



44<sup>th</sup> International Conference on  
Environmental Systems



TEXAS TECH  
UNIVERSITY.



# Microorganism Utilization for Synthetic Milk Production

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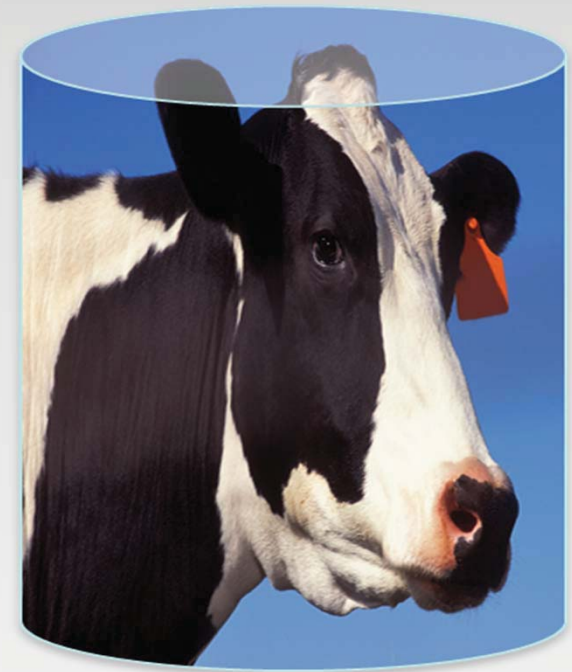
July 13-17, 2014



# History

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- Astronauts spend extended periods of time in space aboard the ISS
- Experience the effects of microgravity
- Dietary requirements require a certain amount of plant and animal material not readily available
- Grow vegetable crops as lettuce, radish, and tomato aboard the ISS, creating a large amount of plant waste





# History

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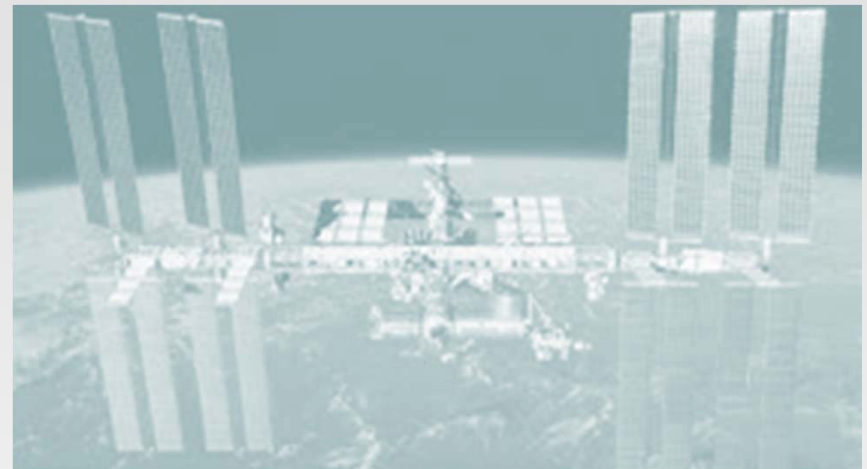
- Use of biological agents may serve as a synthetic food processor
  - Cellulolytic microbes
    - Converts the waste into multiple, high value, synthetic 'animal' products
    - Milk
- Bovine milk components include protein, sugar, lipid
- Multi-stage bioprocessing required to create the fuel for each successive stage.



# History

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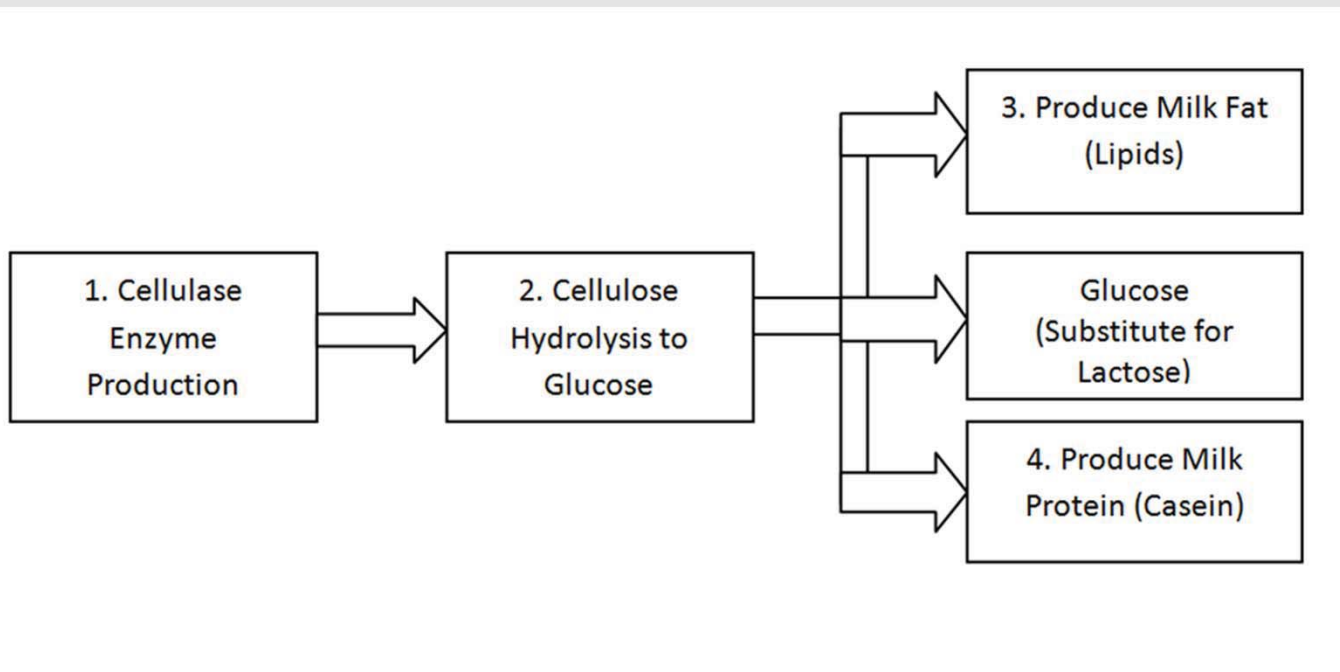
- Concept of converting crop residue to a useable product
- Could be adapted for other space mission solid waste
- Provides the benefit of reducing solid waste in space.
- TA06-Human health, life support, and habitation systems> ECLSS and Habitation Systems>Waste Management





# Challenges

Explore the use of biological agents- bacteria, fungi,  
and yeast- as synthetic food processors

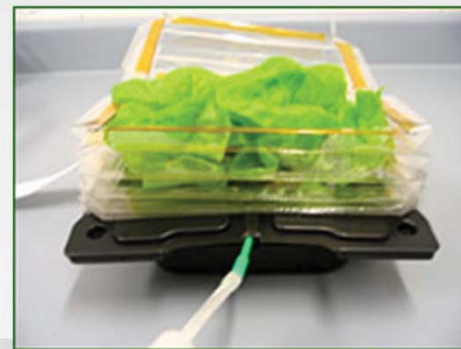




# Introduction

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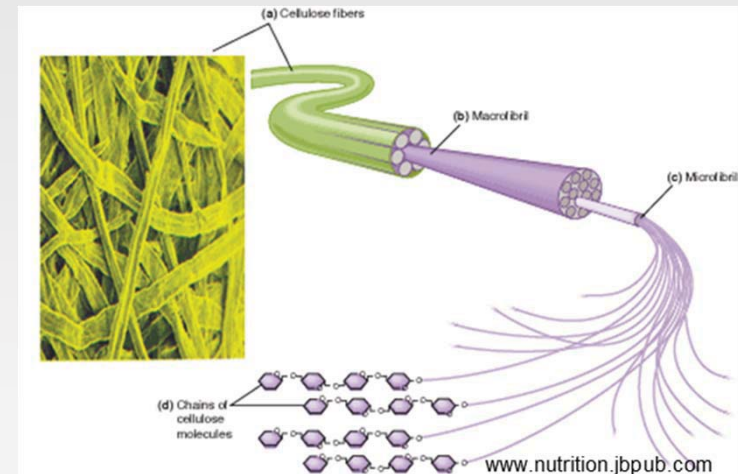
- Desired architecture for long duration spaceflight,
  - International Space Station (ISS)
  - Future missions to Mars
- Provide a supply of fresh food crops
  - Crops create a high proportion of inedible plant waste.





# Background

- Fungi, bacteria, and yeasts possess metabolic pathways fueled by cellulosic biomass
  - Fungi can produce glucose from cellulose
  - Oleaginous yeasts accumulate upwards of 80% of their dry weight as lipids.
  - Microbes produce proteins





# Background

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- Fungi produce a number of polysaccharide hydrolyzing enzymes and enzyme complexes.
  - Monosaccharide glucose useful as fuel for other organisms
- Yeast can be fueled by glucose to produce lipids.
  - Milk lipids are the fatty portion of milk and make up 33 g total lipid/L in bovine milk





# Goals

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- Produce the components of milk from inedible plant waste by utilizing microorganisms
  - Sugar - glucose
  - Lipid - milk fats
  - Protein – casein
- Utilize fungi that possess cellulolytic metabolic pathways
  - produce glucose from cellulose to fuel future reactor stages
  - substitute glucose for lactose component of milk
- Production of milk lipids through oleaginous yeasts.





## Materials and Methods: Environmental Conditions

Environmental conditions optimized:

### TIME COURSE BATCH STUDIES

- pH
- Temperature
- Carbon source
- Aeration
- Choice microorganisms





## Materials and Methods - Conditions

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



## Materials and Methods: Microorganisms

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- *Trichoderma reesei*, cellulolytic fungus, BSL I
  - QM 9414 (ATCC 26921), Rut C30, (ATCC 56765)
  - Drive the production of glucose
  - Known for its ability to convert native and derived cellulose
  - Produces a number of polysaccharide hydrolyzing enzymes
- *Rhodosporidium toruloides*, yeast, BSL I
  - Strain Y-1091 (ATCC 10788), USDA ARC
  - Lipid production found in milk fats
  - Can accumulate up to 76% dry weight as lipid



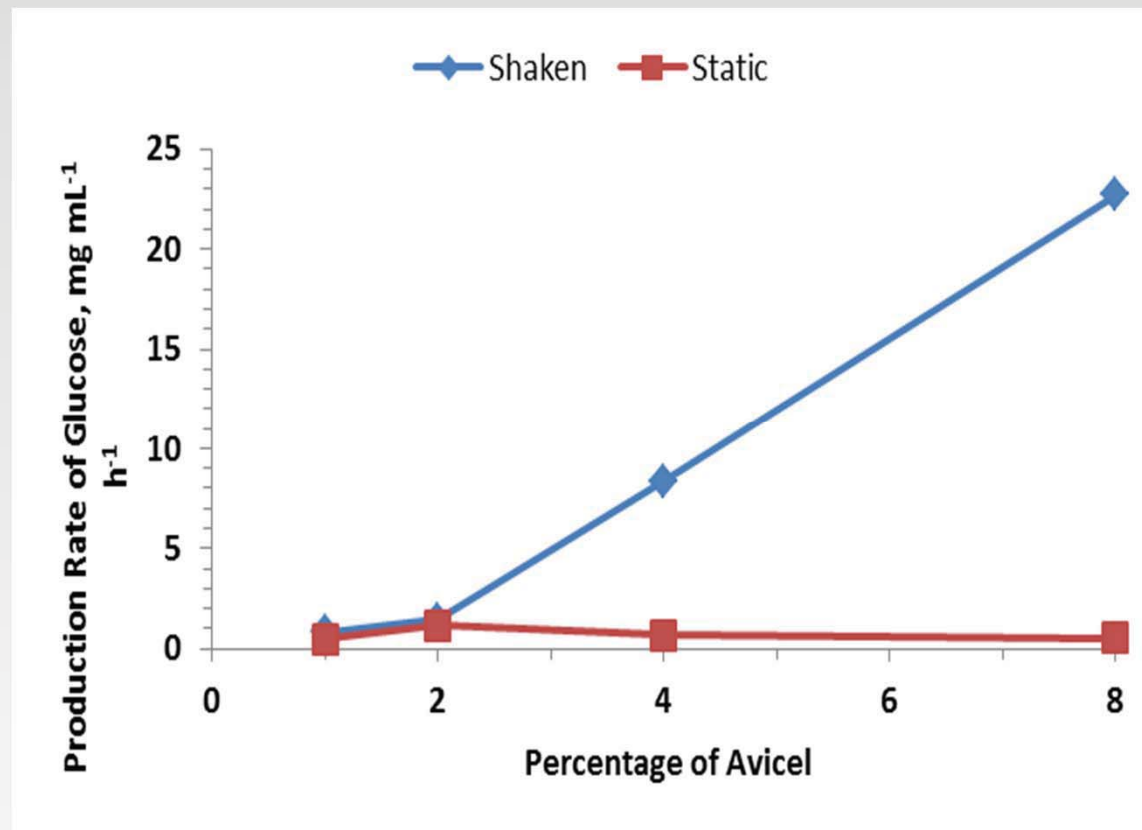
## Materials and Methods: Sugar Production

- Time course batch study of *T. reesei* cultured in Mandel's media
  - supplemented with 0 – 8 % Avicel
  - Incubated at 30° C, static or shaken at 125 rpm
  - Monitored environmental conditions
- Glucose and cellobiose detected over time
  - Agilent 1100 HPLC with an Aminex HPX-87H ion exclusion column



## Results: Environmental Conditions - Glucose

### Glucose Production Rate & Avicel

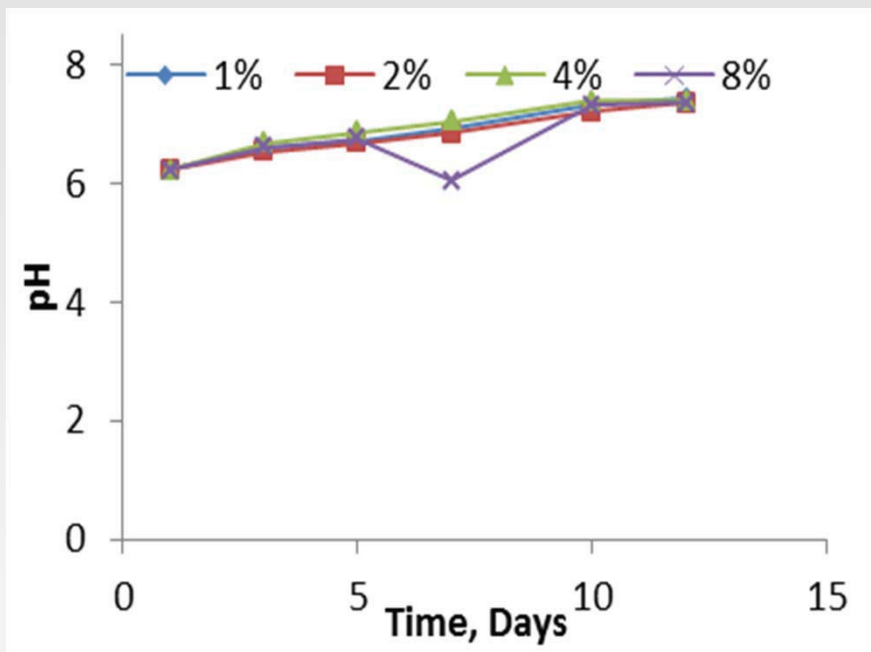




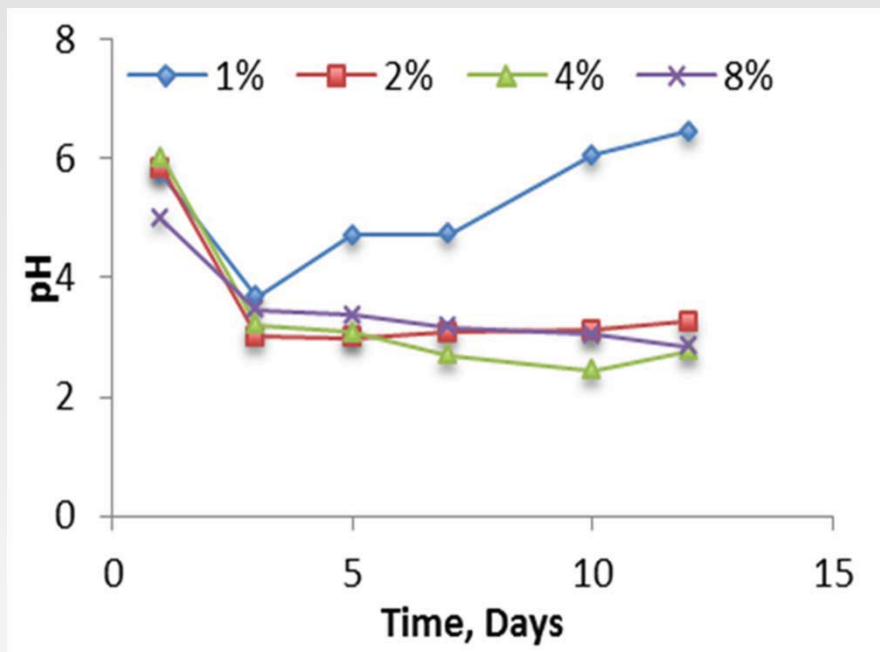
## Results: Environmental Conditions, pH

### Glucose Production

Static

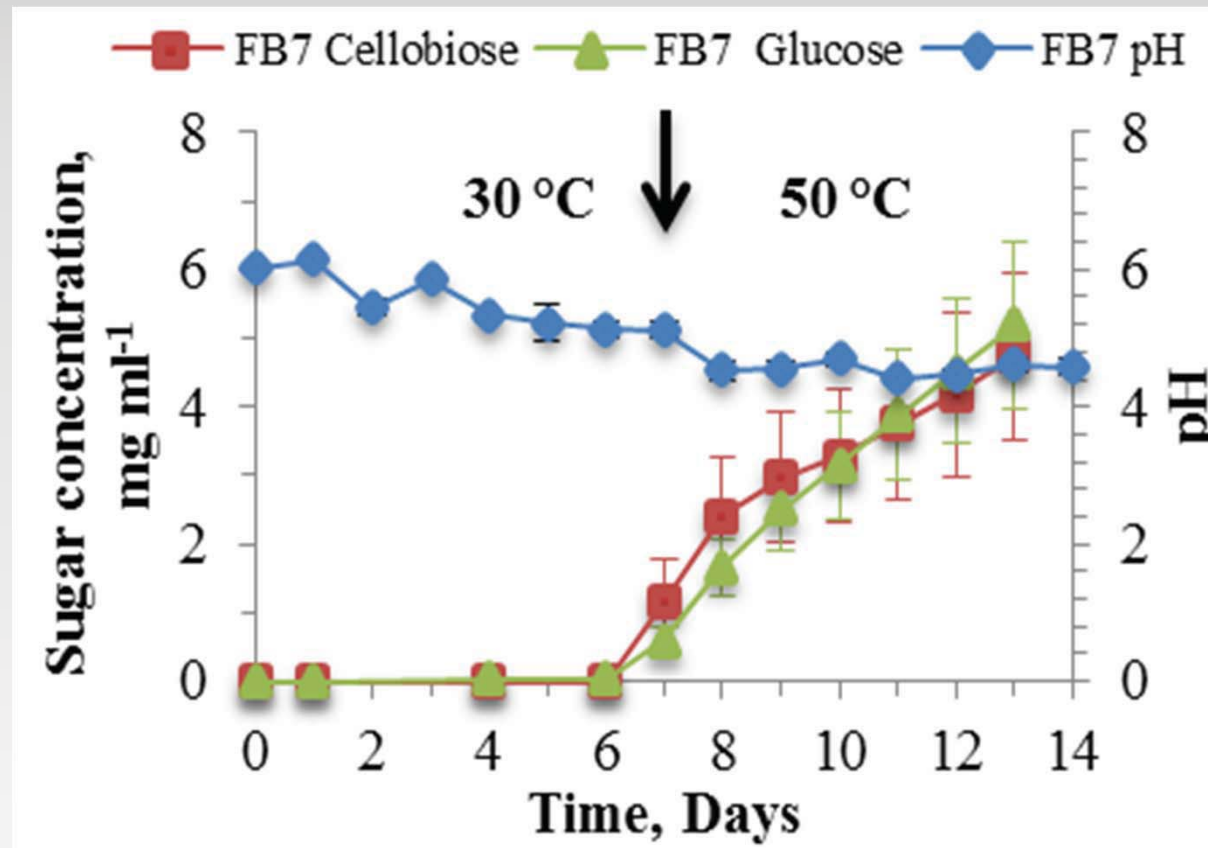


Shaken





## Results: Environmental Conditions & Sugar Production

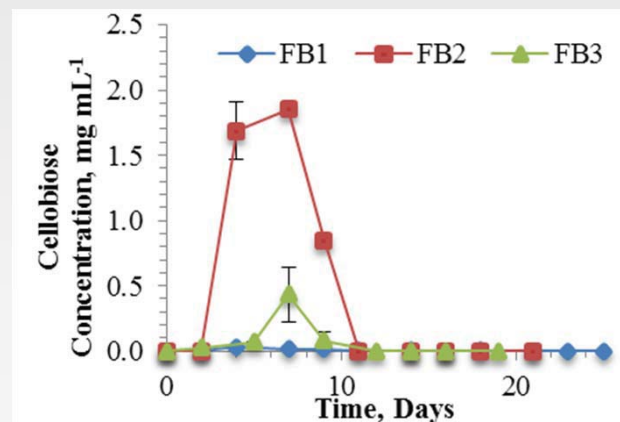
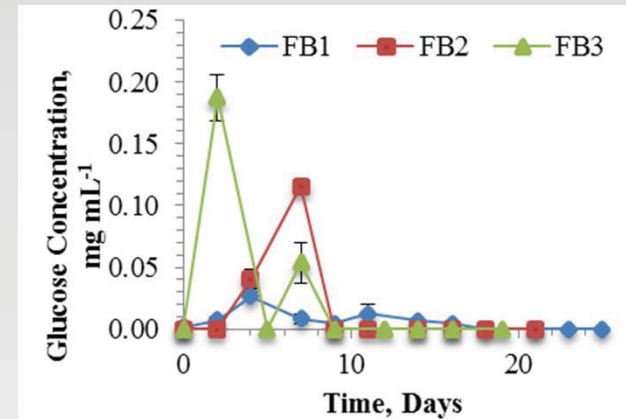
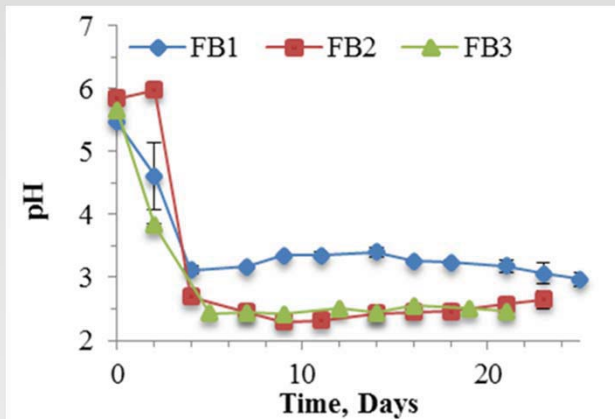






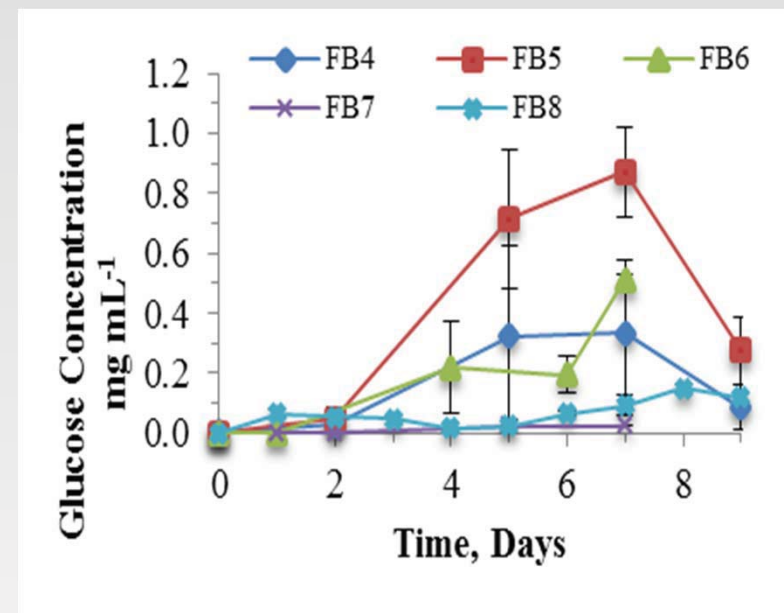
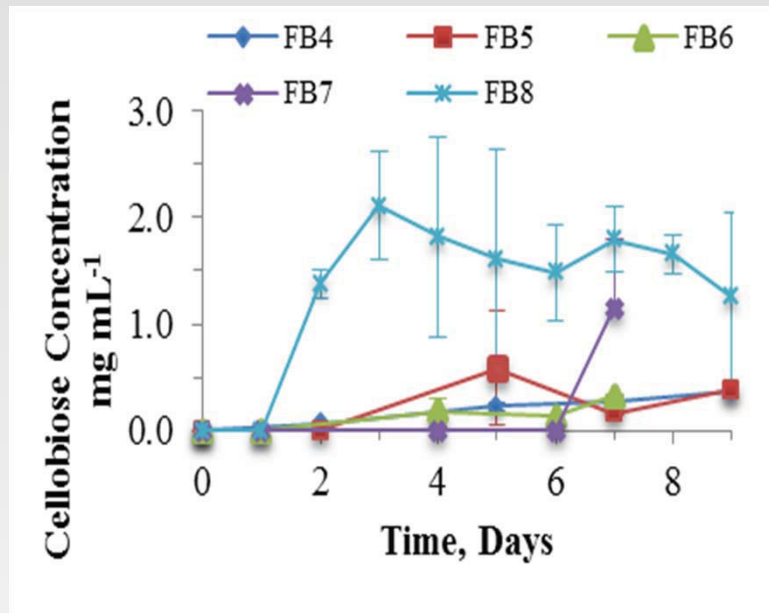
## Results: Overall Glucose Production

- glucose production below what was expected





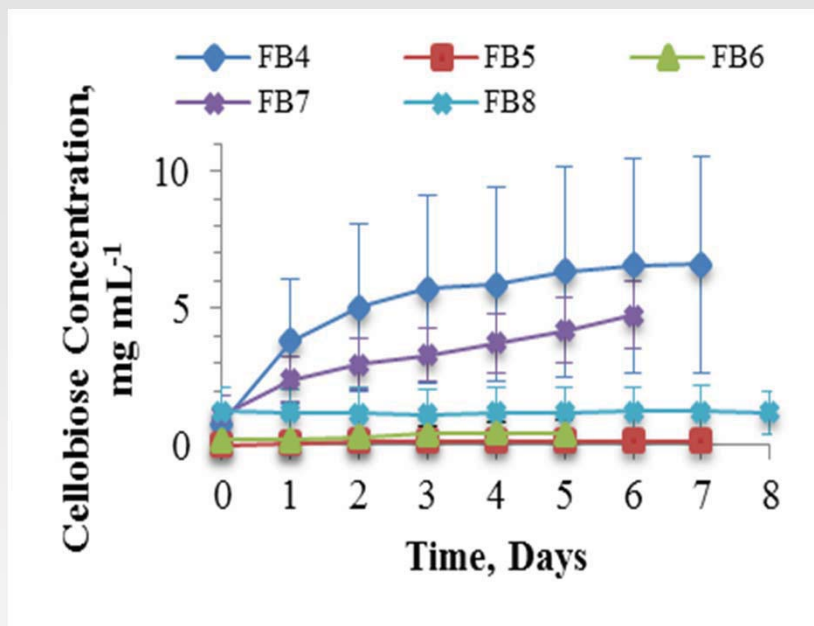
## Results: Glucose Averages



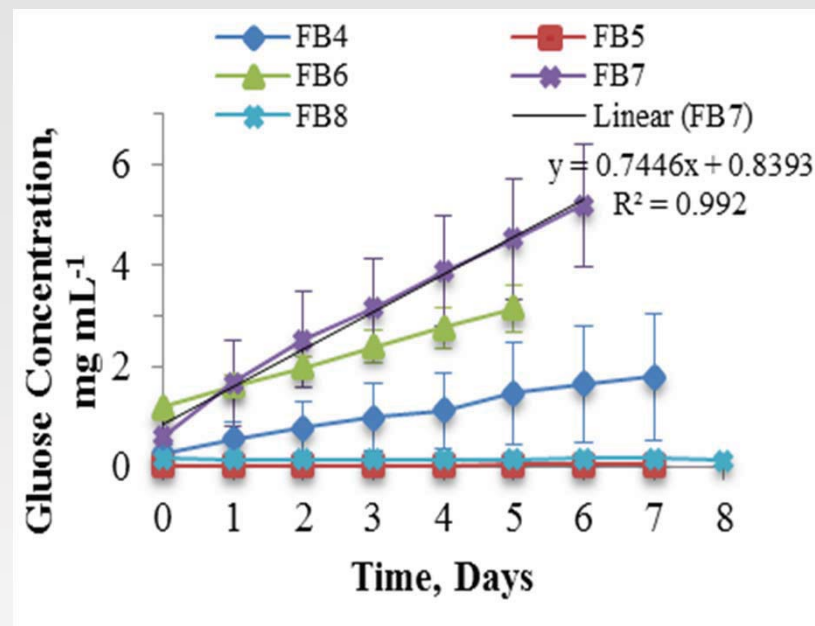


## Results: Enzymatic Hydrolysis

### Cellobiose



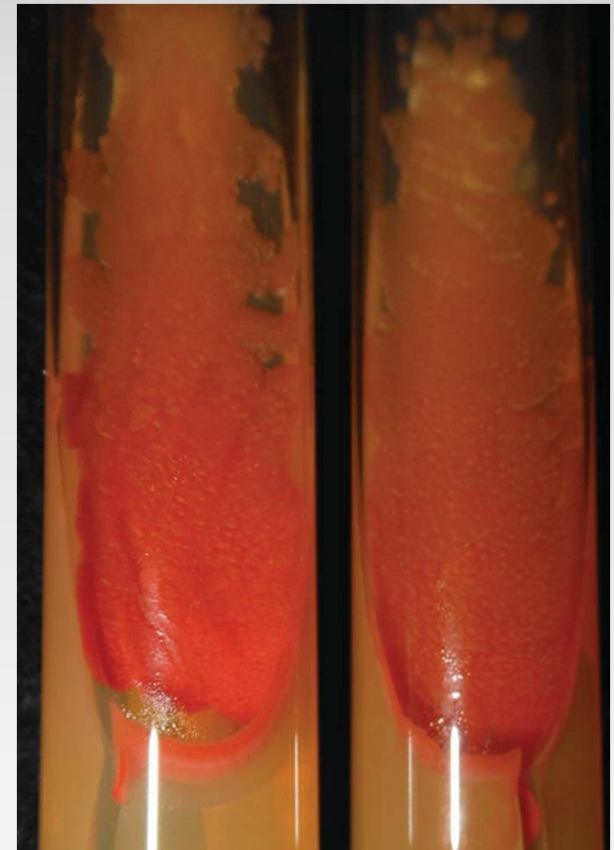
### Glucose





## Materials and Methods: Lipid production

- *Rhodospiridium toruloides*
  - Cultured in 1 L minimal media supplemented with glucose
  - 30 ° C with a rotary rate of 200 rpm
  - Optical density (600 nm) used to determine concentrations
  - Environmental conditions monitored & controlled
  - Glucose & lipid levels monitored over time as previously described





## Materials and Methods - Conditions

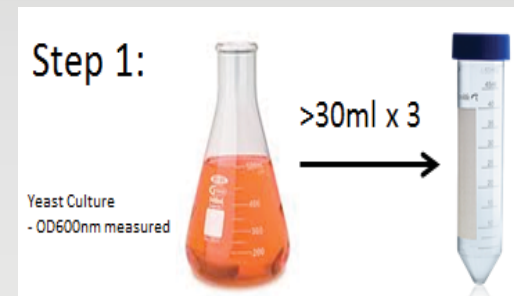
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<b>Experiment</b>	<b>Experimental Conditions</b>	<b>Duration (d)</b>
<b>RB 1</b>	90 g/L glucose in minimal media; no pH control	14
<b>RB 2</b>	60 g/L glucose in modified minimal media; pH control D7-14	21
<b>RB 3</b>	60 g/L glucose in minimal media;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



## Materials and Methods: Lipid detection

- Lipid composition occurred in three stages
  - freeze drying
  - lipid extraction
  - trans-esterification/methylation





## Materials and Methods: Lipid Analysis

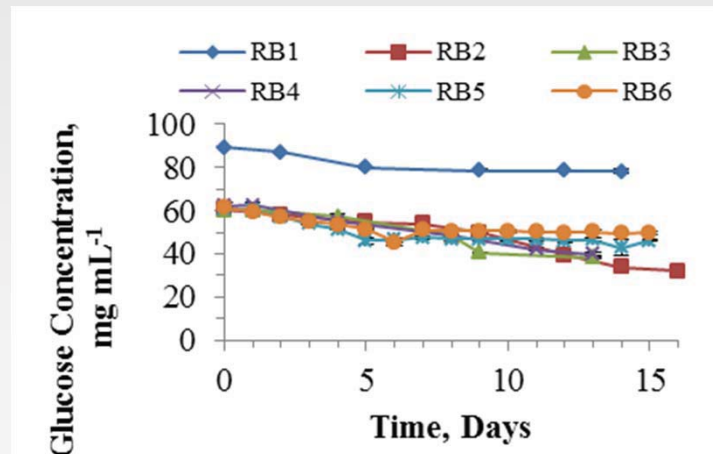
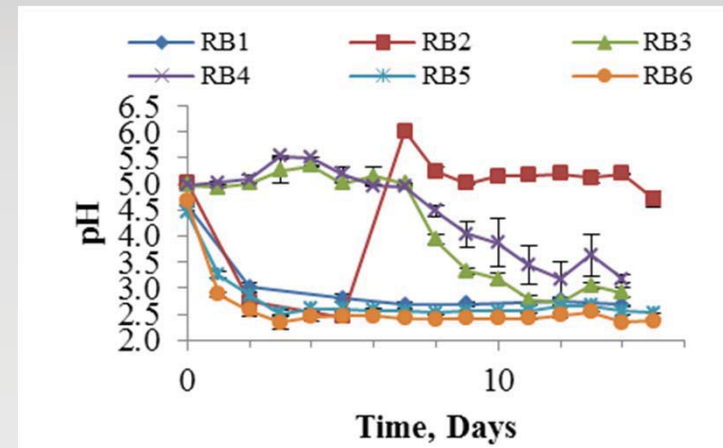
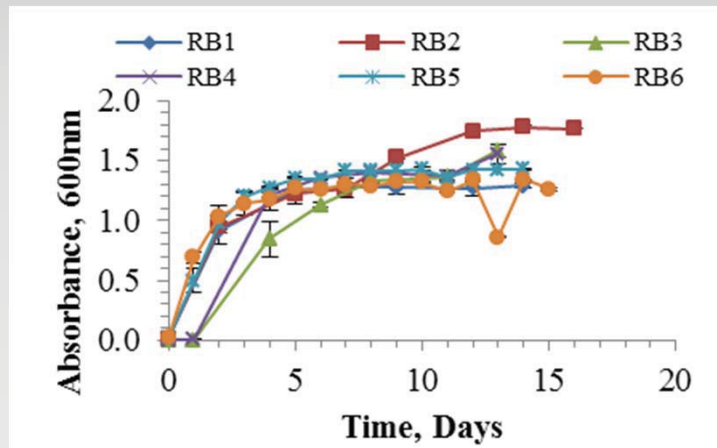
- Fatty acids analyzed with a Trace Ultra gas chromatograph (GC)
  - fitted with a 30m x 0.25mm x 0.25 $\mu$ m DB-225ms column
- Determined % total lipid recovery of dry cell weight
- Quantified 6 fatty acids of interest


oleic acid	palmitic acid
stearic acid	myristic acid
linoleic acid	linolenic acid





## Results: Growth & Environmental Conditions - Lipids

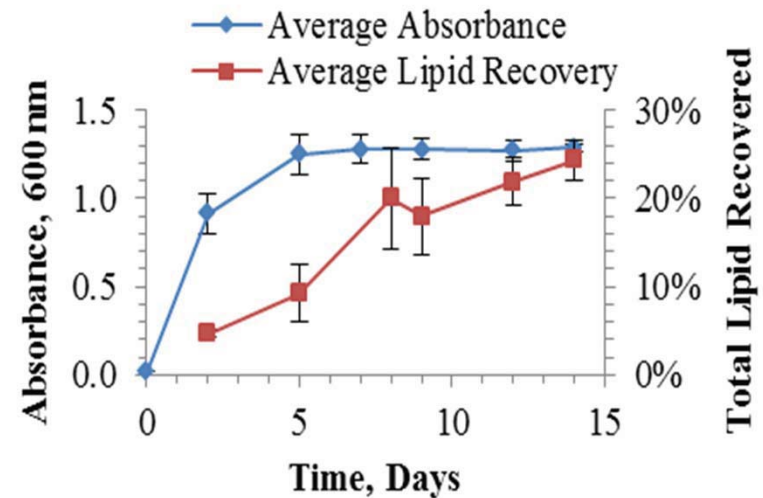
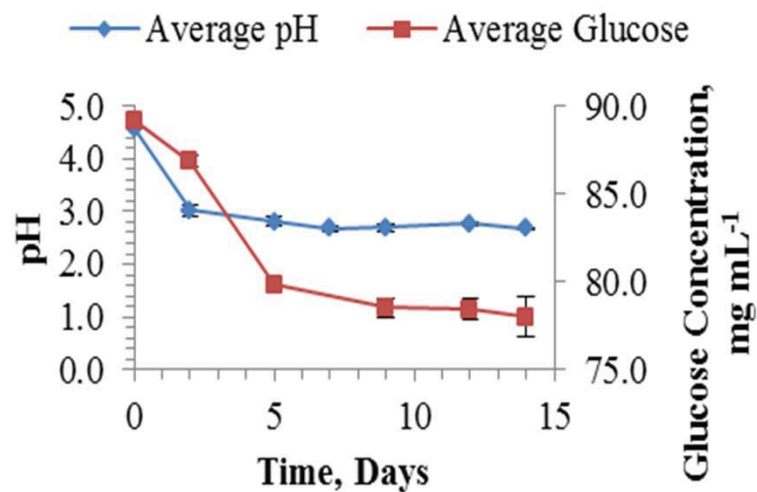






## Results: Environmental Factors & Lipid Production

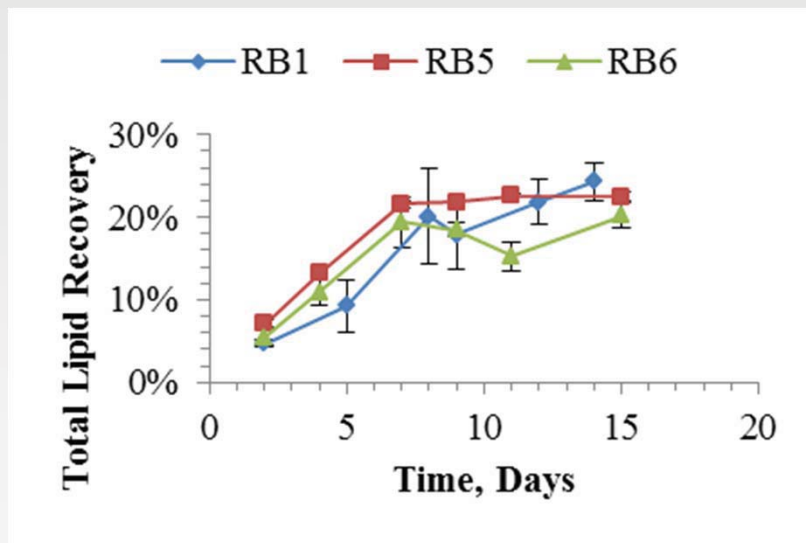
- pH level decreased to about 3.0 or below
- Glucose decreased indicating culture growth





## Results: Lipid Production & Fatty Acid Analysis

- Total lipid content below what expected
- Individual analysis of six fatty acids revealed the percentage of each fatty acid was lower than naturally produced bovine milk.



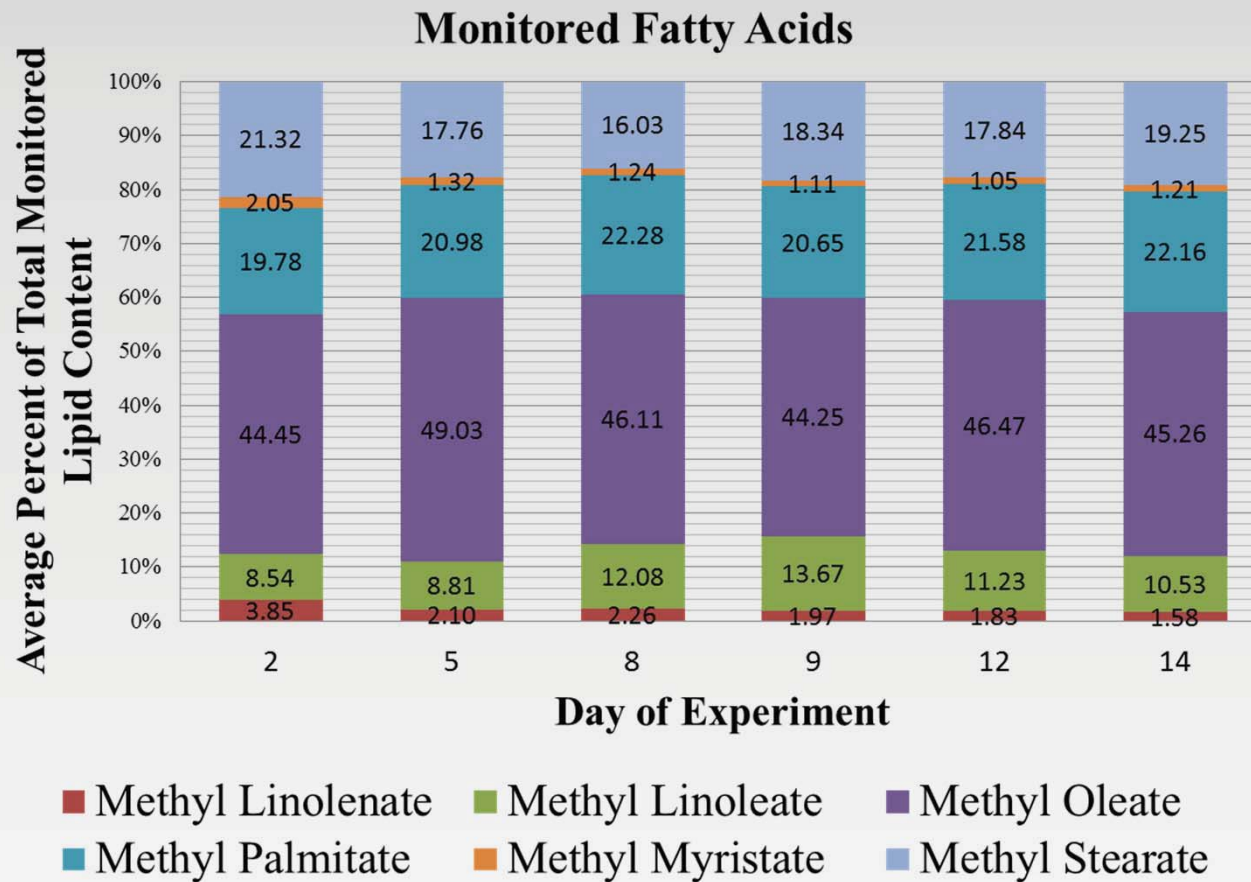
RB1 -minimal media  
with 90g/L glucose.

RB5 minimal media with  
60g/L glucose.

RB6 modified minimal  
media with 60g/L  
glucose.



## Results: Lipid Production – Average Fatty Acid Content





## Results: Lipid Production & Bovine Comparison

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



## Discussion and Conclusions

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- Glucose and fatty acid production were lower than expected
- Sugar produced by breakdown of cellulose could serve as a resource for other microorganisms to produce lipids
- Of 6 fatty acids investigated, all were present but in lower percentage than bovine milk



## Discussion and Conclusions

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- Environmental parameters were crucial to continued performance of cultures
  - Fed-batch culture with a biphasic pH and temperature regime required.
- Cultures need to increase biomass at optimal pH before recovery of glucose
- Microorganisms could be utilized to breakdown inedible solid waste to produce glucose



## Further Studies:

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- Continue with fed-batch experimentation, glucose extraction and reactor scale studies
- Proceed to fed-batch & reactor scale studies, as well as lipid extraction techniques for use in simulant milk.
- Investigate additional organisms for increased lipid production
- Remaining third component, casein or milk protein became the missing piece of the puzzle.
  - Required a GMO, beyond the scope of this project



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# Questions?

