ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Ethanol and Hydrogen Production by Two Thermophilic, Anaerobic Bacteria Isolated From Icelandic Geothermal Areas

Perttu E.P. Koskinen,[1] Steinar R. Beck,[2] Jóhann Örlygsson,[2] Jaakko A. Puhakka[1]

[1]Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FIN-33101, Tampere, Finland; telephone: +358-3-3115-11; fax: +358-3-3115-2869; e-mail: perttu.koskinen@tut.fi

[2]Faculty of Natural Resource Sciences, University of Akureyri, Borgir Nordurslod, Akureyri, Iceland

Received 5 November 2007; revision received 18 February 2008; accepted 10 April 2008

Published online ? ? ? ? in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21942

ABSTRACT: Microbial fermentations are potential producers of sustainable energy carriers. In this study, ethanol and hydrogen production was studied by two thermophilic bacteria (strain AK15 and AK17) isolated from geothermal springs in Iceland. Strain AK15 was affiliated with *Clostridium uzonii* (98.8%), while AK17 was affiliated with *Thermoanaerobacterium aciditolerans* (99.2%) based on the 16S rRNA gene sequence analysis. Both strains fermented a wide variety of sugar residues typically found in lignocellulosic materials and some polysaccharides. In the batch cultivations, strain AK17 produced ethanol from glucose and xylose fermentations of up to 1.6 mol-EtOH/mol-glucose (80% of the theoretical maximum) and 1.1 mol-EtOH/mol-xylose (66%), respectively. The hydrogen yields by AK17 were up to 1.2 mol-H_2/mol-glucose (30% of the theoretical maximum) and 1.0 mol-H_2/mol-xylose (30%). The strain AK15 produced hydrogen as the main fermentation product from glucose (up to 1.9 mol-H_2/mol-glucose [48%]) and xylose (1.1 mol-H_2/mol-xylose [33%]). The strain AK17 tolerated exogenously added ethanol up to 4% (v/v). The hydrogen and ethanol production performance from glucose by a co-culture of the strains AK15 and AK17 was studied in a continuous-flow bioreactor at 60°C. Stable and continuous ethanol and hydrogen co-production was achieved with ethanol yield of 1.35 mol-EtOH/mol-glucose, and with the hydrogen production rate of 6.1 mmol/h/L (H_2 yield of 0.80 mol-H_2/mol-glucose). PCR-DGGE analysis revealed that the AK17 became the dominant bacterium in the bioreactor. In conclusion, strain AK17 is a promising strain for the co-production of ethanol and hydrogen with a wide substrate utilization spectrum, relatively high ethanol tolerance, and ethanol yields among the highest reported for thermoanaerobes.

Biotechnol. Bioeng. 2008;9999: 1–12.

KEYWORDS: bioethanol; biohydrogen; thermophilic microorganisms; biological ethanol and H_2 co-production; *Clostridium uzonii*; *Thermoanaerobacterium aciditolerans*

Introduction

Microbial fermentations offer an attractive alternative to produce sustainable energy. Fermentations can use various kinds of biomass or organic waste to produce energy carriers such as ethanol, butanol, methane, or hydrogen (for a review, see Claassen et al., 1999; Zeikus, 1980). Recently, the production of ethanol and butanol has received increasing attention due to their use as a vehicle fuel supplement. The technology for methane production through anaerobic digestion has been developed and widely utilized (Claassen et al., 1999), while in recent years increasing efforts has been aimed at the fermentative H_2 production. Hydrogen is considered as the energy carrier of the future due to its superior properties and diverse application possibilities compared to any other carriers (Das and Veziroğlu, 2001; Nath and Das, 2003). Hydrogen is also used as reductant in many industrial processes (Nath and Das, 2003).

Some thermophilic bacteria belonging to the genera *Clostridium*, *Thermoanarobacter*, or *Thermoanaerobacterium* are known for their capabilities to produce high

Correspondence to: P.E.P. Koskinen

Contract grant sponsor: Academy[Q1] of Finland (HYDROGENE)
Contract grant number: 107425
Contract grant sponsor: Nordic Energy Research (BIOHYDROGEN)
Contract grant number: 28-02
Contract grant sponsor: Tampere University of Technology Graduate School
Contract grant sponsor: RANNÍS
Contract grant sponsor: The Icelandic Centre for Research
Contract grant sponsor: The Research Fund of the University of Akureyri
This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/0006-3592/suppmat.

BIT-07-892.R1(21942)

quantities of ethanol from carbohydrates (Ng et al., 1981; Weimer, 1985; Wiegel and Ljungdahl, 1981). These bacteria use Entner–Mayerhof (phosphoroclastic) pathway in the oxidation of substrates, which results in the production of ethanol and hydrogen along with organic acids (e.g., acetate, butyrate, and lactate; Zeikus, 1980). Hydrogen production from carbohydrates is considered to occur along with the production of acetate (Eq. 1) or butyrate (Eq. 2), while the ethanol production alone results in no H_2 production (Eq. 3) (Thauer et al., 1977; Wiegel, 1980).

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+ \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2H_2 + 2HCO_3^- + 3H^+ \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (3)$$

This implies that when H_2 production is optimized (acetate production), ethanol production decreases and vice versa. Depending on the organism, the ethanol (and hydrogen) yields vary substantially, from traces to nearly quantitative amounts (Wiegel, 1980; Zeikus, 1980). Equation (3), called the decarboxylastic (or Entner–Doudoroff) pathway, results in nearly complete conversion of glucose to ethanol, but is rare amongst bacteria and reported only in *Zymomonas mobilis* (Wiegel, 1980; Zeikus, 1980).

Although the ethanol tolerance and hexose conversion rates of thermoanaerobes remain less than those of the yeast *Saccharomyces cerevisiae* or mesophilic bacterium *Z. mobilis*, the advantage of these bacteria is their metabolic diversity, that is, the capability of degrading great variety of carbohydrates of lignocellulosic feedstock (Sommer et al., 2004). Lignocellulosic materials found in a variety of wastes (agricultural, municipal, and pulp and paper industry) and forestry residues are low-cost and abundant raw materials for ethanol and hydrogen production (Lynd, 1989; Zaldivar et al., 2001). The economically feasible production of ethanol from lignocellulosic materials requires efficient conversion of all the main carbohydrate constituents of this complex material to ethanol, and therefore, such microorganisms would be desirable (Galbe and Zacchi, 2002; Ingram et al., 1999; Olsson and Hahn-Hägerdal, 1996). Several thermoanaerobes, capable of utilizing a variety of sugar constituents of lignocellulosic material, produce ethanol along with H_2, another valuable energy carrier (Koskinen et al., 2008; Mistry and Cooney, 1989). In the co-production of ethanol and H_2, substantial amounts of both biofuels can be obtained (Wu et al., 2007).

High temperatures favor the thermodynamics and kinetics of H_2 fermentation (van Groenestijn et al., 2002; van Niel et al., 2003). Further, the recovery of ethanol is enhanced at high temperatures favoring the use of continuous removal of ethanol though the application of mild vacuum or gas stream (Wiegel, 1980). In addition, thermophilic ethanol and H_2 production processes can utilize high temperature wastewaters, such as coffee processing, palm oil mill, cannery or distillery wastewaters (Yu et al., 2002a). There are, however, only a few reports on continuous H_2 and ethanol producing processes with thermophilic pure cultures (Mistry and Cooney, 1989). Generally, pure culture processes are not considered viable for industrial-scale ethanol and H_2 production from waste materials, since this would require costly sterilization procedures of the feed material (Valdez-Vazquez et al., 2005). However, in high-temperature bioprocesses, the growth of contaminating mesophiles present in the waste fractions is inhibited (van Groenestijn et al., 2002), and the ethanol utilizing thermoanaerobes are rare (Wiegel, 1980).

Geothermal springs are a potential source for saccharolytic, ethanol-, and hydrogen-producing thermophiles (Koskinen et al., 2008; Sommer et al., 2004; Wiegel and Ljungdahl, 1981). The objective of this study was to characterize the ethanol and hydrogen co-production potential by two thermophilic bacteria isolated from geothermal areas in Iceland. The ethanol and H_2 production of the isolates was studied in a batch assays and in a continuous-flow bioreactor. This study contributes to the knowledge on the diversity of ethanol and hydrogen producing thermoanaerobes, and to the design and operation of continuous, thermophilic ethanol and H_2 co-production processes

Materials and Methods

Isolation and Characterization of Bacterial Strains

The two bacterial strains (AK15 and AK17) studied were earlier isolated from two sediment samples collected from geothermal springs in the Krafla area (Víti) in NE-Iceland (Orlygsson and Baldursson, 2007). The sampling and isolation procedures, and the determination of carbon source utilization patterns are described in detail in Orlygsson and Baldursson (2007).

Glucose (20 mM) and xylose (20 mM) fermentation patterns of the isolates were characterized in batch assays in 120 mL serum bottles (40 mL of growth media) or in 25 mL serum tubes (10 mL of growth media) with anaerobic head space (N_2). The sterilized medium, prepared in tap water, was modified from Örlygsson et al. (1993) and contained buffers (g/L) (NaH_2PO_4, 5.5; Na_2HPO_4, 0.6; KH_2PO_4, 0.6); minerals (g/L) (NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 0.1), micronutrients (mg/L) ($FeCl_2 \cdot 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.038; $MnCl_2 \cdot 2H_2O$, 0.041; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $NiCl_2 \cdot 6H_2O$, 0.05; EDTA, 0.5; $Na_2SeO_3 \cdot 5H_2O$, 0.026; $NaWO_4 \cdot 2H_2O$, 0.033); vitamins (DSMZ medium No141, German Collection of Microorganisms and Cell Cultures); Resazurin, 0.025 mg/L; Cystein-HCl $\cdot 1H_2O$, 0.56 g/L; Na_2S, 0.24 g/L and yeast extract, 2 g/L. The effect of exogenously added ethanol on

the growth of strain of AK17 was studied in batch assays in 25 mL serum tubes using 10 mL of the growth media described above with 20 mM of glucose. Strain AK15 was incubated at 60°C at pH 7.0 and strain AK17 at 60°C at pH 6.0. Bacterial growth in batch assays was determined as optical density at 600 nm with a Ultraspec 500 Pro Visible spectrophotometer (Amersham Biosciences, Piscataway, NJ), and the gas production was determined based on Owen et al. (1979).

Bioreactor Set-Up

A completely mixed (by liquid recycle), suspended-cell bioreactor (total volume of 0.3 L, height to diameter ratio of 7) was used for continuous EtOH and H_2 production at 60°C. A synthetic feed was as described above, except that glucose concentration was either 12.6, 17.7 or 25.2 mM, 4 g/L of $NaHCO_3$ was added, and the yeast extract concentration was 0.2 g/L. The feed was prepared in two separate tanks, one containing the glucose and the other containing buffers, nutrients, minerals and vitamins. The feed tanks were kept at +4°C, and the feed flows were combined prior to the reactor inlet. The reactor pH was maintained at around 6.0 by adjusting feed pH by 5 M NaOH or 37% HCl. The bioreactor was inoculated with 25 mL each of batch cultures of AK15 and AK17. The inoculum cultures were prepared by incubating overnight at 60°C in the media described above with 20 mM of glucose. After the inoculation, bioreactor was operated in a batch mode for 24 h. After this, the bioreactor was operated continuously for 90 days by stepwise increasing the glucose loading rate (LR) and decreasing the hydraulic retention time (HRT; Fig. 2; Table II). The bioreactor was operated as an open system, that is, the feed was not sterilized. Bacterial community in the bioreactor was monitored for enrichment of new species. Gas production in bioreactor was measured with a wet gas meter (Ritter Apparatebau, Bochum, Germany).

Chemical Analyses

The composition of product gas from batch assays and bioreactor was measured using either a HP 5890II or a Perkin Elmer gas chromatographs (GCs) equipped with thermal conductivity detectors. The HP5890II GC-system had a 6 ft Porapak N packed column (80/100 mesh; Varian, Inc., Palo Alto, CA), and N_2 as the carrier gas. Oven, injector, and detector temperatures were 80, 110, and 110°C, respectively. The Perkin Elmer GC-system had a Supelco 1010 Carboxen GC Plot capillary column (Sigma-Aldrich, St. Louis, MO), and argon as the carrier gas. Oven, injector and detector temperatures were 65, 200, and 200°C, respectively. The concentrations of organic acids and alcohols were measured using a HP 5890II gas chromatograph with a DB-FFAP capillary column (Agilent Industries, Inc., Palo Alto, CA; dimensions 30 m × 0.53 mm × 1.0 mm), and a flame ionization detector. Glucose, lactate and formate were analyzed using a Waters 510 liquid chromatograph pump with a Shodex® Sugar SH1011 column (Showa Denko K.K., Tokyo, Japan) and a Δn -1000 refraction index detector (WGE Dr. Bures GmbH & Co KG, Dallgow, Germany). Mobile phase was 5 mM H_2SO_4. Biomass from the bioreactor was analyzed as volatile suspended solids (VSS) according to APHA standard method (APHA, 1995).

Molecular Characterization of the Isolates and the Bacterial Community Dynamics

Almost complete 16S rRNA gene sequences of the isolates (1,469 bp for AK15 and 1,454 bp for AK17) were analyzed for their phylogenetic characterization. Analyses were done by the Prokaria Company, Ltd (Reykjavik, Iceland) as previously described (Skirnisdottir et al., 2000).

Bacterial community of the bioreactor was monitored using DNA extraction and PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) of partial 16S rRNA genes followed by their sequencing. Duplicate samples of reactor liquid were taken during the operation, and stored at −20°C. DNA was extracted from 1 mL of samples with a VIOGENE Blood and Tissue Genomic DNA kit (Proteogenix SA, Fegersheim, France). Partial bacterial 16S rRNA genes of the community DNA were amplified using a primer pair GC-BacV3f (Muyzer et al., 1993) and 907r (Muyzer et al., 1996) as previously described (Koskinen et al., 2007). DGGE was performed with an INGENYphorU2x2-system (Ingeny International BV, GP Goes, The Netherlands) using a 8% polyacrylamide gel (acrylamide/bisacrylamide gel stock solution 37.5:1) with a denaturing gradient from 40% to 60% (100% denaturing solution contains 7 M of urea and 40% formamide). The gel was run at 60°C in 1 × TAE with 100 V for 21.5 h, and stained with SYBR® Gold (Molecular Probes, Inc., Eugene, OR). The dominant bands were excised from the gels, eluted in sterile H_2O (25 μL, overnight at +4°C), and re-amplified for sequencing as previously described (Koskinen et al., 2007). Sequence data was analyzed with a Bioedit software (version 7.0.5.2; Hall, 1999), and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/). The existence of chimeras was analyzed using a CHIMERA_CHECK software (version 2.7; Center for Microbial Ecology, Michigan State University [http://rdp.cme.msu.edu/cgis/chimera.cgi?su=ssu]). Bacterial 16S rRNA gene sequences were aligned and phylogenetic trees constructed with an ARB software (Ludwig et al., 2004) by using distance matrix and neighbor joining algorithms with 300 bootstraps.

The accession numbers of the gene sequences submitted to GenBank were EU262599; EF088330; EU255569; EU255570; EU255571; EU255572; EU255573; EU255574; EU255575; EU255576.

Results and Discussion

Phylogeny of the Isolates AK15 and AK17

Based on the almost complete 16S rRNA gene sequence analysis, the strains AK15 and AK17 were both members of

the family *Thermoanaerobacteriaceae* in the order *Thermobacteriales* in the class *Clostridia* of the phylum *Firmicutes* (Garrity et al., 2004). AK15 fell within the genus *Thermoanaerobacter* with the closest affiliation being *Clostridium uzonii* (98.8%; Table I, Fig. 1). Genus *Thermoanaerobacter* comprises of saccharolytic, thermophilic, anaerobic bacteria some of which have high ethanol production capabilities, for example, *T. ethanolicus* (Wiegel and Ljungdahl, 1981) and *T. thermohydrosulfuricus* (Wiegel et al., 1979). The closest relative of AK15, *C. uzonii*, was originally isolated from hot springs in the Kamchatka Peninsula, Russia. Obligately anaerobic, spore-forming *C. uzonii* has the optimum growth temperature and pH of 65°C and 7.0, respectively (Krivenko et al., 1990). The optimum growth condition for AK15 was 60°C and initial pH of 7.0 (unpublished results).

Strain AK17 fell within the genus *Thermoanaerobacterium* with the closest affiliations being *Thermoanaerobacterium aciditolerans* (99.2%) and *T. aotearoence* (98.1%; Table I, Fig. 1). The genus *Thermoanerobacterium* is characterized by saccharolytic, thermophilic, anaerobic bacteria, most of which have the ability to reduce thiosulfite to elemental sulfur (Cann et al., 2001; Lee et al., 1993). Several *Thermoanaerobacterium* species are known for their H_2 and ethanol production capabilities, such as *T. aciditolerans* (Kublanov et al., 2007), *T. aotearoense* (Liu et al., 1996), *T. polysaccharolyticum* (Cann et al., 2001), *T. saccharolyticum*, *T. thermosulfurigenes*, *T. xylanolyticum* (Lee et al., 1993) and *T. zeae* (Cann et al., 2001). The closest relative of AK17, *T. aciditolerans*, was originally isolated from hot springs in Kamchatka Peninsula, Russia. Obligate anaerobe, spore-forming, *T. aciditolerans* has the optimal growth temperature and pH of 55°C and 5.7, respectively (Kublanov et al., 2007). The optimum growth condition for AK17 was 58°C and initial pH of 6.0 (unpublished results).

Carbon Source Utilization by the Isolates AK15 and AK17

In the presence of yeast extract, AK15 utilized fructose, galactose, glucose, mannose, ribose, xylose, sucrose, xylan, and pyruvate. Substrates that were not utilized included arabinose, lactose, cellulose, and pectin (Orlygsson and Baldursson, 2007). In the presence of yeast extract, AK17 utilized arabinose, fructose, galactose, glucose, mannose, ribose, xylose, lactose, and sucrose. Limited growth was observed with cellulose and pectin. Substrates that were not utilized included xylan and pyruvate (Orlygsson and Baldursson, 2007).

The majority of the biomass in all green plants is lignocellulose, the structural polymer (cellulose, hemicellulose, pectin, and lignin) that comprises the cell wall (Ingram et al., 1999). The sugar residues of hemicellulose contain a varying mixture of hexoses (e.g., glucose, mannose and galactose), and pentoses (e.g., arabinose and xylose), of which pentose sugars are more rarely utilized by microorganisms (Ingram et al., 1999). Strain AK17 was able to utilize all these main hexose and pentose residues of hemicellulose, while AK15 did not utilize arabinose. Further, AK17 grew on pectin and cellulose. These results demonstrate that AK17 has a wide substrate utilization spectrum for the sugar residues in hydrolysates of lignocellulosic material.

Batch Fermentation Patterns From Glucose and Xylose by the Isolates AK15 and AK17

In the batch fermentation of glucose by AK15, hydrogen was the main fermentation product yielding 1.1–1.9 mol-H_2/mol-glucose (28–48% of the theoretical maximum). The main soluble metabolites were ethanol (0.6–0.8 mol-EtOH/mol-glucose) and acetate, while lactate was produced

Table I. Affiliations[Q2] of the 16S rRNA gene sequences of the strains AK15 and AK17 isolated from Icelandic geothermal springs, and bands excised from DGGE gel of the continuous-flow bioreactor inoculated with the strains AK15 and AK17 at 60°C.

OTU (acc)[a]	BL[b]	SL[c]	Family[d]	Affiliation (acc)[e]	Sim (%)[f]
AK15 (EU262599)		1452	*Clostridiaceae*	*Clostridium uzonii* DSM 9752 (Y18182)	98.8
AK17 (EF088330)		1442	*Clostridiaceae*	*Thermoanaerobacterium aciditolerans* (AY350594)	99.2
TRAK-A (EU255569)	A	519;	*Clostridiaceae*	*Clostridium thermoamylolyticum* DSM 2335 (X76743)	99.8
		519		*Thermoanaerobacterium aciditolerans* (AY350594)	99.4
TRAK-B (EU255570)	B	523	*Clostridiaceae*	*Clostridium uzonii* DSM 9752 (Y18182)	98.3
TRAK-C (EU255571)	C	523	*Clostridiaceae*	*Clostridium uzonii* DSM 9752 (Y18182)	98.3
TRAK-D (EU255572)	D	523	*Clostridiaceae*	*Clostridium uzonii* DSM 9752 (Y18182)	98.5
TRAK-E (EU255573)	E	523	*Clostridiaceae*	*Clostridium uzonii* DSM 9752 (Y18182)	98.5
TRAK-F (EU255574)	F	524	*Clostridiaceae*	*Clostridium butyricum* (AY442812)	99.4
TRAK-G (EU255575)	G	524	*Clostridiaceae*	*Clostridium butyricum* DSM 2478 (X68177)	99.4
TRAK-H (EU255576)	H	549	*Enterobacteriaceae*	*Escherichia coli* K-12 MG1655 (U00096 AE000111-AE000510)	100

[a]Operational taxonomic unit with accession number.
[b]Band label in Figure 3.
[c]Sequence length (bp).
[d]Family according to Ribosomal Database Project II.
[e]Closest species in GenBank with accession number.
[f]Similarity (%).

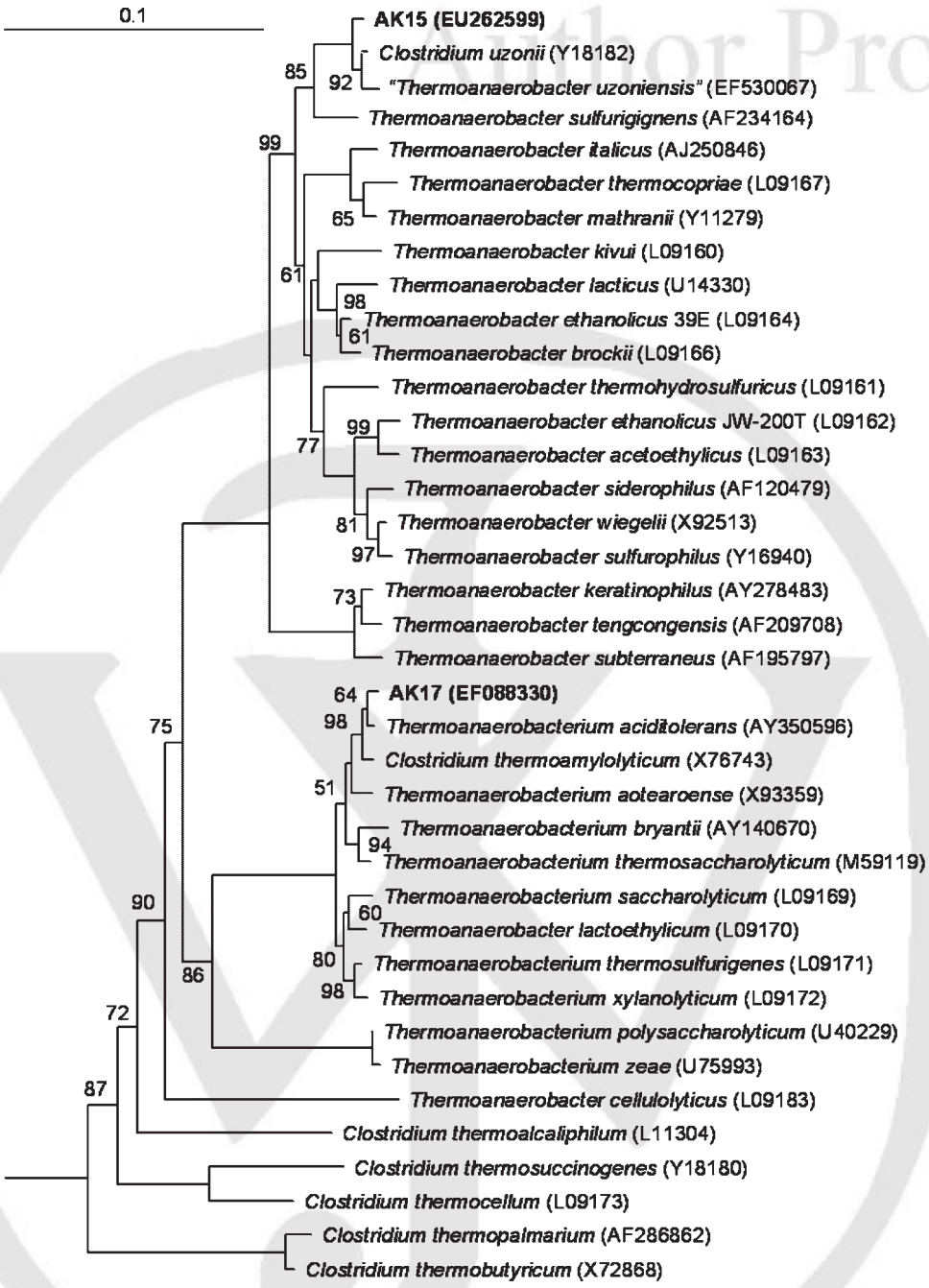


Figure 1. Phylogeny of the strains AK15 and AK17 isolated from Icelandic geothermal springs based on the 16S rRNA gene sequences (1,454–1,469 bp). The phylogenetic tree was generated using a distance matrix and neighbor joining algorithms with 300 bootstraps. Only supported bootstrap values (>50%) are shown. *Escherichia coli* (AE000406) was selected as an out-group. The scale bar indicates 0.1 substitutions per nucleotide position.

to a lesser extent. The AK15 produced H_2 yields of 1.0–1.1 mol-H_2/mol-xylose (30–33% of the theoretical maximum) from xylose. Acetate was the main soluble metabolite from xylose followed by ethanol (0.4 mol-EtOH/mol-xylose) and lactate.

Ethanol was the main product of batch fermentation of glucose by AK17 yielding typically 1.2–1.6 mol-EtOH/mol-glucose (60–80% of the theoretical maximum). The other fermentation products included hydrogen (0.4–1.2 mol-H_2/mol-glucose) and acetate. In the batch fermentation of xylose by AK17, ethanol yields of 1.0–1.1 mol-EtOH/mol-xylose (60–66% of the theoretical maximum) were typically obtained. Hydrogen (0.9–1.0 mol-H_2/mol-xylose) and acetate were also produced.

Continuous Ethanol and Hydrogen Production From Glucose

In the continuous-flow bioreactor maintained with a co-culture of strains AK15 and AK17, hydrogen production started during the 1 day batch mode after the inoculation. When the continuous feed was started, H_2 production rates slowly increased, and the steady-state was achieved after 30 days from the start-up (Fig. 2). After this when the glucose loading was stepwise increased, H_2 production rate in the bioreactor increased along with the increasing LR, until the HRT 2.8 h (last HRT studied; Fig. 2; Table II). The highest steady-state hydrogen production rate of 6.1 mmol/h/L (H_2 yield of 0.80 mol-H_2/mol-glucose) was obtained with the HRT of 3.1 h corresponding to a glucose LR of 8.1 mmol/h/L. On day 83, feeding tube for the nutrient feed containing buffering agents was clogged. This resulted in a pH decrease and H_2 production ceased momentarily. After the feed had been recovered, H_2 production started rapidly and H_2 production rates were resumed at the same levels preceding the technical failure. Hydrogen concentrations in the product gas varied generally from 30% to 50% throughout the experiment.

Ethanol was the main soluble metabolite in the bioreactor, followed by acetate and lactate (Fig. 2; Table II). Molar ethanol to acetate ratios varied between 2.6 and 3.7 throughout the

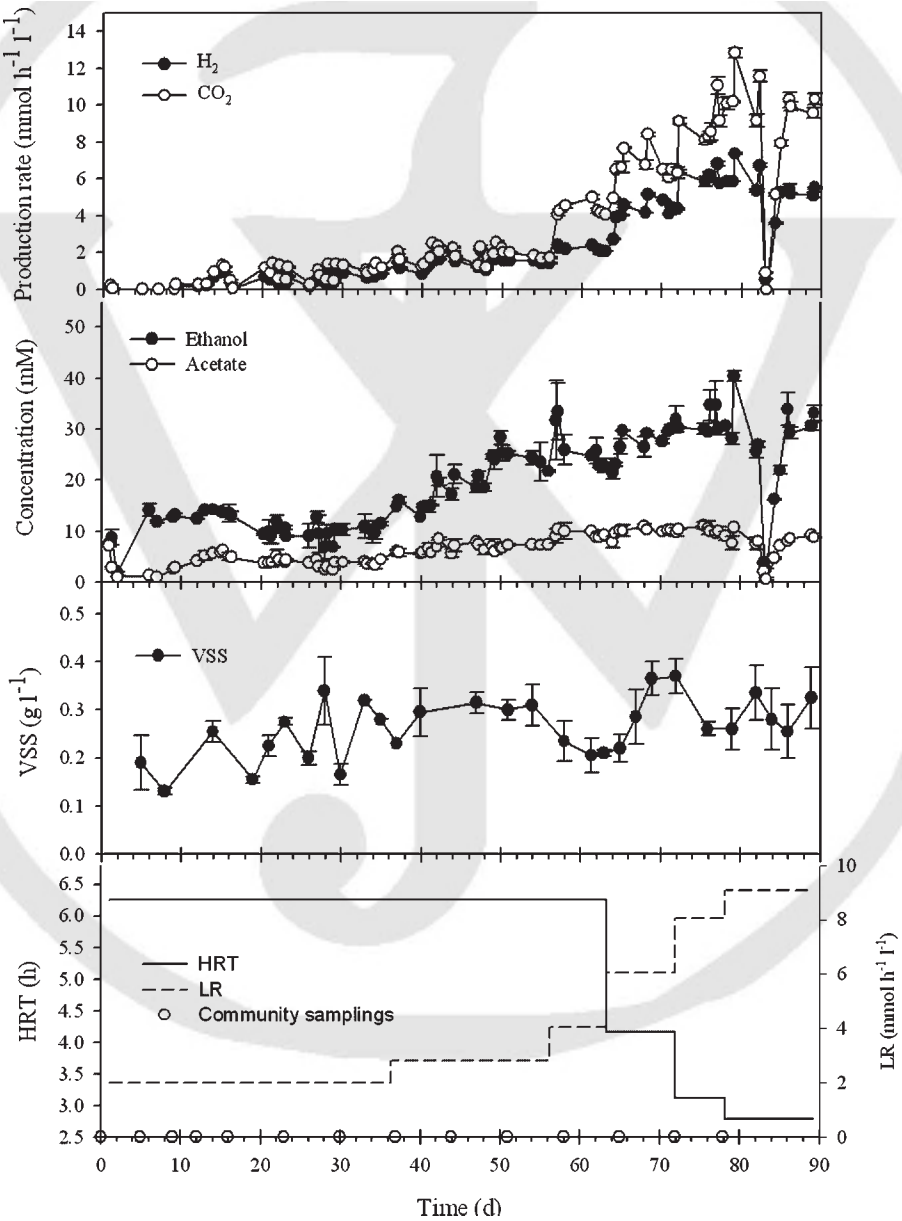


Figure 2. Performance data for the continuous-flow bioreactor maintained at 60°C with the isolates AK15 and AK17.

Table II. Summary of performance data for the continuous-flow bioreactor maintained at 60°C with isolates the AK15 and AK17 at different HRT and LR (±standard deviation).

	Time period (measurements)					
	30–35 (6)	50–56 (5)	58–63 (5)	70–72 (4)	75–78 (6)	86–89 (4)
HRT (h)	6.25	6.25	6.25	4.2	3.1	2.8
Glucose LR (mmol/h/L)	2.0	2.8	4.0	6.1	8.1	9.1
H_2 % in product gas	40.2 (1.9)	46.0 (1.3)	33.4 (1.3)	40.7 (1.0)	39.2 (3.3)	34.7 (0.6)
HPR (mmol/h/L)	0.78 (0.14)	1.50 (0.08)	2.18 (0.13)	4.43 (0.32)	6.10 (0.39)	5.31 (0.19)
Ethanol (mM)	10.50 (0.66)	24.31 (1.76)	24.47 (1.52)	29.85 (1.76)	31.66 (2.45)	31.77 (2.19)
Acetate (mM)	3.91 (0.39)	7.26 (0.06)	9.46 (0.60)	10.12 (0.21)	10.12 (0.73)	8.72 (0.51)
Lactate (mM)	4.57 (0.52)	4.80 (0.46)	9.61 (0.22)	3.10 (0.14)	2.43 (0.23)	4.21 (0.35)
H_2 yield (mol-H_2/mol-glucose)[a]	0.39 (0.07)	0.53 (0.03)	0.55 (0.03)	0.80 (0.06)	0.80 (0.05)	0.63 (0.03)
Ethanol yield (mol-ethanol/mol-glucose)[a]	0.84 (0.05)	1.37 (0.08)	0.98 (0.06)	1.29 (0.07)	1.35 (0.09)	1.35 (0.10)
Lactate yield (mol-lactate/mol-glucose)[a]	0.36 (0.04)	0.27 (0.04)	0.39 (0.01)	0.13 (0.01)	0.1 (0.01)	0.18 (0.02)
Ethanol/acetate	2.69 (0.13)	3.33 (0.30)	2.59 (0.18)	2.95 (0.19)	3.15 (0.38)	3.66 (0.42)
Glucose degradation efficiency (%)	99.4 (0.1)	99.6 (0.1)	98.9 (0.6)	91.9 (1.2)	94.8 (2.3)	93.4 (2.6)

LR, loading rate; HRT, hydraulic retention time; HPR, hydrogen production rate.
[a]Yields were calculated per glucose degraded.

experiment. The steady-state ethanol yields were from 1.29 to 1.37 mol-EtOH/mol-glucose, except when the glucose LR was 2.0 or 4.0 mmol/h/L. With LRs of 2.0 and 4.0 mmol/h/L, the ethanol yields were less than 1 mol-EtOH/mol-glucose and were associated with elevated lactate yields (Table II). On the other hand, during the highest ethanol yield (1.37 mol-EtOH/mol-glucose) obtained with LR of 2.8 mmol/h/L the lactate production was also relatively high. Lactate production decreased when the HRT was shortened. Highest steady-state ethanol concentration of 31.8 mM (ethanol yield of 1.35 mol-EtOH/mol-glucose) was obtained with the HRT of 2.8 h (LR of 9.1 mmol/h/L). Butyrate concentrations remained below 1 mM and formate concentrations below 3 mM throughout the experiment (data not shown).

Stable and continuous ethanol and H_2 co-production from glucose was achieved in the bioreactor inoculated with the co-culture of thermophilic isolates AK15 and AK17. Table III summarizes the performance of some continuous H_2 production processes reported in the literature. The maximum hydrogen production rate of this study (6.1 mmol/h/L) is comparable with the majority of studies with thermophilic systems regardless of the fact that the majority of electrons were directed to ethanol production instead of H_2 production. Oh et al. (2004) reported substantially higher H_2 production rates by using a thermophilic trickling biofilter system. The H_2 production rate in this study, however, remains far less than the highest reported in mesophilic systems, obtained in granular reactors with extremely short HRTs of 0.25 (Zhang et al., 2008) or 0.5 h (Wu et al., 2006) and with high substrate loading. The H_2 yield achieved in this study (0.80 mol-H_2/mol-glucose) remains low due to the high ethanol production.

In the batch culture, AK17 produced ethanol from glucose up to 1.6 mol-EtOH/mol-glucose and from xylose up to 1.1 mol-EtOH/mol-xylose. In the continuous fermentation, the AK17 dominated culture (see next section) produced up to 1.37 mol-EtOH/mol-glucose. These ethanol yields are among the highest reported for thermoanaerobes. Of the thermoanaerobes, *T. ethanolicus* has the highest

Table III. The comparison of hydrogen production performance of some continuous-flow bioreactors reported in the literature.

Substrate	Temperature (°C)	H_2 yield (mol-H_2/mol-hexose)	H_2 production rate (mmol/h/L)	References
Glucose	60	0.80	6.1	This study
Glucose	60	1.11	43.8[a]	Oh et al. (2004)
Sugar factory wastewater	60	2.57	8.3[a]	Ueno et al. (1996)
Winery wastewater	55	2.14	6.6[a]	Yu et al. (2002b)
Glucose	70	2.47	2.1[a]	Kotsopoulos et al. (2006)
Glucose	74	0.42	1.4	Koskinen et al. (2008)
Cellulose powder	60	2.00	1.2[a]	Ueno et al. (2001)
Sucrose	40	1.59[a]	627[a]	Wu et al. (2006)
Glucose	37	1.71	311[a]	Zhang et al. (2008)
Fructose	35	0.56	33.0	Wu and Chang (2007)
Glucose	35	1.71[a]	29.6[a]	Lin and Chang (1999)
Glucose	30–34	0.86[a]	15.0[a]	Lin and Chang (2004)

[a]Calculated based on the information provided.

ethanol yields from glucose and xylose. Ethanol yields from glucose of 1.9 mol-EtOH/mol-glucose have been reported for *T. ethanolicus* in both batch (Wiegel and Ljungdahl, 1981) and continuous cultures (Lacis and Lawford, 1991). From xylose, *T. ethanolicus* produces ethanol up to 1.37 mol-EtOH/mol-xylose (Lacis and Lawford, 1988). Another high ethanol-producing thermoanaerobe, *T. thermohydrosulfuricus* (previously known as *Clostridium thermohydrosulfuricum*), produces ethanol from glucose up to 1.5 mol-EtOH/mol-glucose (Wiegel et al., 1979). Wu and Chang (2007)[Q3] reported yields of 0.65 mol-EtOH/mol-fructose and 0.56 mol-H_2/mol-fructose in continuous, mesophilic co-fermentation of ethanol and hydrogen. In the bioreactor of this study, high ethanol yields (and substantial hydrogen production) were obtained by the AK17 dominated culture when operated with short HRTs (2.8 and 3.1 h) indicating the applicability of the culture to continuous co-production of ethanol and H_2.

Glucose Utilization, Biomass Concentration, and Carbon Balances in the Continuous-Flow Bioreactor

With the HRT of 6.25 h, glucose was nearly completely degraded regardless of the increase in glucose concentration (Table II). When the HRT was shortened, glucose was more than 90% degraded. Biomass concentrations (as VSS) in the bioreactor varied from 0.15 to 0.37 g/L (Fig. 2).

Carbon mass balances of the bioreactor were calculated for each glucose LR used (Table IV). Carbon included in the yeast extract (0.2 g/L) was not accounted for in the carbon balance, and the composition of biomass was assumed to be $C_5H_7O_2N$ (Fang and Liu, 2002). Carbon recoveries for different LRs varied from 84% to 96% (Table IV). The carbon from glucose was mainly directed to ethanol production followed by CO_2, acetate, biomass, and lactate production in the bioreactor.

Dynamics of Bacterial Community Composition

Bacterial community dynamics of the continuous-flow bioreactor was monitored to reveal the fate of the thermophilic isolates (AK15 and AK17), and to monitor whether new species became enriched in the open system without feed sterilization. After the start-up of continuous operation, both AK15 (*C. uzonii* affiliated strain) and AK17 (*C. uzonii* affiliated strain) were detected in the community profile (Fig. 3, Table I). AK15 had four slightly different 16S rRNA gene sequences seen as distinctive band positions in the DGGE gel, while the AK17 had distinctive band positions in the gel, but the bands shared the same 16S rRNA gene sequence. Within three weeks after the start-up, AK15 affiliated bands disappeared from the community profile whereas strain AK17 affiliated bands were seen throughout the experiment time course. This indicates that AK17 was the dominant thermophile in the bioreactor (Fig. 3; Table I). The dominance of AK17 and disappearance of AK15 was likely due to the growth conditions, for example, the feed composition, pH, temperature or hydrodynamic conditions were more suitable for AK17 than AK15. In batch assays at pH 6 and 60°C, the AK17 had higher growth rate and shorter doubling time than AK15 (unpublished results). Further, the bioreactor feed had 0.2 g/L of yeast extract which

Table IV. Carbon balances for the continuous-flow bioreactor maintained at 60°C with the isolates AK15 and AK17 at different HRT and glucose LR (±standard deviation).

	Time period (measurements)					
	30–35 (6)	50–56 (5)	58–63 (5)	70–72 (4)	75–78 (6)	86–89 (4)
HRT (h)	6.25	6.25	6.25	4.2	3.1	2.8
Glucose LR (mmol/h/L)	2.0	2.8	4.0	6.1	8.1	9.1
Carbon in substrates (mmol C/h/L)[a]						
Glucose	12.10	16.95	24.20	36.31	48.42	54.47
Residual glucose	0.07 (0.01)	0.06 (0.02)	0.26 (0.15)	2.93 (0.42)	2.53 (1.11)	3.60 (1.44)
Glucose–carbon consumption rate	12.03 (0.01)	16.88 (0.02)	23.95 (0.15)	33.38 (0.42)	45.89 (1.11)	50.87 (1.44)
Carbon in products (mmol C/h/L)						
Ethanol	3.36 (0.21)	7.71 (0.47)	7.83 (0.48)	14.33 (0.85)	20.26 (1.57)	22.88 (1.58)
Acetate	1.25 (0.12)	2.32 (0.09)	3.03 (0.19)	4.86 (0.10)	6.48 (0.47)	6.28 (0.37)
Lactate	2.19 (0.25)	2.31 (0.22)	4.61 (0.10)	2.23 (0.21)	2.33 (0.22)	4.55 (0.37)
Butyrate	0.03 (0.01)	0.02 (0.01)	0.14 (0.07)	0 (0)	0.02 (0.02)	0.04 (0.03)
Formate	0.12 (0.14)	0 (0)	0.42 (0.06)	0 (0)	0 (0).	0 (0)
CO_2[b]	1.08 (0.17)	1.84 (0.16)	4.41 (0.38)	6.36 (0.19)	9.20 (1.16)	10.02 (0.35)
Biomass[c]	2.12 (0.20)	2.16 (0.05)	1.47 (0.03)	3.90 (0.04)	3.68 (0.00)	4.68 (0.08)
Carbon recovery (%)[c]	84.1 (4.0)	96.5 (3.7)	91.1 (4.4)	87.2 (1.3)	86.7 (4.3)	88.8 (2.8)

LR, loading rate; HRT, hydraulic retention time.
[a]Carbon in yeast extract (0.02 g/L).
[b]CO_2 in the liquid phase were not taken into account.
[c]Biomass was calculated based on VSS assuming the biomass composition of $C_5H_7O_2N$ (Fang and Liu, 2002).

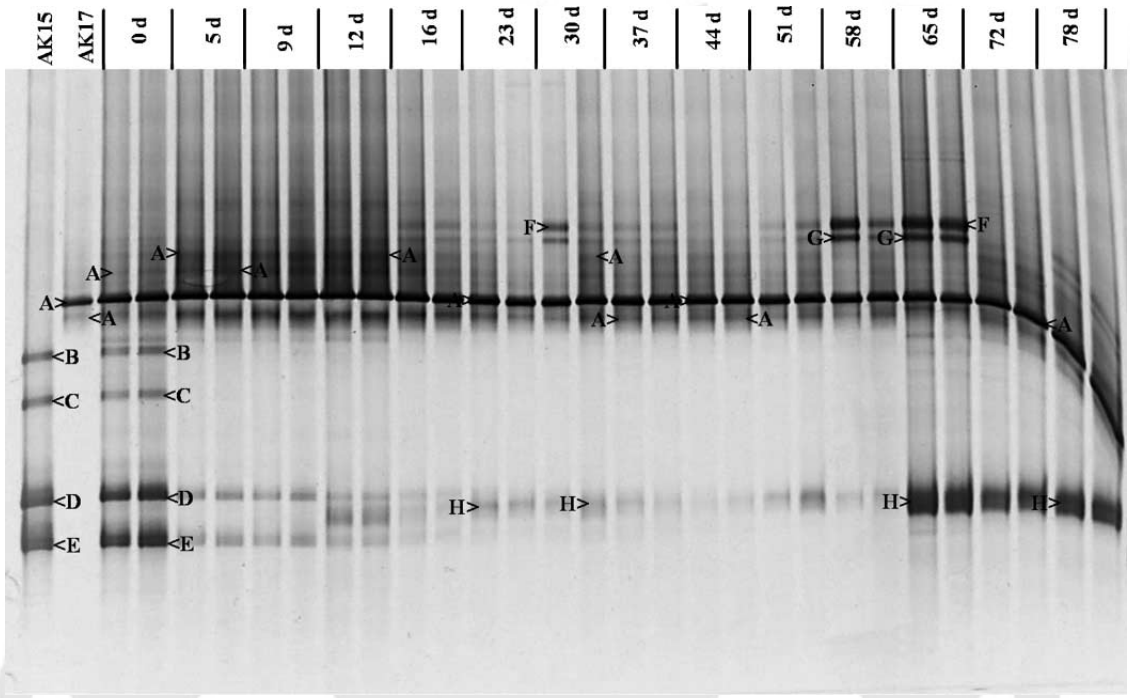


Figure 3. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes (519–549 bp) of the continuous-flow bioreactor maintained with the isolates AK15 and AK17. See Table I for the labeled bands. The bioreactor was operated at 60°C as an open system without feed sterilization.

possibly limited the growth of AK15, while the AK17 did not require yeast extract for growth.

After 2 weeks of operation, new organisms were seen in the bioreactor community profiles (Fig. 3; Table I). These organisms were closely affiliated with mesophilic *Clostridium butyricum* and *Escherichia coli*. The mesophiles likely grew in the feed tank or in the feed tubings. The glucose degradation in the feed tank was likely very low as the feed was kept at +4°C, and the glucose feed did not contain nutrients (nutrients were fed from a separate tank). Further, the concentration of butyrate, the main fermentation product of *C. butyricum* (Crabbendam et al., 1985), remained low (<1 mM) in the bioreactor throughout the experiment. In a similar manner, the concentration of formate, a major fermentation product of *E. coli* (for a review, see Nandi and Sengupta, 1998), was low in the bioreactor (<3 mM). These results demonstrate that the degradation of glucose in the bioprocess by the mesophilic

Table V. Summary of growth of strain AK17 in the presence of exogenously added ethanol.

Ethanol concentration (%, v/v)	Lag time (h)[a]	Maximum OD[b]	Growth rate (h^{-1})[c]	H_2 yield (mol-H_2/mol-glucose)
0	1	1.58 (0.05)	0.54 (0.01)	1.20 (0.01)
0.5	8	1.59 (0.06)	0.52 (0.01)	ND
1.0	15	1.70 (0.08)	0.50[d]	1.10 (0.09)
1.5	17	1.52 (0.07)	0.50[d]	1.02 (0.23)
2.0	26	1.52 (0.05)	0.52 (0.03)	1.17 (0.07)
2.5	28	1.49 (0.09)	0.49 (0.05)	1.09 (0.21)
3.0	32	1.32 (0.01)	0.42 (0.01)	1.00 (0.30)
3.5[d]	ND	0.63	ND	0.72
4.0[d]	>52	0.47	ND	0.32

The data represent averages of triplicate measurements (±standard deviation).

Cultivations were performed in 25 mL serum tubes (10 mL of medium) with 20 mM of glucose at 60°C and at initial pH of 6.0. The optical density (OD) was measured at 600 nm. Hydrogen production was measured at the end point, after the cultures had reached maximum OD.

ND, not determined.

[a]Determined as the time when the OD of the sample exceeded that of a no-inoculum control by 0.1 units.

[b]Determined as the maximum OD of the sample minus the OD of the no-inoculum control.

[c]Determined based on the OD values directly by using the Monod equation (Georgieva et al., 2007).

[d]Based on a single tube measurement.

bacteria was insignificant. These results also show that long-term maintenance of ethanol and hydrogen production activity by thermophilic pure cultures is possible in an open system.

Ethanol Tolerance of Strain AK17

The tolerance to exogenously added ethanol was determined for the strain AK17. The AK17 tolerated up to 4% (v/v) of exogenously added ethanol, while the growth was completely inhibited at 5% (v/v). The increased ethanol concentration progressively prolongated the lag phase of AK17 (Table V) as also previously reported for *Thermoanaerobacter* A10 (Georgieva et al., 2007). The ethanol concentration of up to 2.5% (v/v) did not substantially inhibit the growth of AK17. Ethanol addition of 3% (v/v) resulted in a slight decrease in maximum OD and growth rate, while the ethanol concentrations of 3.5% and 4% (v/v) decreased the growth drastically (Table V). Similarly, the hydrogen yields decreased with ethanol additions of 3.5% and 4.0% (v/v). With ethanol supplementation of up to 3% (v/v), the hydrogen yields remained between 1.0 and 1.2 mol-H_2/mol-glucose.

Thermoanaerobes should be able to sustain ethanol concentrations above about 4–5% (v/v) in order to obtain commercially viable separation of ethanol from bioprocess (Lynd et al., 2001; Sudha Rani and Seenayya, 1999). The ethanol tolerance of the strain AK17 (4%, v/v) was substantially higher than generally considered among wild-type thermoanaerobes (<1–2%, v/v; Burdette et al., 2002; Lynd, 1989). The highest ethanol tolerances for wild-type thermoanaerobes include 5.1% (v/v) for *Thermoanaerobacter* A10 (Georgieva et al., 2007) and 5% (v/v) for *Clostridium thermocellum* SS22 (Sudha Rani and Seenayya, 1999), respectively. The ethanol tolerance of several thermoanaerobes has been successfully increased by batch or continuous cultivation at high ethanol concentrations resulting in adaptation or generation of ethanol-resistant mutants (Baskaran et al., 1995; Burdette et al., 2002; Sudha Rani and Seenayya, 1999).

Conclusions

Ethanol and hydrogen production was studied by two bacterial strains, AK15 affiliated with *C. uzonii* (98.8%) and AK17 affiliated with *T. aciditolerans* (99.2%), isolated from Icelandic geothermal springs. The strains utilized a wide variety of sugar monomers found in the lignocellulosic materials, and also some polysaccharides. Strain AK17 had high ethanol yields from glucose and xylose fermentations of up to 1.6 mol-EtOH/mol-glucose and 1.1 mol-EtOH/mol-xylose, respectively, while hydrogen was produced to a lesser extent. The strain AK15 produced hydrogen as the main fermentation product from glucose and xylose. The AK17 tolerated exogenously added ethanol up to 4% (v/v). Stable and continuous ethanol and hydrogen co-production from glucose was demonstrated in a bioreactor dominated by the strain AK17. The bioreactor produced up to 1.37 mol-EtOH/mol-glucose, and the highest H_2 rate was 6.1 mmol/h/L (H_2 yield of 0.80 mol-H_2/mol-glucose). In summary, the results indicate that strain AK17 is a promising ethanol (and hydrogen) producer with high ethanol yields, relatively high ethanol tolerance, and the capability of utilizing a variety of hydrocarbons found in the lignocellulosic materials.

This research was funded by the Academy of Finland (HYDROGENE project, no. 107425), Nordic Energy Research (BIOHYDROGEN project, no. 28-02), Tampere University of Technology Graduate School (P. E. P. Koskinen), RANNÍS, The Icelandic Centre for Research, and The Research Fund of the University of Akureyri.

References

APHA. 1995. Standard methods for the examination of water and wastewater, 19th edn. Washington DC, U.S.: American Public Health Association/American Water Works Association/Water Environment Federation.

Baskaran S, Ahn H-J, Lynd LR. 1995. Investigation of the ethanol tolerance of *Clostridium thermosaccharolyticum* in continuous culture. Biotechnol Prog 11:276–281.

Burdette DS, Jung S-H, Shen G-J, Hollingsworth RI, Zeikus JG. 2002. Physiological function of alcohol dehydrogenases and long-chain (C30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. Appl Microbiol Biotechnol 68:1914–1918.

Cann IKO, Stroot PG, Mackie KR, White BA, Mackie RI. 2001. Characterization of two novel saccharolytic, anaerobic thermophiles, *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobacterium zeae* sp. nov., and emendation of the genus *Thermoanaerobacterium*. Int J Syst Evol Microbiol 51:293–302.

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA. 1999. Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52:741–755.

Crabbendam PM, Neijssel OM, Tempest DW. 1985. Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. Arch Microbiol 142:375–382.

Das D, Veziroğlu TN. 2001. Hydrogen production by biological processes: A survey of literature. Int J Hydrogen Energy 26:13–28.

Fang HHP, Liu H. 2002. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour Technol 82:87–93.

Galbe M, Zacchi G. 2002. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618–628.

Garrity GM, Bell JA, Lilburn TG. 2004. Taxonomic outline of the prokaryotes Bergey's manual® of systematic bacteriology, 2nd edn. Release 5.0. New York: Springer, 401 p. http://141.150.157.80/bergeysoutline/main.htm.

Georgieva TI, Skiadas IV, Ahring BK. 2007. Effect of temperature on ethanol tolerance of a thermophilic anaerobic ethanol producer *Thermoanaerobacter* A10: Modelling and simulation. Biotechnol Bioeng (in press[Q4]), available on-line, 10.1002/bit. 21536.

Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98.

Ingram LO, Aldrich HC, Borges ACC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano LP, York SW, Zaldivar J, Zhou S. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnol Prog 15:855–866.

Koskinen PEP, Kaksonen AH, Puhakka JA. 2007. The relationship between instability of H_2 production and compositions of bacterial

communities within a dark fermentation fluidized-bed bioreactor. Biotechnol Bioeng 97:742–758.

Koskinen PEP, Lay C-H, Beck SR, Tolvanen KES, Kaksonen AH, Örlygsson J, Lin C-Y, Puhakka JA. 2008. Bioprospecting thermophilic microorganisms from Icelandic hot springs for hydrogen and ethanol production. Energy Fuels 22:134–140.

Kotsopoulos TA, Raymond JZ, Angelidaki I. 2006. Biohydrogen production in granular up-flow anaerobic sludge blanket reactors with mixed cultures under hyper-thermophilic temperature (70°C). Biotechnol Bioeng 94:296–302.

Krivenko VV, Vadachloriya RM, Chermykh NA, Mityushina LL, Krasil'nikova EN. 1990. *Clostridium uzonii* sp. nov., an anaerobic thermophilic glycolytic bacterium isolated from hot springs in the Kamchatka Peninsula. Microbiologiya 59:1058–1066.

Kublanov IV, Prokofeva MI, Kostrikina NA, Kolganova TV, Tourova TP, Wiegel J, Bonch-Osmolovskaya EA. 2007. *Thermoanaerobacterium aciditolerans* sp. nov., a moderate thermoacidophile from a Kamchatka hot spring. Int J System Evol Microbiol 57:260–264.

Lacis LS, Lawford H. 1988. Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. Arch Microbiol 150:48–55.

Lacis LS, Lawford H. 1991. *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. Appl Environ Microbiol 57:579–585.

Lee YE, Jain MK, Lee CY, Lowe SE, Zeikus JG. 1993. Taxonomic distinction of saccharolytic thermophilic anaerobes; description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium Thermohydrosulfuricum* E 100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. Int J System Bacteriol 43:41–51.

Lin C-Y, Chang R-C. 1999. Hydrogen production during the anaerobic acidogenic conversion of glucose. J Chem Technol Biotechnol 74:498–500.

Lin C-Y, Chang RC. 2004. Fermentative hydrogen production at ambient temperature. Int J Hydrogen Energy 29:715–720.

Liu SY, Rainey FA, Morgan HW, Mayer F, Wiegel J. 1996. *Thermoanaerobacterium aotearoense* sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and emendation of the genus *Thermoanaerobacterium*. Int J System Bacteriol 46:388–396.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar ???[Q5], Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann A, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H. 2004. ARB: A software environment for sequence data. Nucleic Acids Res 32:1363–1371.

Lynd LR. 1989. Production of ethanol from lignocellulosic materials using thermophilic bacteria. Critical evaluation of potential and review. Adv Biochem Eng Biotechnol 38:1–52.

Lynd LR, Baskaran S, Casten S. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of *Thermoanaerobacterium thermosaccharolyticum* HG-8 at elevated feed xylose concentrations in continuous culture. Biotechnol Prog 17:118–125.

Mistry FR, Cooney CL. 1989. Production of ethanol by *Clostridium thermosaccharolyticum*: I. Effect of cell recycle and environmental parameters. Biotechnol Bioeng 34:1295–1304.

Muyzer G, De Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction—Amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700.

Muyzer G, Hottenträger S, Teske A, Waver C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—A new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans ADL, van Elsas JD, De Bruijn F, editors. Molecular microbial ecology manual. Dordrecht: Kluwer Academic Publishers, p 3.4.4[Q6]/1–23.

Nandi R, Sengupta S. 1998. Microbial production of hydrogen: An overview. Crit Rev Microbiol 24:61–84.

Nath K, Das D. 2003. Hydrogen from biomass. Curr Sci 5:265–271.

Ng TK, Ben-Bassat A, Zeikus JG. 1981. Ethanol production by thermophilic bacteria: Fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 41:1337–1343.

Oh Y-K, Kim SH, Kim M-S, Park S. 2004. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnol Bioeng 88:690–698.

Olsson L, Hahn-Hägerdal B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enz Microb Technol 18:312–331.

Orlygsson J, Baldursson SRB. 2007. Phylogenetic and physiological studies of four hydrogen producing thermoanaerobes from Icelandic geothermal areas. Icel Agric Sci 20:93–105.

Örlygsson J, Houwen FP, Svensson BH. 1993. Anaerobic degradation of protein and the role of methane formation in steady state thermophilic enrichment cultures. Swed J Agric Res 23:45–54.

Owen WF, Stuckey DC, Healy JB, Jr., Young LY, McCarty PL. 1979. Bioassay for monitoring methane potential and anaerobic toxicity. Water Res 13:485–492.

Skirnisdottir S, Hreggvidsson GO, Hjörleifsdottir S, Marteinsson VT, Petursdottir SK, Holst O, Kristjansson JK. 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. Appl Environ Microbiol 66: 2835–2841.

Sommer P, Georgieva T, Ahring BK. 2004. Potential for using thermophilic anerobic bacteria for bioethanol production from hemicellulose. Biochem Soc Trans 32:283–289.

Sudha Rani K, Seenayya G. 1999. High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22. World J Microbiol Biotechnol 15:173–178.

Thauer RK, Jungermann K, Decker K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180.

Ueno Y, Otsuka S, Morimoto M. 1996. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. J Fermen Bioeng 82:194–197.

Ueno Y, Haruta S, Ishii M, Igarashi Y. 2001. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol 57:555–562.

Valdez-Vazquez I, Rios-Leal E, Esparza-Garcia F, Cecchi F, Poggi-Varaldo HM. 2005. Semi-continuous solid substrate anaerobic reactors for H_2 production from organic wastes: Mesophilic versus thermophilic regime. Int J Hydrogen Energy 30:1383–1391.

van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int J Hydrogen Energy 27:1141–1147.

van Niel EWJ, Claassen PAM, Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol Bioeng 81:255–262.

Weimer PJ. 1985. Thermophilic anaerobic fermentation of hemicellulose and hemicellulose-derived aldose sugars by *Thermoanaerobacter* strain B6A. Arch Microbiol 143:130–136.

Wiegel J. 1980. Formation of ethanol by bacteria. A pledge for use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experientia 36:1434–1446.

Wiegel J, Ljungdahl LG. 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Arch Microbiol 128:343–348.

Wiegel J, Ljungdahl LG, Rawson JR. 1979. Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. J Bacteriol 139:800–810.

Wu S-Y, Hung C-H, Lin C-N, Chen H-W, Lee A-S, Chang J-S. 2006. Fermentative hydrogen production and bacterial community structure

in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng 93(5):934–946.
Wu K-J, Chang C-F, Chang J-S. 2007. Simultaneous production of biohydrogen and bioethanol with fluidized-bed and packed-bed bioreactors containing immobilized anaerobic sludge. Process Biochem 42:1165–1171.
Yu HQ, Fang HHP, Gu GW. 2002a. Comparative performance of mesophilic and thermophilic acidogenic upflow reactors. Process Biochem 38:447–454.
Yu H, Zhu Z, Hu W, Zhang H. 2002b. Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. Int J Hydrogen Energy 27:1359–1365.
Zaldivar J, Nielsen J, Olsson L. 2001. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34.
Zeikus JG. 1980. Chemical and fuel production by anaerobic bacteria. Ann Rev Microbiol 34:423–464.
Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J. 2008. Enhanced continuous biohydrogen production by immobilized anaerobic microflora. Energy Fuels 22:87–92.

Q1: Author: Please check the grant sponsors.

Q2: Author: Please check the presentation of the tables.

Q3: Author: Please add in the reference list.

Q4: Author: Please update.

Q5: Author: Please provide the first name (initials) of the author.

Q6: Author: Please check the page range.

WILEY
Publishers Since 1807

111 RIVER STREET, HOBOKEN, NJ 07030

*****IMMEDIATE RESPONSE REQUIRED*****

Your article will be published online via Wiley's EarlyView® service (www.interscience.wiley.com) shortly after receipt of corrections. EarlyView® is Wiley's online publication of individual articles in full text HTML and/or pdf format before release of the compiled print issue of the journal. Articles posted online in EarlyView® are peer-reviewed, copyedited, author corrected, and fully citable via the article DOI (for further information, visit www.doi.org). EarlyView® means you benefit from the best of two worlds--fast online availability as well as traditional, issue-based archiving.

Please follow these instructions to avoid delay of publication.

☐ **READ PROOFS CAREFULLY**

- This will be your only chance to review these proofs**. Please note that once your corrected article is posted online, it is considered legally published, and cannot be removed from the Web site for further corrections.**
- Please note that the volume and page numbers shown on the proofs are for position only.

☐ **ANSWER ALL QUERIES ON PROOFS** (Queries for you to answer are attached as the last page of your proof.)

- Mark all corrections directly on the proofs. Note that excessive author alterations may ultimately result in delay of publication and extra costs may be charged to you.

☐ **CHECK FIGURES AND TABLES CAREFULLY** (Color figure proofs will be sent under separate cover.)

- Check size, numbering, and orientation of figures.
- All images in the PDF are downsampled (reduced to lower resolution and file size) to facilitate Internet delivery. These images will appear at higher resolution and sharpness in the printed article.
- Review figure legends to ensure that they are complete.
- Check all tables. Review layout, title, and footnotes.

☐ **COMPLETE REPRINT ORDER FORM**

- Fill out the attached reprint order form. It is important to return the form even if you are not ordering reprints. You may, if you wish, pay for the reprints with a credit card. Reprints will be mailed only after your article appears in print. This is the most opportune time to order reprints. If you wait until after your article comes off press, the reprints will be considerably more expensive.

RETURN ☐**PROOFS**
☐**REPRINT ORDER FORM**
☐**CTA (If you have not already signed one)**

RETURN IMMEDIATELY AS YOUR ARTICLE WILL BE POSTED ONLINE SHORTLY AFTER RECEIPT; FAX PROOFS TO 201-748-6825 or 8852

QUESTIONS?

Jeffrey Collins, Production Editor
Phone: 201-748-8864
E-mail: jecollin@wiley.com
Refer to journal acronym and article production number
(i.e., BIT 00-001 for Biotechnology and Bioengineering ms 00-001).

WILEY
Publishers Since 1807

COLOR REPRODUCTION IN YOUR ARTICLE

Color figures were included with the final manuscript files that we received for your article. Because of the high cost of color printing, we can only print figures in color if authors cover the expense.

Please indicate if you would like your figures to be printed in color or black and white. Color images will be reproduced online in Wiley *InterScience* at no charge, whether or not you opt for color printing.

You will be invoiced for color charges once the article has been published in print.

Failure to return this form with your article proofs will delay the publication of your article.

JOURNAL **BIOTECHNOLOGY AND BIOENGINEERING**

MS. NO. _____

NO. OF COLOR PAGES _____

TITLE OF MANUSCRIPT _____

AUTHOR(S) _____

No. Color Pages	Color Charges	No. Color Pages	Color Charges	No. Color Pages	Color Charges
1	**500**	**5**	**2500**	**9**	**4500**
2	**1000**	**6**	**3000**	**10**	**5000**
3	**1500**	**7**	**3500**	**11**	**5500**
4	**2000**	**8**	**4000**	**12**	**6000**
Please contact the Production Editor for a quote if you have more than 12 pages of color					

☐ Please print my figures in black and white

☐ Please print my figures in color

☐ Please print the following figures in color:

BILLING ADDRESS: _____

WILEY
Publishers Since 1807

111 River Street
Hoboken, NJ 07030, USA
201-748-8864
FAX: 201-748-6825

COPYRIGHT TRANSFER AGREEMENT

Date:

To:

Production/Contribution ID#_____ Publisher/Editorial office use only

Re: Manuscript entitled _____
_____ (the "Contribution")
for publication in _____ (the "Journal")
published by Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. ("Wiley").

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the publishing process and enable Wiley to disseminate your work to the fullest extent, we need to have this Copyright Transfer Agreement signed and returned to us as soon as possible. If the Contribution is not accepted for publication this Agreement shall be null and void.

A. COPYRIGHT

1. The Contributor assigns to Wiley, during the full term of copyright and any extensions or renewals of that term, all copyright in and to the Contribution, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution and the material contained therein in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the Contribution or any material contained therein, in any medium as permitted hereunder, requires a citation to the Journal and an appropriate credit to Wiley as Publisher, suitable in form and content as follows: (Title of Article, Author, Journal Title and Volume/Issue Copyright © [year] Wiley-Liss, Inc. or copyright owner as specified in the Journal.)

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's Employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution, and the right to make oral presentations of material from the Contribution.

C. OTHER RIGHTS OF CONTRIBUTOR

Wiley grants back to the Contributor the following:

1. The right to share with colleagues print or electronic "preprints" of the unpublished Contribution, in form and content as accepted by Wiley for publication in the Journal. Such preprints may be posted as electronic files on the Contributor's own website for personal or professional use, or on the Contributor's internal university or corporate networks/intranet, or secure external website at the Contributor's institution, but not for commercial sale or for any systematic external distribution by a third party (e.g., a listserve or database connected to a public access server). Prior to publication, the Contributor must include the following notice on the preprint: "This is a preprint of an article accepted for publication in [Journal title] © copyright (year) (copyright owner as specified in the Journal)". After publication of the Contribution by Wiley, the preprint notice should be amended to read as follows: "This is a preprint of an article published in [include the complete citation information for the final version of the Contribution as published in the print edition of the Journal]", and should provide an electronic link to the Journal's WWW site, located at the following Wiley URL: http://www.interscience.Wiley.com/. The Contributor agrees not to update the preprint or replace it with the published version of the Contribution.

2. The right, without charge, to photocopy or to transmit online or to download, print out and distribute to a colleague a copy of the published Contribution in whole or in part, for the Contributor's personal or professional use, for the advancement of scholarly or scientific research or study, or for corporate informational purposes in accordance with Paragraph D.2 below.

3. The right to republish, without charge, in print format, all or part of the material from the published Contribution in a book written or edited by the Contributor.

4. The right to use selected figures and tables, and selected text (up to 250 words, exclusive of the abstract) from the Contribution, for the Contributor's own teaching purposes, or for incorporation within another work by the Contributor that is made part of an edited work published (in print or electronic format) by a third party, or for presentation in electronic format on an internal computer network or external website of the Contributor or the Contributor's employer.

5. The right to include the Contribution in a compilation for classroom use (course packs) to be distributed to students at the Contributor's institution free of charge or to be stored in electronic format in datarooms for access by students at the Contributor's institution as part of their course work (sometimes called "electronic reserve rooms") and for in-house training programs at the Contributor's employer.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/employer which must sign this Agreement (in addition to the Contributor's signature), in the space provided below. In such case, the company/employer hereby assigns to Wiley, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, Wiley hereby grants back, without charge, to such company/employer, its subsidiaries and divisions, the right to make copies of and distribute the published Contribution internally in print format or electronically on the Company's internal network. Upon payment of the Publisher's reprint fee, the institution may distribute (but not resell) print copies of the published Contribution externally. Although copies so made shall not be available for individual re-sale, they may be included by the company/employer as part of an information package included with software or other products offered for sale or license. Posting of the published Contribution by the institution on a public access website may only be done with Wiley's written permission, and payment of any applicable fee(s).

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government Employees: see note at end).

F. COPYRIGHT NOTICE

The Contributor and the company/employer agree that any and all copies of the Contribution or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal as published by Wiley.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work. If the Contribution was prepared jointly, the Contributor agrees to inform the co-Contributors of the terms of this Agreement and to obtain their signature to this Agreement or their written permission to sign on their behalf. The Contribution is submitted only to this Journal and has not been published before, except for "preprints" as permitted above. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in Wiley's permissions form or in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe on the rights or privacy of others, or contain material or instructions that might cause harm or injury.

CHECK ONE:

[____]Contributor-owned work

_____ _____
Contributor's signature Date

_____
Type or print name and title

_____ _____
Co-contributor's signature Date

_____
Type or print name and title

ATTACH ADDITIONAL SIGNATURE PAGE AS NECESSARY

[____]Company/Institution-owned work (made-for-hire in the course of employment)

_____ _____
Company or Institution (Employer-for-Hire) Date

_____ _____
Authorized signature of Employer Date

[____]**U.S. Government work**

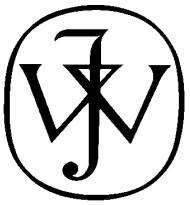
Note to U.S. Government Employees

A Contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication is called a "U.S. Government work," and is in the public domain in the United States. In such case, the employee may cross out Paragraph A.1 but must sign and return this Agreement. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[____]**U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

The rights in a Contribution prepared by an employee of a U.K. government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. In such case, the Publisher will forward the relevant form to the Employee for signature.



WILEY

Publishers Since 1807

BIOTECHNOLOGY AND BIOENGINEERING

Telephone Number: • Facsimile Number:

To: Jeffrey Collins At FAX #: 201-748-6825

From: Dr.

Date:

Re: *Biotechnology* and Bioengineering, ms #

Dear Mr. Jeffrey

Attached please find corrections to ms# _____. Please contact me should you have any difficulty reading this fax at the numbers listed below.

Office phone:
Email:
Fax:
Lab phone:

I will return color figure proofs (if applicable) once I have checked them for accuracy.

Thank you,

Dr.

E-proofing feedback comments:

C1

WILEY

Publishers Since 1807

REPRINT BILLING DEPARTMENT · 111 RIVER STREET, HOBOKEN, NJ 07030
PHONE: (201) 748-8789; FAX: (201) 748-6326
E-MAIL: reprints@wiley.com

PREPUBLICATION REPRINT ORDER FORM

Please complete this form even if you are not ordering reprints. This form **MUST** be returned with your corrected proofs and original manuscript. Your reprints will be shipped approximately 4 weeks after publication. Reprints ordered after printing will be substantially more expensive. **** 25 Reprints will be supplied free of charge.**

JOURNAL *Biotechnology and Bioengineering* VOLUME _____ ISSUE _____

TITLE OF MANUSCRIPT _____

MS. NO. _____ NO. OF PAGES _____ AUTHOR(S) _____

No. of Pages	100 Reprints	200 Reprints	300 Reprints	400 Reprints	500 Reprints
	$	$	$	$	$
1-4	**336**	**501**	**694**	**890**	**1052**
5-8	**469**	**703**	**987**	**1251**	**1477**
9-12	**594**	**923**	**1234**	**1565**	**1850**
13-16	**714**	**1156**	**1527**	**1901**	**2273**
17-20	**794**	**1340**	**1775**	**2212**	**2648**
21-24	**911**	**1529**	**2031**	**2536**	**3037**
25-28	**1004**	**1707**	**2267**	**2828**	**3388**
29-32	**1108**	**1894**	**2515**	**3135**	**3755**
33-36	**1219**	**2092**	**2773**	**3456**	**4143**
37-40	**1329**	**2290**	**3033**	**3776**	**4528**

**REPRINTS ARE ONLY AVAILABLE IN LOTS OF 100. IF YOU WISH TO ORDER MORE THAN 500 REPRINTS, PLEASE CONTACT OUR REPRINTS DEPARTMENT AT (201) 748-8789 FOR A PRICE QUOTE.

☐ Please send me _____ reprints of the above article at $ _____

Please add appropriate State and Local Tax for United States orders only. (Tax Exempt No._____) $ _____

Please add 5% Postage and Handling $ _____

TOTAL AMOUNT OF ORDER** $ _____

***International orders must be paid in currency and drawn on a U.S. bank*

Please check one: ☐ Check enclosed ☐ Bill me ☐ Credit Card

If credit card order, charge to: ☐ American Express ☐ Visa ☐ MasterCard

Credit Card No _____ Signature _____ Exp. Date _____

BILL TO:
Name _____
Institution _____
Address _____

SHIP TO: (Please, no P.O. Box numbers)
Name _____
Institution _____
Address _____

Purchase Order No. _____ Phone _____ Fax _____

E-mail _____

Softproofing for advanced Adobe Acrobat Users - NOTES tool

NOTE: ACROBAT READER FROM THE INTERNET DOES NOT CONTAIN THE NOTES TOOL USED IN THIS PROCEDURE.

Acrobat annotation tools can be very useful for indicating changes to the PDF proof of your article. By using Acrobat annotation tools, a full digital pathway can be maintained for your page proofs.

The NOTES annotation tool can be used with either Adobe Acrobat 4.0, 5.0 or 6.0. Other annotation tools are also available in Acrobat 4.0, but this instruction sheet will concentrate on how to use the NOTES tool. Acrobat Reader, the free Internet download software from Adobe, DOES NOT contain the NOTES tool. In order to softproof using the NOTES tool you must have the full software suite Adobe Acrobat 4.0, 5.0 or 6.0 installed on your computer.

Steps for Softproofing using Adobe Acrobat NOTES tool:

1. Open the PDF page proof of your article using either Adobe Acrobat 4.0, 5.0 or 6.0. Proof your article on-screen or print a copy for markup of changes.

2. Go to File/Preferences/Annotations (in Acrobat 4.0) or Document/Add a Comment (in Acrobat 6.0 and enter your name into the “default user” or “author” field. Also, set the font size at 9 or 10 point.

3. When you have decided on the corrections to your article, select the NOTES tool from the Acrobat toolbox and click in the margin next to the text to be changed.

4. Enter your corrections into the NOTES text box window. Be sure to clearly indicate where the correction is to be placed and what text it will effect. If necessary to avoid confusion, you can use your TEXT SELECTION tool to copy the text to be corrected and paste it into the NOTES text box window. At this point, you can type the corrections directly into the NOTES text box window. **DO NOT correct the text by typing directly on the PDF page.**

5. Go through your entire article using the NOTES tool as described in Step 4.

6. When you have completed the corrections to your article, go to File/Export/Annotations (in Acrobat 4.0) or Document/Add a Comment (in Acrobat 6.0).

7. **When closing your article PDF be sure NOT to save changes to original file.**

8. To make changes to a NOTES file you have exported, simply re-open the original PDF proof file, go to File/Import/Notes and import the NOTES file you saved. Make changes and re-export NOTES file keeping the same file name.

9. When complete, attach your NOTES file to a reply e-mail message. Be sure to include your name, the date, and the title of the journal your article will be printed in.