Meeting Minutes

MacroFuels WP3 meeting

Date: 27-06-2016

Time: 14.05-16.00

Location: Radisson Blu Saga Hotel, Reykjavik, Iceland

Participants

Name	Company
Ana M. López Contreras	DLO
Truus de Vrije	DLO
Anne-Belinda Bjerre	DTI
Xiaoru Hou	DTI
Randi Neerup	DTI
Michele Stanley	SAMS
Angela Hatton	SAMS
Bryndís Björnsdóttir	Matis
Ólafur Friðjónsson	Matis
Antoine Moenaert	Matis

Agenda

1. **Welcome and general matters WP3 (Ana López Contreras)**
   - Minutes from Skype meeting 03-03-2016, action items

2. **Task 3.1. Fermentation of seaweed syrups to ethanol by mesophilic organisms (DTI) – *Anne-Belinda Bjerre***

3. **Task 3.2. Thermophilic anaerobic biorefinery organisms (MATIS) – *Antoine Moenaert***

4. **Task 3.3 Efficient fermentation of seaweeds and seaweed fractions to ABE (DLO)- *Ana López Contreras***

5. **Task 3.4 Anaerobic digestion of seaweed fractions (SAMS)- *Angela Hatton***

6. **General Discussions**
   - Action list
   - Dissemination activities, done and planned in next 6 months
   - Next meeting: suggestion in October on skype

Minutes of meeting

1. General matters. The meeting started with the introduction of the participants. Anne-Belinda Bjerre introduced two new employees at DTI. Randi Neerup (present, chemical and biochemical engineer) will coordinate the analyses of seaweed hydrolysates and fermentation products by HPLC. A senior researcher starts September 1st as Task 3.1 leader and he will be responsible of the ethanol fermentations with meso- and thermophiles.
From SAMS, Angela Hatton is involved in the anaerobic digestion of seaweed residues; Michelle Stanley is taken part in WP1 (cultivation, harvesting, and storage/transportation), 3, and 6 (Techno-economic and sustainability assessment).
From Matis, Antoine Moenaert is a French engineer in biotechnology, and Ólafur Friðjónsson is an expert in molecular biology of thermophiles, and involved in process development of algae.

- Minutes from Skype meeting 03-03-2016

Action items

1. Organization next WP3 meeting, done (ALC)

2. Design pretreatment protocol together with WP2 DLO colleagues, ongoing and to be continued when seaweed biomass arrives (ALC)

3. Requirements of residues from fermentation experiments for biogas production, ongoing. For initial experiments in bottles little amounts (dry weight of residues) are necessary (AH, MS). Results from experiments with residues from ethanol or ABE fermentation might differ because of the medium (supplements) composition. Co-production from both types of residues might be considered (MS).

4. Volume and purity of fuel needed for engine tests, according to Jaap van Hal a minimum of 20 L of distilled alcohols is necessary at a blending of 10% (200 L of fuel). DTI can make use of the large scale distillation facility at the 2nd generation ethanol plant. At DTI a 800L pilot tank for production of

hydrolysate is available. For another project DTI produced hydrolysate from 30 kg of *Laminaria* (per L: free glucose 10 g, oligomeric glucose 10 g, mannitol 3 g, uronic acid not determined) of which approx. 100L was stored. It was used in a 5L reactor for ethanol fermentation. And lab scale (100 mL) test of butanol fermentation (fermentable without inhibition) resulted in high yields of 0.37 g butanol per g glucose + mannitol consumed. The yield is higher than the theoretical one, potentially by consumption of oligosaccharides and/or glucuronic acid. A paper is in preparation. DTI will distribute 1L of this hydrolysate to Matis and 2L to DLO for initial fermentation experiments. If successful DLO will receive approx. 50L of the hydrolysate for ABE fermentation, together with information on the protocol of pretreatment and enzymatic hydrolysis (*note by TV*: approximately 0.5L of butanol can be produced from 50L of hydrolysate).
SAMS will determine the bio-methane potential of the solid residue of the hydrolyzed *Laminaria*. For this 2 – 3 grams dry matter is needed (Xiaoru has to check with the lab technician if there are still enough residues left, as all the solid residues were sent for preparing feed ingredients). Also the fermentation broth of ethanol and ABE fermentations, without the alcohols, will be tested for bio-methane potential. In general, it is agreed to store all residues from hydrolysis and fermentation experiments for anaerobic digestion.

5. Responsible partners for analysis of components, ongoing.

2. Progress in Task 3.1. Activities in this task will start in September with the appointment of a new employee. Experiments on ethanol fermentation will start with dried as compared to ensiled seaweed biomass. MS: Seaweed is ensiled in closed, large barrels with biomass squashed down. Brown seaweeds ensiled for 9 month loose some glucose but alginate and mannitol content do not change. One year of storage was too long, and last Spring was too warm. It is preferred to use a specific inoculum than fermentation by endogenous bacteria. ABB: in WP2 a student will develop an assay to test how much substrate is necessary to stop the fermentation by lowering the pH. Possibly some cheap sugar or lactic acid will be added to save on seaweed substrate.

3. Progress in Task 3.2. see presentation by Antoine Moenaert (in the internal MacroFuels webpage). The thermophilic (60 °C, from hot spring) anaerobic strain *Thermoanaerobacterium* AK17 is an ethanol producer and has a natural competence for genetic modification which is not 'normal' for thermophiles. Three approaches for high yield ethanol production of brown seaweeds: 1) Deletion of side-product pathways (acetic and lactic acid) by knock-out mutants was successful. Increase of ethanol tolerance to 20 g/l restored acetic acid production probably from the butyrate pathway which will now be deleted too. 2) Thermostable laminarase was expressed in AK17 but was not secreted. Another approach will be tested. 3) Two alginate lyase enzymes with endo- and exo-activity are able to hydrolyse 99% of alginate. This will be patented. A DEH reductase will be expressed for metabolism of uronic acids. Transport systems for uronic acids from alginate might not be necessary because transport systems for other uronic acids are present in AK17.
Discussion: XH/BB: H_2 production by AK17 was not measured yet, but will be by another laboratory in the North of Iceland. XH/OF: laminarin is a polymer of β1,3- and β1,6-linked glucans. Cellulose is a polymer of

β1,3- and β1,4-linked glucans. ALC/BB,AM: AK17 has only one copy of a butyrate kinase in contrast to Clostridia. XH/AM,BB: fermentations of real seaweed substrate will start soon. Matis will not produce 20L of ethanol.

4. Progress in Task 3.3. see presentation by Ana López Contreras (in the internal MacroFuels webpage). ABE fermentation by Clostridia from seaweed biomass is studied and 2 approaches, physiological and genetic, are followed. *Clostridium beijerinckii* is inhibited by Na^+ and K^+ salts at concentrations found in seaweed hydrolysates (previous project). *Discussion:* ALC/ABB: These hydrolysates were prepared by hydrochloric acid treatment (without enzymatic hydrolysis) and possibly the acid will extract salts from the seaweed biomass which end up in the hydrolysate. ALC: a thermal, mild treatment (in combination with enzymatic hydrolysis) of Saccharina biomass resulted in a high sugar content hydrolysate (60 g/l) which had to be diluted for fermentation. K^+ and Na^+ content was still high. ABB: the benefit of ensiling is that water and probably also salts are extracted from the biomass which is 50% less wet. XH: with ensiled biomass the lag phase disappeared in biogas fermentations. ALC: salts are entrapped in the hydrocolloids. The potential inhibition by polyphenols (very dark colored hydrolysates) was mentioned. OF: Ca^{2+} and Mg^{2+} content is dependent on time of harvest and differs between seaweed types. High Ca^{2+} contents can be due to shells attached to the seaweed. Under acid conditions Ca^{2+} will end up in hydrolysates. ALC: elemental analyses is important and will be done at a central point, i.e. at ECN.
The genetic approach will be used to develop Clostridia strains suitable for consolidated bioprocessing. For two Clostridium strains the CRISPR/Cas9 system was developed for deletion and insertion of genes in the genome without using markers. A stable mutant with a cellulase gene was obtained which has to be characterized for enzyme activity. One copy is inserted in the genome and activity might be low. *Discussion*: AM/ALC: export of the cellulase out of the cell was achieved by a signal peptide of another Clostridium strain. BB mentioned that a signal peptide of *Thermoanaerobacterium* AK17 was not compatible with a foreign gene.
The strains used in WP3 for alcohol production are: DLO 2 Clostridia strains; DTI *C. beijerinckii*, ethanol production by 2 mesophilic and 1 thermophilic.

5. Progress in Task 3.4. AH: Experiments will start with undigested seaweed biomass.

6. General discussion

- Dissemination activities, done and planned in next 6 months

ABB (and Jaap van Hal) presented MacroFuels at the Seaweed conference in Denmark last week, an overview of MacroFuels (ABB) and analysis of sugars (JH). Future MacroFuels presentations are by ABB and Paulien Harmsen at the Nordic conference in Denmark, Oct 2016, by Paulien Harmsen at Seagriculture in Portugal, Sept 2016. A book chapter is in preparation, coordinated by ALC with contribution of other partners.

- Next meeting: suggestion in October 2016 on skype
- Action list, see table

Noted by Truus de Vrije; Revisions by Ana López Contreras and Xiaoru Hou.

Action Items

#	Action item	Responsible
1	Organize next WP3 skype meeting, October 2016	Ana López Contreras
2	Upload conference and meeting presentations on web site, via Rita Clancy	All
3	*Laminaria* hydrolysate of DTI sent to DLO (2 L) and Matis (1 L), together with pretreatment / enzymatic hydrolysis protocol	DTI
4	*Laminaria* solid residue of hydrolysate preparation by DTI send to SAMS (2 – 3 grams dry matter)	DTI
5	Storage of all residues of hydrolysis/fermentation experiments for anaerobic digestion	DLO, DTI