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Efficient Production of L-Lactic Acid from Xylose by *Pichia stipitis*[▽]

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Microbial conversion of renewable raw materials to useful products is an important objective in industrial biotechnology. *Pichia stipitis*, a yeast that naturally ferments xylose, was genetically engineered for L-(+)-lactate production. We constructed a *P. stipitis* strain that expressed the L-lactate dehydrogenase (LDH) from *Lactobacillus helveticus* under the control of the *P. stipitis* fermentative *ADHI* promoter. Xylose, glucose, or a mixture of the two sugars was used as the carbon source for lactate production. The constructed *P. stipitis* strain produced a higher level of lactate and a higher yield on xylose than on glucose. Lactate accumulated as the main product in xylose-containing medium, with 58 g/liter lactate produced from 100 g/liter xylose. Relatively efficient lactate production also occurred on glucose medium, with 41 g/liter lactate produced from 94 g/liter glucose. In the presence of both sugars, xylose and glucose were consumed simultaneously and converted predominantly to lactate. Lactate was produced at the expense of ethanol, whose production decreased to ~15 to 30% of the wild-type level on xylose-containing medium and to 70 to 80% of the wild-type level on glucose-containing medium. Thus, LDH competed efficiently with the ethanol pathway for pyruvate, even though the pathway from pyruvate to ethanol was intact. Our results show, for the first time, that lactate production from xylose by a yeast species is feasible and efficient. This is encouraging for further development of yeast-based bioprocesses to produce lactate from lignocellulosic raw material.

Lactic acid has numerous industrial applications. One of its expanding uses is for polymerization of lactic acid to polylactic acid (PLA), which is then used to manufacture a variety of products, including biodegradable plastics and textile fibers (14, 47). PLA is an attractive polymer because it can be produced from renewable resources and is biodegradable. These properties have increased interest in developing more efficient production processes for lactic acid. Many PLA-based products are already on the market, where they usually replace petroleum-based consumables (14).

Lactic acid is produced naturally by several species of lactic acid bacteria, which are used for the fermentation of lactic acid from sugars, typically glucose, for commercial purposes (8, 47). The zygomycete fungus *Rhizopus oryzae* also can ferment glucose to lactic acid (26, 50). The bacterial fermentation process is rapid and efficient, but complex fermentation media are used since lactic acid bacteria require complex nitrogen sources and vitamins in the medium, which increases the cost of the process (25, 42, 47). Calcium carbonate or other chemicals are used to neutralize the lactic acid produced and prevent acidification of the medium, which is detrimental to lactic acid bacteria (8, 46). Purification of lactic acid from calcium lactate precipitate is a complex multistep process that produces large quantities of gypsum waste, which then needs to be disposed (8, 46). Thus, it is desirable to eliminate these requirements to decrease the overall process costs.

Interest in yeast species as alternative biocatalysts for lactic acid production has recently increased (1, 4, 7, 10, 17, 29, 30, 41, 44). For example, *Saccharomyces cerevisiae* is an organism

that can be grown in inexpensive growth media and that is relatively tolerant to low pH and organic acids. The ability of yeast to ferment under acidic conditions is also attractive and could reduce or eliminate the need for a neutralizing agent in the low-pH fermentation process. Lactic acid production by strains of *S. cerevisiae*, which is highly efficient in fermenting hexose sugars, expressing lactate dehydrogenases (LDHs) of bacterial (7, 10, 17, 30, 44), bovine (1, 17), and fungal (41) origins has been described. Lactic acid has also been produced by *Kluyveromyces lactis* expressing bovine LDH (4, 29).

During the last couple of decades, more efficient exploitation of lignocellulosic plant biomass has also received increased attention (2, 16, 49). Biomass resources are renewable and abundant, and agricultural and forestry residues, in particular, remain largely unutilized. Plant-derived substrates contain glucose and considerable amounts of pentose sugars, especially xylose and arabinose (21). Efficient utilization of biomass-derived substrates in a production process requires that the fermenting organism convert a wide range of sugars to useful metabolites, such as lactic acid.

The first step in the fungal xylose catabolic pathway is the transport of the sugar into the cell, where it is reduced to xylitol by a xylose reductase and further oxidized to xylulose by a xylitol dehydrogenase (12, 31). These oxidoreductive steps may be circumvented by a direct isomerization step. In many bacteria and in the obligate anaerobic fungus *Piromyces* sp., xylose is converted to xylulose by a xylose isomerase (3, 13). Xylulose is phosphorylated by xylulokinase into xylulose-5-phosphate, which enters the pentose phosphate pathway, where it is converted to C₃ and C₆ compounds that are metabolized further in glycolysis to pyruvate. *S. cerevisiae* is not able to metabolize pentose sugars naturally, although several other yeasts can, e.g., *Candida guilliermondii*, *Candida tenuis*, *Candida tropicalis*,

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Pachysolen tannophilus, and *Pichia stipitis* (22, 38, 43). Although many yeast species assimilate xylose, *P. stipitis* is one of the few yeasts that also efficiently ferments xylose to ethanol (31). *P. stipitis* produces ethanol at a high yield, with very little xylitol accumulation. Metabolic engineering of *P. stipitis* to enhance its xylose metabolism for the production of, e.g., ethanol has been reported (9, 18, 19, 28, 36, 37). Other naturally occurring, xylose-utilizing yeasts (31) and *S. cerevisiae* genetically engineered to utilize xylose (20, 45) produce undesirably large amounts of xylitol.

The main objective of this work was to demonstrate the conversion of xylose to lactate by a nonconventional, xylose-fermenting yeast. In addition, we studied the conversion of glucose to lactate and the effect of pH control on lactate production. We hypothesized *P. stipitis* to be a suitable host for efficient lactate production because of its outstanding capacity to ferment xylose, its moderate tolerance to low pH, and its ability to grow on cheap synthetic media. Lactate production would be accomplished by expressing the L-lactate dehydrogenase gene (*ldhL*) from *Lactobacillus helveticus*, which would convert pyruvate formed in glycolysis to lactate. The study addresses the feasibility of lactic acid production by the yeast *P. stipitis* from sugars other than glucose, e.g., xylose and mixtures of xylose and glucose. We demonstrate that the metabolically engineered *P. stipitis* strain is capable of converting sugars to useful products, such as lactic acid. The results are the first example of lactate production from xylose by a yeast species.

MATERIALS AND METHODS

Microbial strains. *Pichia stipitis* CBS6054 (Centraalbureau voor Schimmelfcultures, Utrecht, The Netherlands) was used as a wild-type reference strain. *P. stipitis* FPL UC7, a *ura3* uracil auxotroph (23) derived from CBS6054, was used for transformation. The lactate-producing strains VTT-C-04588, -04589, -04590, and -05789 and the control strain VTT-C-05788 were constructed for this study. *Escherichia coli* DH5 α (Gibco BRL, Gaithersburg, MD) was used as a host for recombinant DNA work.

Construction of plasmid vectors. The lactate dehydrogenase expression vector pMI344 (Fig. 1) contains the *L. helveticus* *ldhL* gene between the *P. stipitis* *ADH1* promoter and the *S. cerevisiae* *CYC1* terminator; the *P. stipitis* *URA3* gene is the transformation marker, and *P. stipitis* *ARS2* enables autonomous replication. The plasmid was constructed in two steps. First, the *L. helveticus* *ldhL* coding sequence (34) was placed under the control of the *P. stipitis* *ADH1* promoter. The 598-bp promoter of the *P. stipitis* *ADH1* gene (5, 6) (GenBank accession number AF008245) was amplified by PCR with oligonucleotides Ps106ADH (5'-GCGA TCTCGAGAACCGATCCGAGGGGAAAACCGGG-3' [XhoI site is underlined]) and Ps103ADH (5'-CTTGGCCATGCTAATTGGATGCGATCGAC GACT-3' [NcoI site is underlined]) from the genomic DNA of *P. stipitis* CBS6054. The amplified fragment was digested with NcoI and XhoI and ligated to the NcoI and XhoI fragment of pVR1, which contains the L-lactate dehydrogenase-encoding gene (*ldhL*) of *Lactobacillus helveticus* linked to the *S. cerevisiae* *CYC1* terminator. The resulting plasmid, pMI343, was cut with ApaI and XhoI and ligated to the ApaI-XhoI fragment of pJM6 (48), which contains the *P. stipitis* *URA3* gene and an autonomously replicating sequence (PsARS2), to form pMI344 (Fig. 1).

Construction of *P. stipitis* strains. *P. stipitis* was transformed by using a lithium acetate method (11). To facilitate integration for stable maintenance of the heterologous *ldhL* gene, pMI344 (Fig. 1) was digested with XbaI and SnaBI prior to yeast transformation, and the 3.5-kb fragment containing the *ldhL* expression cassette and the *URA3* selection marker but lacking the autonomous replication sequence was isolated from an agarose gel and introduced into *P. stipitis* FPL UC7. Transformants were selected on synthetic complete medium (35) lacking uracil (SCD-Ura), resulting in isolation of the *ldhL* integrants. Uracil prototrophs were tested for lactate production in test tube cultures incubated overnight. The *ldhL* transformants produced 3 to 5 g/liter L-lactate. Southern analysis of genomic DNAs of the transformants with an *ldhL* probe (data not shown) indicated that one copy of *ldhL* had integrated into the genome in the transfor-

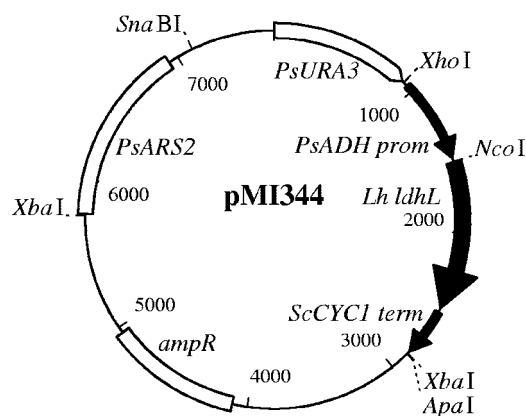


FIG. 1. Map of plasmid pMI344.

mants VTT-C-04588, -04589, -04590, and -05789 but that the integration sites were different, indicating that these transformants resulted from independent transformation events. Hybridization with *URA3* and *ADH1* probes showed that the bands corresponding to the respective endogenous loci were unaltered (data not shown), indicating that the transformed DNA had not integrated at either *URA3* or *ADH1*, in spite of the sequence similarity in the introduced construct. Transformants were also obtained with the circular pMI344 plasmid. However, only the integrants were chosen for further studies since preliminary experiments (data not shown) indicated that they were better lactate producers than were the transformants carrying the autonomously replicating plasmids. Circular pJM6 was transformed into FPL UC7 to generate the *URA*⁺ reference strain VTT-C-05788.

DNA manipulation. Recombinant DNA work was carried out by using standard techniques (33). Plasmid DNA was isolated by using QIAGEN Mini and Midi kits (QIAGEN Corp., Chatsworth, CA). Yeast DNA was isolated by phenol extraction from cells broken with glass beads (15). For Southern analyses, the genomic DNA was cut with HindIII and StuI for hybridization with the *URA3* probe, with SacI and KpnI for hybridization with the *ADH1* probe, and with StuI or with NcoI and NotI for hybridization with the *ldhL* probe and then blotted onto nylon membranes (Hybond N; Amersham Biosciences, Little Chalfont, United Kingdom). Probes for Southern hybridization were labeled with digoxigenin-11-dUTP, and colorimetric detection of the hybridization signals with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) was performed according to the manufacturer's instructions (Roche, Mannheim, Germany).

Media and culture conditions. Strains were grown at 30°C on agar-solidified yeast extract (10 g/liter)-Bacto peptone (20 g/liter) medium supplemented with 20 g/liter glucose (YPD) or xylose (YPX) or on synthetic complete medium (35) lacking uracil and supplemented with 20 g/liter glucose (SCD-Ura) or xylose (SCX-Ura), as appropriate. Inocula for liquid glucose or xylose cultures were grown in the presence of the corresponding sugars.

Uracil prototrophic transformant colonies were initially tested for lactic acid production in test tube cultures incubated overnight in 5 ml YP-plus-glucose (40 g/liter) liquid medium at 250 rpm at 30°C. Lactate production media contained approximately 50 or 100 g/liter (1.7 or 3.3 mol C/liter) xylose or glucose. The maximum theoretical yields of ethanol and lactate are 0.51 and 1.0 g/g, respectively, on both glucose and xylose, and thus equal amounts of the two sugars (in grams or moles of carbon) were added to the media. In shake flask cultures, the yeast extract-peptone medium was supplemented with 55 $\pm$ 2 g/liter glucose (YPD-55) or 53 g/liter xylose (YPX-53). One-stage cultures were inoculated to an optical density at 600 nm (OD₆₀₀) of 0.2 with cells grown on YPD or YPX agar plates. Cultures were incubated at 30°C with shaking at 100 rpm in 250-ml Erlenmeyer flasks containing 50 ml medium. Experiments with all four transformants were carried out in duplicate. Since the results with all four transformants were similar, we combined the results to give eight replicates. For two-stage cultivations, the biomass was grown in yeast nitrogen base medium without amino acids (YNB; Difco, Sparks, MD) plus 50 g/liter glucose or xylose. The pH was buffered to 5.5 with 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES). After overnight growth at 30°C and 250 rpm, cells were harvested and transferred to YNB medium supplemented with 54 $\pm$ 4 (YNBD-54) or 94 (YNBD-94) g/liter glucose, 51 $\pm$ 2 (YNBX-51) or 101 (YNBX-101) g/liter xylose, or a mixture of 47 $\pm$ 1 g/liter glucose and 50 g/liter xylose (YNBDX-97). Cell density was

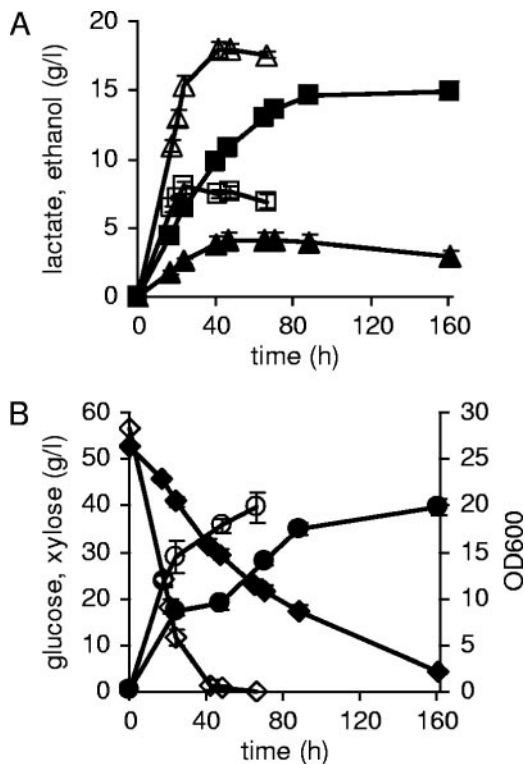


FIG. 2. Production of lactate (A) on xylose (g/liter) (■) and on glucose (g/liter) (□), production of ethanol (A) on xylose (g/liter) (▲) and on glucose (g/liter) (△), consumption of xylose (B) (g/liter) (◆) and glucose (B) (g/liter) (◇), and OD₆₀₀ (B) on xylose (●) and on glucose (○) for *P. stipitis* *ldhL* transformants in nonbuffered YPX-53 and YPD-55 media. Error bars denote standard errors of the means (SEM). The SEM is less than the size of the symbol if no error bars are seen.

adjusted to an OD₆₀₀ of 13 (~3.5 g/liter cell dry weight), and 30 g/liter of calcium carbonate was added for pH control. YNB media supplemented with 47 g/liter glucose (YNBD-47) or 49 g/liter xylose (YNBX-49) were prepared without CaCO₃. The cultures were incubated at 30°C at 100 rpm in 250-ml Erlenmeyer flasks containing 50 ml medium. Two experiments each were carried out with all four *ldhL* transformants (single flasks) in YNBX-51 and YNBD-54 and with two transformants (in duplicate) in YNBX-97. One experiment each with two transformants, VTT-C-04590 and -05789, in duplicate was conducted in YNBX-101, YNBD-94, YNBD-47, and YNBX-49.

Analytical methods. The culture supernatants were analyzed for lactic acid, xylose, glucose, xylitol, pyruvic acid, acetic acid, glycerol, and ethanol by high-

performance liquid chromatography using a Waters 2690 separation module and Waters system interphase module liquid chromatograph coupled with a Waters 2414 differential refractometer and a Waters 2487 dual λ absorbance detector (Waters, Milford, MA). A Fast Juice column (50 × 7.8 mm; Phenomenex, Torrance, CA) and a Fast Acid analysis column (100 × 7.8 mm; Bio-Rad, Hercules, CA) were equilibrated with 2.5 mM H₂SO₄ at 60°C, and samples were eluted with 2.5 mM H₂SO₄ at a 0.5-ml/min flow rate. Data were acquired with Waters Millennium software. L-Lactic acid in the culture supernatants was determined enzymatically by the L-lactic acid UV method (Roche). Lactate and ethanol yields were calculated as the amounts of accumulated products per amount of consumed sugar. Yields are reported for the sample time when the sugar concentration was first recorded as <1.5 g/liter, unless stated otherwise.

RESULTS

Lactate production on xylose and glucose under nonbuffered conditions. Lactate was the main product produced by the *P. stipitis* *ldhL* transformants in the xylose cultivation. Its concentration reached a maximum of 15 g/liter after ~88 h of cultivation, with 20 g/liter xylose remaining (Fig. 2). Maximum ethanol concentrations with the *ldhL* transformants varied from 3 to 7 g/liter, while the control strains CBS6054 and FPL UC7 produced 25 g/liter ethanol (Table 1). Both the *ldhL* transformants (1.5 g/liter) and the control strains (1 g/liter) produced some xylitol. The rates of biomass accumulation and xylose consumption (per volume) were reduced in lactic acid-producing strains relative to those in control strains (Fig. 2B and Table 1; data not shown). Lactic acid production also resulted in more acidic conditions in the medium: at 41 h, the pH was 5.3 for the controls but 3.7 for the lactic acid-producing strains. Although the *ldhL* transformants continued to consume xylose for between 88 and 161 h, lactate and ethanol concentrations did not continue to increase (Fig. 2A). The yields of lactate and ethanol on xylose decreased throughout cultivation, from 0.42 and 0.11 g/g xylose, respectively, at 88 h to 0.30 and 0.06 g/g xylose, respectively, at 161 h. When the cultivation was terminated (161 h), ~4 g/liter residual xylose remained in the medium.

The *ldhL* strains produced 8 g/liter lactate at a 0.14-g/g final yield on glucose (Fig. 2A; Table 1). The maximum ethanol concentration (18 g/liter) was reduced to 80% of the wild-type level (22 g/liter) (Table 1). The control strains utilized all of the glucose within 24 h and produced approximately 20% more biomass than did the *ldhL* strains, which required 48 h to utilize all of the glucose. Production of lactic acid acidified the medium. The final pH in the culture supernatants was 4.9 for

TABLE 1. Lactate and ethanol concentrations and yields on xylose or glucose in YPX-53 and YPD-55 media^a

Carbon source and presence of LDH in strain	Lactate concn (g/liter)	Yield of lactate (g/g of sugar)	Ethanol concn (g/liter)	Yield of ethanol (g/g or sugar)	Time (h) at which all sugar was consumed
Xylose					
+LDH	15 ± 0.4	0.30 ± 0.01 ^b	2.9 ± 0.4 ^b	0.06 ± 0.007 ^b	161 ^c
-LDH	0	0	25 ± 0.2	0.47 ± 0.01	<41
Glucose					
+LDH	7.5 ± 0.3	0.14 ± 0.01	18 ± 0.5	0.33 ± 0.01	46–48
-LDH	0	0	22 ± 0.4	0.40 ± 0.00	<24

^a Average values for four independent *ldhL* transformants (+LDH) and two control strains (-LDH [CBS6054 and FPL UC7]) are shown for the indicated sample times. Data are means ± SEM (*n* = 4 to 8).

^b The values shown have already decreased from the maximum.

^c There was 4 g/liter xylose remaining at this time.

the control strains and 3.7 for the *ldhL* strains. A similar maximum lactate concentration, 8 g/liter, was produced when 97 g/liter glucose was provided (data not shown) relative to that produced when 55 g/liter (Fig. 2A) was provided, but 46 g/liter ethanol was produced in the medium containing the higher initial glucose concentration, compared to 18 g/liter in the medium containing 55 g/liter glucose.

The control strains produced similar final ethanol concentrations and yields on glucose and xylose under nonbuffered conditions (Table 1). However, the volumetric rate of xylose consumption was approximately twofold lower than the glucose consumption rate, since only 27 g/liter xylose was consumed within 24 h, whereas 55 g/liter glucose was consumed. The difference between the volumetric xylose and glucose utilization rates for the *ldhL* strains was greater than the corresponding difference for the control strain, with the xylose utilization rate decreasing more rapidly than the glucose utilization rate (Fig. 2B; Table 1). Low sugar utilization rates correlated with low biomasses. Both the sugar utilization rate and the biomass (OD_{600}) of the *ldhL* strains were approximately twofold lower with xylose than those with glucose during the first 24 h (Fig. 2B).

Lactate production in minimal medium with xylose or glucose buffered with $CaCO_3$. More lactate was produced on xylose in $CaCO_3$ -buffered YNBX-51 (31 g/liter lactate at a 0.60-g/g yield on xylose) than in nonbuffered YNBX-49 (9 g/liter lactate at a 0.29-g/g yield on xylose [data not shown]) or in the nonbuffered YPX-53 (15 g/liter lactate at a 0.30-g/g yield on xylose) cultivation (Fig. 3A and B; Table 2). The final pH in the culture was 6.4. Up to 6 g/liter ethanol was measured, but ethanol levels decreased before all of the xylose was consumed, and the final concentrations were ≤ 3 g/liter. In comparison, control strains CBS6054 and VTT-C-05788 produced 14 g/liter ethanol when grown in buffered YNBX-51 (Table 2).

Since lactate production was efficient under $CaCO_3$ -buffered conditions, two transformants were grown in the presence of a higher xylose concentration (101 g/liter). The final lactate concentration was 58 g/liter lactate, and the yield was 0.58 g/g xylose (Fig. 3C and D; Table 2). The final pH in the culture was 4.3. Xylose was converted to lactate at ~ 0.6 g/liter/h until the xylose concentration was ~ 10 g/liter, after which both the xylose utilization and lactate production rates were reduced (Fig. 3C and D). Ethanol accumulation ceased early in the incubation (24 h) and then remained constant (4 to 5 g/liter ethanol) (Fig. 3C).

Other than ethanol, *P. stipitis ldhL* strains produced relatively few by-products, with ≤ 1.3 g/liter xylitol and ≤ 0.3 g/liter pyruvate and no detectable acetate or glycerol (detection limit, 0.1 g/liter) produced from either 51 or 101 g/liter xylose.

P. stipitis ldhL transformants in $CaCO_3$ -buffered glucose (YNBD-54) (Fig. 3B; Table 2) produced considerably more lactate, at 18 g/liter, than did those under nonbuffered conditions (YNBD-47 [10 g/liter at a 0.24-g/g yield on glucose] [data not shown] or YPD [8 g/liter] [Fig. 2A]). The *ldhL* strains produced $\sim 75\%$ of the ethanol produced by the control strains (Table 2). When the glucose concentration was increased to 94 g/liter, lactate and ethanol concentrations increased proportionately, to 41 g/liter lactate and 21 g/liter ethanol (Fig. 3C; Table 2). Lactate was produced at the same production rate,

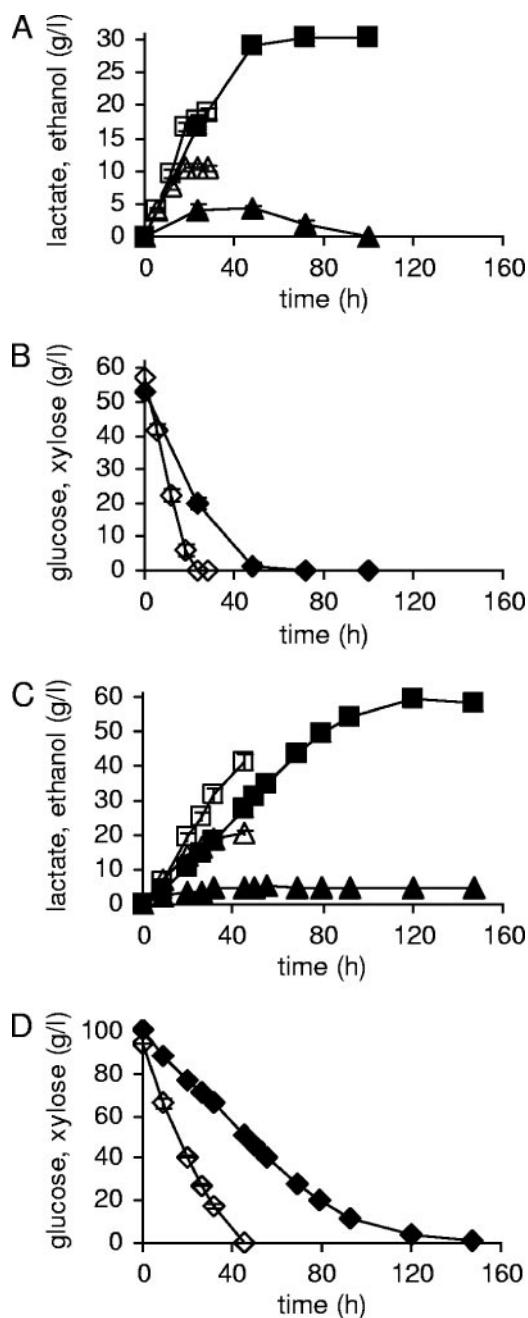


FIG. 3. Production of lactate on xylose (g/liter) (■) (A and C) or on glucose (g/liter) (□) (A and C), production of ethanol on xylose (g/liter) (▲) (A and C) or on glucose (g/liter) (△) (A and C), and consumption of xylose (g/liter) (◆) (B and D) or glucose (g/liter) (◇) (B and D) in $CaCO_3$ -buffered YNBX-51 (A and B), YNBX-54 (A and B), YNBX-101 (C and D), and YNBX-94 (C and D) by *P. stipitis ldhL* transformants. The initial OD_{600} was 13. The averages for four transformants are shown in panels A and B, and the averages for two transformants grown in duplicate are shown in panels C and D. Error bars indicate SEM. The SEM is less than the size of the symbol if no error bars are seen.

0.9 g/liter/h, at both glucose concentrations. In nonbuffered YNBX-47, the rate was <0.1 g/liter/h (data not shown).

Both the *ldhL* transformants and the control strains consumed xylose at a lower rate than that for glucose, as in the

TABLE 2. Lactate and ethanol concentrations and yields on xylose or glucose in YNBX-51, YNBX-101, YNBD-54, YNBD-94, and YNBDX-97 media containing 30 g/liter CaCO_3^a

Carbon source and presence of LDH in strain	Sugar concn (g/liter)	Lactate concn (g/liter)	Yield of lactate (g/g of sugar)	Ethanol concn (g/liter)	Yield of ethanol (g/g or sugar)	Time (h) at which all sugar was consumed
Xylose						
+LDH	51	31 ± 0.6	0.60 ± 0.02	1.2 ± 0.6^d	0.02 ± 0.01^d	72
	101	58 ± 0.6	0.58 ± 0.01	4.5 ± 0.5	0.05 ± 0.00	147
–LDH	51	0	0	14 ± 2.2	0.28 ± 0.04	48–54
Glucose						
+LDH	54	18 ± 0.6	0.33 ± 0.02	12 ± 1.1	0.23 ± 0.03	<24
	94	41 ± 1.9	0.44 ± 0.02	21 ± 0.7	0.22 ± 0.01	45
–LDH	54	0	0	17 ± 0.6	0.31 ± 0.01	<24
Xylose plus glucose						
+LDH	97 ^b	58 ± 0.6	0.60 ± 0.01	4.1 ± 0.3^d	0.04 ± 0.01^d	124
	97 ^c	58 ± 0.8	0.62 ± 0.01	3.4 ± 0.3^d	0.04 ± 0.01^d	124–147

^a Mean values $\pm$ SEM ($n = 4$ to 8) for *ldhL* transformants (+LDH) and two control strains (–LDH [CBS6054 and VTT-C-05788]) are shown for the indicated sample times.

^b Inoculated with xylose-grown cells.

^c Inoculated with glucose-grown cells.

^d The values shown have already decreased from the maximum.

nonbuffered cultivations (cf. Fig. 2 and 3 for *ldhL* strains; data not shown for control strains).

Lactate production in minimal medium containing a mixture of xylose and glucose buffered with CaCO_3 . The *ldhL* transformants consumed xylose and glucose simultaneously in a medium containing a mixture of glucose and xylose (YNBDX-97), even though glucose was consumed more rapidly than xylose (Fig. 4A). The rate of xylose consumption decreased in the presence of glucose relative to that in the cultures containing only xylose. Similarly, the rate of glucose consumption decreased in the presence of xylose. The inocula for the cultures in YNBDX-97 were generated in two different ways, with one grown in glucose-containing medium and the other grown in xylose-containing medium. However, the carbon source on which the inoculum had been growing did not affect lactate production, which was similar to that observed in medium containing only xylose (Fig. 4B; Table 2).

DISCUSSION

P. stipitis strains containing an integrated *ldhL* gene from *L. helveticus* coding for lactate dehydrogenase efficiently converted xylose and glucose to lactate. When *S. cerevisiae* is transformed to produce lactic acid, growth (10) and ethanol production (7, 17, 41) are reduced. Similar reductions in growth and ethanol production also occur in *P. stipitis* cultures growing on media containing either glucose or xylose. *ldhL* expression also decreased the volumetric sugar consumption rate. The effect of lactic acid production was more pronounced in media containing xylose than in media containing glucose.

P. stipitis ldhL strains produced relatively high concentrations of ethanol in nonbuffered cultures grown on glucose. Ethanol production was relatively inefficient in nonbuffered xylose medium, and the lactate concentration did not increase above 15 g/liter, even though only a portion of the xylose was consumed. The pH decreased as the lactic acid concentration increased. We think that the low pH and/or the accompanying intracellular acidification has multiple negative effects on cel-

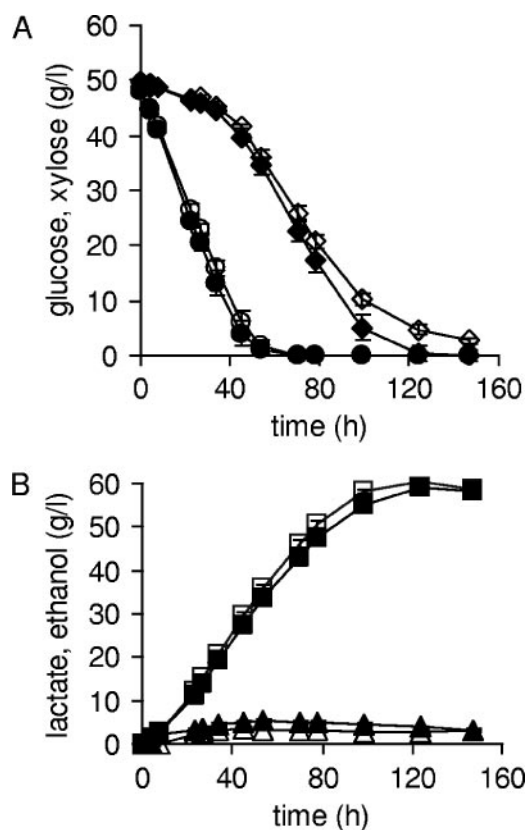


FIG. 4. Consumption of xylose (g/liter) (◆ and ◇) (A) and glucose (g/liter) (● and ○) (A) and production of lactate (g/liter) (■ and □) (B) and ethanol (g/liter) (▲ and △) (B) in a mixture of 50 g/liter xylose and 47 g/liter glucose in CaCO_3 -buffered YNBDX-97 by *P. stipitis ldhL* transformants. The cultures were inoculated to an initial OD_{600} of 13 with cells pregrown on xylose (closed symbols) or glucose (open symbols). The averages for two transformants grown in duplicate are shown. Error bars indicate SEM. The SEM is less than the size of the symbol if no error bars are seen.

lular metabolism and that one of these effects is the inefficient conversion of xylose to lactic acid. Export of lactate is also expected to be more energy demanding at low pH (44), and increased intracellular lactate concentrations may inhibit LDH. When CaCO_3 was added as a neutralizing agent, lactate production was sustained, resulting in higher lactate concentrations in media containing either xylose or glucose as the primary carbon source.

P. stipitis control and *ldhL* strains consumed xylose at a lower rate than that for glucose, as expected for *P. stipitis* and other xylose-utilizing fungi (22). Xylose was predominantly converted to lactate, and a smaller fraction of xylose than of glucose was converted to ethanol. On xylose, LDH competed efficiently for pyruvate, since the lactate yield was high and the ethanol yield was low. The apparent cessation of ethanol accumulation after 24 h by the *ldhL* strains in buffered YNBX-51 was surprising, since the parental control strain produced 14 g/liter ethanol under the same conditions, and there is no particular reason to believe that the ethanol pathway was not functional. Furthermore, *P. stipitis* pyruvate decarboxylase (PDC) and ADH activities are induced by oxygen limitation, not by glucose levels as in *S. cerevisiae*, in media containing either glucose or xylose (6, 24, 27). A significant portion of the ethanol produced on xylose may have been reassimilated, as occurs in *S. cerevisiae* even when the xylose concentration is not limiting (39, 40). On xylose, even with limited oxygen, the carbon metabolism of *P. stipitis* is largely respiratory, and the oxygen level has a strong effect on xylose fermentation (22, 39).

The lactate concentration and yield from 54 g/liter glucose obtained with the *ldhL* strain expressing *P. stipitis*, i.e., 18 g/liter and 0.33 g/g, respectively, are comparable to those reported for *S. cerevisiae* LDH strains with an intact ethanol pathway (7, 10, 30). Furthermore, on 94 g/liter glucose, *P. stipitis* produced 41 g/liter lactate at a 0.44-g/g yield on glucose, which is comparable to the 38 g/liter at a 0.45-g/g yield produced by a diploid *S. cerevisiae* strain expressing LDHA of *R. oryzae* (41). The amounts of ethanol produced by *P. stipitis* and diploid *S. cerevisiae* strains were also similar. High lactate concentrations but only moderate yields, i.e., 40 to 50 g/liter and 0.25 g/g glucose, were also obtained from *S. cerevisiae* cells expressing the LDH gene of *Lactobacillus plantarum* when these transformants were grown on media containing high levels (200 g/liter) of glucose (7). Higher lactate concentrations may also be obtained by modifying the ethanol pathway, e.g., in a *PDC1*-deleted *S. cerevisiae* strain (17, 32) or a *PDC1*-deleted *K. lactis* strain (4, 29). Further metabolic engineering of the *P. stipitis* *ldhL* strains, e.g., the deletion of one or both *PDC* genes and/or optimization of the culture conditions, could further increase lactate production and reduce by-product formation.

The strains we constructed produced up to 58 g/liter lactate acid, with yields of up to 0.58 g/g xylose. These yields are higher than the corresponding values on glucose. Thus, on xylose, the *P. stipitis* *ldhL* strains were as good as or better at producing lactate than the best reported *S. cerevisiae* strains (for glucose to lactate, 38 g/liter with a yield of 0.45 g/g [41], 50 g/liter with a yield of 0.62 g/g [17], and 58 g/liter with a yield of 0.30 g/g [7]).

P. stipitis has the advantage of being able to convert both hexose and pentose sugars to useful metabolites, such as lactic acid or ethanol, in contrast to currently available *S. cerevisiae*

strains, which are very efficient at glucose fermentation but cannot utilize pentose sugars unless they are metabolically engineered. *S. cerevisiae* can be engineered to ferment xylose (20, 45), but the production of lactate from xylose has not been reported previously. To exploit the lignocellulose-derived polymeric carbohydrates cellulose and xylan, whose main constituents are glucose and xylose, the fermenting organisms must be able to consume both sugars and to thus use a larger fraction of the raw material, with correspondingly better yields, than is possible if only glucose is consumed. Our results in the present study show that production of lactate from xylose by yeast is feasible and relatively efficient and that both sugars are simultaneously consumed in a mixture of glucose and xylose, albeit with glucose being exhausted earlier. These strains provide a new benchmark against which other strains can now be measured and should encourage further development of such yeast-based bioprocesses.

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