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Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485

Abstract The gene encoding L-lactate dehydrogenase from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 was cloned, sequenced, and used to obtain an L-*ldh* deletion mutant strain (TD1) following a site-specific double-crossover event as confirmed by PCR and Southern blot. Growth rates and final cell densities were similar for strain TD1 and the wild-type grown on glucose and xylose. Lactic acid was below the limit of detection (0.3 mM) for strain TD1 on both glucose and xylose at all times tested, but was readily detected for the wild-type strain, with average final concentrations of 8.1and 1.8 mM on glucose and xylose, respectively. Elimination of lactic acid as a fermentation product was accompanied by a proportional increase in the yields of acetic acid and ethanol. The results reported here represent a step toward using metabolic engineering to develop strains of thermophilic anaerobic bacteria that do not produce organic acids, and support the methodological feasibility of this goal.

Introduction

Thermoanaerobacterium sp. strain JW/SL-YS485, a thermophilic, anaerobic, Gram-positive bacterium, was originally isolated from Yellowstone National Park, Wyoming (Liu et al. 1996), and subsequently designated *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 (Mai and Wiegel 2000). This organism ferments xylan, but

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not cellulose, and produces ethanol, acetate, lactic acid, CO_2, and H_2 as fermentation products. *T. saccharolyticum* is of interest because of its role in carbohydrate utilization in high-temperature environments and its potential utility for production of ethanol from biomass. In particular, the pentose-fermenting capability of this organism might be used to complement a cellulose-fermenting thermophile such as *Clostridium thermocellum*, in a consolidated bioprocessing configuration featuring fermentation of lignocellulosic biomass in the absence of added saccharolytic enzymes (Lynd et al. 2002). Mai et al. (1997) have previously reported development of an electrotransformation protocol for *T. saccharolyticum* based on the shuttle vector pIKM1 with selection based on kanamycin resistance, and have used this protocol in conjunction with homologous recombination to express a cellobiohydrolase encoded by *cbhA* of *C. thermocellum* (Mai and Wiegel 2000).

Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol (Ingram et al. 1998, 1999; Tao et al. 2001) as well as other products (Altaras and Cameron 1999; Causey et al. 2003; Nakamura et al. 2000; Nie et al. 2002; Vemuri et al. 2002; Zhou et al. 2003). However, such metabolic engineering has been pursued only to a limited extent in gram-positive, mesophilic, obligate anaerobes, and has not been reported for Gram-positive obligate anaerobic thermophiles. Expression of pyruvate decarboxylase and alcohol dehydrogenase in *Clostridium cellulolyticum* resulted in shifts in the distribution of end-products, although the yield of ethanol did not increase (Guedon et al. 2002). Butyrate kinase and phosphotransacetylase knockouts in *Clostridium acetobutylicum* were confirmed genetically but did not eliminate production of butyrate and acetate (Green et al. 1996), perhaps due to the presence of alloenzymes. Antisense RNA complementary to phosphotransbutyrylase and butyrate kinase genes has also been used in *C. acetobutylicum* to alter the yields of catabolic end-products and, although it increased solvent

production, no decrease in acid formation was observed (Desai and Papoutsakis 1999).

In the work reported here, we sought to initiate investigation of metabolic engineering of end-product metabolism in *T. saccharolyticum* by knocking out the lactate dehydrogenase gene and thereby eliminating lactate production.

Materials and methods

Culture origin, maintenance, and cultivation

Thermoanaerobacterium saccharolyticum JW/SL-YS485 (DSM 8691) was kindly provided by Dr. J. Wiegel (University of Georgia, Athens, GA). Single colonies were picked and stored in 25% glycerol and 75% growth medium at −80 °C in 5-ml sealed serum vials under a N_2 atmosphere. Cultures recovered from glycerol stocks were grown in liquid medium prior to use in experiments. Cultures were grown at 55 °C in DSM 61 broth (http://www.dsmz.de/media/med061.htm) supplemented with 0.5 g L-cysteine/l as reducing agent or in MYE medium containing mineral medium as described by Wiegel et al. (1979) supplemented with 2 g yeast extract/l. Xylose or glucose (5 g/l) was added as the growth substrate. DSM medium 61 was used for electrotransformation experiments, and MYE medium for strain characterization experiments. Growth on plates was performed at 55 °C in an anoxic chamber (Coy, Ann Arbor, Mich., USA) using DSM medium 61 or MYE medium with 2% agar. For selection of antibiotic-resistant strains, the medium was supplemented with 50–400 μg kanamycin/ml as noted in the text, and the culture temperature was maintained at 50 °C.

Plasmids and PCR primers

Plasmid pIKM1 (Mai et al. 1997), kindly provided by Dr. J. Wiegel, was used as the source of *aph* conferring thermostable kanamycin resistance (Trieu-Cout and Courvalin 1983) and as a positive control for gene transfer because it contains a thermostable Gram-positive origin of replication. Dr. J. Wiegel also provided a λ-ZAP (Stratagene, La Jolla, Calif., USA) genomic DNA library of *T. saccharolyticum*. Cloning vectors were obtained from commercial suppliers: TOPO pCR2.1 (Invitrogen, Carlsbad, Calif., USA); pUC18 (Invitrogen); pBLUESCRIPT II SK(+) (Stratagene); and pGEM-T (Promega, Madison, Wis., USA). PCR primers were synthesized by IDT (Coralville, Iowa, USA) and were designed based on sequences internal to either L-*ldh* of *T. saccharolyticum* (initialed L) or the *aph* kanamycin marker (initialed K). Primers used in this work were:

L-531–5′ACATGCATGCCTAACTCCAAGGAAAC-GAATAG3′

L′475–5′AACCTGCAGCAGTCCTTGCTTAAAAGG-TATC3′

L527–5′GCTCTAGACAGAGTTTGCAGCATG-GAGC3′

L′959–5′CGGGATCCGAAGCCTCCCAACA-TAATTTG3′

L-560–5′CGATAAAGAACATGGCTGG3′

L′2425–5′AAGAGGCCTCCACCACACATATG3′

K672–5′TATATAGAAGATATCGCCGTATGTAAG-GAT3′

K′1824–5′CTAAATCTAGGTACTAAAATTCATC-CA3′

K′805–5′GGTTATTGTCCTGGGTTTCAAGC3′

K1742–5′GACTTACTGGGGATCAAGCCTG3′

DNA preparation, manipulation, and Southern blot hybridization

DNA fragments were subcloned using standard protocols (Sambrook and Russell 2001). *E. coli* TOP10 (Invitrogen) was grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Plasmid preparation and manipulation, genomic DNA preparation (Qiagen, Valencia, Calif., USA), transformation and hybridization (North2South, Pierce Biochemicals, Rockford, Ill., USA) were carried out using standard procedures or the suppliers' instructions. Restriction enzymes were obtained from New England Biolabs (Beverly, Mass., USA), *pfu* from Stratagene and *Taq* polymerase from Promega. DNA sequencing reactions were conducted using the ABI BigDye terminator kit v3.0 (ABI, Foster City, Calif., USA) at the Dartmouth College Molecular Biology Core Facility (Hanover, N.H., USA).

Cloning of L-lactate dehydrogenase

Conserved regions of L-*ldh* genes from *C. acetobutylicum* ATCC824, *Bacillus megaterium* and *Bacillus stearothermophilus* ATCC12016 were identified as KPGETR, NPVDIL, GEHGD, and KGATYY; degenerate primers were designed with the CODEHOP program (Rose et al. 1998). L-*ldh* fragments within the portion of the gene bounded by conserved KPGETR and KGATYY regions were amplified using degenerate primers with *T. saccharolyticum* genomic DNA as the template and *pfu* as the DNA polymerase. A small fragment of *ldh* between the Sau3AI restriction sites was cloned from the genomic DNA library; the sequence matched the sequence of the corresponding fragment amplified with degenerate primers. The DNA fragments flanking the above-described portion of L-*ldh* were sequenced directly from genomic DNA using ThermoFidelase (Fidelity Systems, Gaithersburg Md., USA) with the BigDye Terminator kit v3.0. The sequence of the entire gene was compiled and deposited with GenBank as follows: *T. saccharolyticum*, accession number JW/SL-YS485-AY278026.

Knockout vector construction

The 5.5-kb suicide vector pSGD8 (Fig. 1) was based on pUC18 using a design approach similar to that reported earlier (Mai and Wiegel 2000). L-*ldh* fragments *ldh-up* (1 kb) and *ldh-down* (0.43 kb) were amplified from genomic DNA using primer pairs L-531-L′475 and L527-L′959, respectively. PCR amplification was performed with *pfu* DNA polymerase. The resulting PCR fragments were A-tailed with *Taq* polymerase and cloned into TOPO pCR2.1 thereby facilitating the subcloning of the fragments. Following standard subcloning procedures, construct pSGD8 was obtained with the kanamycin marker *aph* flanked by *ldh-up* and *ldh-down* fragments. Restriction analysis and DNA sequencing confirmed the presence and orientation of the flanking regions and the *aph* marker.

Transformation of *T. saccharolyticum*

T. saccharolyticum was transformed using electroporation of autoplast-cell preparation, and kanamycin selection as described previously (Mai et al. 1997).

HPLC analysis

Glucose, xylose, acetate, lactate, and ethanol were analyzed by HPLC using an Aminex 87H column (Bio-Rad Laboratories, Hercules, Calif., USA) at 55 °C. The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.7 ml/min. Detection was via refractive index using a Waters 410 refractometer (Milford, Mass., USA). The minimum detection level for lactate was 0.3 mM.

Calculation of carbon and electron recoveries

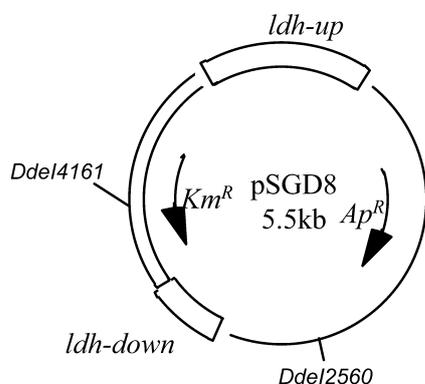Carbon recovery was calculated exclusive of cell synthesis based on yields of organic fermentation products plus stoichiometrically associated CO_2 production accompanying the production of ethanol and acetic acid (Lynd et al. 2002) using the formula:

% Carbon recovery

$$= \frac{3(Y_{E/S} + Y_{A/S} + Y_{L/S})}{N_{C/S}} \times 100 \quad (1)$$

where $Y_{i/S}$ is the yield of product i (mM product i/mM substrate; E for ethanol, A for acetic acid, and L for lactic acid) and $N_{C/S}$ is mM carbon/mM substrate (5 for xylose, 6 for glucose).

Recovery of available electrons was calculated exclusive of cell synthesis based on measured yields of organic fermentation products together with stoichiometrically associated production of H_2 accompanying production of acetic acid (Lynd et al. 2002). Since there are 12 available electrons per mol ethanol, lactic acid, or acetic acid/H_2 produced, and there are 20 available electrons per mol xylose and 24 available electrons per mol glucose (Papoutsakis 1984), available electron recovery is numerically equal to the carbon recovery as calculated using Eq. 1.
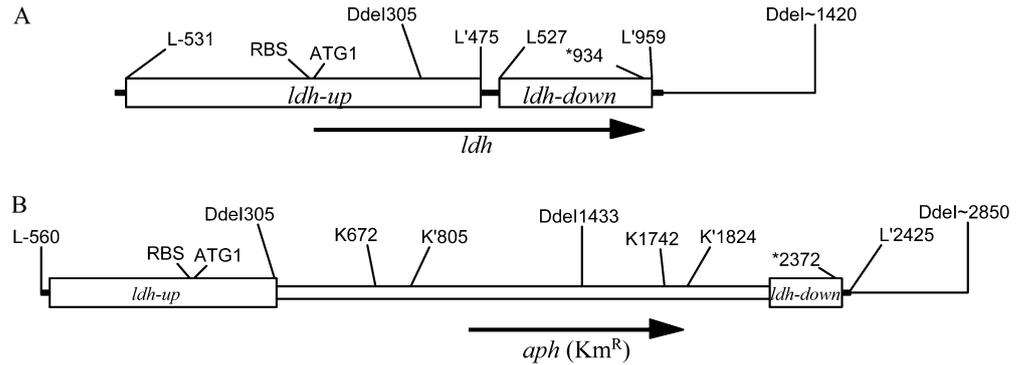
Results

Cloning of L-lactate dehydrogenase

The L-lactate dehydrogenase gene of *T. saccharolyticum* JW/SL-YS485 was cloned and sequenced using degenerate primers and direct sequencing of genomic DNA. Figure 2A shows the map of the sequenced L-*ldh* of *T. saccharolyticum* along with the location of various primers, ribosomal binding site (RBS), translation start and stop sites, and select *Dde*I restriction sites. The L-*ldh* ORF has similarity and identity relative to the L-*ldh* genes of other bacteria as follows: *C. acetobutylicum* ATCC824 (82%, 60%), *B. megaterium* (70%, 49%) and *B. stearothermophilus* 12016 (69%, 47%). A putative RBS rich in GC is located 7 bp upstream of the ATG start codon.

Chromosomal integration and isolation of strain TD1 with a deletion in L-*ldh*

T. saccharolyticum was transformed with the L-*ldh* knockout construct pSGD8 as well as replicative plasmid pIKM1. Electropulsed cell suspensions were recovered and cultured in selective liquid medium, with growth observed after 2 days in cultures transformed with pSGD8 and after 3 days in cultures transformed with pIKM1. Four presumptive chromosomal integrant isolates of *T. saccharolyticum* were picked from plates containing 50 µg kanamycin/ml streaked with cells transformed with pSGD8. All four isolates grew in liquid medium contain-

Fig. 1 Construction of pUC18 based *Thermoanaerobacterium saccharolyticum* L-*ldh* knockout vector pSGD8. Km, *aph* from shuttle vector pIKM1 conferring kanamycin resistance; Ap, *bla* from pUC18 conferring ampicillin resistance; *ldh-up* and *ldh-down*, partial *ldh* fragments. Nucleotides are numbered with respect to the ATG start site of *ldh*

Fig. 2 A *T. saccharolyticum* L-*ldh. RBS* Putative ribosomal binding site. **B** Map of double crossover region with Δ*ldh* and inserted Km^R gene. * Translational stop codon. Nucleotides are numbered with respect to the ATG start site of *ldh*

ing kanamycin at 75, 100, 200, and 400 μg/ml, and yielded identical results following PCR analysis and Southern blots (data not shown). In addition, none of the strains produced detectable lactic acid. One of the four isolates was chosen for further study and designated strain TD1.

Molecular characterization of strain TD1

PCR amplification using primers K672 and K′1824 and genomic DNA from *T. saccharolyticum* strain TD1 as template resulted in a fragment with the expected size of approximately 1.1 kb and confirmed the presence of the kanamycin gene (Fig. 3A, lane 1). PCR was also conducted using primer pairs consisting of one primer from the *ldh* gene that is not present in pSGD8 and one primer from the kanamycin gene. Primer pair L-560-K′805 yielded a 1.3-kb fragment (Fig. 3A, lane 2), which is the expected size for chromosomal integration of *ldh-up* (Fig. 2B). Primer pair K1742-L′2425 yielded a 0.7-kb fragment (Fig. 3A, lane 3), which was the expected size for a double-crossover event (Fig. 2B). All the above three PCR products were sequenced and confirmed. Negative controls with wild-type genomic DNA using the same PCR mix were conducted and none showed any PCR products (data not shown).

Further confirmation of the double-crossover event was obtained from Southern blot analysis. Genomic DNA from the wild-type, strain TD1, and from the wild-type strain transformed with plasmid pIKM1 was digested with *Dde*I,

Fig. 3 A PCR analysis of L-*ldh* deletion mutant *T. saccharolyticum* strain TD1. Genomic DNA from strain TD1 was the template. *Lane 1* Primers K672 and K′1824, *lane 2* primers L-560 and K′805, *lane 3* primers K1742 and L′2425. **B** Southern blot analysis of *T. saccharolyticum* wild-type and L-*ldh* deletion mutant TD1. *Lane 1* Wild-type, *lane 2* wild-type transformed with pIKM1, *lane 3* deletion mutant TD1. *Dde*I genomic DNA digests were hybridized with the 0.43-kb *ldh-down* fragment as the probe

and the 0.43 kb *ldh-down* fragment was used as a probe for hybridization. Both wild-type strains hybridized to a 1.1-kb fragment (Fig. 3B, lanes 1 and 2), whereas the deletion mutant strain TD1 hybridized to a 1.4-kb fragment (Fig. 3B, lane 3). This confirmed that the chromosomal integration was in fact a double-crossover event because a single-crossover event would have resulted in a different fragment size.

Physiological characterization of strain TD1

Figure 4 presents representative batch growth curves for *T. saccharolyticum* wild-type and TD1 strains on glucose (Fig. 4A, B) and xylose (Fig. 4C, D). Two other replicate experiments showed similar trends (data not shown). The time required to achieve substrate exhaustion and the final optical densities achieved in stationary phase were very similar for the wild-type and deletion mutant strains growing on either glucose or xylose. Specific growth rates calculated during exponential phase were 0.4±0.03 h^{-1} for the wild-type on both glucose and xylose and for strain TD1 on glucose, while the growth rate of strain TD1 on xylose was 0.3±0.02 h^{-1}. Lactic acid was below the limit of detection (0.3 mM) for growth of strain TD1 on both glucose and xylose at all times tested, but was readily detected for growth of the wild-type on both glucose and xylose, with average final concentrations of 8.1 and 1.8 mM, respectively. After three transfers in batch culture in the absence of selective pressure, strain TD1 still did not produce lactic acid and did not show any major changes in the yields of ethanol or acetate, suggesting stable gene deletion.

Glucose consumption, fermentation product formation, product and cell yields, and carbon recovery are tabulated in Table 1 for batch cultures of strains *T. saccharolyticum* TD1 and wild-type, in pre-reduced MYE medium. Carbon recoveries were rather similar for the two strains but somewhat in excess of 100%. This excess may be due to the assimilation of components in yeast extract, which is present in the growth medium at a concentration of 2 g/l. Cell yields, evaluated in terms of change in OD per mM substrate consumed, were higher for strain TD1 than for the wild-type for both glucose and xylose as substrates, although the difference was only statistically significant for glucose (*p*= 0.1).
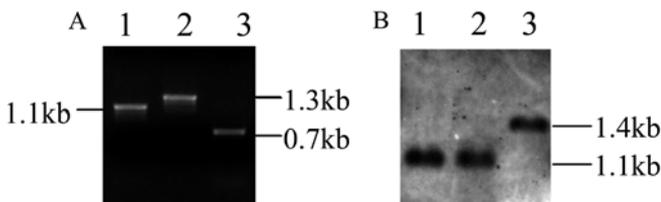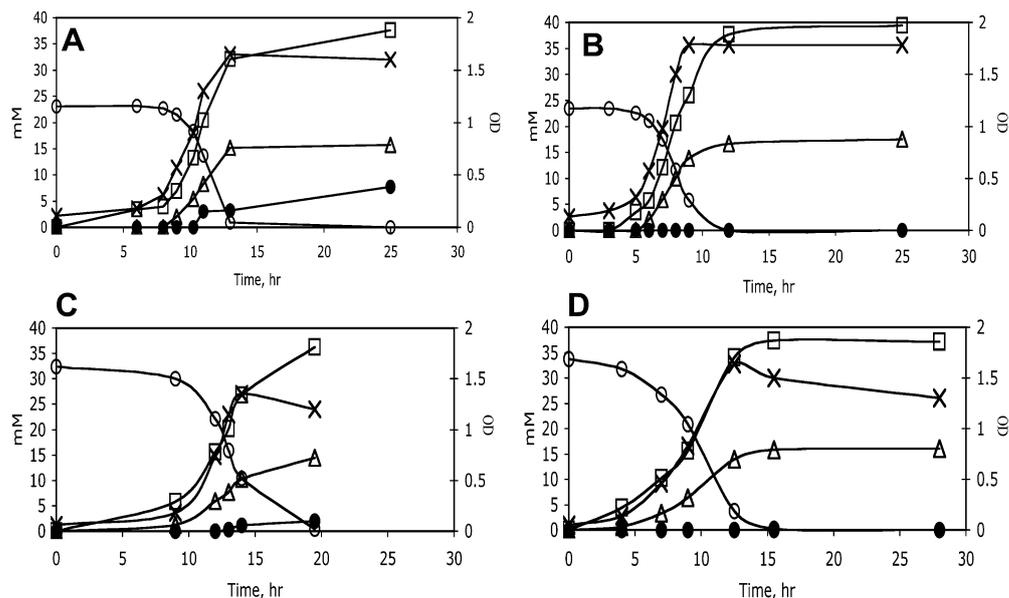
Fig. 4A–D Growth curves for
T. saccharolyticum wild-type
and TD1 strains on glucose and
xylose. **A** Wild-type, glucose; **B**
TD1, glucose; **C** wild-type, xy-
lose; **D** TD1, xylose. Substrate
(○), lactate (●), acetate (△),
ethanol (□) and OD (x)

Discussion

The deduced amino acid sequence encoded by ʟ-*ldh* of *T. saccharolyticum* JW/SL-YS485 exhibited significant similarity relative to previously sequenced ʟ-*ldh* genes from mesophilic and thermophilic bacteria. The observation that three different degenerate primer pairs yielded the same ORF and single bands were obtained upon Southern blotting of genomic DNA confirm that ʟ-*ldh* is present in a single copy in the chromosome of *T. saccharolyticum*.

T. saccharolyticum strain TD1 featuring a homologous recombination-mediated knockout of ʟ-*ldh* was obtained with little difficulty. Data from PCR and Southern blots indicate that deletion of the single-copy ʟ-*ldh* occurred via a double-crossover event. The growth rates of the wild-type strain and the deletion mutant strain TD1 are in the range reported by other researchers for growth at 55 °C (Mai and Wiegel 2000). Lactic acid production was not detectable in stain TD1 during growth on both glucose and xylose. Elimination of lactic acid as a fermentation product resulted in proportionately increased yields of acetic acid and ethanol in replicate experiments. No additional end-products were detected by HPLC in strain TD1 as compared to the wild-type strain, and the magnitude of

the increased ethanol and acetate yields were sufficient to account for the decrease in carbon flow to lactic acid. Mat-Jan et al. (1989) previously examined end-product redistribution in a fermentative *ldh* mutant of *E. coli*. After 4 h of growth, they found no detectable amounts of lactate and a slight decrease in ethanol and acetate formation compared to the parent strain; however, during subsequent incubation substantial amounts of pyruvate accumulated.

The work reported here represents a step toward developing strains of thermophilic anaerobic bacteria that do not produce organic acids. Such strains are doubly desirable in order to pursue advances in conversion of lignocellulosic biomass to ethanol. In addition to increasing ethanol yield, strains in which organic acid production has been eliminated also provide a means to test the hypothesis that higher concentrations of ethanol can be produced when cells are not subject to inhibition by organic acids and their salts (Lynd et al. 2001, 2002).

Table 1 End-product analysis of glucose and xylose fermentation by wild-type and ʟ-*ldh* mutant strains of *Thermoanaerobacterium saccharolyticum*. *ND* Not detected

[a] (final OD - initial OD)/(final substrate concentration - initial substrate concentration), where 'final' denotes the end of exponential growth.
[b] Values in pranthesis are standard deviations for independent replicate experiments.

Strain	Glucose		Xylose	
	Wild type	TD1	Wild-type	TD1
Substrate (S) (mM)	27.8	27.8	33.4	33.4
Lactate (L) (mM)	8.1 (0.49)[b]	ND	1.8 (0.22)	ND
Acetate (A) (mM)	15.5 (0.42)	16.8 (1.06)	15.8 (0.21)	16.4 (0.14)
Ethanol (E) (mM)	36.7 (1.34)	38.6 (1.20)	38.9 (0.85)	40.8 (1.76)
$Y_{L/S}$	0.29	-	0.06	-
$Y_{A/S}$	0.56	0.60	0.47	0.49
$Y_{E/S}$	1.32	1.39	1.16	1.22
C and available electronrecovery (%)	108(2.3)	100(4.1)	101(1.5)	103(3.4)
Cell yield[a]	0.053 (0.003)	0.058 (0.001)	0.036 (0.003)	0.040 (0.004)

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