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Efficient Homolactic Fermentation by *Kluyveromyces lactis* Strains Defective in Pyruvate Utilization and Transformed with the Heterologous *LDH* Gene†

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A high yield of lactic acid per gram of glucose consumed and the absence of additional metabolites in the fermentation broth are two important goals of lactic acid production by microrganisms. Both purposes have been previously approached by using a *Kluyveromyces lactis* yeast strain lacking the single pyruvate decarboxylase gene (*KlPDC1*) and transformed with the heterologous lactate dehydrogenase gene (*LDH*). The *LDH* gene was placed under the control the *KlPDC1* promoter, which has allowed very high levels of lactate dehydrogenase (LDH) activity, due to the absence of autoregulation by KlPdc1p. The maximal yield obtained was 0.58 g g^{-1}, suggesting that a large fraction of the glucose consumed was not converted into pyruvate. In a different attempt to redirect pyruvate flux toward homolactic fermentation, we used *K. lactis LDH* transformant strains deleted of the pyruvate dehydrogenase (PDH) E1α subunit gene. A great process improvement was obtained by the use of producing strains lacking both PDH and pyruvate decarboxylase activities, which showed yield levels of as high as 0.85 g g^{-1} (maximum theoretical yield, 1 g g^{-1}), and with high LDH activity.

Lactic acid is widely used in industry. Pure lactate can be obtained from bacteria: fermentation processes are carried out in buffered conditions at neutral pH in order to avoid metabolic and growth inhibition caused by the accumulation of the acidic product (2, 4, 10). Genetically engineered fermentative yeasts can be used for the production of lactic acid from glucose by transformation with heterologous lactate dehydrogenase (LDH) genes. Conversion of pyruvate to lactic acid by LDH requires cytosolic $NADH/H^+$. A general scheme of pyruvate catabolism in yeast is shown in Fig. 1.

The yeast *Saccharomyces cerevisiae*, which has a predominant fermentative metabolism, has been used for lactic acid production (5, 14). However, the bioprocess yield (grams of lactic acid produced per gram of glucose consumed) was low because of the simultaneous production of ethanol due to the competition for pyruvate by the heterologous LDH and the homologous pyruvate decarboxylase (PDC) activities. *S. cerevisiae* has two active structural *PDC* genes: *PDC1* and *PDC5* (16, 17). A third gene, *PDC6*, is inactive (9). Increased production and yield of lactic acid were obtained by the use of single *pdc1* or *pdc5* mutant strains, but the amount of ethanol could only be slightly decreased (1). On the other hand, *S. cerevisiae* mutant strains with both *PDC1* and *PDC5* genes inactivated are strongly impaired for growth on glucose medium (9) and thus are not useful for production purposes.

The yeast *Kluyveromyces lactis* has a single gene, *KlPDC1*, expressing PDC activity (3). The deletion of *KlPDC1* leads to strains without PDC activity and which do not produce ethanol. In contrast to *S. cerevisiae pdc1 pdc5* double mutant strains, *K. lactis Klpdc1*Δ strains grow at the same rate as the wild-type strains on glucose medium (3). The difference between the two yeasts might be ascribed to active acetyl-coenzyme A (CoA) transport from mitochondria to cytosol in *K. lactis* (Fig. 1). *KlPDC1* is subjected to autoregulation by its own gene product, and it is induced by glucose and repressed by ethanol at the transcriptional level (6). In the presence of both carbon sources, *KlPDC1* promoter-driven expression shows intermediate levels (6). In previous works, we reported the use of wild-type and *Klpdc1*Δ *K. lactis* strains transformed with the bovine *LDH* gene (15), as well as with bacterial *LDH* genes (patent application WO1998EP0005758), for the production of lactic acid.

In aerobic conditions the yeast *K. lactis* has a predominantly respiratory metabolism on glucose media (8, 11, 13) and produces a limited amount of ethanol. In this yeast, pyruvate is largely channeled into the tricarboxylic acid cycle by the pyruvate dehydrogenase (PDH) complex. On the other hand, *K. lactis* strains with no PDH activity have a vigorous fermentative metabolism on glucose in aerobic batch cultures, and only in glucose-limited aerobic chemostat conditions can the *Klpda1*Δ strains metabolize glucose exclusively through the cytoplasmic acetyl-CoA pathway (20).

In this work, we present the results of new metabolic host configurations for the conversion of glucose into lactate, based on engineered rerouting of pyruvate. We used *K. lactis* strains either lacking PDH activity or lacking both PDC and PDH activities (Fig. 1), transformed with the bovine *LDH* gene placed under the transcriptional control of the inducible promoter of *KlPDC1* gene and cloned into a stable multicopy vector. The heterologous LDH enzyme could efficiently com-

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† This work is dedicated to Franco Tato.

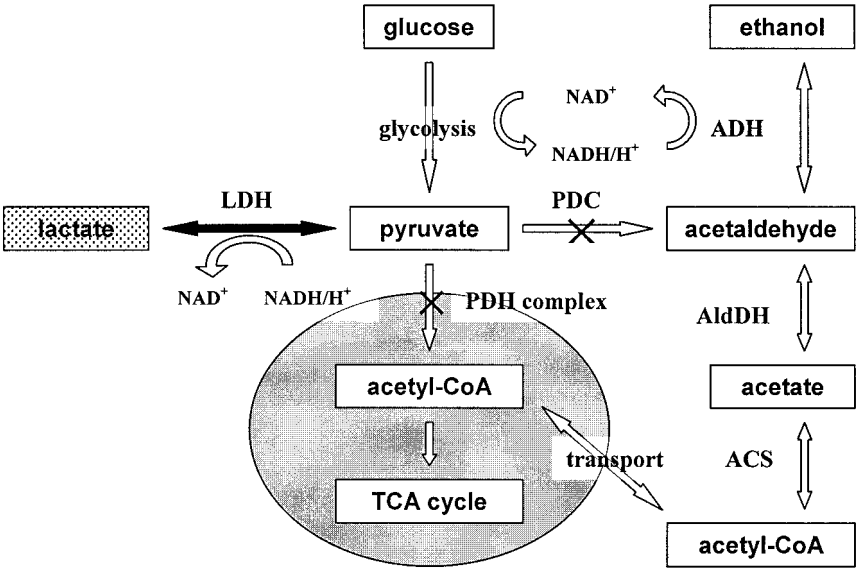

FIG. 1. Scheme of pyruvate metabolism and pyruvate bypass in *K. lactis*. Enzymes: ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase. The gray oval represents the mitochondrial compartment. The black arrow and the dotted box indicate the metabolic step added by transformation with heterologous *LDH* gene and the new metabolite, respectively.

pete for available cytoplasmic $NADH/H^+$ and pyruvate in these strains when they were grown in glucose excess conditions.

MATERIALS AND METHODS

Strains and media. The *Escherichia coli* strain used in the molecular cloning procedures was DH5αF′ [φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deo rec1 end1 sup44* λ *THI-1 gyrA96 relA1*]. The *K. lactis* strains used in this work are listed in Table 1. The deleted strains PMI and MW341-5/*Klpdc1*Δ were obtained from wild-type strains PM6-7A and MW341-5, respectively, by disruption of *KlPDC1* with a deletion cassette containing the marker gene *URA3* from *S. cerevisiae* (3, 6). The deleted strains GG1993 (20) and PM6-7A/*Klpda1*Δ were obtained from wild-type strains CBS2359 and PM6-7A, respectively, by disruption of *KlPDA1* with the bacterial Tn*5BLE* gene from plasmid pUT322 (7). Double-deleted strains were constructed as follows. In strain PM6-7A/DD, the *KlPDC1* gene was deleted and replaced with the *URA3* marker gene by integrative transformation of strain PM6-7A/*Klpda1*Δ with vector pBSU7, as described in Bianchi et al. (3). The deletion of *KlPDC1* was verified by PCR and growth on glucose plus the mitochondrial inhibitor antimycin A. The double-deleted strain BM1-3C was selected as a phleomycin-resistant and antimycin A-sensitive segregant strain issued from a diploid strain obtained by crossing strain MW341–5/*Klpdc1*Δ with the *Klpda1*Δ strain GG1993.

A different approach was followed for the construction of the prototrophic double-deleted strain bearing the heterologous *LDH* gene. The *Klpdc1*Δ strain PMI was transformed with pLAZ10 (see below) and crossed with the wild-type

TABLE 1. Yeast strains

Strain[a]	Genotype	Source or reference
PM6-7A	*MAT***a** *adeT-600, uraA1-1*	18
MW109-8C	*MAT*α *lysA1-1*	18
PMI	*MAT***a** *adeT-600 uraA1-1 Klpdc1*::*URA3*	3
MW341-5/*Klpdc1*Δ	*MAT*α *lac4-8 leu2 lysA1-1 uraA1-1 Klpdc1*::*URA3*	6
7C(pLAZ10)	*MAT*α *lysA1-1 adeT-600 Klpdc1*::*URA3*, pLAZ10$^+$	This work
GG1993	*MAT***a** *ura3-49 Klpda1*::Tn*5BLE*	20
PM6-7A/*Klpda1*Δ	*MAT***a** *adeT-600 uraA1-1 Klpda1*::Tn*5BLE*	H. Y. Steensma
PM6-7A/DD	*MAT***a** *adeT-600 uraA1-1 Klpdc1*::*URA3 Klpda1*::Tn*5BLE*	This work
BM1-3C	*MAT***a** *leu2 Klpdc1*::*URA3 Klpda1*::Tn*5BLE*	This work
BM3-12D (pLAZ10)	*MAT***a** *Klpdc1*::*URA3 Klpda1*::Tn*5BLE, pLAZ10*$^+$	This work

[a] All of the *K. lactis* strains used in this work originate from the standard NRRL strains Y-1140 (CBS2359; *MAT***a** genotype) and Y-1205 (CBS2360; *MAT*α genotype) (19). Auxotrophic markers were introduced by selection of spontaneous or induced mutations or transferred by genetic crosses.

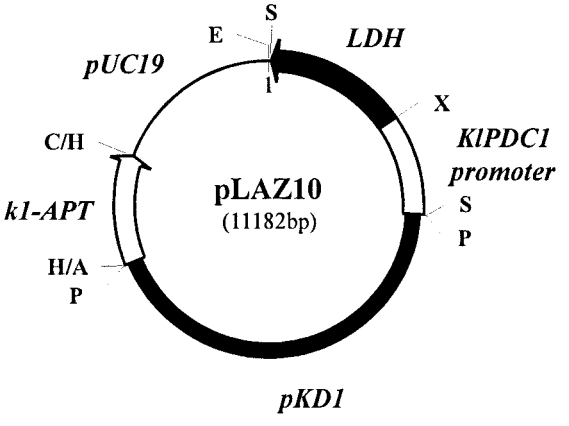

FIG. 2. Map of vector pLAZ10. The elements of pLAZ10 are marked in the figure as follows: thin line, pUC19 bacterial plasmid; black box, entire pKD1 genome; empty arrow, k1-*APT* cassette; black arrow, bovine *LDH-A* cDNA; empty box, *KlPDC1* promoter sequence. Relevant cloning sites: A, *Sca*I; C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; P, *Sph*I; S, *Sal*I; X, *Xba*I.

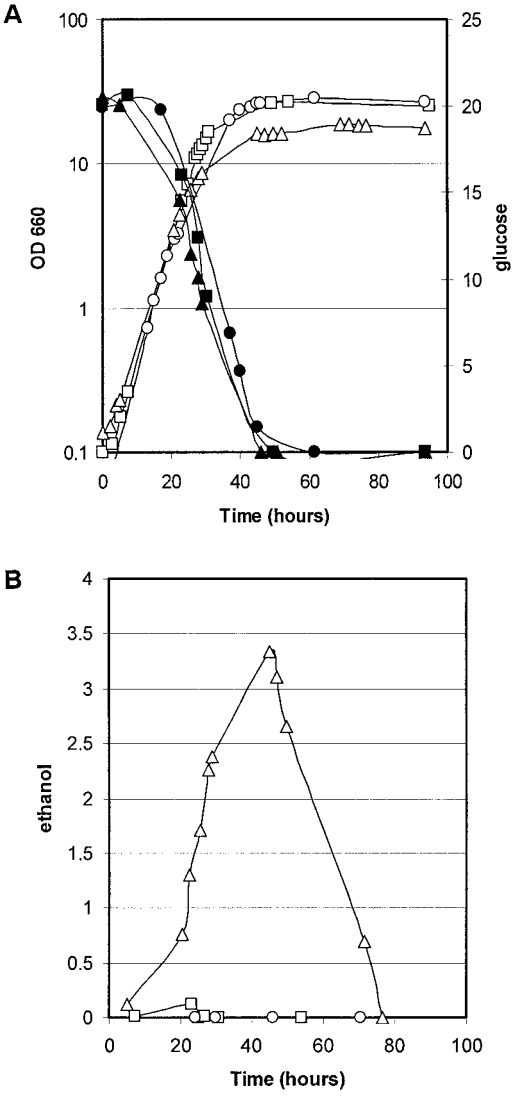

FIG. 3. Growth and metabolites composition of wild-type (squares), *Klpda1*Δ (circles), and *Klpdc1*Δ (triangles) isogenic strain cultures. (A) Cell growth (OD_{660} [open symbols]) and residual glucose (grams liter^{-1} [solid symbols]). (B) Ethanol (grams liter^{-1}). (C) Pyruvate (grams liter^{-1} [open symbols]) and acetate (grams liter^{-1} [solid symbols]).

strain MW109-8C. After sporulation of the resulting diploid strain and tetrad dissection, the strain called 7C(pLAZ10) was selected as a *MAT*α, Geneticin-resistant, and antimycin A-sensitive segregant strain. The double-deleted BM1-3C strain and 7C strain carrying pLAZ10 were then crossed, and haploid segregant strains were isolated after sporulation of the diploid strain. All of them were sensitive to antimycin A (*Klpdc1*Δ) and resistant to Geneticin (pLAZ10^{+}), while the phleomycin-resistance phenotype (*Klpda1*Δ) and auxotrophic markers correctly segregated 2:2. Among these strains, we selected for fermentation processes a prototrophic *Klpda1*Δ segregant strain, BM3-12D(pLAZ10).

Yeast-rich medium (YP) contained 1% (wt/vol) yeast extract and 2% (wt/vol) peptone. Synthetic medium (SM) contained 0.67% (wt/vol) yeast nitrogen base without amino acids. Media were supplemented with 2% (wt/vol) or 5% (wt/vol) glucose (D) and/or 2% (vol/vol) ethanol (E). The YP media used for the selection and identification of the *Klpda1*Δ and *Klpdc1*Δ mutant strains contained 8 mg of phleomycin liter^{-1} and 2% (wt/vol) glucose or 5 μM antimycin A and 5% (wt/vol) glucose, respectively. Geneticin was added to a final concentration of 200 mg liter^{-1}, when needed. Solid media contained 2% (wt/vol) agar.

Construction of the LDH expression vector. Vector pLAZ10 (Fig. 2) was obtained by cloning the 2.8-kbp *Sal*I fragment of vector pEPL2 (15), bearing the *KlPDC1* promoter fused in front of the cDNA of the bovine *LDH-A* gene (14), into the unique *Sal*I site of vector p3K31. Common cloning procedures have been followed. Vector p3K31 is composed of pUC19 DNA and of the kanamycin resistance cassette of vector pKan707 inserted in the unique *Sph*I site of the natural multicopy plasmid pKD1 (12). The kanamycin resistance gene is the aminoglycoside phosphotransferase gene (*APT*) of the bacterial transposon Tn*903*, which confers Geneticin resistance on yeast, fused downstream from the *K. lactis* killer promoter k1. Yeast strains were transformed with pLAZ10 by the electroporation procedure (3), and the transformants were selected on YPD medium containing Geneticin.

Stirred-tank cultures. Growth on glucose medium of strains PM6-7A, PMI, and PM6-7A/*Klpda1*Δ was monitored in a 5-liter Biostat-B stirred-tank bioreactor (B-Braun). Cells were inoculated in 4 liters of SM, containing 2% (wt/vol) glucose and 200 mg of adenine liter^{-1}and supplemented, when required, with 100 mg of uracil liter^{-1}. The bioreactor was kept at 30°C and pH 5 and was aerated at 2 liters min^{-1}. A pO_2 level higher than 40% was maintained throughout the process by controlling the stirring rate. The PM6-7A/DD strain was grown on mixed glucose-ethanol substrate and compared to the wild-type strain PM6-7A grown on the same carbon sources. The process conditions were the same as described above. The medium contained 2% (wt/vol) glucose and 2% (vol/vol) ethanol.

Fermentative processes for lactate production from strain BM3-12D(pLAZ10) were performed in a Biostat-Q 1-liter stirred-tank fermentor (B-Braun) containing 0.8 liter of SM supplemented with 5% (wt/vol) glucose, 2% (vol/vol) ethanol, and 200 mg of Geneticin liter^{-1}. Temperature and stirring were kept at 30°C and 400 rpm, respectively. During the fermentation processes, air was fed at 0.8 liter min^{-1}, and glucose was added to a concentration of 4.5% ± 5% (wt/vol). In the fermentation tests, the pH was maintained at 4.5 by the automatic addition of 2 M KOH.

Measurement of cell concentration, metabolites, and enzymatic activities. Fermentation processes were monitored at regular time intervals. Cell concentrations were determined by measuring the optical density at 660 nm (OD_{660}). Glucose, ethanol, acetate, L-(+)-lactate, and LDH activities were determined by using diagnostic kits (Boehringer Mannheim 716251, 176290, 148261, and 139084 and Sigma DG1340-K, respectively) according to instructions. The concentration of pyruvate was assayed by high-pressure liquid chromatography (Jasco Corporation). Separations were achieved on HPX-87H 300-by-7.8-mm column (Bio-Rad), and peaks were detected at 210 nm with UV-VIS detector (Jasco Corporation). Diluted sulfuric acid (4 mM in water) was used as solvent at 35°C and at a 0.6-ml min^{-1} flow rate. Yields of lactate were calculated by linear regressions obtained by plotting the grams of glucose consumed in the course of fermentation processes versus the grams of lactate produced.

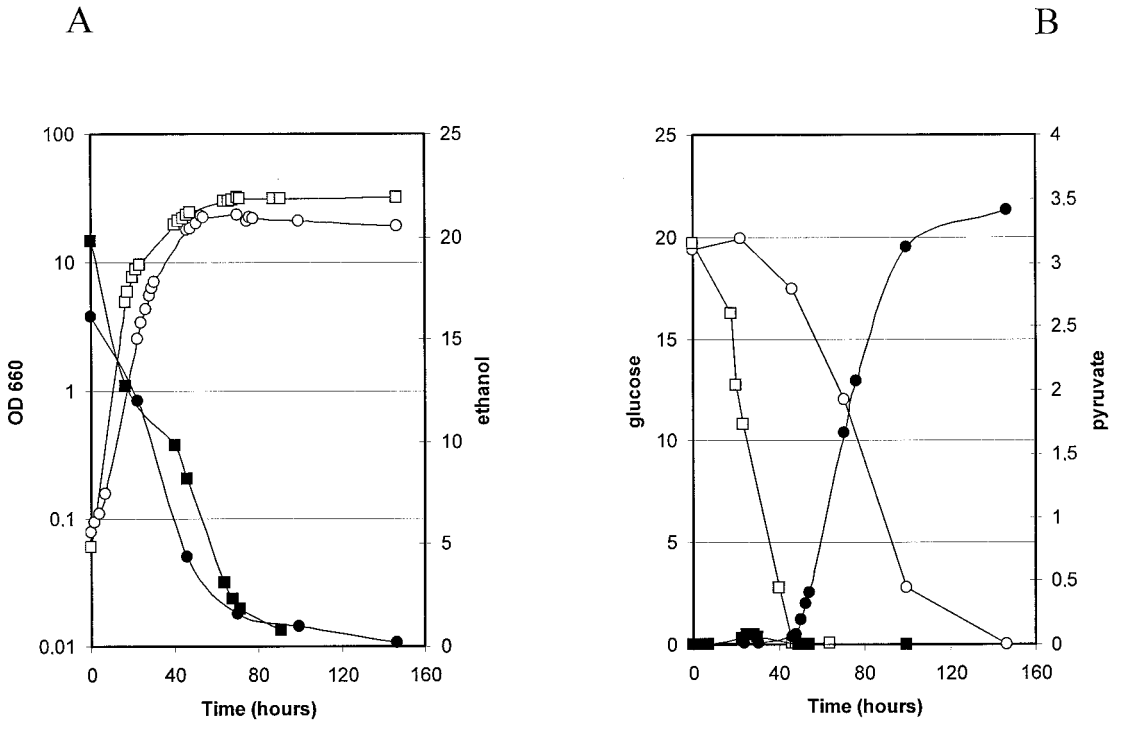

FIG. 4. Growth and metabolite composition of wild-type (squares) and *Klpda1*Δ *Klpdc1*Δ (circles) isogenic strain cultures. (A) Cell growth (OD_{660} [open symbols]) and residual ethanol (grams liter^{-1} [closed symbols]). (B) Residual glucose (grams liter^{-1} [open symbols]) and pyruvate (grams liter^{-1} [closed symbols]).

RESULTS AND DISCUSSION

Growth and metabolite productions of wild-type, Klpdc1Δ, and Klpda1Δ deleted strains. Growth and consumption or production of carbon compounds of strains harboring defects in pyruvate metabolism were studied in stirred-tank fermentor supplemented with defined minimal medium containing glucose as carbon sources. In the first experiment, the parental strain PM6-7A, the *Klpdc1*Δ strain PMI, and the PM6-7A/*Klpda1*Δ strain were compared. These strains were isogenic except for the deletion of the single structural gene encoding for PDC activity in strain PMI and the deletion of *KlPDA1*, which is the gene encoding for the E1α subunit of the PDH complex (Fig. 1). Time courses of the fermentation processes are shown in Fig. 3. Specific growth and glucose consumption rates (Fig. 3A) were similar for the three strains, while the overall biomass production at the stationary phase was lower for the PM6-7A/*Klpda1*Δ strain. Ethanol was produced and accumulated—up to 3.3 g liter^{-1}—during the log growth phase only by strain PM6-7A/*Klpda1*Δ (Fig. 3B). The production of other carbon metabolites (Fig. 3C) showed modest peaks of acetate or pyruvate excretion during the log phase of the wild-type and PM6-7A/*Klpda1*Δ strains and the late log phase of the PMI strain, respectively.

In a second series of experiments, we compared growth and metabolite production or consumption of the wild-type strain PM6-7A and of the double-deleted strain PM6-7A/DD. These processes were carried out on medium containing both glucose and ethanol as carbon sources, because double-deleted strains cannot grow on minimal medium containing only C6 sugars or C3 compounds as the sole carbon sources (data not shown). Results are reported in Fig. 4. Growth profiles (Fig. 4A) were similar for both strains, with only a slightly reduced specific growth rate and a lower cell concentration at the stationary phase for PM6-7A/DD strain. Ethanol and glucose (Fig. 4A and B, respectively) were simultaneously consumed by the wild-type strain PM6-7A. In contrast, the double-deleted strain consumed exclusively ethanol in the first phase of the fermentation process, during which the bulk of biomass was produced. Glucose assimilation was observed only when ethanol was completely consumed. Interestingly, glucose consumption was associated with the accumulation of pyruvate at up to 3.5 g liter^{-1}.

Stirred-tank fermentation of a *Klpda1*Δ *Klpdc1*Δ(pLAZ10) strain. Production of lactic acid by single-deleted *Klpda1*Δ strains transformed with pLAZ10 was tested. The maximum yield obtained (0.35 g g^{-1}) was unsatisfactory because of competition for pyruvate by the PDC enzyme, which channeled carbon flux into the ethanologenic pathway and/or into the pyruvate bypass (not shown). In order to increase the product yield by completely channeling pyruvate flux toward lactate formation, we planned to assay lactic fermentation in strains defective in both PDC and PDH activities. Since the double-deleted strains PM6-7A/DD and BM1-3C were recalcitrant to direct transformation, we genetically transduced pLAZ10 and selected double-deleted transformants as described in Materials and Methods. This procedure also allowed us to select a prototrophic host, BM3-12D, that was more suitable for fermentation processes.

Lactic acid production of BM3-12D(pLAZ10) transformant strain was tested by cultivation in a 1-liter stirred-tank biore-

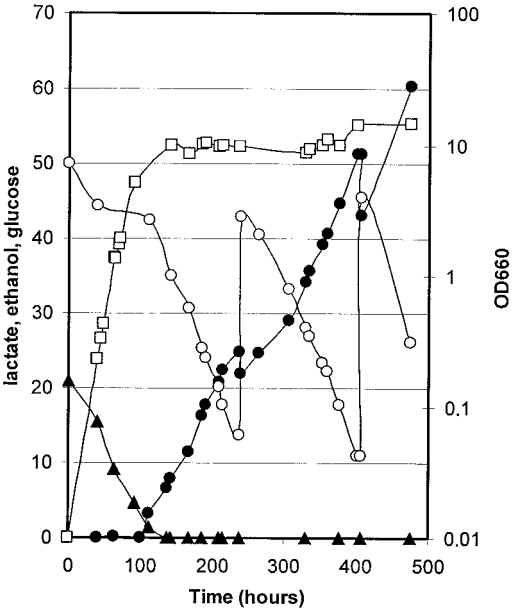

FIG. 5. L-(+)-Lactic acid production in a stirred-tank fermentation process of the *Klpdc1*Δ *Klpda1*Δ strain BM3-12D(pLAZ10). The time courses of product, biomass, and carbon source concentrations are reported. The fermentation was carried out on SM as described in Materials and Methods. The pH was maintained at 4.5 by KOH addition. Symbols: ●, lactate (grams liter^{-1}); ▲, ethanol (grams liter^{-1}); □, cells (OD_{660}); ○, glucose (grams liter^{-1}).

actor at constant pH by KOH addition. The time course of the process is shown in Fig. 5. During the first few hours of the process, cells consumed ethanol for biomass production. Glucose assimilation was observed only when ethanol was completely consumed, as for the nontransformed double-deleted strain (see Fig. 4). However, because of the heterologous LDH activity, in this case the glucose assimilation was associated with lactate production. Further additions of glucose to the bioreactor (after 238 and 405 h of fermentation) resulted in a continuous trend of glucose consumption and lactate accumulation, up to a concentration of 60 g of lactate liter^{-1} at 500 h of incubation, when the process was stopped. Lactate was not produced in control fermentation processes without glucose added in the culture medium (not shown). The yield of product formation (0.85 g g^{-1}), not far from the maximum theoretical value (1 g g^{-1}), was much higher than yields obtained with single *Klpda1*Δ strains (0.35 g g^{-1}) or the single *Klpdc1*Δ strain (0.58 g g^{-1} [15]).

As previously described (15), LDH activity is higher in the *Klpdc1*Δ genetic background (55 to 60 U mg^{-1}) than in wild-type strain (4 to 5 U mg^{-1}), when the *LDH* gene is fused to the *KlPDC1* promoter. This effect is a consequence of the absence of transcriptional autoregulation by KlPdc1p in *Klpdc1*Δ strains (6). We measured values of heterologous LDH activity that were as high as 110 to 150 U mg^{-1} in the *Klpdc1*Δ *Klpda1*Δ BM3-12D(pLAZ10) strain, which contained the same *LDH* expression cassette as in the above-mentioned transformant strains. This finding suggests that the factors involved in the induction or derepression of *KlPDC1* promoter were fully operating also in the double-deleted genetic background. As a whole, the results presented here indicate that the metabolic constraints of carbon flux, growth-phase dependence of substrate assimilation, and upregulation of specific promoters might provide synergetic cooperation in metabolic engineered *K. lactis* strains for highly efficient transformation of glucose into lactic acid.

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