which would be applicable for a future multi-stage milk reactor system, it would also be a suitable substitute for the disaccharide lactose, and would be of benefit to those with a lactose intolerance. With a multi-stage milk reactor system (Fig. 1) in mind for the future, this project would best fit in an aerospace architecture that depends on the growth of crop plants such as wheat, soybeans, potatoes, and other edible agriculture to supply fresh crop food for a space mission crew. The inedible crop portions could serve as an energy resource for a variety of microorganisms to produce other useful products. Of particular interest for this project are inedible residues containing plant polysaccharides, such as cellulose, which could be utilized as fuel for monosaccharide and disaccharide production. Once monosaccharides like glucose are produced, they may serve as the energy source for additional microbes which could fuel the creation of the other components of milk (milk fat and casein protein).

The first goal of this project (later referred to as stage 1) was to utilize fungi that possess cellulytic metabolic pathways to produce glucose from cellulose which could fuel future reactor stages, as well as substitute the lactose component of milk. In order to produce glucose (a monosaccharide) from cellulose (a polysaccharide), specific enzymes, like cellulase, are required to cleave the glycosidic linkages which maintain the polysaccharide (cellulose) structure. Cellulase, a group of enzymes having endo- and exoglucanase functions, as well as β-glucosidase activity is needed to convert cellulose to useable products. Endoglucanase binds to and hydrolyzes internal glycosidic bonds, creating many oligosaccharides, whereas exoglucanases cleave the ends of all oligosaccharides creating shorter molecules such as cellobiose (2-glucose units). The third enzyme in the group, β-glucosidase breaks the glycosidic bond of cellobiose and yields 2 glucose molecules. [1,2]

Many of cellulolytic microbes (microbes that can digest cellulose) studied are utilized in bioethanol production and are focused on one-step consolidated bioprocessing that integrates cellulase production, cellulose hydrolysis, and product fermentation into a single step. These approaches are not useful for our purposes because this project would require a cessation of cellulose hydrolysis and then recovery of the glucose in order to utilize the produced glucose in other stages, or as the milk sugar component. The fungus, *Trichoderma reesei,* was an appealing organism to use for stage 1 for several reasons. First, it is a Bio Safety Level (BSL) I organism, meaning it is a well-known organism that does not usually cause disease in healthy adults, which is ideal for the production of a human consumable. Second, it is a thoroughly studied organism and is known for its ability to convert native and derived cellulose into glucose.[3] In
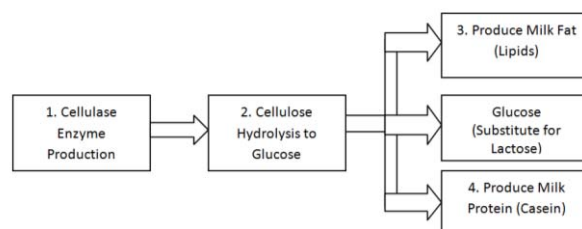
Figure 1. Overarching project vision. *This illustrates the flow of a future multi-stage milk reactor, which inspired fundamental research into growth parameters of possible microorganisms.*

addition to possessing cellulase, *T. reesei,* is capable of producing a number of polysaccharide hydrolyzing enzymes such as xylanase, xylobiosidase, and others which help digest other polysaccharides commonly found in plant material. The variety of polysaccharide hydrolyzing enzymes will allow for the creation and buildup of valuable monosaccharides that can be used in a future reactor system and as the main milk sugar component. Stage 1 experiments with *T. reesei* included; time-course batch studies, which focused on assessing environmental conditions (shaken versus static and cellulose concentration) influence on glucose production; and time-course fed-batch studies, which also focused on assessing environmental conditions' (pH, feed amount, cellulose concentration) influence on glucose production. Future research would need to continue with fed-batch experimentation, in particular the methodology, to expand into glucose extraction and reactor scale studies.

The second goal of this project (later referred to as stage 2) was the production of milk lipids through oleaginous yeasts. Oleaginous yeasts are well known for their ability to accumulate upwards of 80% of their dry weight as lipids.[4,5] Milk lipids are the fatty portion of milk and make up 33 g total lipid/L in bovine milk.[6] Milk fat is composed of a variety of lipids, with the largest proportion existing as triacylglycerides (95-98%) which are derived from three molecules of fatty acid esterified to a glycerol molecule.[7] Over 400 different fatty acids can be found in milk, but only 30 are found in concentrations above 0.1% and their composition varies by species.[7] In order to focus our studies, we only monitored fatty acids found in the largest quantities of bovine milk. These fatty acids are both saturated and unsaturated and include oleic acid (~24%), palmitic acid (~24%), stearic acid (~11%), myristic acid (~9%), linoleic acid (~3.6%), and linolenic acid (~2.3%).[6,7,8] In addition to being the main components of milk fat, many of these lipids have been shown to have positive health effects like preventing coronary heart disease, decreasing low-density lipoprotein (LDL) cholesterol (known as 'bad' cholesterol), increasing high-density lipoprotein (HDL) cholesterol (known as 'good' cholesterol), and serving as a good source for omega-6 and omega-3 fatty acid generation.[6,7]

While reviewing the literature, many references found were focused on the production of biodiesel from cellulosic biomass utilizing oleaginous yeasts. Fortunately, many lipids found in biodiesel are also found in milk fat, and thus many cross references can be made pertaining to their generation. Among the candidate yeasts reviewed, Rhodosporidium *toruloides* was utilized for this project. *Rhodosporidium toruloides* is also a BSL I organism and has documented ability to accumulate up to 70% of their dry weight as lipid. Time-course batch culture studies with the oleaginous yeast were the primary focus of stage 2 testing to assess various environmental conditions like pH and media composition. Future research would expand into fed-batch and reactor scale studies, as well as lipid extraction techniques for use in simulant milk.

The success of this fundamental research may lead to a multi-stage milk reactor system. Additionally, it could lead to other avenues for researching high value synthetic animal products produced by microorganisms. Other possibilities include egg ovalbumin, dietary proteins, high-value fats, and useful carbohydrates like starches and glycogen. Additionally, other space generated waste, both solid and liquid, could be adapted for use in synthetic food processors and would provide the benefit of reducing solid waste in space.

II. Materials and Methods

A. Stage 1: Glucose Production

First stage experiments with the fungi T*richoderma reesei,* strain QM 9414 (ATCC 26921), obtained from the USDA ARC culture collection, and strain Rut C30, (ATCC 56765) obtained from American Type Culture Collection (ATCC) were chosen for their improved cellulase enzyme production and activity. Strain maintenance and inoculum development have followed the procedures and protocols suggested in Parton and Willis (1990).[10] Strains were cultured in Mandel's media (Appendix A) supplemented with Avicel (Sigma), a cellulose compound, and incubated at 30°C with an orbital shaker rotatory rate of 125 rpm.

Initial experimentation included time-course batch studies which focused on assessing the influence of environmental conditions such as shaken versus static culturing and cellulose concentration (1, 2, 4, and 8%) on glucose production. Cellulase activity and culture pH were measured to determine when in the batch cultivation cycle (lag, exponential, stationary) that growth and cellulase enzyme production occurred. The initial glucose production was measured using the 'filter paper unit' assay, which was later replaced with a more direct measure using high-performance liquid chromatography (HPLC) (Agilent 1100). Time-course, fed-batch studies also focused on assessing environmental conditions including pH, feed amount, and cellulose concentration's influence on glucose production (Table 1).

Table 1. Experimental conditions for fed batch studies of *Trichoderma reesei* QM 9414 (ATCC 26921) and Rut C30 (ATCC 56765). *All cultures started in Mandel's media with 8% Avicel; incubated at 30 °C, shaking at 125 rpm. All "b" cultures were incubated at 50 °C and shaken at 140 rpm. All cultures were performed in duplicate.*

Experiment	Experimental Conditions	Duration(d)
FB 1*	2% or 8% Avicel feed; no additional feed, no pH control	25
FB 2	fed 8% Avicel as replacement volume	23
FB 3	Replaced sampled volume every 7 days with 8% Avicel	21
FB 4	No additional feed, pH controlled D1-7	14
FB 4b	8% Avicel added to FB 4 supernatant	7
FB 5	2% Avicel daily feed; pH controlled D1-7	14
FB 5b	8% Avicel added to FB 5 supernatant	7
FB 6	No daily feed; pH controlled D1-7	7
FB 6b	8% Avicel added to FB 6 supernatant	7
FB 7	2% daily feed; pH controlled D1-7	7
FB 7b	8% Avicel added to FB 7 supernatant	7
FB 8	2% Avicel daily feed; no pH control	7
FB 8b	8% Avicel added to FB 8 supernatant	7
FB 9	0% Avicel starting concentration, no pH control	14
FB 10	Temperature changed to 26 °C at D3, no feed, no pH control	14

Fed-batch studies, the "b" cultures for FB4 through FB8, were a means to confirm enzyme production and cellulose hydrolysis. The liquid portion of each culture was removed from each first stage culture at day 7, transferred to a new flask, and additional cellulose added. The incubation temperature during this second phase was 50 °C, which prevented any remaining mycelia of *T. reesei* from utilizing glucose and cellobiose, the desired cellulase enzymatic hydrolysis products. However, the cellulase enzymes present in the first stage liquid would continue to break down the cellulose and produce cellobiose and ultimately glucose. All standards and samples were diluted in water and filtered through a Pall 0.45 micron filter. Glucose and cellobiose were detected over time using the Agilent 1100 HPLC with an Aminex HPX-87H ion exclusion column (Bio Rad) (mobile phase, 4mM sulfuric acid; flow rate at 0.3 mL/m; 15°C; 10μL injection volume). Future research would need to continue with fed-batch experimentation, including methodology pertaining to cellulase isolation and/or *T. reesei* inactivation (i.e. not separating cultures into different flasks), as well as additiona feed parameters, then followed by expansion into glucose extraction and reactor scale studies.

B. Stage 2: Lipid Production

1. Culture Preparation

Second stage experiments with the yeast *Rhodosporidium toruloides,* strain Y-1091 (ATCC 10788), obtained from the USDA ARC culture collection was utilized for its ability to accumulate 76.1% of its dry weight as lipid.[5] *R. toruloides* was maintained on YPD agar (BD Difico) and re-streaked on new media every two weeks. As detailed by Li et al. (2007), sterile minimal media (Appendix A) in 1000 mL volumes was utilized for culturing *R. toruloides*. This minimal media was supplemented with 90g/L glucose, determined by the results described by Li et al. (2007). Their results demonstrated glucose concentrations between 10g/L and 150g/L produced similar optimum growth rates and lipid yields, with 90g/L glucose producing the highest amount of lipid.[5] After the first batch study, a transition from 90g/L glucose to 60g/L glucose occurred in response to research suggesting the carbon-to-nitrogen ratio (C/N) critically affected the potential of lipid production in oleaginous yeasts.[5,10] For similar reasons, the minimal media was modified (referenced as 'modified minimal medium' for the remainder of the report) for some experiments to include 1.5g of ammonium sulfate, instead of the original 12.0g of ammonium sulfate.[5,10]

2. Experimental Conditions

Other environmental parameters suggested by Li et al. (2007) included orbital shaker rotary rate of 200 rpm, 30°C temperature, and Erlenmeyer flasks used for culturing. Samples were taken at different time intervals and the absorbance measured at 600 nm for optical density comparisons.[5] In addition, samples were taken at different time intervals for pH measurements and lipid composition analysis. Some experiments were also subjected to pH control with 5N ammonium hydroxide to maintain a pH between 5-5.5. Glucose concentration was monitored over time utilizing the HPLC method previously described.

3. Lipid Extraction and Analysis

Lipid composition analysis occurred in three stages which included (1) freeze drying, (2) lipid extraction, and (3) trans-esterification/methylation.

Yeast material was freeze-dried and ground into a fine powder. Milligram weights of powdered yeast were weighed and then sandwiched between two layers of Ottawa Sand (PNS23-3, Fisher Scientific) within a 5 mL ASE extraction cell. Extractions were completed with an Accelerated Solvent Extractor (ASE 350, Thermo Fisher, Sunnyvale, CA). The yeast samples were subjected to five static extraction cycles of 8 minutes, at 1500 psi, and 80°C, utilizing a 2:1 chloroform/methanol mixture as the extraction solvent. The extract was filtered with a cellulose filter and a stainless steel frit at the extraction cell outlet, and collected in a 60 mL amber glass vial and weighed again. Samples were frozen in -80°C until trans esterification/methylation could occur.

Samples were thawed, resuspended in 1.5 mL of chloroform, and vortexed for trans esterification/methylation processing. Once mixed, 250 µL samples were transferred to a new amber vial containing 25 µL of internal standard (glyceryl triheptadecanoate); 675 µL of 3N MeOH/HCl was added to each amber vial incubated at 60°C for approximately 15 hours. After incubation, samples were cooled to room temperature, and 350 µL of 6% potassium carbonate was added, followed by 300 µL of hexane with 0.01% BHT; the mixture was then briefly vortexed and placed on ice. Samples were centrifuged at 1100 rpm for 10 minutes in rotors previously stored at -80°C. The aqueous layer was removed and 250µL of the hexane layer containing the lipids was transferred to a new amber vial.

Fatty acids were then analyzed with a Trace Ultra gas chromatograph (GC) (Thermo, Waltham, MA) fitted with a 30m x 0.25mm x 0.25µm DB-225ms column (Agilent Technologies). The GC parameters were as follows: inlet: split/splitless with a 100:1 split ratio set at 200°C; oven: 30°C initial temperature holding for 1 min. 8°C/min to 165°C, then 3°C/min to 200°C, ending with 10°C/min up to 235°C and holding for 2 minutes; detector: FID set at 240°C. Helium was the carrier gas set at 2.9ml/min. A 1µl injection of the extracted sample was used for quantification, and individual fatty acid methyl esters were identified and quantified through comparison to analytical standards. Glyceryl triheptadecanoate was used as an internal standard. This process allowed for determination of total lipid recovery (percentage) of the dry cell weight, and quantification of the fatty acids of interest; oleic acid, palmitic acid, stearic acid, myristic acid, linoleic acid, and linolenic acid.

For the remainder of the report all *Rhodosporidium toruloides* batch studies will be referred to as RB. A description of the varying environmental conditions for RB experimentation can be found in Table 2.

Table 2. Experimental conditions for lipid production in *Rhodosporidium toruloides* (RB1-RB6). *All cultures were grown as batch cultures in minimal media or modified minimal media, supplemented with glucose, and incubated at 30 °C and rotary rate of 200 rpm.*

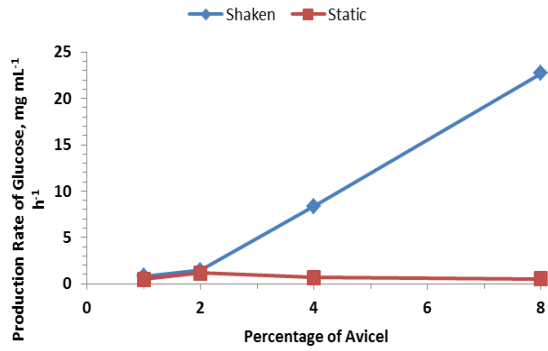| Experiment	Experimental Conditions	Duration (d)
RB 1	90 g/L glucose in minimal media; no pH control	14
RB 2	60 g/L glucose in modified minimal media; pH control D7-14	21
RB 3	60 g/L glucose in minimal media; pH control D1-7	14
RB 4	60 g/L glucose in modified minimal media; pH control D1-7	14
RB 5	60 g/L glucose in minimal media; no pH control	14
RB 6	60 g/L glucose in modified minimal media; no pH control	14

Figure 2. **Cellulase Activity in Shaken and Static Cultures with varying concentrations of cellulose.** *Cellulase enzyme activity in Trichoderma reesei QM 9414 in cultures shaken at 125 rpm (blue diamond) versus static cultures (red square) as determined by the filter paper unit assay (FPU). Percent Avicel refers to the concentration of Avicel in Mandel's medium.*

(A)

(B)

Figure 3. Time course study of pH variation in static and shaken batch cultures. *Cultures of Trichoderma reesei QM9414, in (A) static and (B) shaken flasks. All cultures were grown in Mandel's media with varying cellulose (Avicel) concentration at 30 ˚C for 14 days.*

III.   Results and Discussion

A. Stage 1: Glucose Production

Initial experimentation was a batch, time-course determination of microbial growth and cellulase production under two factors: (1) effects of shaking (125 rpm) versus static (not shaken) incubation conditions and (2) the concentration of cellulose substrate, 10, 20, 40, 80 g L^{-1} Avicel® (n=2). Cellulase activity, measured indirectly using the filter paper unit assay, and culture pH were measured to determine when in the batch cultivation the cellulase enzyme production occurred. Shaken cultures at Avicel concentrations of 4% and 8% produced higher cellulase activity than static cultures, with the 8% Avicel cultures producing the highest cellulase activity (Fig. 2). The increase in the cellulase activity began at day 2 of the incubation. These findings were similar to those determined by Hendy et al. (1984), whose work with *T. reesei* Rut-C30 showed that cultures with a higher concentration of cellulose (10%) produced an increase in cellulase activity.[11] This increase however, occurred after 4 days of incubation. Cultures growing at a lower concentration of cellulose showed an increase in activity at day 1 or 2. Estebauer et al, (1991) determined that enzyme concentration was dependent upon the cellulose or carbon concentration and produced the highest cellulose activity at 8%.[12] The majority of the cultures for this stage of the experimentation were grown at 8% cellulose as 8% had a higher cellulase activity and the increase in the desired product began at day 2 of the cultivation.
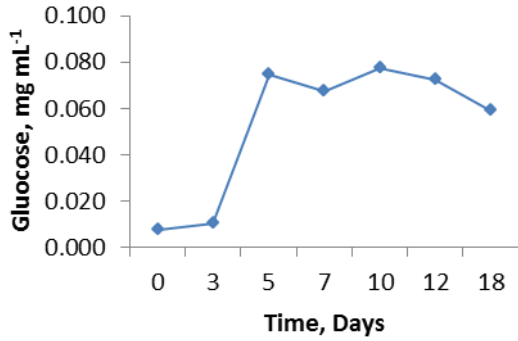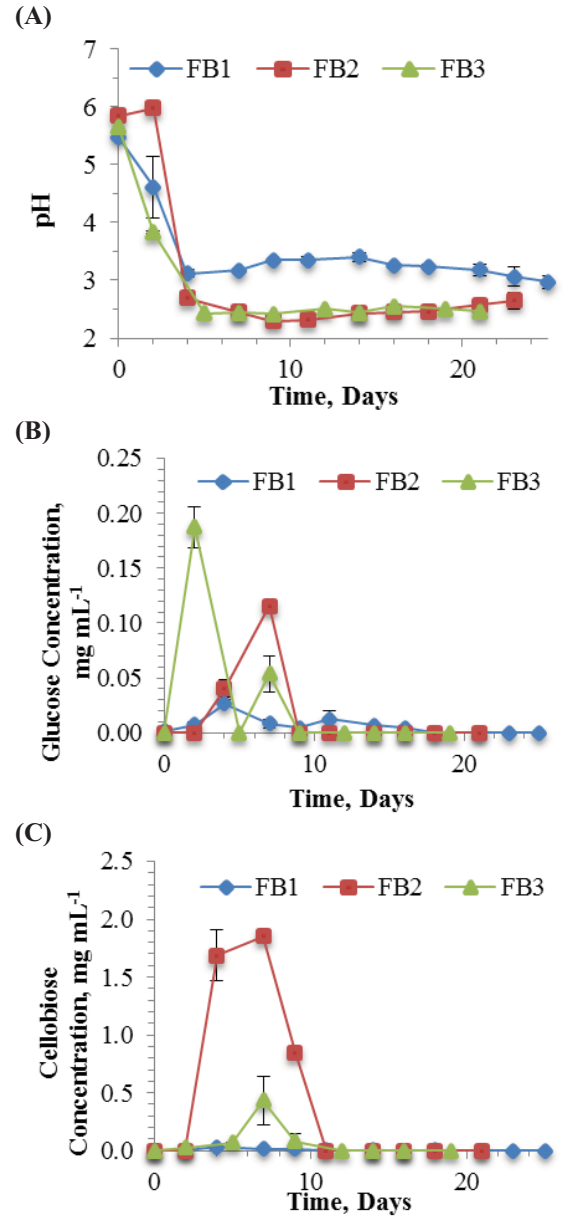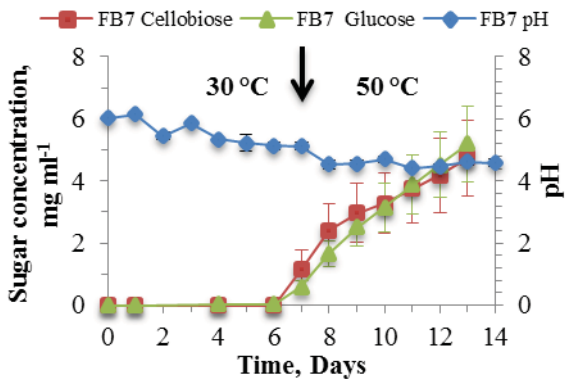
The pH of *T. reesei* cultures was monitored during each day of the 14-day incubation (Fig. 3). The pH of the static cultures increased from pH 6 to approximately 7.   The pH of the shake flask cultures quickly dropped from between 5 and 6 at day one to approximately 3 from day 3 onward for the 2%, 4%, and 8% Avicel concentrations. Only the 1% Avicel culture did not follow this trend. This result fits with literature pH values during *T. reesei* cultivation as demonstrated by Taniguchi et al (1983), and Lejeune et al, (1995).[13,14] Both researchers found an increase in enzyme activity when pH was maintained at or below 4.5. With the results found in this trial, additional cultures were grown at 30 ˚C at 125 rpm with 8% Avicel.
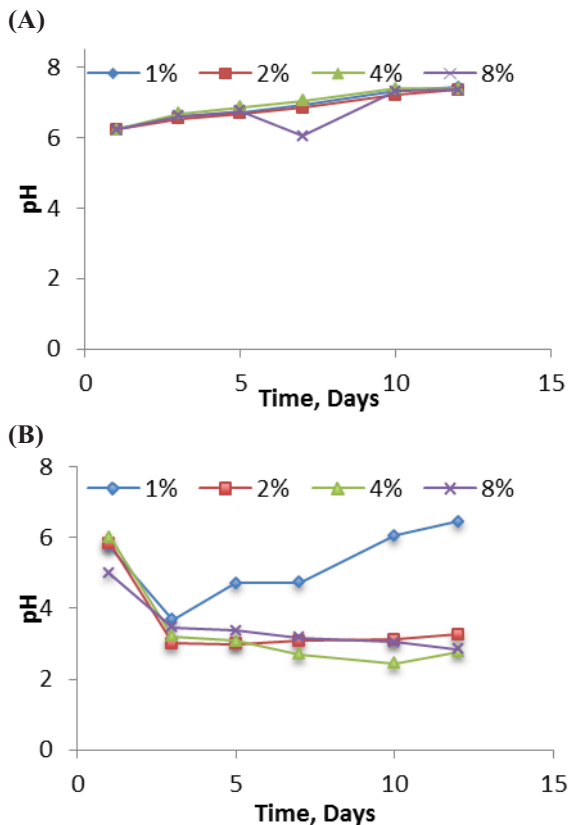
Figure 4. Glucose Oxidase Activity of *Trichoderma reesei*. *Time course study of the β-glucosidase activity/glucose production in Trichoderma reesei QM9414 cultures grown in Mandel's media and 8% Avicel, 30 ˚C, and 125 rpm. Glucose production was monitored for 14 days using the GO assay (Sigma).*

The cellulase activity in *T. reesei* QM9414 is catalyzed by a series of enzymes that are responsible for the digestion of cellulose. One of these enzymes, β-glucosidase, cleaves the cellulose hydrolysis intermediate, cellobiose (a disaccharide) into 2 molecules of glucose. This served as an indirect measurement of the presence of other cellulase enzymes such as the endo- and exo- cellulases needed to split the polysaccharide, cellulose, into oligo- and/or monosaccharides. β-glucosidase was monitored over time using the Glucose Oxidase assay (GO) kit (Sigma). The results of this enzyme assay indicated that there was no production of the β-glucosidase enzyme until after day 3. It is suspected that the biomass of *T. reesei* was accumulating during these first days of cultivation. By day 5, β-glucosidase peaked and fluctuated but at an increased level (Fig. 4). This trend may be indicative of the logarithmic growth curve of the *T. reesei* cultures as the culture size would be restricted based on culture volume and amount of aeration.[6] FB or fed-batch cultures 1 to 3 were cultured at the same conditions as previously described. FB1, was not a fed culture (0% feed) but was maintained at 2% or 8% Avicel starting cellulose concentration. No additional feed was supplied, and no pH control was administered.

FB1 cultures with 2% Avicel were analyzed but further comparisons were only considered between 8% Avicel cultures. In addition, after the completion of FB1 experiment, the *T. reesei* QM9414 was replaced with *T. reesei* Rut-C30 as some researchers suggested that the Rut-C30 strain may produce an increased amount of cellulase

(A)

(B)

(C)

Figure 5. **pH, Glucose Production, and Cellobiose Production for FB1-FB3.** *Fed-batch cultures FB1-FB3 of T. reesei showing pH (A), glucose (B), and cellobiose (C) production. Cultures were 8% Avicel in Mandel's media with no pH control (Table 1). Incubation temperature was 30 °C at 125 rpm.*

enzymes. Fig. 5 illustrates that the pH of FB1 and FB3 immediately decreased to pH 3 and pH 2, respectively. FB2 showed a slight increase in pH but then dropped below pH 3 as did FB3. No apparent trends seemed evident between pH and glucose or cellobiose production. Glucose levels peaked early in the cultivation (day 1-7) for all cultures and dropped to very low concentrations by day 7 and throughout the remainder of the experiment. This may be due to the consumption of the glucose by the *T. reesei* fungi or lack of enzyme activity. Cellobiose, the main disaccharide produced during enzymatic cellulose hydrolysis and converted to glucose by β-glucosidase was not

Figure 6. Average pH, Cellobiose, and Glucose for FB7 Average *values ± standard error of the mean for pH, glucose and cellobiose for fed-batch culture FB-7. Cultures were grown from day 0-7 with pH adjusted to pH 5- 5.5. Incubation temperature was 30 °C for day 1-7, followed by 50 °C from day 7-14 for enzyme hydrolysis as indicated by the arrow (n=2) (Table 1).*
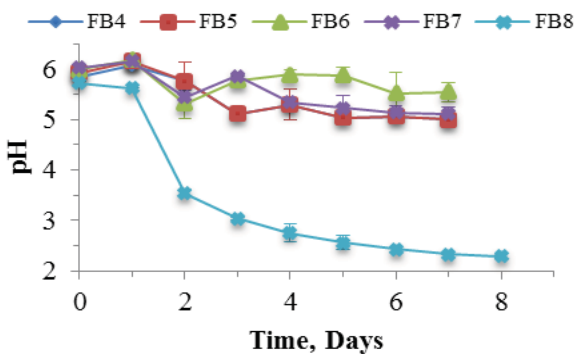
Figure 7. Average pH trends during culture growth. *Changes in average pH over time during culturing of Trichoderma reesei Rut-C30 fed-batch cultures FB4 through FB8. The pH of FB4 to FB7 was adjusted daily for 7 days. The pH of FB8 was not adjusted.*

detected in FB1 cultures and at a low level in FB3. FB2 (cultures fed 8% Avicel equivalent to sample removed) cultures had the highest detection of cellobiose with a dramatic increase at day 3 and reaching its maximum (1.85 mg mL^{-1}) at day 7. The cellobiose levels then decreased and by day 11 were near zero. This may be indicative of culture activity, whereby the cellulase enzyme cleaved the cellobiose producing glucose which was consumed by the *T. reesei*. Evidence for this was inconclusive indicating these cultures would need further study. The remainder of the FB studies, FB4 through FB10, were fed-batch studies with additional Avicel added to each culture phase to increase the cellulase enzyme activity and to produce more glucose. Due to low pH values and low production in FB2 and FB3, cultures FB4 through FB7 were maintained and pH adjusted for the first 7 days to determine if pH was a limiting factor to the amount of enzyme or glucose produced, or to the cellular concentration (low cellular concentration indicates low enzyme concentration). Therefore, the pH of FB4 through FB7 was maintained at pH 5 to 5.5 with a daily adjustment using 5N NH$_4$OH for a period of 7 days (Fig. 6). After 7 days, the pH adjustment was discontinued and the cultures were allowed to proceed naturally. Fig. 6 which illustrates the pH, glucose, and cellobiose production for FB7, is a representative example of the fed-batch cultures.

From the FB7 data, during the time that the culture pH was maintained at a higher level, there was no detectable glucose or cellobiose, however, as the pH began to drop the levels of glucose and cellobiose began to increase in similar fashion. At day 7, the liquid culture was removed, transferred to a new culture flask, 8% Avicel added, and incubated at 50 °C inhibiting growth of *T. reesei* but allowing the enzymes to act on the cellulose substrate. Both sugars continued to increase until the completion of the experiment at day 14 suggesting that the enzymes were produced in the original culture and that pH may have an influence on the production of cellulase enzymes. It also indicated that the glucose end product may be available for use by additional microorganisms.

Fig. 7 describes the trends in pH in an adjusted culture versus a non-adjusted culture. The natural trend for pH was a sharp decline after approximately 1 day to a pH level of approximately 3.5 by day 2. It was suspected that a low pH would slow culture growth and hence produce less biomass. Since fungal biomass was not measured in these cultures, additional studies would need to be completed to confirm this, however, the trends strongly suggested pH should be maintained at a higher level for the first few days of culture growth.

Fig. 8 displays the sugars glucose and cellobiose produced in each of the fed-batch cultures. Glucose levels were slightly higher in cultures that had the pH adjusted from days 1-7.

The cellobiose and glucose concentrations were monitored using HPLC in each of two replicates and averaged in FB 4 through FB8 (Fig. 8). Cellobiose concentrations were highest in FB8 cultures and began to increase after day 1 and reached a maximum of approximately 2 mg mL^{-1} by day 3 fluctuating at about 1.5 mg mL^{-1} thereafter. The glucose concentration in the same cultures remained very low and was monitored at levels below 0.2 mg mL^{-1}. The cellobiose accumulation in FB8 could indicate that β-glucosidase, responsible for converting cellobiose to glucose, was not present in sufficient quantities to keep up with the rate of cellobiose production by the endo-and exo-
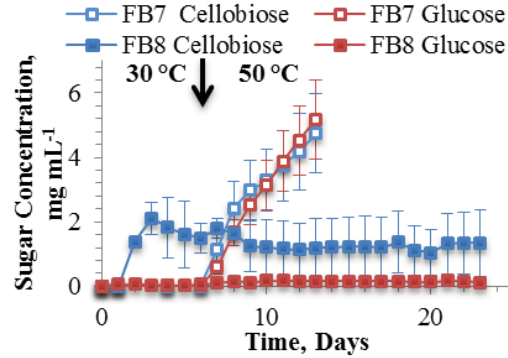
(A)

(B)
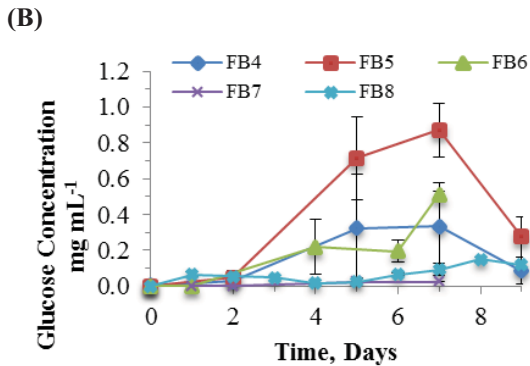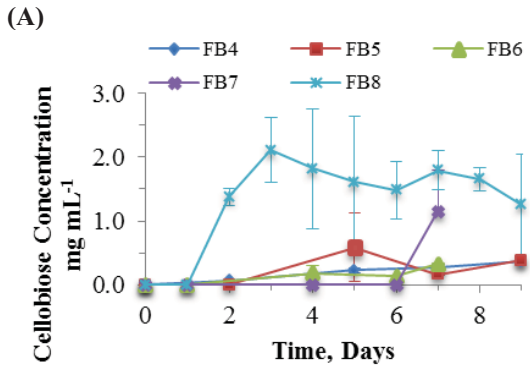
Figure 8. Average cellobiose and glucose production for all experiments. *Average production values ± standard error of the mean for cellobiose (A) and glucose (B) during the growth phase, days 0–7 for FB4 through FB8. FB4 and FB6 were not fed additional Avicel while FB5, FB7, and FB8 had 2% cellulose added daily. All cultures were incubated at 30°C from day 0-7 followed by 50 °C from day 7-14.*

Figure 9. Comparison of average sugar concentrations with pH adjustment (FB7) and without pH adjustment (FB8). *Comparison of the average, ±SEM glucose and cellobiose concentration with pH adjustment (FB7) and without pH adjustment (FB8) during the growth phase, days 0 to 7. At day 7 enzymatic hydrolysis of cellulose occurred at 50 °C, days 7 - 14 (FB7) or 7 – 23 (FB8).*

glucanase enzymes. The low concentration of the β-glucosidase may also be due to a low pH. The pH of the FB8 culture, which was not adjusted at any time, dropped to below 3 by day 4. Enzyme activity is reduced when environmental conditions range outside of the optimal indicating that further experimentation may be required to determine an optimal pH range for these cultures.

FB5 and FB7 also showed small increases in the cellobiose concentration though these increases appeared at day 5 and 6, respectively, indicating that enzyme may have been present early in culture growth cleaving the cellulose bonds to produce cellobiose and glucose. Glucose concentration increased during this culture growth phase and reached a maximum in FB5 at or about day 7. This increase could indicate a slowing in the culture growth and more glucose was produced than was needed by the cells, or that the cells produced more of the enzyme complex. It should be noted that the pH of FB5 and FB7 was maintained at a higher level during the early stages of the culture growth (Fig. 7). This should have increased the biomass of the fungi, which would have delayed the production of enzyme. When the pH was allowed to drop, detectable enzyme activity would have been seen. Again, additional studies would need to be completed to make this determination.
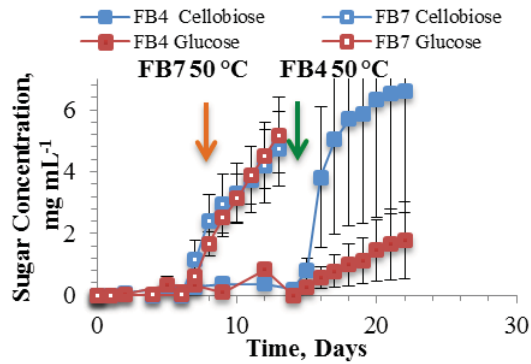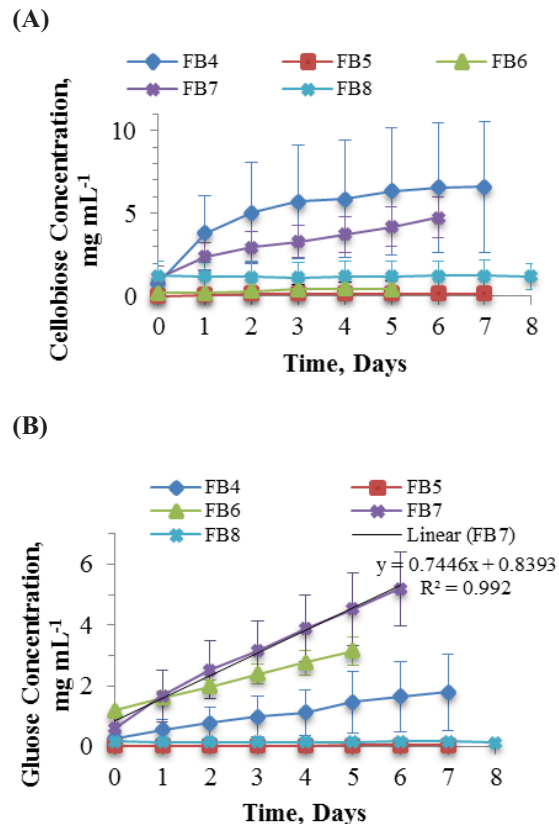
It appeared that culture pH may have been a limiting factor to the production of sugars in the fungal cultures; it seemed appropriate to directly compare a representative culture with pH adjustments (FB7) to a culture where no pH adjustments occurred (FB8) (Fig. 9). Fig. 9 compares cellobiose and glucose production during the growth phase (day 1-7) and the hydrolysis phase (day 7-14). During the growth phase pH was adjusted to 5-5.5 in the FB7 culture, whereas FB8 experienced the natural trend in pH drop. During the first 6-7 days, no glucose or cellobiose was detected. However, in FB8 cellobiose was detected at 2 mg mL^{-1} concentration within three days of culture growth then proceeded to decrease to approximately 1 mg mL^{-1} and remained at this level even after the culture liquid was transferred to a hydrolysis reaction. This indicated that there was a very low concentration of the cellulase enzymes in this culture or the low pH inactivated the enzymes. FB7, though no cellobiose or glucose was detectable during the growth phase, as the liquid culture was transferred to a hydrolysis flask and additional cellulose added, the glucose and cellobiose both increased dramatically indicating that the cellulase enzyme complex was present in the

Figure 10. Sugar Production during growth phase and enzymatic hydrolysis. *Comparison of the average concentration ± SEM for glucose and cellobiose produced during the growth phase, day 0-7, and enzymatic hydrolysis of cellulose after culture transfer and incubation at 50 °C. FB4 culture was monitored to day 14, or 7 days after transfer while FB7 was monitored to day 23, or 16 days after transfer.*

(A)

(B)

Figure 11. Enzymatic Hydrolysis of Cellobiose and Glucose. *Comparison of average values ± SEM for cellobiose (A) and glucose (B) concentration during enzymatic hydrolysis (after day 7) in FB4 through FB8 cultures. The slope of the line for FB7 was used to determine the highest rate of glucose production.*
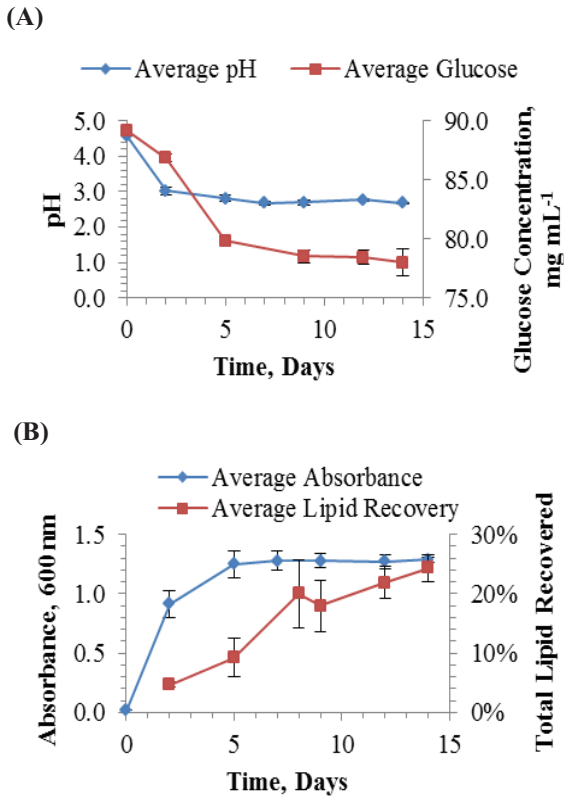
cultures. It is also possible that glucose was produced to a level sufficient for use by the culture concentration, and thus the cells may have not needed to produce more enzymes.

Another factor to consider between cultures was the amount of feed presented to each fed-batch culture during the first 7 days of growth. FB4 received no additional cellulose during the growth phase of the culture while FB7 received an additional 2% daily addition of cellulose (Fig. 10). During the first 7 days of growth neither culture produced glucose or cellulose even though an adequate source of cellulose was available to both cultures. However, once the liquid culture was removed from the growing cultures and fed an equal amount of cellulose in the form of Avicel, the FB7 fed culture began to produce both cellobiose and glucose in equal amounts indicating a sufficient amount of cellulase enzymes present in the culture. The FB4 culture whose initial 8% Avicel was not supplemented on a daily feed schedule, delayed the production of glucose and cellobiose until day 14 when a pronounced concentration of cellobiose could be detected. Glucose, however, was produced in a much lower concentration indicating that the β-glucosidase enzyme required to cleave the cellobiose was present in low concentration and the cellobiose not glucose accumulated in the cultures.

Simple sugars such as glucose are only one of the three constituents in synthetic bovine milk and it was demonstrated that glucose was produced in varying concentrations dependent upon environmental factors (Fig. 11). Enzymatic hydrolysis of each culture was completed when the liquid media portion of each culture was removed after the suspended cellulose was allowed to settle to the bottom of the flasks for varying amounts of time (30 minutes to overnight). The liquid media was transferred to new flasks and the recovered volume measured to determine the amount of Avicel required for 8%. The new culture was incubated at 50 °C at 140 rpm as described by Esterbauer, et al., 1991.[12] The 50 °C temperature inhibits the growth of the fungi; therefore glucose production would not be utilized by the organisms. The pH and concentrations of glucose and cellobiose were monitored. For all FB studies, pH varied little within the cultures however, the pH for FB6 and FB7 was slightly below 5, while the pH of FB4, FB5, and FB8 fell between pH 2.2 and 3.2. Percin and Jarak (1995) reported the optimal pH for β-glucosidase to be 4.8.[15] The best glucose levels were achieved in FB6 and FB7, cultures where the pH remained at or near the optimal pH of 4.8. When the pH dropped below the optimal the glucose concentrations also decreased as

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(A)

(B)

Figure 12. RB1 Monitored Parameters. *Average values (n=2) ± standard error of the mean for pH and glucose concentration (A), and culture absorbance and total lipid recovery (B) for RB1. pH was not adjusted for RB1 experimentation.*

the enzyme was no longer performing optimally (FB4) or not at all (FB5 and FB8). The cellobiose concentrations followed a different trend.

FB4, which had the lowest glucose concentration, had the highest cellobiose concentration. This suggested the loss of activity of the β-glucosidase enzyme. However, FB7 also had a high concentration of cellobiose which continued to increase with time. FB5, FB6, and FB8 had low concentrations of cellobiose and glucose with no increase throughout the time-course. The glucose produced was the desirable end-product for this project as it was to serve as the substitute for lactose, the naturally occurring sugar in bovine milk, and serve as a fuel source for future reactor stages. Cellobiose, though it can be metabolized by some microorganisms, other microorganisms such as those used in the next stage of this project have little or no ability to utilize the unprocessed cellobiose.

B. Stage 2: Lipid Production

As defined in Table 2, RB1 differed in initial glucose concentration (90g/L) from RB2-RB6 (60g/L). Additionally, some experiments were pH controlled with 5N ammonium hydroxide (RB2, RB3, RB4) while others were not controlled (RB1, RB5, RB6). Of those that were pH controlled, pH alteration either occurred from day 1 to day 7 (RB3, RB4) or from day 7 to day 14 (RB2). Fig. 12 represents the RB studies with regard to lipid production, the goal of stage 2.

As depicted in Fig. 12, the time-course of growth for *R. toruloides* exhibited a typical parabolic growth curve for a single-celled microorganism (represented by absorbance recorded at 600nm). As growth reached the stationary phase (the plateau ~day 5-7), a dramatic increase in total lipid content was observed. This would be expected as biomass of the culture was increasing and nutrient content, like glucose, was being utilized. As stationary phase continued, lipid content increased, and glucose concentration in the culture decreased.This experiment terminated on day 14, resulted in approximately 25% lipid recovery from the dry cell weight, but in reference to the literature, lipid content should continue to increase as nutrients like nitrogen and carbon continue to be utilized.[5,16]

A comparison of all RB experimentation and the monitored parameters-culture growth, pH, glucose concentration, and total lipid recovery- can be observed in Fig. 13. The time-course of culture pH (Fig. 13 C), showed that pH rapidly dropped from approximately 4.5 at day 0 to approximately 3.0 or lower over the first several days and remained depressed for the remainder of experimentation for the non-pH adjusted cultures (RB1, RB5, RB6). Similarly, RB2 which did not have pH adjustment over the first 7 days, followed the same initial decrease in pH, however following pH adjustment from day 7 to day 14, the pH decreased more gradually and not nearly to the same extent as other cultures with a pH of approximately 4.0. The other cultures (RB3, RB4) which were pH controlled from day 1 to day 7, also exhibited a more gradual pH decrease after conclusion of adjustment, but eventually reached the lower pH values like the unaltered cultures exhibited.

Overall, cultures that were not adjusted for pH (RB1, RB5, RB6) seemed to exhibit normal growth curves, whereas those that were adjusted from day 1 to day 7 (RB3, RB4) took slightly longer to reach stationary phase (Fig. 13 A). In the case of RB2, when pH adjustment began on day 7, the culture had already reached stationary phase like the unaltered cultures and was able to increase its growth past this normal stationary phase, and eventually reached a new plateau after conclusion of pH adjustment on day 14. This increase in culture growth of RB2 was most likely due to the addition of ammonium hydroxide to control the pH, which ultimately served as a nitrogen nutrient source. Cultures that were controlled for pH (RB2, RB3, RB4) also appeared to consume glucose at a slightly greater rate than those that were not controlled (RB1, RB5, RB6), as determined by the decreasing slopes in the glucose concentration plot (Fig. 13 B). This may also be a result of using ammonium hydroxide to adjust pH, thus providing *R. toruloides* with additional nitrogen for use.

With regard to total lipid recovery, those cultures that had pH adjustments from day 1 to day 7 (RB3, RB4) had the lowest amounts of lipid recovery, 12.84% (RB4) and 14.52% (RB3), and also took longer to accumulate lipid for recovery (Fig. 13 D). In contrast, cultures that were not adjusted for pH (RB1, RB5, and RB6) had the larger amounts of sustained lipid recovery; 20.21% (RB6), 22.58% (RB5), and 24.36% (RB1), at a quicker accumulation rate. Further contrast is seen in the unique lipid recovery pattern for RB2. During the first 7 days of RB2 experimentation, there was no pH adjustment, but lipid recovery was approximately double that of the other non-pH adjusted cultures (RB1, RB5, RB6), reaching a maximum of 43.37% on day 7. Upon addition of ammonium hydroxide from day 7 to day 14, lipid recovery dramatically decreased by more than half of the previously observed values initially (16.84%), then rose to 27.83%, but then returned to a depressed level of 13.48 to 16.8% for the remainder of experimentation. This depressed level fell between the maximum values found in the pH controlled cultures (12.84%-14.52%) and the maximum values found in the unaltered cultures (20.21%-24.36%). The initial elevated levels of lipid recovery in RB2 were unexpected, and in theory should have been similar to RB6 which contained the same modified medium with 60g/L glucose and lacked pH control. This discrepancy might be due to natural variation in the microorganisms. The decrease in lipid recovery of RB2 observed on day 7 is most likely due to addition of ammonium hydroxide, which served as an additional nitrogen source for the yeast to utilize, and has
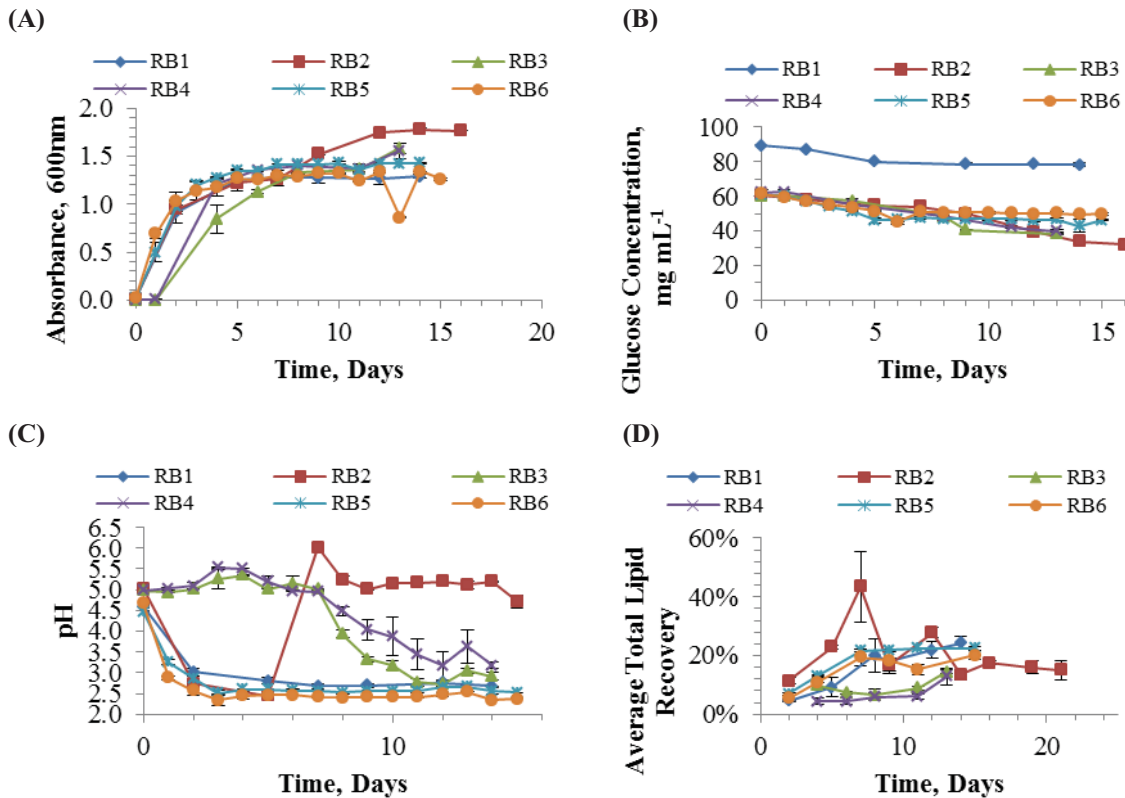

Figure 13. RB Culture Comparisons. *Average values (n=2) ± SEM for RB culture growth (A), glucose concentration (B), pH (C), and total lipid recovery (D), over time.*
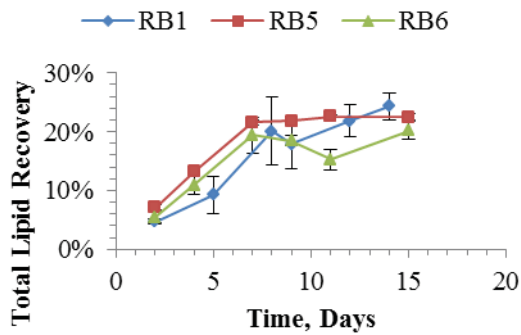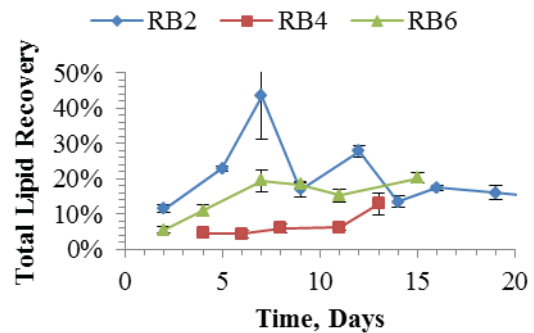
Figure 14. Media Comparison of Total Lipid Recovery. *Average values (n=2) ± standard error of the mean for RB total lipid recovery over time. All depicted experiments were not pH controlled. RB1 contained minimal media with 90g/L glucose. RB5 contained minimal media with 60g/L glucose. RB6 contained modified minimal media with 60g/L glucose.*
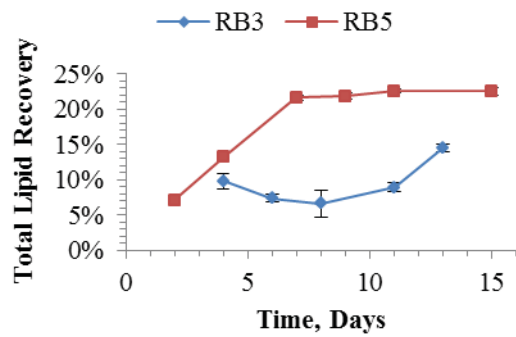
Figure 15. pH Adjustment Effect on Total Lipid Recovery in Cultures Grown in Minimal Media with 60 g/L Glucose. *Average values (n=2) ± standard error of the mean for RB total lipid recovery over time. RB3 was pH controlled from day 1 to day 7. RB5 did not have pH adjustment.*

been shown to restrict lipid accumulation.[5, 16] Additional experimentation that permits longer experiment duration would be needed to see the full effects of nitrogen depletion on lipid accumulation. In addition, experimentation utilizing different pH buffers would be of benefit to support some of these claims.

Another parameter varied in RB studies was media composition. As seen in Fig. 14, cultures not subject to pH control but containing different media compositions performed very similarly with regard to lipid recovery. RB5, which contained minimal media with 60g/L glucose, produced slightly more lipid than RB1 and RB6 over the first 7 days but then experienced a plateau of lipid recovery at approximately 22% for the remainder of experimentation. In comparison, RB1, which contained minimal media with 90 g/L glucose, performed similarly to RB5 but with a lower amount of total lipid for the first 7 days, and then experienced a slight decrease followed by an increasing trend, to a maximum amount of 24.36%. It is unclear if total lipid recovered from RB1 would continue to increase or if it would also plateau like RB5. Longer duration experiments would be required to observe these

Figure 16. pH Adjustment Effect on Total Lipid Recovery in Cultures Grown in Modified Minimal Media with 60 g/L Glucose. *Average values (n=2) ± standard error of the mean for RB total lipid recovery over time. RB2 was pH controlled from day 7 to day 14. RB4 was pH controlled from day 1 to day 7. RB6 did not receive any pH adjustment.*

trends. RB6, which contained modified media with 60g/L glucose, was not significantly different from RB5 for the first 7 days of experimentation. However after day 7, RB6 experienced a longer and ultimately larger decrease in lipid content, followed by what appears to be an increasing trend, resulting in a maximum recording of 20.21% lipid recovery. As previously stated, longer duration experiments would be needed to delineate whether nitrogen concentration or carbon concentration in culture medium has significant effects on lipid recovery.

If we consider experimentation based solely by media type, cultures containing minimal media with 60 g/L glucose, had significantly increased lipid recovery when pH was not controlled (Fig. 15). For cultures containing modified minimal media with 60 g/L glucose, RB6, which exhibited no pH control, did significantly better over time when compared to others of the same media type (Fig. 16). RB2 which also contained minimal media with 60 g/L glucose, performed similarly to RB6 by the end of experimentation, but still had less lipid recovery when compared to RB6 (Fig. 16). RB4 which was pH controlled and contained minimal media with 60 g/L glucose produced significantly less total lipid throughout experimentation (Fig. 16). The major conclusion from these comparisons

was that regardless of culture medium type, pH control initiated at the beginning of culturing is detrimental to increased lipid production. Further studies are needed to determine the impact of culture medium on lipid production.

Aside from monitoring the total lipid recovery from cultures over time, lipid composition was also monitored over time. Six fatty acids (oleic acid, palmitic acid, stearic acid, myristic acid, linoleic acid, and linolenic acid) were monitored out of the total lipid in order to assure the lipids being produced correlated to those found in bovine milk. This was essential since the oleaginous yeast being utilized has historically been researched in reference to biodiesel production.

In particular, we were interested in those that are able to mimic the composition of bovine milk fat, which, as mentioned previously, is composed of oleic acid (~24%), palmitic acid (~24%), stearic acid (~11%), myristic acid (~9%), linoleic acid (~3.6%), and linolenic acid (~2.3%). When composition of the individual fatty acids were compared to the amount of fatty acid recovered from extraction, all RB experiments were producing fatty acids found in bovine milk (Table 4). However, the relative compositions that were produced were far from similar to that of bovine milk. Several reactors seemed to produce the right proportional order, but again did not equate to the correct percentages found in bovine milk. More experimentation that would allow for longer duration and higher lipid accumulation might help align these proportions to those observed in bovine milk.

Table 4. Average percentage of each fatty acid from the total recovered lipid.
Percentages are the average recovered over the entire course of experimentation. Bovine milk percentages are the averages found in the literature.

Experiment	Linolenic	Linoleic	Myristic	Stearic	Oleic	Palmitic
RB1	0.27	1.45	0.16	2.37	6.09	2.79
RB2	0.12	0.45	0.08	3.63	3.25	1.38
RB3	0.24	0.93	0.13	1.37	3.20	1.47
RB4	0.38	1.19	0.19	2.43	6.62	2.69
RB5	0.12	0.58	0.05	0.80	21.45	1.07
RB6	0.19	0.73	0.06	1.11	3.15	1.41
Bovine Milk	**2.30**	**3.60**	**9.00**	**11.00**	**24.00**	**24.00**

IV. Conclusion

Astronauts spend extended periods of time in space aboard the ISS and experience the effects of microgravity during this time. Dietary requirements for all humans require a certain amount of plant and animal material which is not readily available, if at all, to the crew. Current experimentation proposes to grow vegetable crops such as lettuce, radish, and tomato aboard the ISS, which would create a large amount of plant waste. The vision for this project was to explore the use of biological agents as a synthetic food processor that could take space mission solid wastes as input(s) through several columns of immobilized microbes (fungi, yeast, and bacteria) and convert the waste into multiple, high value, synthetic 'animal' products. The aerospace architecture most relevant to this approach would be one where most of the crew food is supplied by growth of crop plants such as wheat, soybeans, potatoes, etc. which produce inedible crop residues which become waste for disposal. This concept of converting crop residue to a useable product could also be adapted for other space mission solid waste input(s) such as any biodegradable trash, garbage, or paper, providing the benefit of reducing solid waste in space.

Microbial production of a synthetic/simulated milk product was selected as the first demonstration. In its simplest formulation, a synthetic milk would contain casein (milk protein, ~ 3.2%), lactose (milk sugar, ~4.8%), and butterfat (milk fat, ~3.9%). We chose to replace the lactose with glucose, which is easier to produce (and still sweet), could be readily available to any organism as an energy or carbon source, and could be used as the substrate for making casein and butterfat.

The findings of this project were numerous including 1) that fed-batch culture with a biphasic pH and temperature regime gave the best results for glucose production; 2) glucose should be recovered after cultures had time to increase the biomass at optimal pH; 3) Glucose production was steady and at detectable concentrations when pH levels were near the reported optimum of 4.8 versus near 2-3; and 4) glucose levels at day 6 to 7 were high enough to use as a milk sugar for simulated milk. However, it is important to note that glucose levels were probably

too low to serve as the sole source of growth substrate for casein or butterfat production where at least 20 mg mL^{-1} may be needed vs. the 5 mg mL^{-1} produced. This would require further investigation and a substantial increase in culture size transitioning to bioreactor capabilities.

Though lipid material was recovered in adequate amounts, it was determined that the initial makeup of the total lipid recovered varied in its fatty acid composition from bovine milk. It may be necessary to investigate additional microorganisms or analyze the total lipid material for additional fatty acids. Overall, the best 'sustainable' total lipid content was about 20%.

Finally, though two of three phases were investigated, the remaining component, casein or milk protein became the missing piece of the puzzle. After a thorough literature and scientific community search, it became evident that the required GMO needed for the last step was out of reach. However, a scientific protocol, published in the literature, is available and this GMO could be manufactured in the laboratory. This process was not within the scope of this project but would be a recommendation for further studies into the productin of synthetic milk products.

Appendix

A. Culture Media Compositions

4. *Mandel's Media:*
   Avicel (by percent)
   0.3 g Urea
   0.75 g Peptone
   0.25 g Yeast extract
   2.0 g KH2PO4 (potassium diphosphate)
   1.4 g (NH4)2SO4 (ammonium sulfate)
   0.3 g CaCl2 (calcium chloride)
   0.3 g MgSO4 (magnesium sulfate)
   0.1% Tween 80
   Trace elements: 5.0mg FeSO4 7H2O (iron sulfate heptahydrate)
                    1.6 mg Mn SO4 4H2O (manganese sulfate tetrahydrate)
                    1.4 mg Zn SO4 7H2O (zinc sulfate heptahydrate)
                    20.0 mg CoCl2 6H2O (cobalt chloride hexadydrate)

5. *Minimal Media:*
   pH 5.5
   1.5 g MgSO4 7H2O
   12.0 g NH4SO4
   1.0 g KH2PO4
   0.5 g yeast extract
   Glucose

6. *Modified Minimal Media:*
   pH 5.5
   1.5 g MgSO4 7H2O
   1.85 g NH4SO4
   1.0 g KH2PO4
   0.5 g yeast extract
   Glucose

Acknowledgments

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