Technische Universität Hamburg-Harburg

Thermische
Verfahrenstechnik

Technical Note 86.3.3

on the MAP-Project

"A Total Converting and Biosafe Liquefaction Compartment for MELISSA"

Work Package 3.300: Production and characterization of substrate coming from hyperthermophilic reactor

Tobias Albrecht, Gerd Brunner

TU Hamburg-Harburg, Arbeitsbereich Thermische Verfahrenstechnik,
Eißendorfer Str. 38, 21073 Hamburg, Germany

E-mail: albrecht@tu-harburg.de, Fax: +49 40 42878 4072

Technical Note 3.3 Jürgen Kube, Herbert Märkl

1. Introduction

In the last technical notes were shown that a liquefaction of the ESA substrate is partly achieved with the hyperthermophilic consortium from the Azores. The liquefaction efficiency was 25% during continuous fermentation, 40 % in batch fermentations and 60% in dialysis batch experiments. However these values show that a total liquefaction is still not achieved. In this technical note two possibilities are shown to increase the liquefaction level further. The first way is dialysis fermentation with a continuous dialysate exchange. Biller [2002] showed that it is possible to increase the cell density dramatically by the use of dialysate membranes.

Another way to achieve a better liquefaction is to lower the temperature to 70°C and work with new microorganisms. After a literature study two bacteria were found which are able to degrade cellulose, hemicellulose and pectin anaerobicly. These bacteria are related to the clostridia family though they are not spore forming. The absence of resistant dormant forms allows the application in the ESA-cycle.

Additionally it was tested if mesophilic pre-treated substrate form a methanogenic step can be liquefied further in a hyperthermophilic step. The residue of the first and second closed loop experiment was inoculated with the consortium from the Azores, though hyperthermophilic acetate oxidation was never monitored before. The results

showed that no liquefaction was achieved in first closed loop and only a minor liquefaction in second closed loop appeared.

2. Material and Methods

2.1 Substrate

2.1.1 ESA-Substrate

The ESA-Substrate consists of wheat straw, soy pellets, cabbage, algae, and faecal matter. To draw balances, the carbon- and COD-content of each constituent was determined.

The COD-values and TC-values of the ESA-substrate were calculated to COD_{feed}= 19.44g_{COD}/l, TC_{feed}=6.00g_C/l, and TN_{feed}=0,53g_N/l (without faeces, TS=1.5%). The C/N ratio is calculated to 11.9.

2.2 Determination of total solids (TS)

2.2.1 Evaporation at 105°C

Two samples of 10 ml each are filled in previously dried and weighed ceramic beakers. The samples are weighed again. Then the beakers are dried at 105°C for 48 hours. After this the beakers are cooled down and weighed again. The TS-value is calculated by dividing the mass difference after drying by the mass difference before drying. The arithmetic average of the two measurements is determined.

2.2.2 Filtration with 0.45µm filter

A 0.45µm cellulose-filter is dried for 24h at 105°C. The filter cools down to ambient temperature in an exicator and is weighted. The sample is filtrated with a vacuum pump and flushed twice with 5 ml demineralised water and once with 5 ml 5% HCl Solution to remove adhered salts. The loaded filter is dried afterwards for 8h and weighted again.

2.3 Minimal Media

For Fermentation of pure cultures the following media were used

2.3.1 *Pyrococcus furiosus*

The medium was described in the last technical note

2.3.2 *Caldocellulosiruptor lactoaceticus*

Medium 671 from DSMZ was used. The medium contains the following substances [mg/l]: 1000 NH4Cl, 100 NaCl, 100 MgCl2x6H2O, 50 CaCl2x6H2O, 400 K2HPO4x3H2O, 2600 NaHCO2, 750 Yeast extract, 500 Cysteine, 10ml Trace element solution from DSMZ Medium 141, 1ml vitamin Solution from *Pyrococcus furiosus* medium, 1ml Resazurin solution.
For pre-cultures 2g/l cellulose was used as main carbon source.

2.3.3 *Anaerocellum thermophilum*

Medium 516 from DSMZ was used. The medium contains the following substances [mg/l]: 330 NH4Cl, 330 KH2PO4, 330 KCl, 330MgCl2x6H2O, 330 CaCl2, 500 Yeast extract, 1500 NaHCO3, 500 Cysteine, 1ml Resazurin Solution, 10ml Trace element solution, 1 ml vitamin solution.
For pre-cultures 5g/l cellobiose was used as main carbon source.

2.4 Microorganisms

2.4.1 *Pyrococcus furiosus*

The hyperthermophilic Archaeon *P. furiosus* (DSZM 3836) was isolated by Fiala and Stetter [1986] from geothermal vents. The substrate spectrum covers proteins and α-1,4 and α-1,6. It is able to degrade cellobiose, but shows no activity on cellulose. The Fermentation of *Pyrococcus furiosus* is well known and described in several publications, Krahe et.al. [1986], Raven and Sharp [1996].

2.4.2 *Caldocellulosiruptor lactoaceticus*

The extremthermophilic Bacterium *C. lactoaceticus* (DSMZ9545) was isolated by Mladenovska et al. from alkaline hot springs in Iceland. The bacterium is not spore forming. The optimal growing conditions are 70°C and pH7. *C. lactoaceticus* is able to break down cellulose and degrade it to CO2 (8%), acetic acid (18%), lactate (52%), ethanol (1%), hydrogen (1%) and glucose (16%). The turnover was 95% after 3 days. *C. lactoaceticus* is also able to degrade pectin and xylose.

2.4.3 *Anaerocellum thermophilum*

The extremophilic bacterium *A. thermophilum* (DSMZ6527) was isolated by Svetlichny et al. [1990] from geysers in Kamchatka, Russland. The bacterium is not

spore forming. The optimal growing conditions are 75°C and pH7.2. *A. thermophilum* degrades Cellulose to lactate and acetic acid, CO2 and H2. *A. thermophilum* uses a wide range of carbon sources, such as glucose, mannose, glycogen, fructose, maltose, starch, and sucrose.

2.4.4 Consortium from the Azores

The hyperthermophilic consortium was isolated from hot sources at the Azores, Portugal. Fermentation of ESA-Substrate showed a liquefaction of 40%. Lactate was only produced in traces. The main metabolic products are acetate, propionate, butyrate and valerianate.

2.5 Fermentation setup

2.5.1 Batch fermentation

For the fermentation a 2l Visual Safety Reactor from Bioengineering (Wald, Switzerland) was used [Biller et al. (2002); Krahe (1998)]. The fermentor was equipped with temperature and pH-control. The titration was done with 2M NaOH. The fermentation parameters were: T=90°C, stirrer speed=500rpm, pH on-line 7.0, fermentation volume 1.5 litres, if not otherwise mentioned. The fermentor set up is shown in figure 1.

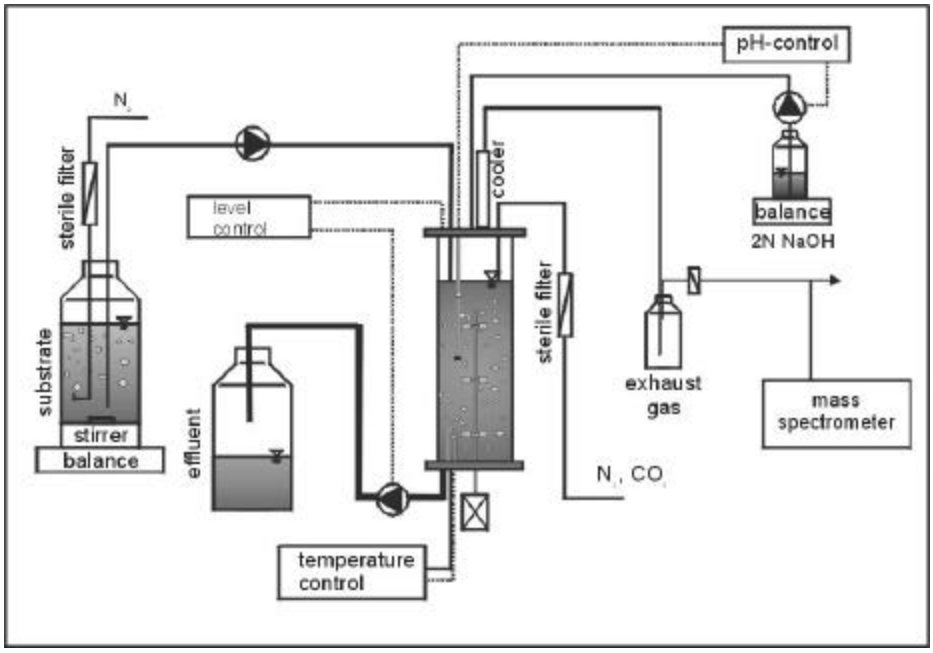

Figure 1 Hyperthermophilic pre-treatment of fibrous material, experimental set up

2.5.2 Dialysis batch fermentations

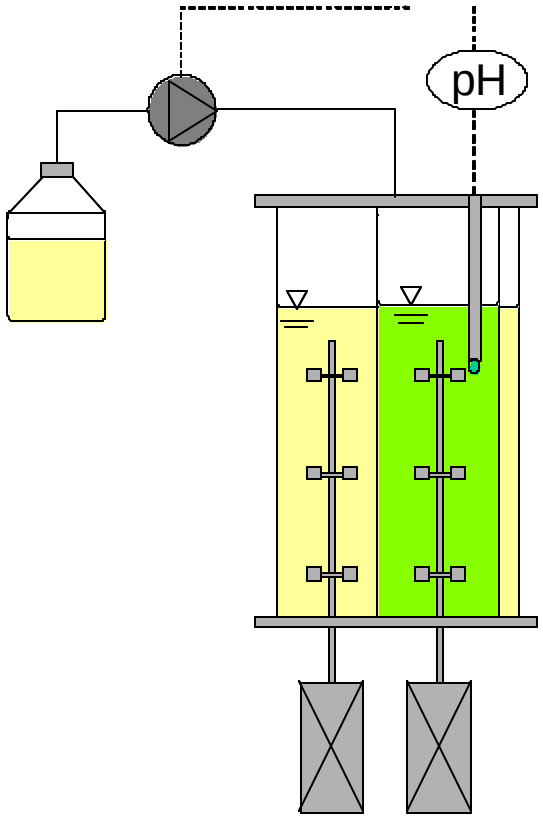

Figure 2 P&I of a dialysis batch experiment

Dialysis fermentations were performed in a 6l and 10l dialysis reactor. The principle of dialysis is shown in figure 2.

2.5.3 Dialysis fermentation with fixed dialysate exchange rate

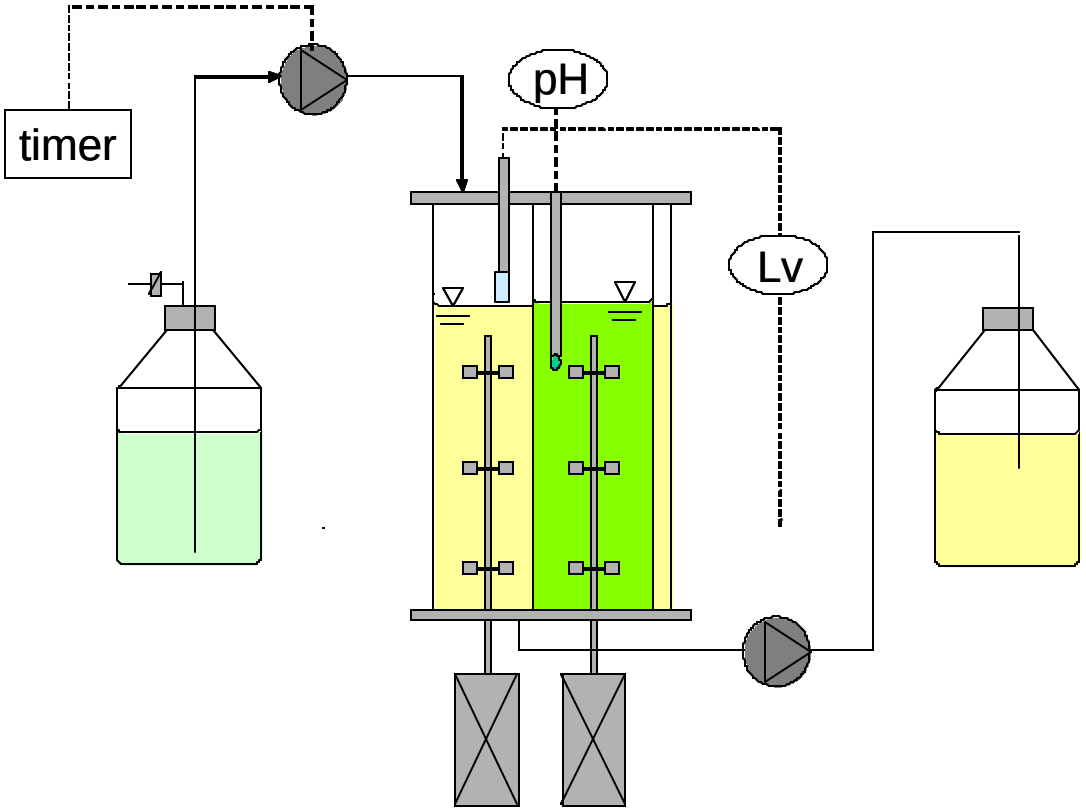

Figure 3 Dialysis fermentation with fixed dialysate exchange rate

In figure 3 dialysis fermentation with fixed dialysate rate is shown. The pH-value is just monitored. No pH-control is applied. A level-controller maintains a constant reaction volume.

2.5.4 Dialysis fermentation with pH-controlled dialysis exchange rate

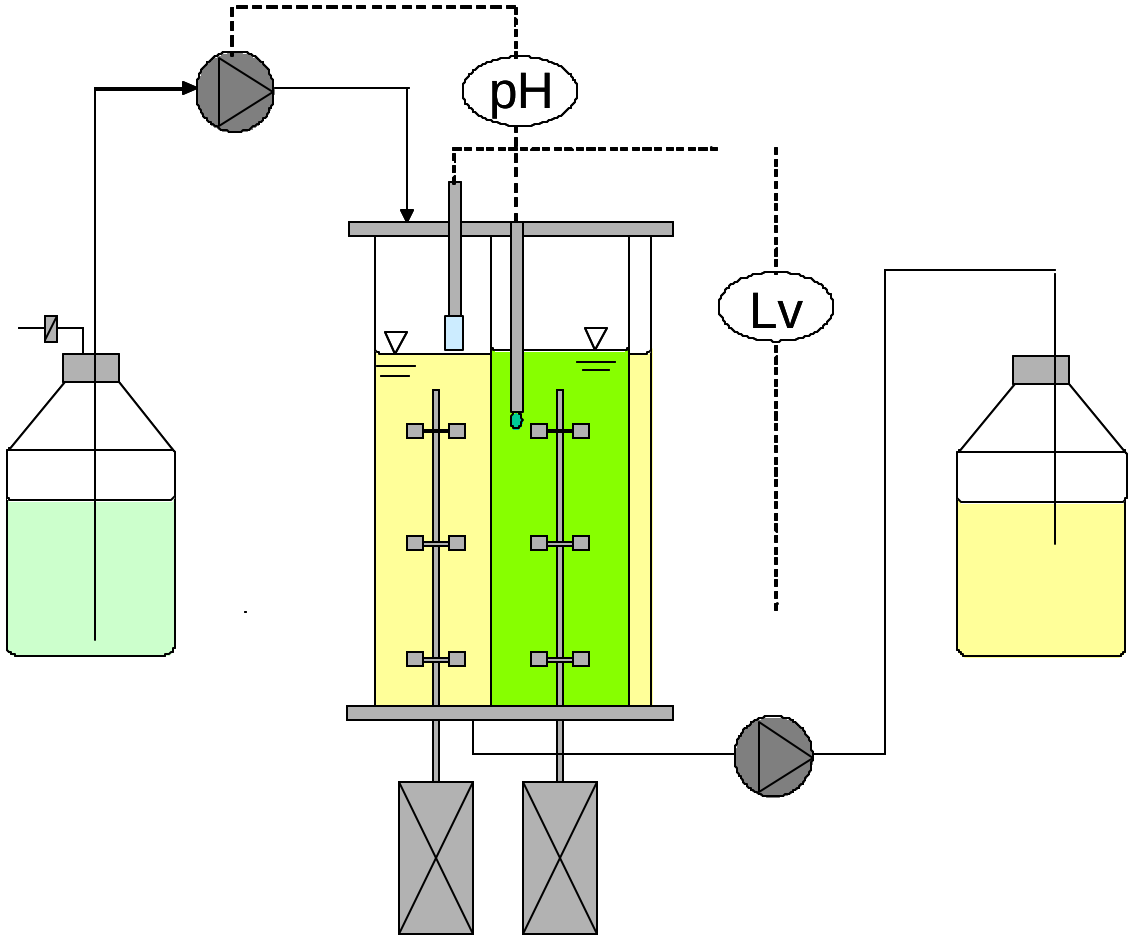

Figure 4 reactor scheme of a dialysis culture with pH control due to dialysis rate

In figure 4 dialysis fermentation is shown with a variable dialysis rate. As soon as the pH value drops below the setpoint fresh dialysate is pumped into the dialysate chamber and loaded dialysate leaves the dialysate chamber. This setup can only be applied to microorganisms which cause a decrease in pH during the fermentation. Produced acids are removed from the fermentation broth without being neutralised with NaOH titration.

2.4 Chemical Oxygen Demand (COD)

For the determination of the COD the tests LCK 414, LCK014, LCK154 from Dr. Lange with a sample volume of 200 and 1000µl were used.

2.5 Dissolved Organic Carbon (DOC), Total Inorganic Carbon (TIC)

The DOC and TIC value of the samples was determined with TOC + TN_b from Elementar.

2.6 Total Carbon (TC)

The TC value of the samples were determined with Leco CNS 2000 Analyser

2.7 Analyses of exhaust gas

The fermentor's exhaust gas is analysed qualitatively with a mass spectrometer (Omni Star, Blazers Instruments).

2.8 Lactate and Glucose analysis

Lactate and Glucose are determined simultaneously with YSI 2700 select, YSI inc. Yellowsprings USA. The measurement is done enzymaticly.

2.9 volatile fatty acids determination

VFA are determined with a headspace gas chromatograph Chrompack CP9001. A 30mx0.32mm Nukol capillary from Supulco is used. The samples are acidified with H3PO4. The detector is a FID.

2.10 dialysis membrane modules

Dialysis Membrane modules from Fresenius Medical Care were used. The modules are manufactured from Polysulphone. The capillary diameter is 200µm. The membrane area is 0.65m² for the F4/40 Module and 1.2 m² for the F6/60 module. Two types of modules are available at the moment: a high flux Membrane with a cut off of 30kDa and a low flux membrane with a cut off of 8kDa. These modules are the first which can be autoclaved with 1bar gauge at 121°C.

3. Treatment of pre-treated ESA-Substrate

ESA Substrate was treated by partner 1 in a methanogenic reactor with a hydraulic residence time of 15-20 days. Non degradable solids were removed from this reactor

after one year (first loop) and two month (second loop) and sent to partners 2 to 4 for further liquefaction. The analysis of the residue is shown in table 1.

Table 1 Elemental analysis of residue from first closed loop experiment

	C [%]	N [%]	S [%]
Closed loop residue	36,1	0,9	4,5

The first loop residue was fermented in a 6l dialysate reactor with a cultivation volume of 1.5 litres and a dialysate volume of 4.5 litres. A volumetric load of 20g/litre dry matter was adjusted. Because the residue was just the dry matter, amino acids and vitamins from *Pyrococcus furiosus* medium were added. Figure 5 shows the DOC-time plot. The figure shows that after inoculation no additional carbon is transported from solid to liquid phase. The experiment was run for 20 days, but no more carbon liquefaction occurred. Also no nitrogen was liquefied as shown in figure 6.

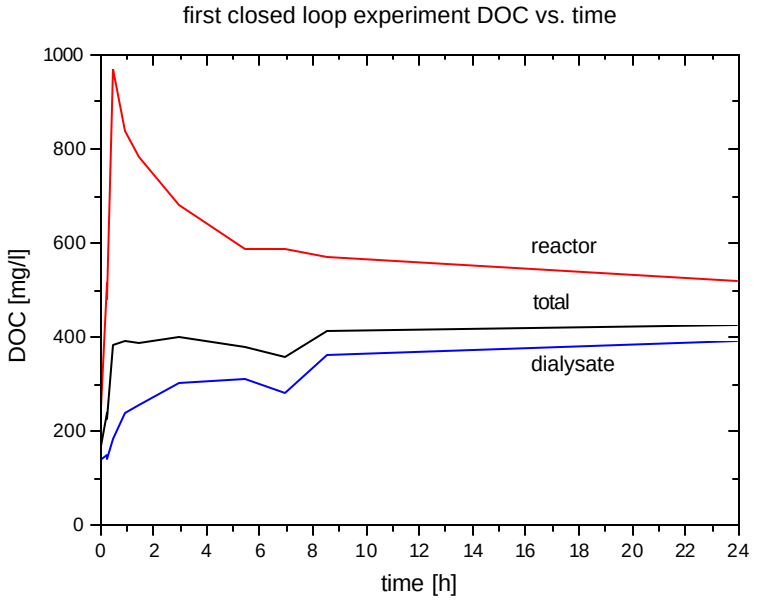

Figure 5 DOC vs. time plot in first closed loop experiment during hyperthermophilic liquefaction the red line indicates the DOC in the inner cultivation chamber; the blue line is the DOC in the dialysate. After

the inoculation DOC from the inoculum diffuses through the membrane into the outer chamber. The black line shows the average DOC content and is calculated from the volumes and DOC-levels.

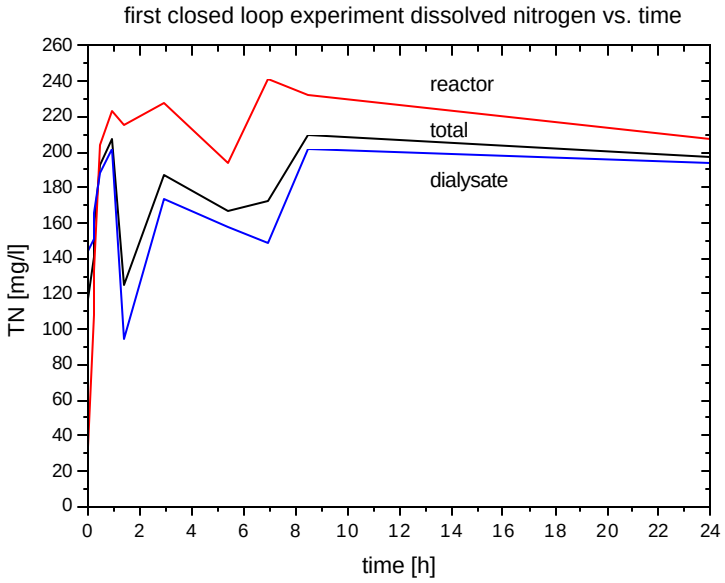

Figure 6 dissolved nitrogen vs. time plot during hyperthermophilic liquefaction of first closed loop experiments. For comments see figure 5.

The residue from the second closed loop experiment was tested in 50ml vials with *Pyrococcus furiosus* and the consortium from the Azores. The volumetric load was adjusted to 1% and 2% (w/v). The pH was set to 7 with NaOH; no other additional chemicals were added. The vials were inoculated for 14 days at 90°C without shaking. Figure 7 shows the liquefaction efficiency of the second closed loop experiment.

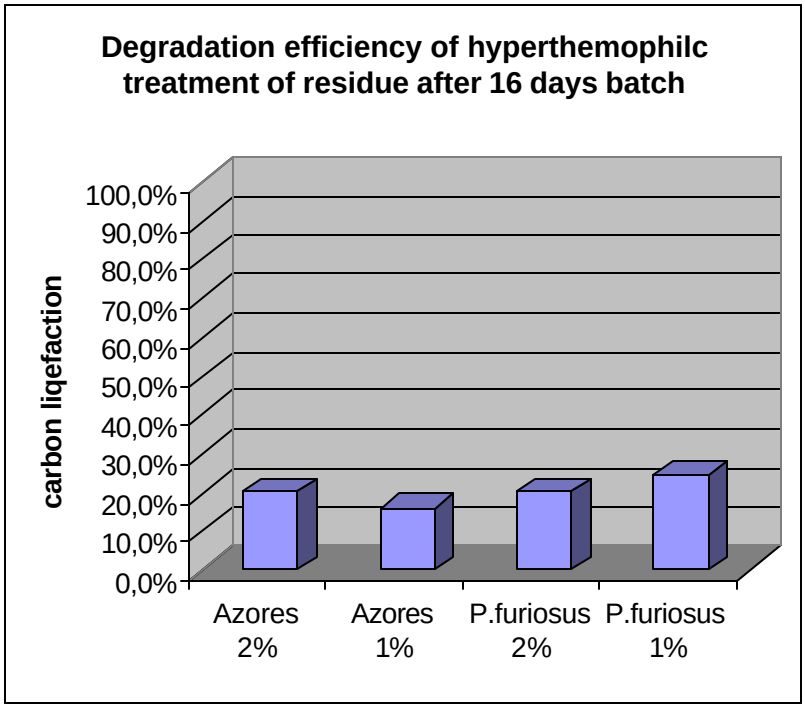

Figure 7 Hyperthermophilic degradation of second closed loop residue at 90°C pH7.

The carbon liquefaction ranges between 12% and 22%. Probably dialysis fermentation of second closed loop residue would lead to higher liquefaction rates but it will certainly not reach 100% liquefaction and was therefore not tested.

4. Hyperthermophilic Dialysis Fermentation

Hyperthermophilic Fermentations were carried out with the Consortium from the Azores at 90°C and pH7. Fermentations with other hyperthermophiles showed that these microorganisms are strongly influenced by inhibiting substances. One way to remove these inhibitors is dialysis fermentation. But not only inhibitors can be removed through the membrane, but also metabolites such as acetic acid, propionic acid and lactate or semi digested sugars (glucose and cellobiose) are removed. These metabolites have also an inhibiting effect but the inhibition of growth is only light (Krahe, 1996; Pietsch 2000, Friedmann, Märkl 1994). Nevertheless these metabolites are the main part of liquefied solids and the target molecules in the liquefaction process.

ESA substrate with a volumetric load of 1.6% (w/v) was fermented for three days with a setup according to figure 4. The pH controller regulated the dialysis flow rate. A pH setpoint of 6.5 was applied. The dialysate was demineralised water with a NaCl content of 0.5% (w/v). Technical problems with the level controller caused the level to

drop below the pH probe several times. The pH was not constant but varied from 7.0 to 5.5. After the fermentation the residue was filtered and weighted. A dry matter mass balance showed a dry matter liquefaction of 55%. Through the membrane only 33% of the total carbon was removed as shown in figure 8.

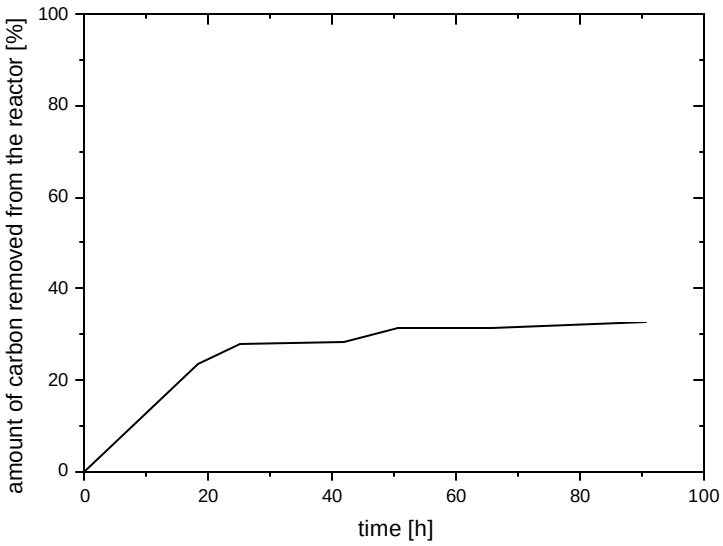

Figure 8 hyperthermophilic liquefaction experiment of ESA-substrate with pH controlled dialysate rate. The figure shows the carbon which is removed via the dialysate from the system.

The figure shows that not all of the liquefied carbon can be removed from the reactor after 4 days. At this time the membrane broke.

A membrane breakage also caused the end of an experiment according to figure 3. To prevent membrane breakage external membrane modules were considered. To describe the mass transport through a dialysis membrane eq. 1 is used.

$$\frac{dn}{dt} = P \cdot A \cdot (c_1 - c_2) \qquad (1)$$

Where the first derivative of n is the mole flux [mol/s], P is the Permeability [m/s], A is the Membrane area [m²] and c_1 and c_2 are the concentrations on either side of the membrane [mol/m²]. The permeability of the new modules was measured for glucose (M=180g/mol) and lactate (M=90g/mol). Figure 9 shows the permeability compared to a cuprophane membrane.

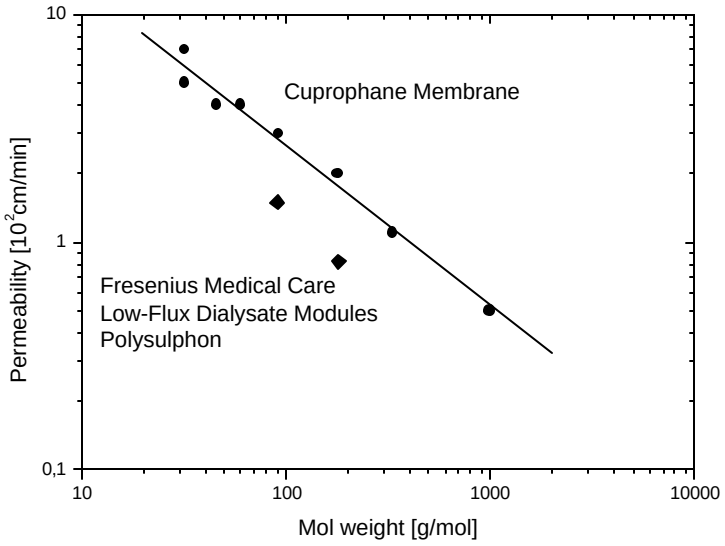

Figure 9 Permeability determination of FMC Low-Flux modules. The values for Cuprophane were listed in [Pörtner, Märkl ,1998]

Though the permeability of the Polysulphone membrane is only half of the permeability of the Cuprophane membrane the later one can not be used because it is manufactured from regenerated cellulose and will be destroyed by cellulases and esterases.

The lesser permeability is matched with a bigger membrane area in dialysis modules. The total mass transport resistance of the smallest module is seven times lower than in a membrane reactor with cuprophane membrane.

5. First cultivations with Caldocellulosiruptor lactoaceticus and Anaerocellum thermophilum.

As the hyperthermophilic consortium from the Azores could not be identified successfully new bacteria were applied for the thermophilic liquefaction step. A literature research led to 11 microorganisms for potential application. The microorganisms should be able to degrade cellulose, hemicellulose or pectin anaerobicly at high temperatures. Most of the microorganisms have a temperature optimum near 65°C, while only five are able to grow at temperature at or above 70°C optimally. All of these microorganisms are related to the clostridia family. The non spore forming bacteria were selected and ordered from the DSMZ.

The bacteria were first grown on defined medium with cellulose or cellobiose as main carbon source the growth is shown in figure 10 and 11.

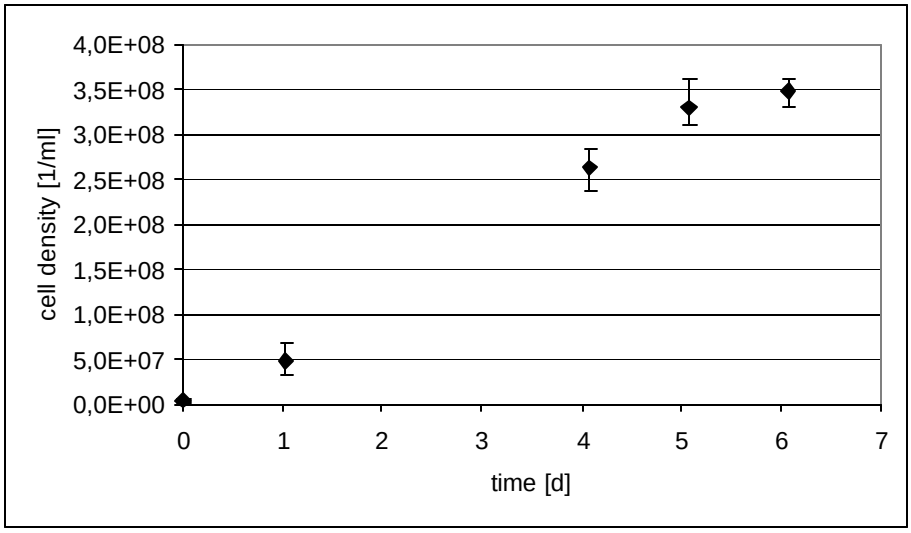

Figure 10 Growth of *caldocellulosiruptor lactoaceticus* on defined medium with 2g/l cellulose

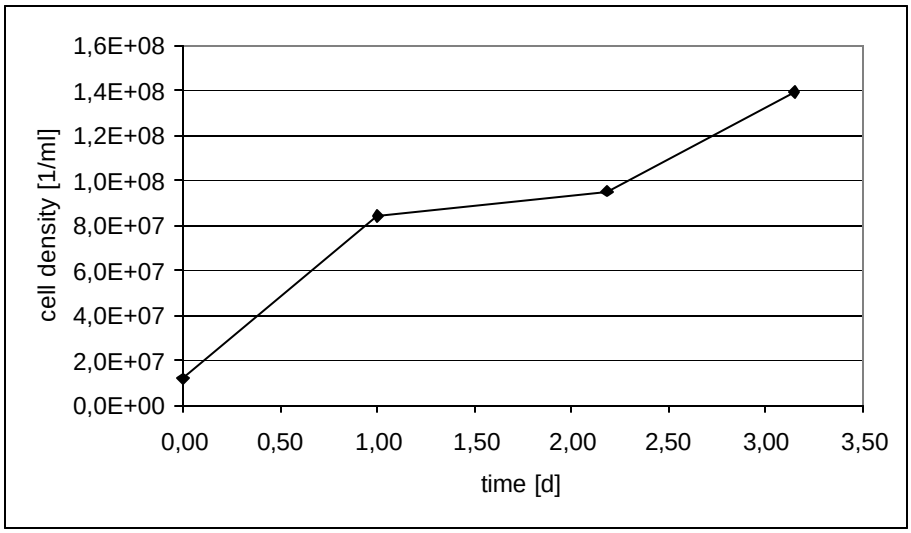

Figure 11 Growth of *Anaerocellum thermophilum* on defined m edium with 5 g/l cellobiose

Both fermentations show a decreasing µ or i.e. an increasing doubling time. The growth seems to be inhibited very early either by metabolites or by inhibitors. Lysis was detected neither at *C. lactoaceticus* nor at *A. thermophilum*. Both strains started with a doubling time of 5 to 7 hours. *C. lactoaceticus* reached a cell density of $3.5x10^8$ cells per ml and *A. thermophilum* reached a cell density of $1.4x10^8$ cells/ml.

The strains showed promising results for an application with ESA-substrate.

The liquefaction efficiency of *C. lactoaceticus* was tested in 50 ml vials for the parts of the ESA-substrate which are hard to degrade. Figure 12 shows the degradation

efficiency in terms of total solid (TS) liquefaction and total carbon (TC) liquefaction. The produced CO_2 which escaped into the gas phase was not considered in the TC liquefaction.

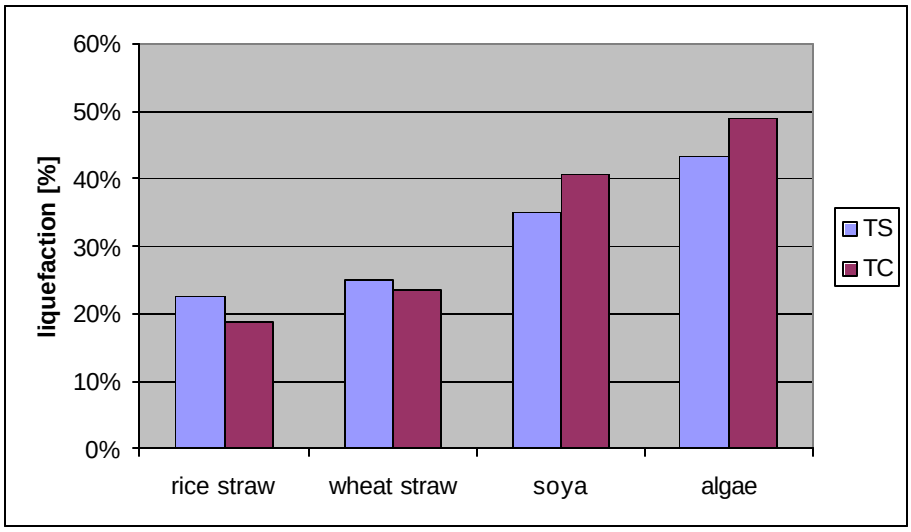

Figure 12 liquefaction efficiency of *Caldocellulosiruptor lactoaceticus* in 50 ml vials after inoculation at 70°C for 7 days .

The liquefaction ranges from 20% for straw to 35% for soy and 45% for algae. The analysis of the liquid phase for is shown in figure 13.

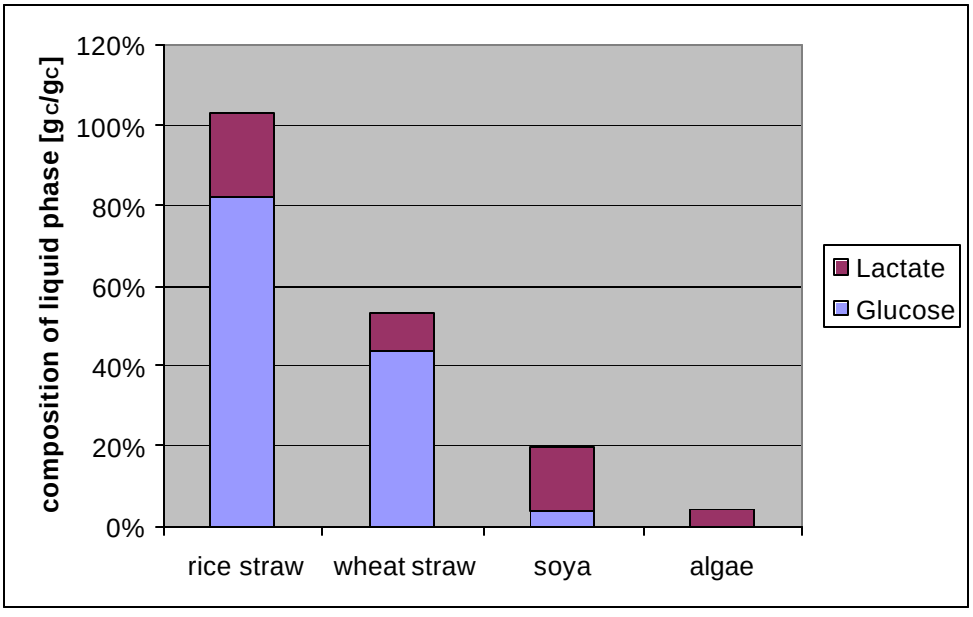

Figure 13 Composition of liquid phase in terms of carbon balance. Acetic acid and ethanol were not measured. Due to measuring errors the sum of glucose and lactate equals more than 100% (103%)

The high quantity of glucose in the liquid phase shows that the bacteria were very soon inhibited. *C. lactoaceticus* produces extra cellular cellulases which finally cut the

cellulose into glucose monomers. The glucose is used as main carbon and energy source for the bacteria and is digested to acetic acid, ethanol and favourable lactate. The highest total amount of lactate was visible during the fermentation of soy (11.5 mmol/l) which goes along with the highest pressure in the vials due to CO_2 production.

To see, if the liquefaction could be improved *C. lactoaceticus* was tested in a 2l fermentor with 900 rpm, no gassing at 70°C. The fermentor was loaded with 10g/l straw and 10g/l soy. The reactor was inoculated with 150ml pre-culture. The starting cell density was $2.8x10^7$ cells/ml. The pH was kept constant by titrating with 1M NaOH.

Figure 14 shows the DOC and TIC vs. time plot. After one week 23% of the substrate's carbon was found in the liquid. Additional 4% was dissolved carbon dioxide in the liquid. Half of the liquefaction was achieved during the first 24 hours of the experiment. The dissolved CO2 content increased during the whole experiment.

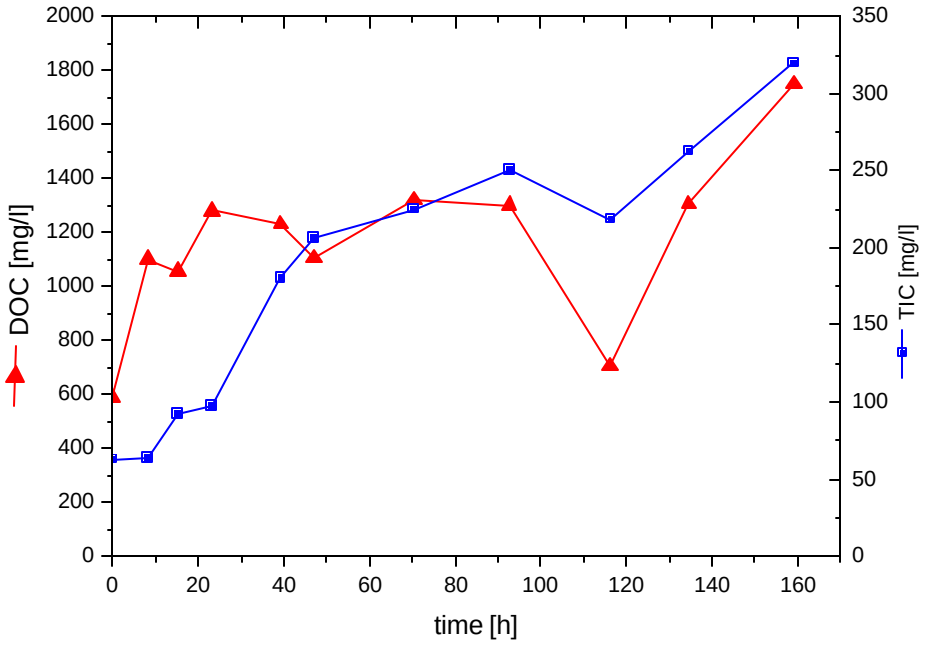

Figure 14 DOC, TIC vs. time plot. TIC represents the dissolved CO2 in the liquid phase.

Further information about the experiment is given in figure 15. There two metabolites, glucose and lactate are displayed. Additionally the amount of NaOH added for titration is visible.

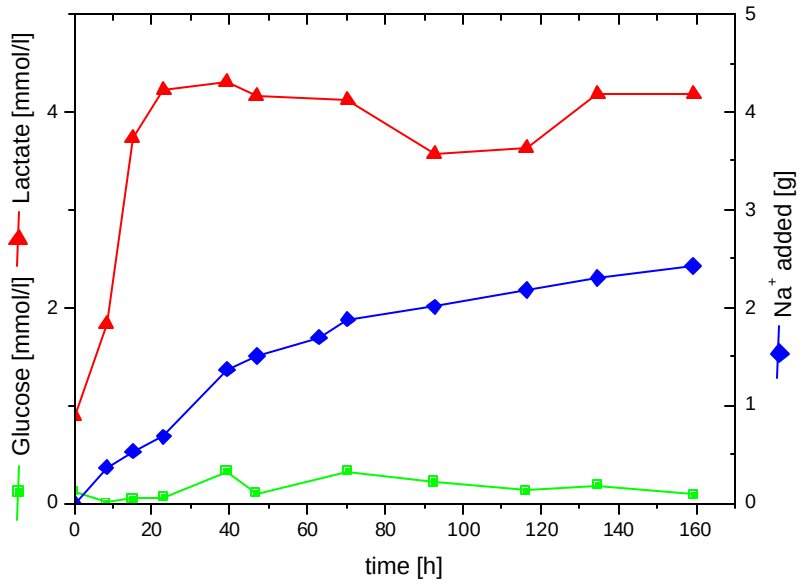

Figure 15 metabolites during fermentation of straw and soy with *C. lactoaceticus* Glucose, lactate and used NaOH are shown

In figure 15 is visible that lactate production stops after 24 hours. Glucose was measured just in very small concentrations during the whole fermentation. It is reported, that *C. lactoaceticus* is inhibited by salt concentration of more than 1g/l. The medium already contains more than 720mg/l Cl and 920mg Na^+ ions. After 30 hours a total amount of 1g Na^+ ions was added.

It seems that all these effects are coupled and are caused by the NaOH addition. When a certain level of Na^+ was added the lactate production stops, the DOC production stops, and the TIC production starts. As the gasified carbon is not wanted the process should be redesigned to keep a low salt content during the fermentation.

6. Conclusions and Outlook

The fermentation of the first closed loop residue with the consortium from the Azores showed no liquefaction even with dialysis fermentation. The second closed loop experiment yielded poor liquefaction efficiencies of about 20%. Here the hyperthermophilic consortium from the Azores and *Pyrococcus furiosus* was applied.

Two new dialysis fermentation set ups were tested. Both experiments failed due to technical problems, but these problems are now known and can be avoided in future fermentations. Additionally new temperaturestable membrane modules manufactured

from Polysolphone were tested. Measurements of the permeability showed that the Polysolphone membrane has got the same permeability as the well known Polyethersulphone membrane. The usage of membrane modules can lower the mass transport resistance by the factor of seven to ten compared to a cuprophane membrane-reactor.

Fermentations of the two new microorganisms showed that both *Caldocellulosiruptor lactoaceticus* and *Anaerocellum thermophilum* grow on ß-1,4 linked glucosepolymeres. A maximum cell density of $3.5x10^8$ and $1.5x10^8$ cells per ml is reached in 50 ml vials on defined medium.
Fermentation of compounds of the ESA-substrate showed a medium liquefaction of 20-50%. Also glucose is produced in reasonable quantities (up to 2,3g/l). Glucose is normally easily used by microorganisms. So the high glucose levels showed that the cells were inhibited quite fast and only the cellulases were still active.
The fermentation in the 2litre fermentor showed no significant glucose levels in the medium. It seems that the titration of NaOH caused the stop of the liquefaction process. In the fermentor a reasonable amount of CO_2 was produced which can be monitored by the increase of TIC in the liquid. So the total degradation consists here also of the gasified part, which cannot be quantified when using a 2l foil-rector.

A combination of the new fermentation technique and the new microorganisms seems to be promising, as the cells are very fast inhibited by NaOH which is added to keep a constant pH. Also a combination of *C. lactoaceticus* and *A. thermophilum* can lead to a faster liquefaction, as both bacteria do not cover exactly the same substrate spectrum.

Literature:

Pörtner, Märkl 1998. Dialysis cultures. Appl Microbiol Biotechnol 50: 403-414

Krahe et al. 1996. Fermentation of extremophilic Microorganisms. FEMS Microbiology Reviews 18 271-285

Krahe 1998. Kultivierung von Mikrooganismen zu hohen Zelldichten. Thesis at the Technical University of Hamburg Harburg

Pietsch 2000 Rückhalt suspendierter Biomasse und örtliche Verteilung gelöster Gase in einem Biogas-Turmreaktor. Thesis at the Technical University of Hamburg Harburg

Friedmann, Märkl (1994) Der Einfluss der Produktgase auf die mikrobielle Methanbildung. gwf Wasser Abwasser135,302-311

Mladenovska, Mathrani, Ahring 1994 Isolation and characterization of *Caldocellulosiruptor lactoaceticus* sp.nov., an extremely thermophilic, cellulolytic, anaerobic bacterium. Arch Microbiol (1995) 163:223-230

DSMZ. www.dsmz.de

Raven, Sharp (1997) Development of defined and minimal media for the growth of the hyperthermophilic archaeon *Pyrococcus furiosus* Vc1. FEMS Microbiol. Lett. 146, 135-141

Biller (2002) Fermentation hyperthermophiler Mikroorganismen Thesis at the Technical University Hamburg Harburg