TECHNISCHE UNIVERSITÄT HAMBURG-HARBURG

biotechnologie

Bioprozeß- und Bioverfahrenstechnik

A TOTAL CONVERTING AND BIOSAFE LIQUEFACTION COMPARTMENT FOR MELLISSA

TECHNICAL NOTE 86.3.1

Hyperthermophilic Liquefaction

Kai F. Biller, Herbert Märkl

June 2001

Biochemical and Bioprocess Engineering
Technische Universität Hamburg- Harburg
Denickestr. 15; 21071 Hamburg
Tel.: +49 40 42878 3017
Fax.: +49 40 42878 2909

1 Introduction

In the degradation process hydrolysis of the lignocellulosic fraction of vegetable residues is always the limiting step [Converti et al. 1999]. In the latest past hyperthermophilic microorganisms showed great potential in the hydrolysis of organic compounds [Bustard et al. 2000; Huber & Stetter 1998]. Hyperthermophilic organisms like *Pyrococcus furiosus* are able to grow at temperatures around 100°C with high metabolic rates [Fiala et al. 1986, Krahe et al. 1996]. The objective of our work is the establishment of a "liquefaction" process for solid botanical material in order to simplify the degradation in a following methanogenetic reactor. Liquefaction means the degradation of the solids to soluble organic compounds like oligosachcharids and fatty acids.

1.1 Potentials and physiology of hyperthermophiles

Organisms with outstanding properties are required to develop novel microbial processes in industry. The interest in hyperthermophiles and the rate of discovery of new species increases. Hyperthermophiles are heat-loving Archaea and Bacteria isolated from terrestrial and marine biotopes at temperatures between 80 an 113°C. Despite the outranging potentials little information about applications in industry is available to date. More than 70 hyperthermophilic species have been found at the end of 1999 [Hicks et al. 1999; Huber & Stetter 1998; Perttula et al. 1989; Stetter 1996; Vielle & Zeikus 2001].

The main advantages of hyperthermophilic over mesophilic microorganisms are (a) reduction of cooling costs, because thermophilic cultivations do not have to be cooled usually, (b) reduced viscosity of media, which increases efficiency of mixing and harvesting, (c) increase in solubility of reactants allows higher concentrations of less soluble components, (d) volatile products, such as ethanol, may be removed through application of a mild vacuum, (e) reactor operation at elevated temperatures reduces contamination by other microorganisms, allowing long periods of stable operation, (f) decreased solubility of oxygen aids cultivation of anaerobic organisms, (g) thermophile enzymes are more resistant to detergents or solvents [Bustard et al. 2000].

The majority of hyperthermophilic microorganisms, especially from marine sites, are chemolithoautotrophic, but many of such organisms exhibit heterotrophic metabolism. A selected number of hyperthermophilic microorganisms with their metabolic requirements are shown in table 1.

Investigation into the physiology and biochemistry of hyperthermophiles was limited by difficulties in cultivation so far. A lot of experience and special fermentation techniques have enabled our group to optimise cultivation conditions and to cultivate hyperthermophiles like *Pyroccocus* species to high cell densities [Rüdiger et al. 1992; Holst et al. 1997; Krahe et al. 1996; Krahe 1998; Pörtner & Märkl 1998]. A scheme of *Pyrococcus furiosus* metabolism is shown in figure 1.

Table 1: Selected hyperthermophiles and its characteristics [Bustard et al. 2000; Huber & Stetter 1998]

Genus	T_{max} (°C)	Substrate	Reference
Bacteria			
Thermotoga	90	Peptides, carbohydrates	Huber 1986; Jannasch 1988
Archaea			
Archaeglobus	92	Carbohydrates, (H_2)	Stetter 1988; Burggraf 1990
Pyrolobus	113	H_2	Blöchl 1997
Aeropyrum	100	Peptides	Sako 1996
Pyrobaculum	103	Peptides, H_2	Huber 1987
Pyrococcus	103	Peptides, carbohydrates	Fiala 1986; Zillig 1987b; Erauso 1993
Pyrodictium	110	Peptides, carbohydrates, H_2	Stetter 1983; Pley 1991
Staphylothermus	98	Peptides	Fiala 1986b
Sulfolobales	95		Segerer 1986; Zillig 1987a

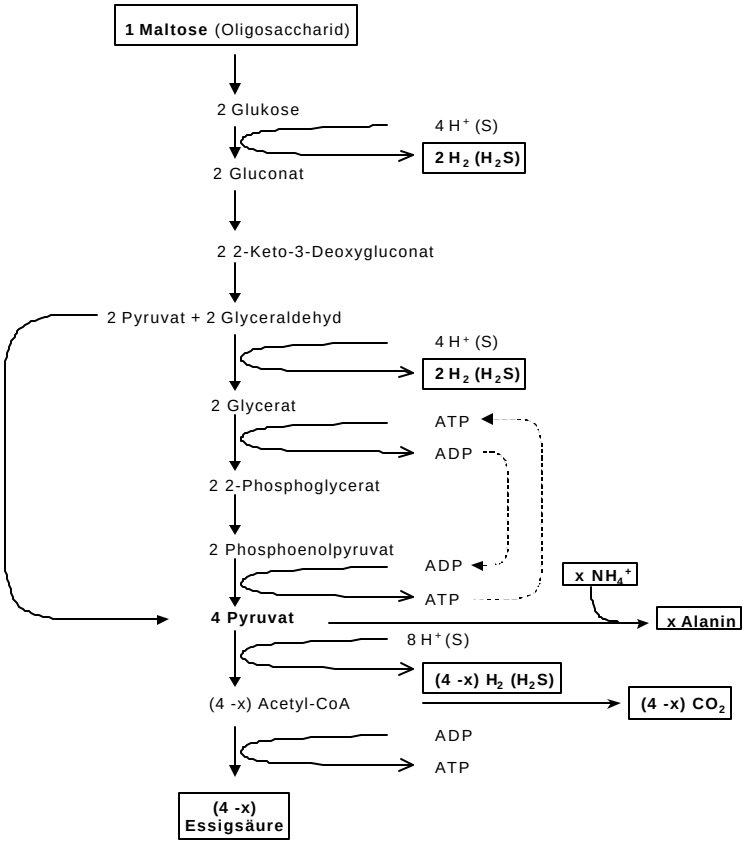

Figure 1: Metabolism of *Pyrococcus furiosus* [Krahe 1998]

1.2) Objectives of work package

The objectives of workpackage 3.100 were the determination of a hyperthermophilic micro-organism consortium for the degradation of ESA-substrate and the implementation of a stable degradation process. First test on the COD removal potential of the new consortium had to be done.

2) Bioreactor and Analysis

Analysis of hot springs
The temperature was measured with a transportable digital thermometer 9010 from testo term. The pH was determined with universal indicator paper from Macherey-Nagel 90202 and in the samples with a Broadley and James pH-Fermprobe F-635-B225-DH.

Fermentor and fermentation set up (Fig. 2)
For the fermentations a 2l-foil fermentor from Bioengineering (Wald, Switzerland) was used [Biller et al. (2001); Krahe (1998)]. The fermentor was equipped with temperature and pH-control. The titration was done with a 2N-NaOH solution.
The fermentation parameters were: T=90°C; stirrer speed 600 rpm; pH on-line 7.0; fermentation volume 1.5 liter.

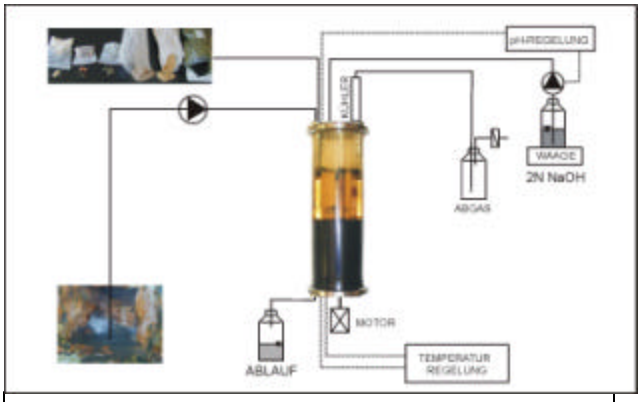

Fig. 2: Scheme of the fermentation set-up for consortium search on the Azores. On top left the medium (except of the faeces) can be seen, on bottom left one of the springs is shown as a sample.

Medium

For the consortium search
The search took place before the first project meeting in April. Therefore the medium could not be adjusted to the ESA-substrate agreed on in Ghent. The medium used for the search was oriented on the original information from Christophe Lasseur, given in February.
The medium was made of demineralized water with 3% dry material. The dry material consisted of 10% human feces, 30% algae *Spirulina platensis* (fodder grade) and 60% plant waste. The plant waste was composed of 50% wheat straw, 25% potato leaves, 5% soy bean pellets, 5% salad, 5% spinach, 5% onion and 5% tomato leaves.
The human feces were self collected. The algae were bought from BlueBiotech (Hamburg), the soy bean pellets were a gift from the Ölmühle Hamburg and the remaining plant wastes were bought from local farmers at Hamburg.
All the dry matter was milled, so all the particles introduced into the fermentor were smaller than 1mm side length.

For the degradation tests
For the first degradation tests the dry matter of the medium was reduced to algae (60%) and wheat straw (30%), the hardest to degrade parts of the medium.

ESA-substrate
Demineralized water with 2% dry matter. The dry matter consists of 20% human feces, 10% algae *Spirulina platensis* fodder grade, 23.33% wheat straw, 23.33% soy bean waste, 23.33% green cabbage. The algae, the wheat straw and the soy bean pellets are added dry (water content below 5%) into the water. The green cabbage and the human feces are added "fresh", what means they do not get dries before addition to the medium. Dry mass of the fresh parts was determined and input corrected, so composition of the ESA-substrate is insured in order of the agreed on values mentioned above.

Measurement of redox value
The redox value was measured on-line with a Broadley and James platinum redox probe D1107A-PBA.

Measurement of gas production
The exhaust gas was collected in a graduated cylinder.

Determination of cell number
Cells were counted directly with a Neubauer chamber (depth 0.02 mm) under a phase contrast microscope.

Chemical Oxygen Demand (COD)

For the determination of the COD the test LCK 114 from Dr. Lange () with 1 ml sample volume was used.

Diluted Organic Carbon (DOC)
The DOC value of the samples were determined with a highTOC + TN_b from Elementar.

3 Establishment of hyperthermophilic consortium

3.1 Selection and description of sampling sites

The search was focussed on hyperthermophilic microorganisms growing optimally under neutral or slightly alkaline conditions. Hydrothermal sites with water at temperatures over 80°C and neutral or alkaline pH can be found e.g. at places on the Island of Montserrat [Atkinson et al. (2000)], at Termas de Chile [Antranikian, personell note], at Whakatrach (New Zealand) and on the Azores [Victor Hugo Forjaz (1997); de Carvalho (1955)].

On São Miguel, an Azorian island centered in the middle of the Atlantic Ocean at 37° 45´´ N and between 25° and 26° W, sites which match the desired conditions could be found near Ribeira Grande, in the river bed of Ribeira Quente, at Furnas lake and in Furnas itself. For the search and enrichment eight springs in Furnas with temperatures between 80 and 98°C and a pH between 6,5 and 8,5 were chosen.

3.2 Enrichment in a fermentor fed with samples directly from the springs

The most common way of isolating new microorganisms is by taking samples and trying to cultivate organisms in a lab usually not situated at the same location. After isolation the organisms are characterized and in case of novel microbes the results will be published [Atkinson et al. (2000); Fiala & Stetter (1986)].

For an in situ enrichment a complete fermentor was transported to Furnas and assembled in the Thermal Center located very close to the main spring area. The Center provided everything necessary, like shelter, current, cleaning and cooling water. A scheme of the fermentation set-up can be seen in figure 2. After the assembly, the fermentor was filled with 1,5 liter fresh, hot spring samples. The temperature in the fermentor was then adjusted to 90°C and the stirrer speed set to 600 rpm. Thirty grams of dry medium (algae, plant wastes, feacal matter) were added. Three times a day, at 9, 15 and 21 o'clock, 500 ml of the reactor content were filled into a glass bottle. This bottle was sealed gas tight, isolated to keep the temperature and stored. After 30 minutes of sedimentation the bottle was opened and 250 ml of the liquid were poured away. The bottle was filled up with fresh samples from the springs and 5 grams dry medium were added. After mixing, the content of the bottle was filled back into the fermen-

tor. The first 4 days the dry medium was only added once a day, from day 5 to 10 twice a day and from day 11 on every time.

During the enrichment the use of sodium hydroxide, the gas production and cell number were controlled to see if the enrichment is successful. Living cells could be seen under the microscope from the beginning on. From day 10 the pH dropped below 7, therefore titration had to start. Gas production was almost 0 until the end of the enrichment. After 14 days the enrichment was stopped, the reactor content was collected into clean and sterilized bottles and sent to Germany for the tests.

4 Effectiveness of the new consortium

To test the effectiveness of the new consortium, batch fermentations in the 2-l foil fermentor were carried out. The medium consisted of the hardest to degrade parts of the ESA-medium, algae cells (1.33 g/l) and wheat straw (0,67 g/l), dissolved into demineralized water. The medium was titrated with 2N- NaOH solution to pH 7.0. NO FURTHER COMPONENTS WERE ADDED. The cultures were gassed with 0,2vvm N_2, to avoid contamination with oxygen.

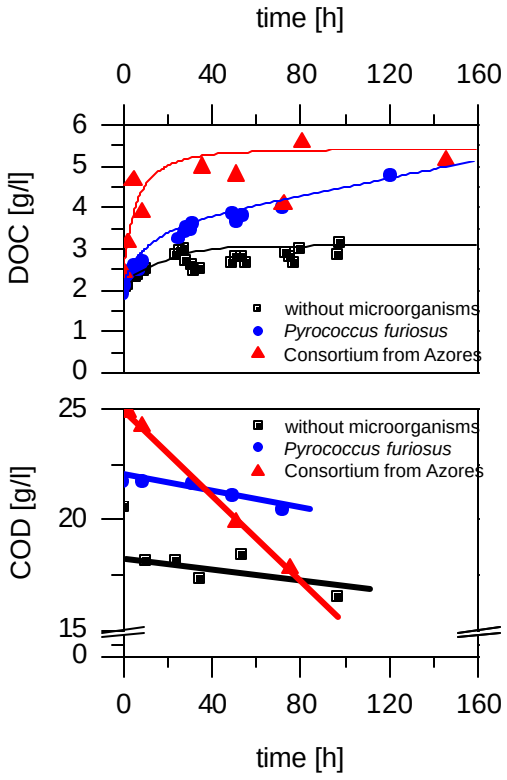

Fig 3: Trends of DOC and COD values in batch fermentations

In the first experiment, it was tested if the solids were degraded only by temperature. In figure 3 the COD and DOC values of the experiment are shown. No significant change in the values took place over a five day reaction. In conclusion a significant degradation of algae cells and wheat straw does not take place by temperature only.

In a second experiment a equal medium was inoculated with *Pyrococcus furiosus* cells. The stirrer speed was adjusted to 1800rpm for best growth [Krahe 1998]. For the inoculation *P. furiosus* was cultivated in 50ml serum bottles on defined medium [Krahe et al.1996] with minerals, amino acids and maltose. 2 bottles (100ml) were used for the inoculation. Growth of *P. furiosus* could be detected but was low, the cell number increased over a period of 120 hours from $2x10^8$ cells /ml to $1x10^9$ cells/ml. The slow but constant growth of *P. furiosus* indicates a strong substrate limitation but the existence

of at least a minimum of substrate, because usually *P. furiosus* grows fast and dies fast after all substrate is used [Biller et al. 2001]. A further indication for growth or some kind of metabolism is the production of organic acids detected by use of NaOH for titration and the increase in the dissolved organic carbon (DOC). Therefore *P. furiosus* is able to degrade algae cells or fibers from the wheat straw, but degradation performance is low as can be seen in figure 3 by the slow increase in the DOC value and no reduction of the COD value and in figure 4 where the algae cell number over cultivation time is shown.
The slight increase in the COD values in the first two experiments is due to evaporation loss and therefore increasing concentration of solids.

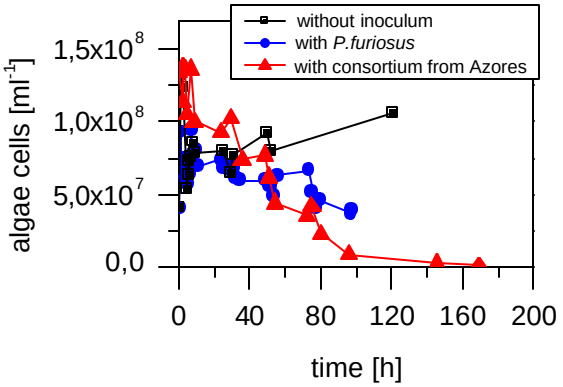

Figure 4: Degradation of algae cells in batch cultures

In a third experiment finally a equal medium as before was inoculated with 100ml from the enrichment culture from the Azores. The sample from the enrichment culture was taken from 4°C and put directly into the fermentor. Stirrer speed was 600rpm again. The good degradation performance of the new consortium can be seen in figure 3. The DOC value and therefore the value of dissolved carbon, mainly organic acids, increased very fast and stayed constant for more than 5 days, whilst the COD decreased from 25 g/l to 20 g/l, a reduction of 20% over five days. The solids, mainly the algae cells were degraded by the new Azorian consortium. Over a period of 5 days 99% of the algae cells were degraded as can be seen in figure 4. This degradation took only place in the presence of the new consortium and was not due to temperature effects.

The COD removal potential of the new Azorian consortium could be confirmed with continuous experiments with retention times of 2,6 and 4,8 days. The decrease of the COD value between the influx and the effluent was also 20%.

A batch fermentation with the complete ESA-substrate agreed on was done to test the degradation performance and the sanitation potential of the new hyperthermophilic consortium. Due to technical problems this experiment had to be stopped 12 hours after inoculation, so no degradation data could be collected. For the sanitation test samples of the medium were taken from the fresh ESA-substrate and 8 hours after heating up and inoculation with the new consortium. The samples were than plated on special agar for *E. coli* and coliformes and on special agar for the growth of yeasts and funghi. The coliforme agar was heated at 37°C for 24 and 48 hours respectively and the test on yeasts and funghi was cultivated at 20°C for one week. The tests with the ESA-substrate showed a lot of growth of coliformes and yeasts and

funghi respectively. On the plates with the samples taken after 8 hours of inoculation did nothing grow at all. The kind and the number of the colony forming units on the tests were not detected. Therefore this was only a qualitative test on the sanitation. The sanitation effect on coliformes, yeasts and funghi is probably due to the temperature of the fermentation only [Dichtl 1997].

The new consortium found can hardly be compared to other hyperthermophiles, because no data is available on any hyperthermophile growing on feces, botanical waste or algae cells. Compared to thermophile consortia in the waste water cleaning industry [Burtscher et al. 1998; Converti et al. 1999; Dichtl 1997; Krugel et al. 1998; Roberts et al. 1999] it shows the expected features, good sanitation effectiveness, no production of methane and production of organic acids, which makes it a good pre-fermentation step before the methanogenic reactor, and a great potential of degrading cells.

5 Conclusion

The objectives of the workpackage were completely fulfilled. A new hyperthermophilic microorganism consortium for the degradation of botanical waste and human feces was looked for and found in Furnas on the Azorian island Sao Miguel. Batch and continuous experiments showed great degradation potential, especially for the algae cells, of the new consortium.

A stable and continuous process could be established.

From next period on, effluent from fermentations with the new consortium could be sent to the partners for further tests, especially for tests in the methanogenic reactor, to see if the performance of the complete system can be improved with the hyperthermophilic fermentation step. Also the undegradable parts from Ghent will be sent to us and tested if they are degradable in the hyperthermophilic fermentation.

References

Antranikian G (1990) Physiology and enzymology of anaerobic bacteria degrading starch. FEMS Microbiol Rev 75: 201-218

Atkinson T, Cairns S, Cowan DA, Danson MJ, Hough DW, Johnson DB, Norris PR, Raven N, Robinson C, Robson R, Sharp RJ (2000) A microbial survey of Montserrat Island hydrothermal biotopes. Extremophiles 4: 305-313

Biller KF, Kato I, Märkl H (2001) Effect of glucose, maltose, soluble starch and CO_2 on the growth of the hyperthermophilic Archaeon *Pyrococcus furiosus*. Handed in

Blöchl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1: 14-21

Burggraf S, Jannasch HW, Nicolaus B, Stetter KO (1990a) *Archaeoglobus profundus* sp. nov. Represents a new species within the sulfate-reducing archaebacteria. Syst Appl Microbiol 13: 24-28

Burtscher C, Fall PA, Christ O, Wilderer PA, Wuertz S (1998) Detection and survival of pathogens during two-stage thermophilic/mesophilic anaerobic treatment of suspended organic waste. Wat Sci Tech 38 (12): 123-126

Bustard MT, Burgess JG, Meeyoo V,Wright PC (2000) Novel opportunities for marine hyperthermophiles in emerging biotechnology and engineering industries. J Chem Technol Biotechnol 75: 1095-1109

Converti A, Del Borghi A, Zilli M, Arni S, Del Borghi M (1999) Anaerobic digestion of the vegetable fraction of municipal refuses: mesophilic versus thermophilic conditions. Bioprocess Engineering 21: 371-376

Dichtl N (1997) Thermophilic and Mesophilic (Two-Stage) Anaerobic Dgestion. Water Environmental Management 11 (2): 98-104

Erauso G, Reysenbach A-L, Godfroy A, Meunier JR, Crump B, Partensky F, Baross JA, Marteinsson V, Barbier G, Pace NR, Prieur D (1993) *Pyrococcus abyssii* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydsrothermal vent. Arch Microbiol 145: 56-61

Fiala G, Stetter KO (1986a) Pyrococcus furiosus sp. nov. represents a novel genus of marine hyperthermophilic archaebacteria growing optimally at 100°C. Arch Microbiol 145: 56-61

Fiala G, Stetter KO, Jannasch HW, Langworthy TA, Madon J (1986b) *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic archaebacteria growing up to 98°C. Syst Appl Microbiol 8: 106-113

Forjaz V H (1997) Alguns Vulcãoes da Ilha de São Miguel, Ponta Delgada ISBN 972-97466-1-3

Hicks PM, Adams MWW, Kelly RM (1999) Thermophilic microorganisms: 2536-2552. In: Flickinger MC, Drew SW (ed.) Encyclopedia of bioprocess technology: fermentation and bioseperation. John Wiley & Sons, Inc, New York NY

Holst O, Manelius A, Krahe M, Märkl H, Raven N, Sharp R (1997) Thermophiles and fermentation technology. Comp Biochem Physiol 118A: 415-422

Huber R, Kristjansson JK, Stetter KO (1987) *Pyrobaculum* gen. nov. a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100°C. Arch Microbiol 149: 95-101

Huber R, Dyba D, Huber H, Burggraf S, Rachel R (1998) Characterization of the sulfur-inhibited *Thermosphera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after ist prediction from environmentally derived 16S rRNA sequences. Int J Syst Bacteriol 48, 31-38

Huber H, Stetter KO (1998) Hyperthermophiles and their possible potential in biotechnology. J Biotechnol 64: 39-52

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana*, sp. nov. of the extremely thermophilic, eubacterial genus *thermotoga*. Arch Microbiol 150: 103-104

Kengen SWM, Stams AJM, de Vos WM (1996) Sugar metabolism of hyperthermophiles. FEMS Microbiol Rev 18: 119-137

Krahe M, Antranikian G, Märkl H (1996) Fermentation of extremophilic microorganisms. FEMS Microbiol Rev 18: 271-285

Krahe M (1998) Kultivierung von Mikroorganismen zu hohen Zelldichten. Ph.D. Thesis at the Technical University Hamburg-Harburg

Krugel S, Nemeth L, Peddie C (1998) Extending thermophilic anaerobic digestion for producing class A biosolids at the greater Vancouver regional districts Annacis Island waste water treatment plant. Wat Sci Tech 38: 409-416

Perttula M, Viikari L, Kristjansson J, Pere J, Kondradsdottir M (1989) Treatment of evaporator condensates using thermophilic bacteria. VTT Symp 103, Non-waste technol. 2: 353-358

Pley U, Schipka J, Gambacorta A, Jannasch JW, Fricke H, Rachel R, Stetter KO (1991) *Pyrodictium abyssi* sp. nov. represents a novel heterotrophic marine archael hyperthermophile growing at 110°C. Syst Appl Microbiol 14: 245-253

Pörtner R, Märkl H (1998) Dialysis cultures. Appl Microbiol Biotechnol 50: 403-414

Roberts R, Davies WJ, Forster CF (1999) Two-stage, thermophilic-mesophilic anaerobic digestion of sewage sludge. Trans IChemE 77(2 B): 93-97

Rüdiger A, Ogbonna JC, Märkl H, Antranikian G (1992) Effect of gassing, agitation, substrate supplementation and dailysis on the growth of an extremely thermophilic archaeon *Pyrococcus woesei*. Appl Microbiol Biotechnol 37: 501-504

Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T (1996) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int J Syst Bacteriol 10: 1070-1077

Segerer A, Neuner A, Kristjansson JK, Stetter KO (1986) *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. Nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. J Bacteriol 36: 559-564

Stetter KO (1986) Diversity of extremely thermophilic archaebacteria. In: Brock TD (ed) *Thermophiles*: General, Molecular, and Applied Microbiology. Wiley, New York: 40-74

Stetter KO (1988) *Archaeglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. Syst Appl Microbiol 10: 172-173

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fiedler M, Cash H, Vance I (1993) Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature – 300, 743-745

Siskin M, Katritzky AR (1991) Reactivity of organic compounds in hot water: geochemical and technological implications. Science 254: 231-237

Verstraete W, Vandevivere P (1998) New and broader applications of anaerobic digestion. Critical Reviews in Environmental Science and Technology 28: 151-173

Vieille C, Zeikus GJ (2001) Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. Microbiol Mol Biol Rev 65 (1): 1-43

Weemaes MPJ, Verstraete WH (1998) Evaluation of current wet sludge disintegration techniques. J. Chem. Technol. Biotechnol. 73: 83-92

Zillig W, Yeates S, Holz I, Böck A, Gropp F, Simon G (1987a) *Desulfurolobus ambivalens*, gen. nov., sp. nov., an autotrophic archaebacterium facultatively oxidizing or reducing sulfur. Syst Appl Microbiol 8: 197-203

Zillig W, Holz I, Klenck HP, Trent J, Wunderl S, Janekovic D, Imsol E, Haas b (1987b) *Pyrococcus woesei* sp. nov., an ultra-thermophilic marine archaebacterium, represents a novel order, *Thermococcales.* Syst Appl Microbiol 9, 62-70