

**Southeastern Sun Grant Center Quarterly Progress Report  
Q2, Yr1**

**Project Title:** Conversion of agricultural materials to biofuels and bioproducts by *Thermotoga neapolitana*, a hyperthermophilic, anaerobic bacterium

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**Project Location:** Clemson University

**Reporting Period:** April 1, 2008 to June 30, 2008

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**IMPORTANT NOTE:** If any part of your quarterly report contains **proprietary/confidential information**, or details that should not be released to the general public, the specific sections of the report should be marked as such, by clearly marking the beginning and end of the confidential information. The marked sections will not be released to the general public or any unauthorized parties.

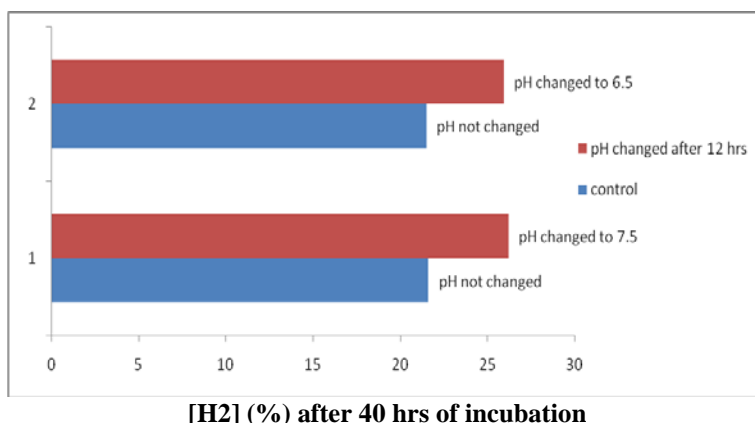
**There is no confidential information in this quarterly report.**

1. **Planned Activities:** The following outline provides the planned activities for Q2 of 2008.
  - a. The hydrogen production from peaches will pursue the following:
    - i. Media optimization studies to develop minimal cost media per mol of hydrogen produced.
    - ii. Assessment of hydrogen production from continuous bioreactor operation using peach media.
  - b. The assessment of hydrogen production from switchgrass will pursue the following:
    - i. Enrich the *T. neapolitana* on the milled switchgrass,
    - ii. Assess the effects of pretreatment, such as lignin removal, on hydrogen production by *Thermotoga. neapolitana*,

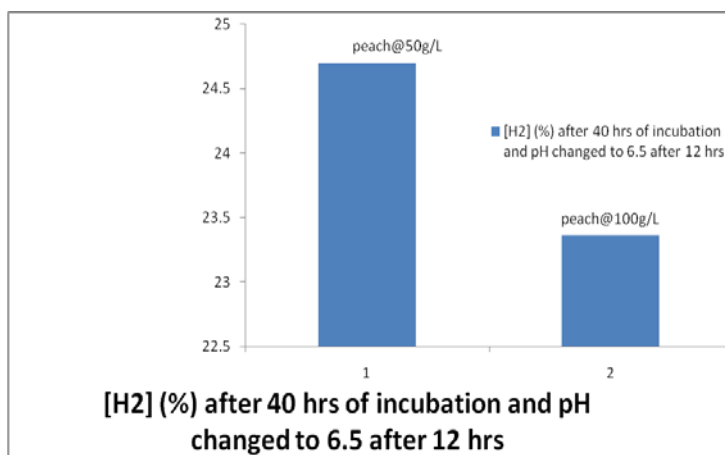
2. **Actual Accomplishments:**

Peaches of the Redheaven variety were processed by blending and adding with other media components per liter as follows- 2.0 g of yeast extract, 2.0 g of trypticase, 1g of NH<sub>4</sub>Cl, 0.3 g of K<sub>2</sub>HPO<sub>4</sub>, 0.3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of MgCl<sub>2</sub>·2H<sub>2</sub>O, 0.1 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 10.0 g of NaCl, 0.1 g of KCl, 1.0 g of cysteine HCl, 0.121 g of trizma base and 10.0 mL of vitamin solution and 10.0mL of trace element solution as specified in DSMZ 141 media.

- A. Three replicates of each of batch reactors containing autoclaved and unautoclaved media with 100 ml working volume and containing 50g/L of blended peaches with added nutrients were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm. There was no difference in hydrogen production after 20 hrs of incubation ( $\alpha=.05$ ). Unautoclaved peach media has thus been used for rest of the work reported here.
- B. Three replicates of each of batch reactors with 100 ml working volume and containing 50g/L of blended peaches with added nutrients were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm. The pH was set to 6.5 after 12 hrs of incubation using 5 N NaOH in the respective reactors. There was an increase of 20.6% approximately in hydrogen production after 40 hours of incubation.
- C. Three replicates of each of batch reactors with 100 ml working volume and containing 50g/L of blended peaches with added nutrients were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm. The pH was set to 7.5 after 12 hrs of incubation using 5 N NaOH in the respective reactors. There was an increase of 21.2% approximately in hydrogen production after 40 hours of incubation.



- D. Three replicates of each of batch reactors with 100 ml working volume and containing 50g/L and 100g/L of blended peaches respectively with added nutrients were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm for 40 hours. The pH was set to 6.5 after 12 hrs of incubation using 5 N NaOH in the respective reactors. There was a decrease of 5.4% approximately in hydrogen production in reactors containing blended peaches at 100g/L as compared to reactors containing blended peaches at 50g/L.



- E. Three replicates of each of batch reactors with 100 ml working volume and containing 50g/L of blended peaches respectively with soyabean meal at 5.0g/L as nitrogen source (and NH<sub>4</sub>Cl, trypticase and yeast extract were not added to the media) were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm. There was a decrease of 27% approximately in hydrogen production as compared to reactors with trypticase, yeast extract and ammonium chloride together as nitrogen source, after 20 hours of incubation.
- F. Three replicates of each of batch reactors with 100 ml working volume and containing 50g/L of blended peaches with added nutrients containing NaCl at 2.5g/L were prepared at initial pH of 8.0 at room temperature and inoculated with *T. neapolitana* (2% inoculum) and incubated at 77°C and 200 rpm. There was no hydrogen production observed in these reactors after 20 hours of incubation.

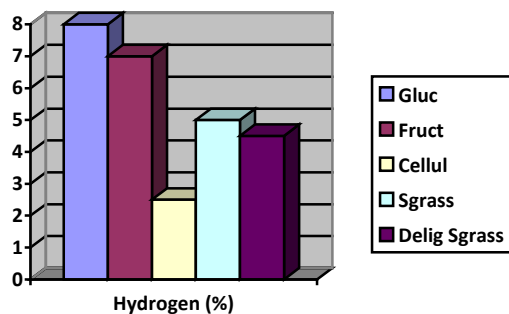
A second substrate being evaluated as a potential feedstock for biofuel production is switchgrass. Dried switchgrass, obtained from the Clemson University Pee Dee Research and Education Center, was milled using a Thomas Wiley Model 4 lab mill to obtain particles of about 4 mm size. For delignified switchgrass evaluations, the

milled switchgrass was treated with 15% ammonium hydroxide overnight at 60°C. The delignified switchgrass was then washed with distilled water until a neutral pH, approximately 7, was obtained.

The medium for *T. neapolitana* was prepared as follows: switchgrass, 5.0g; delignified switchgrass, 8.0g; yeast extract, 2.0g; trypticase, 2.0g; NH<sub>4</sub>Cl, 1.0; K<sub>2</sub>HPO<sub>4</sub>, 0.3g; of KH<sub>2</sub>PO<sub>4</sub>, 0.3g; MgCl<sub>2</sub>·2H<sub>2</sub>O, 0.2g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1g; NaCl, 10.0g; KCl, 0.1g; cysteine HCl, 1.0g; trizma base, 0.121g; vitamin solution, 10 mL; and, 10.0mL of trace element solution as specified in DSMZ 141 media. The pH of the medium was adjusted to 8.5, at room temperature, with 5N NaOH, placed into serum bottles, sparged with nitrogen gas, closed with butyl rubber stoppers, sealed with aluminum crimps, and autoclaved at 121°C for 15 minutes.

The following paragraphs provide experiments conducted to assess the conversion of switchgrass to hydrogen. Inoculation of media with *T. neapolitana* was by sterile needle and syringe that had the atmosphere replaced with either an inert gas or reduced medium. The serum bottles were incubated with shaking, 150 rpm, in a floor shaker at 77°C.

- A. Three replicates of each of the following were prepared and the headspace of each serum bottle was analyzed by gas chromatography. Uninoculated controls neither produced hydrogen nor exhibited turbidity. Each of the carbohydrate substrates were provided at a final concentration of 5g/L. The following data provide the percent hydrogen observed after 3 days of incubation.



- B. To assess the effect that autoclaving switchgrass may have by possibly releasing growth substrates for *T. neapolitana*, media were prepared with switchgrass and delignified switchgrass as described above. After autoclaving, the liquid portion of the medium was removed and added to sterile serum bottles in which the headspace had been replaced with nitrogen. These triplicate serum bottles were inoculated with *T. neapolitana* while uninoculated serum bottles served as controls. After 3 days, the headspace for each serum bottle was analyzed by gas chromatography. Hydrogen was not observed in the medium containing the liquid portion from the autoclaved delignified switchgrass medium. However, the medium containing the liquid portion from the autoclaved switchgrass medium produced approximately 5 percent hydrogen in the headspace.
- C. The base growth medium contains both yeast extract and trypticase, each at 0.2% concentration. An evaluation as to whether these organic components could be a source for growth and hydrogen production was performed. Medium was prepared without added a source of switchgrass, triplicate serum bottles were inoculated with *T. neapolitana*, and allowed to shake at 77°C for 3 days. Headspace analysis indicated that the concentration of hydrogen was approximately 4%.
- D. *T. neapolitana* was enriched using the culture medium with untreated switchgrass. Upon enrichment, the culture showed turbidity after 24 hours and produced hydrogen. The culture was allowed to continue growth for 6 or more days until visible changes in the switchgrass particles were observed. This enrichment was transferred aseptically several times to enhance the cellulose-degrading ability. To assess the visible changes in the particles, three enrichments and three uninoculated controls were filtered through dried and preweighed 0.8mm filters. After drying at 60°C, the filters were weighed until a constant dry weight was observed. The difference in dry weight between the two treatments indicated removal of 9.4% of the switchgrass in the enrichment culture.

- E. Preliminary assessments of the conversion of cellulose and hemi-cellulose to carbohydrates were initiated. Samples were prepared and injected into an HPLC equipped with a Bio Rad Aminex HP-87X column and a Refractive Index Detector. Carbohydrates were not detected.

3. **Explanation of Variance:** The planned tasks were accomplished in Q2, Yr1.

4. **Plans for Next Quarter:**

- a. The hydrogen production from peaches will pursue the following:
  - i. Continue to evaluate possible changes to the medium to reduce the cost per mol of hydrogen produced.
  - ii. Assess hydrogen production from continuous bioreactor operation using peach media.
- b. The assessment of hydrogen production from switchgrass will continue as follows:
  - i. Continue to enrich the *T. neapolitana* on the milled switchgrass,
  - ii. Continue to evaluate the carbohydrates present in the medium as the switchgrass is converted from cellulose and hemi-cellulose,
  - iii. Assess the production of organic acids as *T. neapolitana* grows, and
  - iv. Evaluate cellulase activity for cultures of *T. neapolitana*.

5. **Patents:** None

6. **Publications / Presentations:** None