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Genetic Engineering of *Candida utilis* Yeast for Efficient Production of L-Lactic Acid

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Polylactic acid is receiving increasing attention as a renewable alternative for conventional petroleum-based plastics. In the present study, we constructed a metabolically-engineered *Candida utilis* strain that produces L-lactic acid with the highest efficiency yet reported in yeasts. Initially, the gene encoding pyruvate decarboxylase (*CuPDC1*) was identified, followed by four *CuPDC1* disruption events in order to obtain a null mutant that produced little ethanol (a by-product of L-lactic acid). Two copies of the L-lactate dehydrogenase (L-*LDH*) gene derived from *Bos taurus* under the control of the *CuPDC1* promoter were then integrated into the genome of the *CuPdc1*-null deletant. The resulting strain produced 103.3 g/l of L-lactic acid from 108.7 g/l of glucose in 33 h, representing a 95.1% conversion. The maximum production rate of L-lactic acid was 4.9 g/l/h. The optical purity of the L-lactic acid was found to be more than 99.9% e.e.

Key words: *Candida utilis*; pyruvate decarboxylase; L-lactate dehydrogenase; L-lactic acid

Lactic acid, a monomer of polylactic acid (a material of plastic), can be produced by fermentation from plant biomass, and this should reduce the use of petroleum and generate no net increase in carbon dioxide emissions.

L-Lactic acid is commonly produced by fermentation using lactic acid bacteria, such as *Lactobacillus* species, which have numerous growth requirements and produce D-lactic acid as well, resulting in an optical purity of about 95% L-lactic acid.[1–3] Since optical purity affects physical characteristics, including thermostability and crystallization of polylactic acid, it is desirable to have the highest purity of L-lactic acid possible.

Yeast normally produces ethanol *via* alcoholic fermentation, in which pyruvic acid (the final product of glycolysis) is converted to acetaldehyde by decarboxylation *via* pyruvate decarboxylase (PDC; EC 4.1.1.1), followed by reduction to ethanol *via* alcohol dehydrogenase. L-Lactic acid is not a product of this process in yeast. However, pyruvic acid can be converted into L-lactic acid by heterologous expression of L-lactate dehydrogenase (L-LDH; EC 1.1.1.27). When both L-LDH and PDC are expressed simultaneously, the production or yield of L-lactic acid is low, presumably due to the competition for pyruvic acid by the two enzymes.[4] Decreasing PDC activity has been found to boost the production of L-lactic acid in *Saccharomyces cerevisiae*[4–8] and *Kluyveromyces lactis*.[9,10]

In the case of *S. cerevisiae*, which is known as a Crabtree-positive yeast, and which has three *PDC* genes (*ScPDC1*, *ScPDC5*, and *ScPDC6*), reduction/inactivation of PDC activity causes a severe growth defect. Increasing the copy number of L-*LDH* and the use of EMS-generated mutants in which the growth defect is suppressed has resulted in better strain performance. For example, a yield of 122 g/l of L-lactic acid from a 61% cane-juice sugar medium after 48 h of fermentation under neutral conditions using NaOH has been reported.[11,12] A yield of 82.3 g/l of L-lactic acid, representing an 81.5% conversion of D-glucose within 168–216 h, has also been achieved.[13] While further attempts have been reported, *e.g.*, in which *ScADH1* (alcohol dehydrogenase 1; EC 1.1.1.1) was disrupted in addition to the deletion of *ScPDC1*,[14] and in which *ScJEN1*, encoding a lactate transporter, was overexpressed,[15] improvements in yield and duration of fermentation are still needed.

In the case of *K. lactis*, a Crabtree-negative yeast, the growth rate of a PDC-null mutant was comparable to that of a wild-type strain,[16] but the yield of a strain expressing L-*LDH* and lacking *KlPDC1*, which was reported by Porro *et al.*,[10] was still far from the maximum theoretical yield: 0.58 lactic acid produced/g-glucose consumed rather than 1 g/g. Bianchi *et al.* modified the strain by additional disruption of *KlPDA1*, encoding the pyruvate dehydrogenase (PDH; EC 1.2.4.1) E1α subunit, which converts pyruvic acid into acetyl-coenzyme A.[9] The resulting strain produced L-lactic acid with a higher yield, 0.85 g/g, under neutralizing conditions, but the glucose consumption rate was extremely low, less than 0.3 g/l/h even with adequate cell density. Slow consumption was probably related to disruption of *KlPDA1*, because this disruption in the wild-type strain resulted in poor growth in glucose medium.[17]

The industrially important yeast *Candida utilis*, classified as a Crabtree-negative yeast, is currently used to produce several valuable chemicals, such as glutathione and RNA.[18–20] *C. utilis* can grow on inexpensive substrates, *e.g.*, pulping-waste liquors from the paper industry, while most other yeasts cannot.[21] Since the development of an efficient electroportation-based method for transforming *C. utilis*,[22] this yeast has been used in the heterologous production of monellin,

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Abbreviations: kb, kilo-base pair; HygB, hygromycin B; PDC, pyruvate decarboxylase; L-LDH , L-lactate dehydrogenase; AcH, acetaldehyde

α-amylase, and carotenoids such as lycopene.[23–26] Recently, we developed new strategies for performing efficient multiple transformations of *C. utilis*. One is co-transformation,[27] and the other is based on the Cre-*loxP* recombination system.[28] Using the latter method, a four-round deletion of the *CuURA3* gene, encoding orotidine-5′-phosphate decarboxylase, yielded an uracil auxotroph.[28]

In the present study, we describe a genetically-engineered strain of *C. utilis* which to our knowledge produces L-lactic acid more efficiently than previously reported in yeasts.

Materials and Methods

Strains and media. *Candida utilis* NBRC0988, its derivatives (described below), and *Saccharomyces cerevisiae* S288C were used. Cells were cultured at 30 °C in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose), unless otherwise specified. Solid media were made with 2% agar. G418 (Geneticin, Sigma-Aldrich, St. Louis, MO) was added to YPD to a final concentration of 200 μg/ml to select transformants expressing the *APT* gene, which encodes an aminoglycoside phosphotransferase. Hygromycin B (HygB, Wako Pure Chemical Industries, Osaka, Japan) was added to YPD to a final concentration of 600 μg/ml to select for transformants carrying the *HPT* gene, encoding HygB phosphotransferase.

E. coli DH5α (Toyobo, Osaka, Japan) served as a plasmid host. *E. coli* was grown in LB (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) containing 50 μg/ml of ampicillin, and was transformed by standard methods.[29]

Recombinant DNA techniques. Standard recombinant DNA techniques were used,[29,30] or the procedures recommended by the suppliers. PCR (polymerase chain reaction) was performed with LA-Taq polymerase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The primers used are listed in Table 1. Nucleotide sequences were determined using an ABI 3130 xl DNA analyzer (Applied Biosystems, Foster City, CA).

Southern blot hybridization analysis. DNA was digested with selected restriction enzymes and separated in 1% agarose gels. After electrophoresis, the gels were incubated in 0.25-M HCl for 15 min with shaking, followed by denaturation and capillary blotting of DNA onto a Hybond N+ nylon membrane (GE Healthcare UK, Buckinghamshire, UK). Probe DNA labeled with a random primer labelling kit (Takara Bio) and [α-^{32}P] dCTP (110 TBq/mmol) was added to the hybridization solution, and hybridization was performed at 60 °C for 16 h. After hybridization, the membranes were washed in 1 × SSC containing 0.1% SDS, followed by a wash in 0.5 × SSC containing 0.1% SDS at 60 °C for 2 h. The membranes were then subjected to autoradiography.

Molecular cloning. Construction of a *C. utilis* genomic DNA library has been described.[22] In order to obtain fragments containing the *PDC* gene, PCR was performed using *C. utilis* genomic DNA as template. Oligonucleotides IKSM-29 and IKSM-30 were used as primers. The amplified DNA fragments (207 bp) were cloned into pCR2.1-TOPO vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA), followed by sequencing. The PCR product was used as a probe in colony hybridization and Southern blot hybridization in order to clone the *PDC* gene from *C. utilis*. Colony hybridization was conducted as previously described.[29]

Construction of plasmids. The downstream region of the *CuPDC1* gene was amplified with primer set IM-345 and IM-346, followed by digestion with *Bss*HII. The DNA fragment was ligated into the *Bss*HII site of pBluescriptIISK+ (Stratagene Products Division, Agilent Technologies, La Jolla, CA) to construct plasmid pCU670.

Plasmid pGKHPT1[31] was used as a template in a PCR with primers IM-283 and IM-57 in order to amplify a DNA fragment that contained a 34-bp *loxP* sequence, the approximately 800-bp *PGK* promoter (*CuPGKpr*) and the *HPT* gene, which is responsible for HygB-resistance. Plasmid pGAPPT10[23] was used as a template in a PCR with primers IM-54 and IM-55 to amplify a DNA fragment containing a *GAP* terminator (*CuGAPtr*) and *loxP*. The two PCR products were mixed, and, due to the complementarity of primers IM-57 and -54, were used as a template in the next round of PCR with primers IM-1 and IM-2, resulting in a 2.5-kb DNA fragment (the LHL module) consisting of *loxP*, *CuPGKpr*, *HPT*, *GAPtr*, and *loxP*, which was cloned into the pCR2.1-TOPO vector, yielding plasmid pCU621.

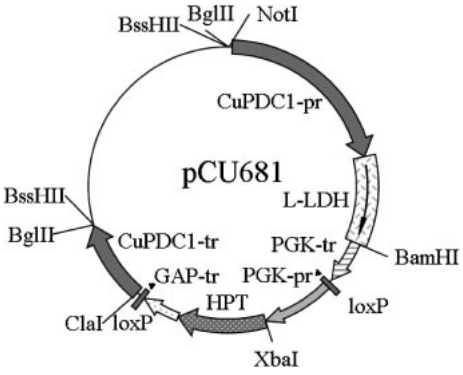

Fig. 1. Map of Constructed Plasmid pCU681 (see "Materials and Methods").

Two individual PCRs were performed, as follows: one fragment was amplified using primers IM-347 and -348 and template DNA pPGKPT2;[32] the other was done with primers IM-349 and -350, and pCU621. Because primers IM-348 and IM-349 have complementary regions at their 5′-ends, the two resulting fragments were mixed and used as a template in the next PCR with primers IM-347 and IM-350. The amplified fragment (approximately 3 kb) was digested with *Bam*HI and *Cla*I, and ligated into the *Bam*HI-*Cla*I gap of pCU670 to construct plasmid pCU675.

The *Bos taurus* (bovine) L-*LDH* gene sequence (DDBJ/EMBL/GenBank accession no. AAI46211.1)[33] was modified based on major codon usage in *Pichia jadinii* (*C. utilis* is an anamorph of *P. jadinii*; Codon Usage Database: http://www.kazusa.or.jp/codon/) and synthesized (Takara Bio) in such a way that the original amino acid sequence was unchanged. Using the L-*LDH* gene as template, a fragment of about 1-kb DNA was amplified with primers IM-343 and IM-379. The promoter region of the *CuPDC1* gene was amplified using oligonucleotides IM-341 and IM-342 as primers and *C. utilis* genomic DNA as template. Due to the complementarity of primers IM-342 and -343, the two resulting products were mixed and used as a template in the next PCR with primers IM-341 and IM-379, after which the amplified fragment (approximately 2.2 kb) was digested with *Not*I and *Bgl*II, and ligated into the *Not*I-*Bam*HI gap of pCU675 to construct pCU681 (Fig. 1).

Breeding of yeast strains. Transformations of *C. utilis* were carried out by electroporation as previously described.[28] In order to construct a null disruptant of the *CuPDC1* gene, designated *Cupdc1Δ4*, a fourth deletion was carried out using the Cre-*loxP* system essentially as described for constructing the *CuUra3*-null deletant.[28] Two different gene disruption cassettes consisting of the aforementioned LHL module flanked by the 5′- and 3′-regions adjacent to the *CuPDC1* gene for targeted replacement were generated in two-step PCRs. Initially, the fragments forming these cassettes were amplified separately. The LHL module was constructed using primers IM-1 and 2 and pCU563[28] as template. A fragment upstream of *CuPDC1*, the targeted region, was amplified with primers IM-277 and -278, with *C. utilis* genomic DNA as template. Two different downstream regions for *CuPDC1*, one for the first and second deletions, and the other for the third and fourth deletions, were generated with primer pairs IM-279/-280 and IM-185/-168 respectively, with *C. utilis* genomic DNA as template. Plasmid pCU595,[28] which harbors the Cre-expressing module with the *APT* gene for G418-resistance, was introduced after every deletion in order to excise the *HPT* gene. The resulting strains were then grown in non-selective YPD to allow loss of pCU563.

Table 1. PCR Primers Used in This Study

Name	Sequence (5′ to 3′)
IKSM-29	CARGTYTTRTGGGGTTCYATYGGTTT
IKSM-30	TTCAATRGTGTARCCMYYGTTGTTCAA
IM-345	ACTCGCGCGCAAGATCTAAGCGGCCGCTAATGGATCCAATAATCGATGCTGTCTTTCTTCTTCATGGG
IM-346	ACTCGCGCGCAAGATCTGAACTTCTCCAACAGGTAGC
IM-283	CGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTCGACAACCCTTAATATAACTTCGTATAATGTATGCTATACGAAGTT-ATCCTTTGCTGTGTTCTACC
IM-57	CATGAGGATCATAATTTATAACGTAATCCCATAAATAAAAGTCATACAATCATTCCTTTGCCCTCGGA
IM-54	ATTGTATGACTTTTATTTATGGGATTACGTTATAAATTATGATCCTCATG
IM-55	TAGGCCACTAGTGGATCTGATATCACCTAATAACTTCGTATAGCATACATTATACGAAGTTATTCATTCATCCCTCACT-ATCG
IM-1	GGCCGCCAGCTGAAGCTTCG
IM-2	AGGCCACTAGTGGATCTGAT
IM-347	ACTCGGATCCCTGCAAGCTACTTTGTAATTAAACAAATAACGGG
IM-348	GGAGACTCTTCACACTGTTGGCGTCTATGATTCAAGATTGTCAGTTTCCATCGTGGATTGGAATAGTTGTGGTGACCTTG
IM-349	ACAACTATTCCAATCCACGATGGAAACTGACAATCTTGAATCATAGACGCCAACAGTGTGAAGAGTCTCCAGCTGAAG-CTTCGTACGCTG
IM-350	ACTCGGCCGGCCATCGATCACTAGTGGATCTGATATCACC
IM-343	CCCGTTATACACAACAAACAAACAAAACTAAAACAATCGATACCATGGCTACCTTGAAGGACCA
IM-379	ACTCAGATCTTCATCAGAACTGCAATTCCTTCTGG
IM-341	ACTCGCGGCCGCTCTAGACACCAACTTTGAAGATAGGG
IM-342	TGGTCCTTCAAGGTAGCCATGGTATCGATTGTTTTAGTTTTGTTTGTTTGTTGTGTATAACGGG
IM-277	AGTTGGACTCGGATCATCTC
IM-278	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAACATTCCTACGCTCAGAGC
IM-279	ATTAGGTGATATCAGATCCACTAGTGGCCTACAGAACGCTCCTAAACACG
IM-280	GAACTTCTCCAACAGGTAGC
IM-185	ATTAGGTGATATCAGATCCACTAGTGGCCTTGCAATTGACCGTCCAAGAG
IM-168	TCTTGTACGTGTTTAGGAGC

In order to construct strains harboring the L-*LDH* gene, the strains were transformed with *Bgl*II-digested pCU681. The *CuPdc1*-null disruptant *Cupdc1Δ4* was transformed with the plasmid to construct a strain harboring a single copy of L-*LDH* (named *Cupdc1Δ4-LDH1*). After removal of *HPT* using pCU563, the resulting clone was transformed with the same plasmid to obtain a strain carrying two copies of L-*LDH* (named *Cupdc1Δ4-LDH2*).

Fermentation. In pre-cultivation, cells from a 2-d-old colony taken from a YPD plate at 30 °C were transferred to flasks containing 50 ml of YPD medium in 500-ml Sakaguchi-flasks and incubated for 15–22 h at 30 °C on a shaker (130 rpm). The cells were harvested by centrifugation at 3,000 × *g* for 5 min at 4 °C, washed twice with fresh fermentation medium, and then inoculated into the fermentation medium. Fermentations were performed at 25/30/35 °C in 100-ml spherical flat-bottom flasks containing 15 ml (unless specified otherwise) of YPD10 medium (1% Bacto yeast extract, 2% Bacto peptone, 10–11% glucose) with gentle shaking (80 rpm). They were performed with an initial OD_{600} of 10, otherwise described. Calcium carbonate (45 g/l) was added to neutralize the acidic products (*e.g.*, lactic acid and pyruvic acid), otherwise described. The yield of L-lactic acid was calculated according to the following equation: (amount of L-lactic acid at sampling time/amount of glucose at 0 h) × 100. Optical purity (% e.e.) was calculated as follows: optical purity = (L-lactic acid − D-lactic acid)/(L-lactic acid + D-lactic acid) × 100.

Analytical methods. Glucose and L-lactic acid were measured with an immobilized enzymatic membrane method using the Biochemistry Analyzer YSI-2700 System (YSI, YellowSpring, OH). D-Lactic acid was determined using a diagnostic kit (Roche Diagnostics, Mannheim, Germany) according to the instructions provided.

Ethanol and acetaldehyde (AcH) were analyzed as described previously[34] with slight modifications. The supernatant solution was diluted and subjected to high-pressure liquid chromatography (HPLC) analysis using an ICSep-ION-300 column (CETAC Technologies, Omaha, NE). The column was operated at 60 °C at a 0.4 ml/min flow rate, with in 0.005 M sulphuric acid as the solvent.

The concentration of pyruvic acid was also assayed by HPLC. Separation was achieved using a Shim-pack SPR-H column (Shimazu, Kyoto, Japan), with 5-mM p-toluenesulfonic acid as the mobility buffer. A solution consisting of 5-mM p-toluenesulfonic acid, 0.08-mM EDTA, and 20-mM Bis-Tris was applied as the reaction buffer. The column was operated at 45 °C at a 0.8-ml/min flow rate.

Nucleotide sequence accession numbers. The DNA sequences of the *CuPDC1* gene have been deposited in the DDBJ/EMBL/GenBank database under accession no. AB489119. The nucleotide sequence of the bovine L-*LDH* gene, whose codon-usage was modified for expression in *C. utilis*, has been given accession no. AB489120.

Results

Cloning of PDC *genes in* C. utilis

While enhancement of the production of pyruvate decarboxylase in *C. utilis* has been well studied in terms of culture conditions, *e.g.*, oxygen supplied and medium pH,[35–38] the *PDC* genes of *C. utilis* (*CuPDC*) have not previously been identified. In the present study, we cloned a *CuPDC* gene. Genes encoding PDC have been cloned and sequenced from various yeast species, including *S. cerevisiae*, *K. lactis*, *Pichia stipitis*, and *Candida glabrata*.[16,39–41] Comparison of their amino acid sequences indicates that their carboxyl termini are highly conserved (data not shown). Based on the conserved region, mixed oligonucleotide primers (IKSM-29 and IKSM-30; see "Materials and Methods") were synthesized and used in PCRs. The nucleotide sequences of the two primers were deduced from the conserved catalytic domains in *S. cerevisiae PDC1* (*ScPDC1*) and *K. lactis PDC1* (*KlPDC1*), located at the carboxy terminus. Sequence analysis of the amplified fragment (207 bp) indicated significant similarity to the known *ScPDC1* gene (approximately 80% identity in deduced amino acid sequence). One of these DNA fragments was used as a probe in Southern and colony hybridization.

As shown in Fig. 2, this probe hybridized to three different bands in *Hind*III digests of *S. cerevisiae*

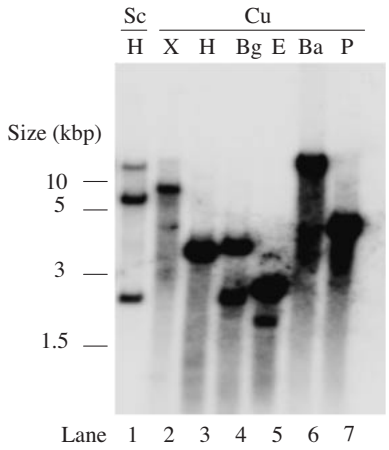

Fig. 2. Detection of *PDC* Genes by Southern-Blot Hybridization Analysis.

Lane 1, *Hind*III-digested genomic DNA from *S. cerevisiae* S288C. Lanes 2 to 7, digested genomic DNA from *C. utilis* NBRC0988: *Xba*I (lane 2), *Hind*III (lane 3), *Bgl*II (lane 4), *Eco*RI (lane 5), *Bam*HI (lane 6) and *Pst*I (Lane 7). A 0.2-kb fragment amplified with primers IKSM-29 and -30 was used as a probe (see "Materials and Methods"). *Abbreviations*: Sc, genomic DNA of *Saccharomyces cerevisiae*; Cu, genomic DNA of *Candida utilis*; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; X, *Xba*I

genomic DNA, and these were deduced to be *ScPDC1*, *ScPDC5*, and *ScPDC6*. *C. utilis* DNA digests with *Xba*I, *Hind*III, *Bam*HI, or *Pst*I yielded a single strong band, and the *Bgl*II digest gave two clear bands, in good agreement with a *Bgl*II site in the probe. Two bands were also observed in the *Eco*RI digest, but sequence analysis indicated no *Eco*RI site in the probe. These results suggest that *C. utilis* possesses one or two types of *PDC* genes, or that it has two or more copies due to its polyploidy.

Colony hybridization yielded several clones harboring *CuPDC* genes, and their sequences were determined. The *C. utilis PDC* gene, designated *CuPDC1*, consists of a 1,692-bp open reading frame (ORF) encoding a polypeptide of 563 amino acids. The deduced amino acid sequence shared 76 and 77% identity with that of ScPdc1p and KlPdc1p respectively. In the 1.2-kb region upstream of the *CuPDC1* ORF, a presumptive promoter region was found containing putative *cis*-elements including a TATA element and a UAS-PDC sequence reported to be necessary for transcriptional activation in *S. cerevisiae* and *K. lactis*.[42,43] In *S. cerevisiae*, reduction of PDC activity by disruption of both *ScPDC1* and *ScPDC5* has been reported to cause a severe growth defect in YPD,[44] but expression of *CuPDC1* under the control of a *ScPDC1* promoter complemented the lethality (data not shown), strongly suggesting that CuPdc1p has PDC activity.

Characterization of the Cupdc1*-null mutant*

We evaluated the fermentation ability of the *Cupdc1*-null mutant (*Cupdc1Δ4*), constructed by performing four individual *CuPDC1* disruptions (see "Materials and Methods"). In aerobic culture on YPD medium, *Cupdc1Δ4* did not show severe growth deficiency, but the glucose consumption and growth of *Cupdc1Δ4* were poorer than those of the wild type (Fig. 3). In microaerobic culture, *Cupdc1Δ4* consumed only 20% of the initial glucose provided, using just 20 g/l (Table 2). *Cupdc1Δ4* produced little AcH and ethanol, while a significant amount of pyruvic acid, a precursor of AcH, accumulated as observed in *S. cerevisiae* strains with low PDC activity.[45,46] This profile apparently differed from that of the wild-type strain, which produced more ethanol and AcH. These data suggest that CuPdc1p provides the highest activity among any potentially functional PDCs in *C. utilis*.

Table 2. Fermentation Analysis of the Wild-Type and the *Cupdc1*-Null Strain (*Cupdc1Δ4*)

Strain	NBRC0988	*Cupdc1Δ4*	Medium without cells
OD_{600}	59.9 ± 2.0	2.8 ± 0.2	Not determined
Glucose (g/l)	<0.1	80.0 ± 0.6	100.2 ± 0.3
Ethanol (g/l)	3.96 ± 0.04	<0.01	<0.01
AcH (mg/l)	26.2 ± 9.0	1.14 ± 0.06	0.30 ± 0.14
Pyruvic acid (mg/l)	462.4 ± 5.5	3659.9 ± 25.6	<0.1

Fermentation was performed at 30 °C for 48 h in 100-ml spherical flat-bottom flasks with an initial OD_{600} of 0.5. The medium used was 50 ml of YPD10 without neutralization reagent. Values are means for independent triplicate experiments.

Fermentation

Next we constructed strains *Cupdc1Δ4-LDH1* and *Cupdc1Δ4-LDH2*, which harbor one and two copies of bovine L-*LDH* under the control of the *CuPDC1* promoter and integrated at the *CuPDC1* locus, respectively (see "Materials and Methods"). In aerobic culture, their growth was poorer than that of the wild-type strain, but was comparable to that of *Cupdc1Δ4* (Fig. 3B). The maximum production rate of L-lactic acid was calculated in order to investigate the effects of L-*LDH* copy number and temperature (Table 3). When fermentation was carried out at 30 °C, production of L-lactic acid by *Cupdc1Δ4-LDH1* was lower than by *Cupdc1Δ4-LDH2*. Subsequently, *Cupdc1Δ4-LDH2* was tested at 25 and 35 °C, and the production at 35 °C was found to be the highest among the three temperatures. Similarly, the yield of *Cupdc1Δ4-LDH2* at 35 °C was the highest compared to that under the other conditions. Based on these data, we analyzed fermentation conducted by *Cupdc1Δ4-LDH2* at 35 °C in YPD10 media over a 36-h time course (Fig. 4). Glucose was completely consumed at 36 h, and the concentration of L-lactic acid produced was equivalent to the concentration of glucose at 0 h. Ethanol was scarcely produced (<0.01 g/l), and the concentration of pyruvate was 291 ± 20 mg/l. Although the fermentation conditions were not identical, the pyruvate concentration was much lower than that of *Cupdc1Δ4* (Table 2), suggesting that the introduced L-*LDH* effectively metabolized pyruvate into L-lactic acid. The yield of L-lactic acid was highest at 33 h, 95.1 ± 1.1%, and its optical purity was more than 99.9% (L-lactic acid, 103.3 ± 0.94 g/l; D-lactic acid, 0.0214 ± 0.004 g/l). We also analyzed production in another medium (0.1% yeast extract, 0.2% peptone, and 10% glucose), and obtained a yield of 91.0% at 33 h. We conclude that *Cupdc1Δ4-LDH2* can produce L-lactic acid efficiently even in a relatively nutrient-poor medium, which would simplify subsequent purification.

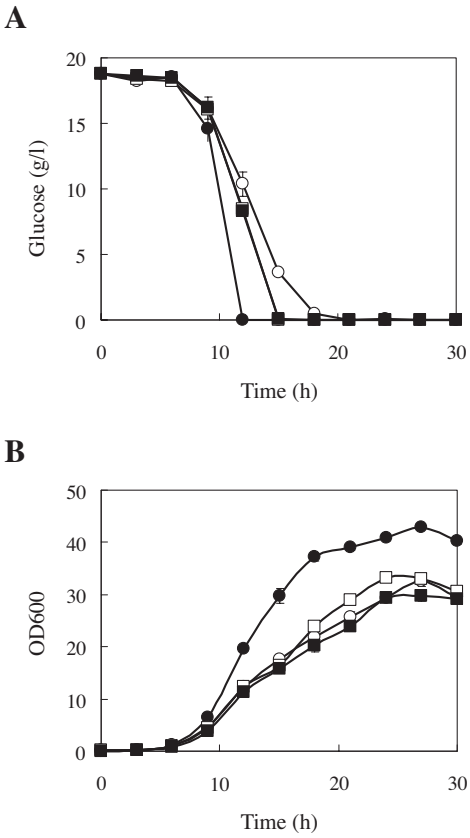

Fig. 3. Time Course in Aerobic Yeast Culture.

A, Glucose-Concentration in the Media; B, Cell-Density (OD_{600}). Solid circles, open circles, open squares, and solid squares represent the wild-type strain, *Cupdc1Δ4*, *Cupdc1Δ4-LDH1*, and *Cupdc1Δ4-LDH2* respectively. The glucose-concentration values of L-*LDH*-carrying strains were too similar to be distinguished. Data are means for two independent experiments. Error bars (mean deviations) are too small to be seen. Cells from 2-d-old YPD plates grown at 30 °C were added to 50-ml of YPD pre-cultures in 500-ml Sakaguchi flasks and incubated overnight at 30 °C on a shaker (130 rpm). The cells were harvested by centrifugation at 3,000 × *g* for 5 min at 4 °C, and then added to fresh medium to initiate the time course at 30 °C in 50 ml of YPD in 500-ml Sakaguchi flasks (130 rpm).

Table 3. Effect of L-*LDH* Copy Number and Temperature on the Production Rate of L-Lactic Acid

Strain name/Temperature	L-Lactic acid (g/l/h)*	Glucose (g/l/h)*	Ratio (%)**
Cupdc1Δ4-LDH1/30 °C	3.5 ± 0.1	3.8 ± 0.3	86.2 ± 4.3
Cupdc1Δ4-LDH2/30 °C	4.2 ± 0.1	5.2 ± 0.2	88.1 ± 2.9
Cupdc1Δ4-LDH2/25 °C	2.9 ± 0.1	3.1 ± 0.1	77.3 ± 2.5
Cupdc1Δ4-LDH2/35 °C	4.9 ± 0.2	5.3 ± 0.1	93.9 ± 2.3

Values are means and standard deviations for at least three independent experiments.

*The maximum production rate of L-lactic acid/the maximum consumption rate of glucose were calculated from concentrations between 4, 6, 8, 10 and 12 h, using a linear regression method.

**This ratio was calculated according to the following equation: (g-L-lactic acid produced for 12 h)/g-glucose consumed for 12 h) × 100.

Discussion

The industrially-important yeast *Candida utilis* is widely used to produce heterologous reagents, but to our knowledge the elimination of a major metabolic end-product and its replacement by another *via* recombinant DNA technology has not yet been reported in this species. Here we conducted genetic engineering of a

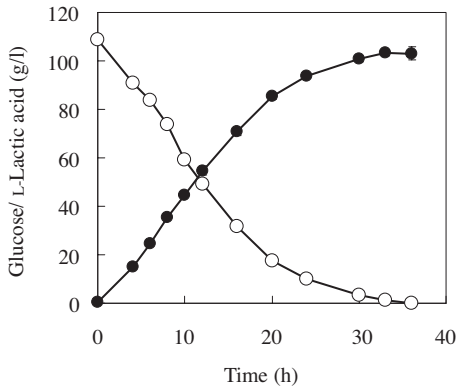

Fig. 4. Time Course for Micro-Aerobic Yeast Culture.

Data are means for at least three independent experiments. Error-bars (standard deviations) are hidden by the symbols. The concentration of glucose at 0 h was 108.7 ± 0.5 g/l. At 33 h, a maximum concentration of L-lactic acid was obtained, 103.3 ± 0.94 g/l. The purity of the L-lactic acid in all samples was 99.9% e.e. or higher.

C. utilis strain, whose ploidy was suggested to be tetraploid because four rounds of disruption were required to inactivate *CuPDC1*. This observation accords with the copy number of *CuURA3*, determined in a previous study.[28] The strain constructed in the present study, *Cupdc1Δ4-LDH2*, had extremely high L-lactic acid productivity compared to other genetically-modified yeasts.

When a *CuPDC1* promoter-driven L-*LDH* gene was introduced into a wild-type strain, glucose was converted into both L-lactic acid and ethanol (data not shown). By contrast, recombinants lacking a functional *CuPDC1* (*Cupdc1Δ4*, *Cupdc1Δ4-LDH1*, and *Cupdc1Δ4-LDH2*) produced little ethanol, indicating that disruption of *CuPDC1* effectively decreased ethanol production. Although inactivation of PDC activity in *S. cerevisiae* prevented growth in YPD, this severe growth defect was not observed in the corresponding *C. utilis* construct, similarly to what has been reported for the Crabtree-negative yeast *K. lactis*.[16] Additional inactivation of PDH activity was required to increase the yield of L-lactic acid in *K. lactis*,[9] although this was accompanied by a significant growth defect, which is not considered desirable, particularly in fermentation processes. In *C. utilis*, however, inactivation of PDC activity alone was sufficient to obtain high yields of L-lactic acid, the yield being 95.1% (103.3 g/l; >99.9% e.e.) from 108.7 g/l of glucose in a 33-h fermentation.

It is still not fully evident why *Cupdc1Δ4-LDH2* can produce L-lactic acid efficiently, but we suppose that the principal reason is derived from the high ability to convert glucose into ethanol in the wild-type strain (Table 2). It was higher than that of *K. lactis*, as reported by others.[10,16] Ethanol production was almost completely abolished in *Cupdc1Δ4*, and its capacity appeared to be efficiently shifted to L-lactic acid production by the introduction of two copies of exogenous L-*LDH*. In addition, a certain level of metabolic capacity from pyruvate to the citric acid cycle in the strain might also affect L-lactic acid production, because ATP generation in the cells is important in increasing L-lactic acid.[47] We observed that *Cupdc1Δ4-LDH2* showed higher production in the micro-aerobic culture than in the static culture (data not

shown), and this is consistent with recent reports that lactic acid-producing *S. cerevisiae* strains require oxygen for the generation of ATP.[12,47] Since the growth rate of *Cupdc1Δ4-LDH2* was comparable to that of *Cupdc1Δ4* (Fig. 3B), it was assumed that *Cupdc1Δ4-LDH2* had the ability to generate enough respiration-metabolism to obtain sufficient ATP under micro-aerobic conditions. However, further study is needed to confirm these hypotheses.

Cupdc1Δ4-LDH2 had the highest ability to produce L-lactic acid at 35 °C, although it produced high concentrations of L-lactic acid over a range of temperatures, spanning 25 to 35 °C. This feature is similar to a previous report that PDC in *C. utilis* was induced only at permissive growth temperatures, 5 to 35 °C, and that the highest activity was detected at 35 °C.[48] Accordingly, the high expression of *CuPDC* at 35 °C might contribute to the large production of L-lactic acid at that temperature. This relatively high optimal temperature is also advantageous because lactic acid at high concentrations easily becomes solidified in the medium due to low solubility. Usually, fermentation of *S. cerevisiae* and *K. lactis* is limited at lower temperatures from 30 to 32 °C.[5–7,9–14]

The pH value of the medium also appeared to be important for the production of L-lactic acid, since a previous study showed that adjustment of pH was effective for the high expression of PDC in *C. utilis*.[35] However, the limited research equipment in the present study did not enable us to control this during the fermentation shown in Fig. 3 (strain, *Cupdc1Δ4-LDH2*; medium, YPD10 with $CaCO_3$), and the initial pH of 6.6 finally dropped to 4.0 (data not shown). This decrease in pH was more likely due to the accumulation of a major acidic product, L-lactic acid (pKa = 3.86). Therefore, exact control of pH might further improve the efficiency of L-lactic acid production.

There are other advantages to fermentative production by *C. utilis*. For example, it is known to produce significant amounts of invertase,[49] which is valuable when sucrose-containing molasses is used as a growth medium. *Cupdc1Δ4-LDH2* was found to produce L-lactic acid with a yield of 94.8% when grown in YP-based media containing 100 g/l of sucrose for 33 h (data not shown). Also, *C. utilis* can ferment at high cell densities,[50,51] which should contribute to increasing production efficiency. Taking all this, the recombinant *C. utilis Cupdc1Δ4-LDH2* strain probably holds great promise for the industrial production of L-lactic acid.

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