Efficient Homofermentative L-(+)-Lactic Acid Production from Xylose by a Novel Lactic Acid Bacterium, Enterococcus mundtii QU 25

Running title: HOMO-L-(+)-LACTATE PRODUCTION FROM XYLOSE

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Abstract

Enterococcus mundtii QU 25, a newly isolated lactic acid bacterium, efficiently metabolized xylose into \textit{L}-lactate. In batch fermentations, the strain produced 964 mM \textit{L}-(+)-lactate from 691 mM xylose, with a yield of 1.41 mol/mol-xylose consumed and an extremely high optical purity of 99.9% or higher without acetate production.

Text

Lactic acid bacteria (LAB) ferment sugars through different pathways, resulting in homo-, hetero-, or mixed acid fermentation. Few LAB strains produce lactate from xylose, the major constituent of hemicelluloses in lignocellulosic biomass and the second most abundant sugar, next to glucose, in nature (14). The phosphoketolase (PK-) pathway in LAB converts 2 of the 5 carbons in xylose to acetic acid, increasing the purification cost of lactic acid (6, 16, 20). Hence, the maximum theoretical yield of lactate is limited to 1 mole/mole of xylose (0.6 g lactate per gram of xylose). Therefore, LAB strains using the PK- pathway for xylose metabolism are not effective for the industrial-scale production of lactic acid. The lack of industrially suitable LAB for efficient conversion of xylose into lactate has been cited as a major technical obstacle in developing a poly-(lactic acid) industry.

\textit{Lactococcus lactis} IO-1 (JCM 7638), which was isolated and characterized in our laboratory, was reported to produce \textit{L}-lactate at high yield of 0.68 g/g of consumed xylose (exceeding 1.0 mol/mol). This result was achieved by fermenting xylose to lactate via 2 different pathways, the PK pathway and the pentose phosphate (PP)/glycolytic pathway (20). The PP/glycolytic pathway quantitatively converts xylose into a 3 carbon intermediate, pyruvate, providing the potential to produce 1.67 mol of lactate per mole of pentose (15, 16) (homo-
lactate-fermentation). In rare cases, homo-lactate fermentation of pentose is carried out by some streptococci. Fukui et al. (5) reported that Streptococcus sp. (Enterococcus sp.) and Lactobacillus thermophilus T1 metabolized D-xylose, L-arabinose and D-ribose to only lactate under anaerobic culture conditions. In 1978, Barre (2) isolated 1 Lactobacillus species from wine, named Lactobacillus MONT4. This species exclusively fermented arabinose and ribose, yielding DL-lactate. To the best of our knowledge, few researchers have performed studies on the development of engineered LAB strains to increase the lactate yield from xylose. Recently, Okano et al. (12, 13) used Lactobacillus plantarum NCIMB 8826. This species is deficient in the L-LDH gene, and its PK genes have been replaced with a heterologous transketolase gene. Owing to this development, L. plantarum ΔldhL1-xpk::tkt strain had been used to produce 41.2 g/L (458 mM) of D-lactate from 46.4 g/L (309 mM) xylose after 60 h of fermentation. However, to our knowledge, there has been no report on homo-L-(+)-lactate fermentation from xylose by LAB, in particular wild-type strains. Our laboratory has recently isolated a variety of new LAB from many natural sources. Here, we report the efficient fermentation of xylose to L-(+)-lactate with less by-products by one of these isolates, discovered from an ovine fecal sample collected from Fukuoka Zoo, Japan and identified as Enterococcus mundtii QU 25 NITE BP-965 (1).

For growth of E. mundtii QU 25, 1 mL of glycerol stock was transferred into 9 mL of mMRS medium (MRS broth with xylose instead of glucose) supplemented with 1% xylose in a 15-ml screw-capped tube and incubated at 30°C for 24 h. A 4mL aliquot of this culture was then transferred to a 50-mL vial containing 36 mL mMRS medium. The inoculum was incubated at 30°C for 8 h before inoculation at 10% (v/v) in a jar fermentor. Batch fermentations were carried out at 200 rpm in a 1-L jar fermentor (Biott, Tokyo, Japan), with a working volume of 0.4 L mMRS medium supplemented with 25 g/L xylose (final concentration, 166 mM). Analysis of
cell growth and dry cell weight (DCW) was evaluated as described previously (19). Xylose and fermentation products were determined using high-performance liquid chromatography as described previously (11, 19). The optical purity of the lactate product was measured using a BF-5 biosensor (Oji, Hyogo, Japan) according to the manufacturer's protocol. The purity of L-lactate was evaluated as follows: % optical purity = (L-lactate concentration – D-lactate concentration)/(L-lactate concentration + D-lactate concentration) × 100. To prepare the crude cell extract for enzyme assays, cells cultured at low (ca. 166 mM) or high (ca. 666 mM) xylose concentration for 12 h were harvested, suspended with respective buffers, disrupted using a French Pressure Cell, and centrifuged at 7,190×g for 15 min at 4°C. The obtained supernatants were used as crude cell extract. Activity of phosphoketolase in PK pathway was measured spectrophotometrically as ferric acetyl hydroxamate produced from the enzymatically generated acetyl phosphate at 43°C as previously described (10, 17). One unit of phosphoketolase activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of acetyl phosphate per min from xylose-5-phosphate. Activities of transaldolase and transketolase in PP/glycolytic pathway were measured at 43°C by coupling glyceraldehyde-3 phosphate production from either fructose-6-phosphate and erythrose-4-phosphate or ribulose-5-phosphate and xyluose-5-phosphate to NADH oxidation via triose-phosphate isomerase and glycerol phosphate dehydrogenase, as previously described (20). The enzyme activities were determined by subtracting NADH oxidase activity from the resulting rate of absorbance decrease at 340 nm. The activity of NADH oxidase was measured at 43°C in the same reaction buffer with transaldolase and transketolase containing NAD as sole substrate. One unit of enzyme activity is defined as the amount of the enzyme catalyzing the formation of 1 µmol glyceraldehyde-3 phosphate for transaldolase and transketolase, and 1 µmol NAD⁺ for NADH oxidase per min.
Under non-pH-controlled batch fermentations at 30°C, the rates of biomass accumulation and xylose consumption were very slow, with a maximum lactate concentration of 35 mM after 24 h of cultivation (Table 1). The residual xylose concentration was 131 mM. The inefficient conversion of xylose to lactate may be attributed to the low pH (5.0 after 24-h fermentation), which has a negative effect on cellular metabolism.

pH-controlled fermentations were carried out at 30°C to reduce the inhibitory effects of free lactate on the producer cells according to the method described previously (21). pH 7.0 provided the highest lactate produced (131 mM) and the maximum lactate productivity (12.1 mM/h), which increased by 274% and 365%, respectively, compared to non pH-controlled batches (Table 1). Furthermore, by optimizing the cultivation temperature, the flux to lactate increased at elevated temperatures. When the temperature was increased from 30°C to 43°C at pH 7.0, the amount of lactate produced and the maximal lactate productivity were increased by 52% and 81% (200 mM and 21.9 mM/h), respectively (Table 1).

In an attempt to obtain higher lactate concentrations, fermentations were carried out at pH 7.0 and 43°C with 3 levels of initial xylose concentrations: 334 mM (50.1 g/L) (Fig. 1A), 480 mM (72.0 g/L) (Fig. 1B), and 691 mM (103 g/L) (Fig. 1C). Table 2 summarizes the results of batch cultures at various xylose concentrations. The optical purity of L-lactate was ≥99.9% at all xylose levels. With 334 mM xylose, fermentation was almost completed within 24 h with a production of 490 mM lactate at a high yield of 1.51 mol/mol of xylose consumed (0.9 g of lactate per gram of xylose consumed), which is substantially higher than the highest reported yield of lactate (1.37 mol/mol) from xylose by wild-type LAB (5). Very small amounts of by-products were detected, i.e., ≤17 mM acetic acid, ≤13 mM formic acid, and ≤34 mM ethanol.

With 480 mM xylose, fermentation was almost completed at 48 h, with a production of 668 mM lactate.
lactate at a yield of 1.47 mol/mol of xylose consumed (0.88 g of lactate per gram of xylose consumed). There was a remarkable decrease in by-product formation, with maximum concentrations of 12, 9, and 15 mM for acetic acid, formic acid, and ethanol, respectively. With the highest level of xylose tested (691 mM), complete fermentation of xylose to 964 mM (86.7 g/L) lactate was achieved after 96 h of incubation. Lactate yield was calculated to be 1.41 mol/mol of xylose consumed (equivalent to 0.83 g of lactate per gram of xylose consumed), and no acetate was detected at the end of fermentation. The maximum specific productivity was almost the same at all xylose levels (Table 2). Furthermore, to investigate lactate production by strain QU 25 in nitrogen/vitamin-poor medium, we used a medium containing lower amounts of nitrogen and vitamin sources (5 g/l yeast extract) with the other components (K₂HPO₄, triammonium citrate, MgSO₄•7H₂O, MnSO₄•5H₂O, and Tween 80) than mMRS media (10 g/l peptone, 8 g/l beef extract, 4 g/l yeast extract). As the result, a slight decrease in lactate (771±5.02 mM) was observed at high yield of 1.41±0.009 mol/mol from 658 mM xylose under the optimum conditions, which indicated the economic feasibility of lactate production using strain QU 25.

Ilmen et al. (8) constructed *Pichia stipitis* that produced 644 mM (58 g/L) lactate, with a yield of up to 0.58 g/g of xylose. The recombinant *Corynebacterium glutamicum* produced lactate from xylose under anaerobic conditions with a yield of 0.54 g/g (9). During our manuscript preparation, Wang et al. (23) reported the highest concentration, 752 mM (67.7 g/L), of lactate in batch fermentation by a newly isolated *Bacillus* sp. strain; however, this product did not show optical purity. In addition, they used very high concentrations of calcium carbonate (60% w/w of xylose) as a neutralizing agent, which would probably produce a considerable amount of calcium sulfate (gypsum) during the conversion of calcium lactate to free L-lactate in...
addition to the high consumption of sulfuric acid during this process. Moreover, the resulting
gypsium poses considerable economic and ecological problems \((3, 4, 18, 22)\). Furthermore, their
strain showed substrate inhibition at higher xylose concentration, with a sharp decrease in lactate
production, and produced less than 333 mM (<30 g/L) of lactate from 681 mM (102 g/L) xylose.
Our strain produced a higher lactate concentration of 964 mM from 691 mM xylose with very
high optical purity \((\geq 99.9\%)\).

An analysis of products at the end of fermentation suggested a metabolic pathway of
strain QU 25 from xylose. Strain QU 25 could be presumed to utilize the PP/glycolytic pathway,
not the PK pathway, as the main pathway for xylose metabolism. This hypothesis was proposed
because lactate represented 95–97% of the total fermentation products, and the yield of lactate to
xylose reached up to 1.51 mol/mol (Table 2). This value is very close to the maximum
theoretical yield obtained through the PP/glycolytic pathway (1.67 mol/mol) and is comparable
to 1.48 mol/mol of the yield of lactate produced via only the PP/glycolytic pathway by PK gene-
disrupted \(L.\ plantarum\) mutant in homo-lactate fermentation with xylose \((12)\). In addition, very
small amounts of formic acid, acetic acid, and ethanol were produced during xylose fermentation
by the QU 25 strain, which are probably formed by pyruvate format lyase or pyruvate
dehydrogenase, phosphotransacetylase/acetate kinase, and aldehyde and alcohol dehydrogenases,
respectively, as previously reported \((14, 20)\). Note that these low by-products would probably
result in lower purification cost \((7)\). To prove our hypothesis, we investigated the key enzymes
relating to PK- and PP/glycolytic-pathways in the respective cells grown at low and high xylose
concentrations (166 mM and 666 mM). Phosphoketolase activity was detected with 17.7±0.390
U/mg-protein in cells grown at low xylose concentration but not at high xylose concentration.
Higher activities of transaldolase and transketolase (0.377±0.016, and 0.366±0.057 U/mg-protein,
respectively) were detected in cells grown at high xylose concentration rather than that at low
xylose concentration (0.314±0.011, and 0.286±0.062 U/mg-protein, respectively). These results
indicate that only PP/glycolytic-pathway is used for xylose metabolism of strain QU 25 at high
xylose concentration, however, PK- and PP/glycolytic-pathways are used for xylose catabolism
at low xylose concentration. To the best of our knowledge, no wild LAB strain was previously
reported to utilize PP-pathway only for the fermentation of xylose, which facilitates the near-
complete conversion of xylose into optically pure L-lactic acid.

In conclusion, the production of L-lactate from xylose by *E. mundtii* QU 25 is feasible
and more efficient than that of any other strains reported thus far. This strain may provide an
ideal wild-type microorganism for economical L-lactate production from renewable biomass
substrates. It should be especially emphasized that this is the first report on homo-L-(+)-lactate
fermentation with xylose by LAB.

**Acknowledgment**

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   Isolation and characterization of lactic acid bacterium for effective fermentation of


FIG 1. Profiles of lactate fermentation with xylose by *Enterococcus mundtii* QU 25.

Fermentations were conducted in a 1-L jar fermentor with 0.4-L working volume at 43°C, pH 7.0, and 200 rpm. The initial xylose concentrations were 334 mM (A), 480 mM (B), and 691 mM (C). Symbols: open circles, xylose concentration; closed circles, lactate concentration; open triangles, acetic acid concentration; closed triangles, formic acid concentration; open squares, ethanol concentration; closed squares, dry cell weight. Data points represent the means and standard deviations of results from 3 independent experiments. The standard deviation is less than the size of symbol if no error bars are seen.
TABLE 1

L-Lactate fermentation with xylose by *Enterococcus mundtii* QU 25 under different pHs and temperatures.

<table>
<thead>
<tr>
<th>pH controlled</th>
<th>Temperature (°C)</th>
<th>Maximum lactate production at the indicated time (mM) ± SD</th>
<th>Maximum lactate productivity (mM/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>30</td>
<td>35.0 ± 0.1</td>
<td>24</td>
</tr>
<tr>
<td>6.0</td>
<td>30</td>
<td>66.0 ± 7.6</td>
<td>24</td>
</tr>
<tr>
<td>6.5</td>
<td>30</td>
<td>126 ± 15.0</td>
<td>24</td>
</tr>
<tr>
<td>7.0</td>
<td>30</td>
<td>131 ± 1.8</td>
<td>24</td>
</tr>
<tr>
<td>7.5</td>
<td>30</td>
<td>87.0 ± 1.2</td>
<td>24</td>
</tr>
<tr>
<td>7.0</td>
<td>37</td>
<td>141 ± 20.0</td>
<td>24</td>
</tr>
<tr>
<td>7.0</td>
<td>41</td>
<td>155 ± 2.9</td>
<td>24</td>
</tr>
<tr>
<td>7.0</td>
<td>43</td>
<td>200 ± 3.1</td>
<td>16</td>
</tr>
<tr>
<td>7.0</td>
<td>45</td>
<td>168 ± 0.7</td>
<td>16</td>
</tr>
<tr>
<td>7.0</td>
<td>47</td>
<td>15.0 ± 0.1</td>
<td>24</td>
</tr>
</tbody>
</table>

Xylose concentration at the beginning of fermentation is 166 mM. Averages with standard deviations are based on three independent fermentations.
TABLE 2. Lactate fermentation with high concentration of xylose by *Enterococcus mundtii* QU 25

<table>
<thead>
<tr>
<th>Initial xylose concentration (mM)</th>
<th>Maximum cell mass (g/L)</th>
<th>Maximum lactate produced (mM)</th>
<th>Yield of products (mol-product/mol of xylose consumed)</th>
<th>Maximum specific productivity&lt;sup&gt;b&lt;/sup&gt; (mmol/g [DCW] of cells per h)</th>
<th>Maximum lactate productivity (mM/h)</th>
<th>Lactate/acetate (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>334</td>
<td>5.24</td>
<td>490</td>
<td>1.51±0.18 0.08±0.01 0.03±0.01 0.14±0.03 16.2 42.7</td>
<td>18.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>480</td>
<td>4.47</td>
<td>668</td>
<td>1.47±0.06 0.01±0.004 0.04±0.03 0.03±0.02 14.0 32.1</td>
<td>134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>691</td>
<td>3.56</td>
<td>964</td>
<td>1.41±0.09 0.00 0.02±0.01 0.009±0.001 16.4 38.0</td>
<td>unlimited</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fermentations were done at 43°C and pH 7. Averages with standard deviations are based on three independent fermentations.

<sup>a</sup> The maximum theoretical yield for lactate by pentose phosphate/glycolytic pathway (1.67 mol of lactate per mol of xylose).

<sup>b</sup> Maximum specific productivity for lactate.
Figure 1. Fermentation products and xylose (mM)

Dry cell weight (g/L)

Time (h)