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L-(+)-Lactic acid production by co-fermentation of cellobiose and xylose without carbon catabolite repression using *Enterococcus mundtii* QU 25

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The use of lignocellulosic biomass for the production of optically pure lactic acid remains challenging because it requires efficient utilisation of mixed sugars without carbon catabolite repression (CCR). *Enterococcus mundtii* QU 25, a novel L-lactic acid-producing strain, was used in this study to ferment mixed sugars. This strain exhibited apparent CCR in a glucose–xylose mixture; however, replacement of glucose by cellobiose (cellobiose–xylose mixture) led to simultaneous consumption of both sugars without CCR. The production of lactic acid and activity of enzymes related to xylose metabolism were also investigated. Xylose isomerase and xylulokinase specific activity in cellobiose–xylose grown cells was three times higher than that in glucose–xylose grown cells. The addition of yeast extract and ammonium hydroxide effectively improved sugar utilisation and cell growth. Under the optimal conditions with simulated lignocellulosic hydrolysates, a high L-lactic acid concentration (up to 163 g L^{-1}) was produced with a yield of 0.870 g g^{-1} and maximum productivity of 7.21 g L^{-1} h^{-1} without CCR in the fed-batch fermentation. Thus, we could establish rapid and simultaneous consumption of hexose and pentose sugars by using a lactic acid bacterium strain, which significantly increased production of high-purity L-lactic acid.

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Introduction

Lactic acid (2-hydroxypropionic acid, $CH_3CHOHCOOH$) is one of the most widely used organic acids in the food, pharmaceutical, cosmetic, and chemical industries. Lactic acid production is currently a subject of active research and development because it serves as feedstock in the synthesis of polylactic acid, which can be used for manufacturing of biodegradable materials.[1] Lactic acid can be produced by either chemical synthesis or microbial fermentation. Recently, lactic acid fermentation from non-food feedstock, such as renewable biomass, has been the focus of intense research interest as an environmentally friendly method of lactic acid production because of the low temperature and energy requirements, as well as the high optical purity of the produced lactic acid.[2,3]

Among the variety of potential renewable biomass sources, lignocellulosic biomass is available in large quantities with widespread distribution and comparatively low prices.[2] The major components of lignocellulose are cellulose (insoluble fibres of β-1,4-glucan), hemicellulose (polysaccharides such as xylans, mannans, and glucans other than β-1,4-glucan), and lignin (amorphous phenylpropanoid polymer).[2,3] The hydrolysates of lignocellulosic biomass after pretreatment and saccharification are mainly composed of mixed sugars, including hexoses (such as glucose and cellobiose) and pentoses (such as xylose and arabinose). Furthermore, the composition of mixed sugars differs depending not only on the type of lignocellulosic biomass but also on the method of pretreatment and saccharification.[1,3] Efficient fermentation of mixed sugars derived from lignocellulosic biomass to lactic acid by lactic acid bacteria (LAB) is hampered by the following problems: (1) few LAB can utilise xylose, (2) most of the xylose-utilising LAB produce by-products such as acetic acid and ethanol, and (3) LAB, as many other bacteria, tend to utilise a preferred (rapidly metabolisable) sugar (such as glucose) first, while inhibiting catabolism of sugars other than that preferred (such as xylose); a phenomenon well known as carbon

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catabolite repression (CCR).[4,5] As a result of CCR, the fermentation of xylose in sugar mixtures is inhibited by glucose, which becomes a major obstacle to efficient utilisation of all sugars derived from lignocellulosic biomass.[2]

In regard to lactic acid fermentation using LAB, various studies have reported the approaches to overcome CCR caused by glucose and improve fermentation of mixed sugars to lactic acid, such as (1) usage of two LAB strains specific for xylose and glucose,[6] (2) establishment of a fermentation process based on controlling glucose concentration at lower than the CCR threshold level,[7] and (3) genetic engineering of LAB strains by introduction of the genes related to xylose metabolism.[8,9] However, in those studies, wild type LAB exhibited not only simultaneous consumption of mixed sugars but also heterolactic acid fermentation with production of by-products, such as acetic acid and ethanol, resulting from xylose utilisation of heterofermentative LAB.[2] To date, there are no reports on production of lactic acid by wild type LAB through homolactic fermentation of mixed sugars derived from lignocellulosic biomass.

We recently reported a unique fermentation strategy for butanol production by using wild type clostridial bacterium based on replacement of glucose by cellobiose without CCR of xylose consumption.[10] On the other hand, *Enterococcus mundtii* QU 25, isolated and characterised in our laboratory, is a homofermentative LAB that can utilise xylose[11] and cellobiose[12] as a sole substrate to produce optically pure L-lactic acid. No studies on lactic acid fermentation using mixtures of cellobiose and xylose have yet been reported. Thus, the aim of the present work was to establish an efficient L-lactic acid production process without CCR by using wild type *E. mundtii* QU 25 and sugar mixtures containing cellobiose and xylose derived from lignocellulosic biomass.

Experimental

Microorganism and media

E. mundtii QU 25 was used throughout this study. The stock culture was stored at −80 °C in vials containing 15% (v/v) glycerol until use.

Unless otherwise stated, cell growth, inoculum preparation, and fermentation was conducted using a modified Man, Rogosa, and Sharpe (mMRS) medium containing the following components (L^{-1}): 10 g peptone (Becton, Dickinson and Company; Sparks, MD, USA), 8 g beef extract, 4 g yeast extract, 2 g K_2HPO_4, 5 g $CH_3COONa \cdot 3H_2O$, 2 g tri-ammonium citrate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 1 mL Tween 80 (all from Nacalai Tesque; Kyoto, Japan). As indicated in each experimental description, glucose, xylose (both from Nacalai Tesque), and cellobiose (Carbosynth; Berkshire, UK) were added at various concentrations as carbon sources. The medium pH was adjusted to 7.0 by 10 M NaOH prior to sterilisation at 115 °C for 20 min.

Fermentation process

For inoculum preparation, 1 mL of glycerol stock was inoculated into 9 mL mMRS medium containing 15 g L^{-1} cellobiose and 15 g L^{-1} xylose and refreshed for 24 h at 43 °C. Then, the pre-culture was performed by transferring 4 mL of the refreshed culture to a 100 mL flask with 36 mL mMRS medium and incubating at 43 °C for 8 h. All main cultures, except for those used in investigation of nitrogen sources, were grown at 43 °C in a 1 L jar fermenter (Biott; Tokyo, Japan) containing 0.4 L mMRS medium or in a test tube with 15 mL mMRS medium including 10% (v/v) of pre-culture grown under different conditions, as described for each experiment. Samples were taken at different time intervals and analysed for cell growth and composition of sugars and fermentation products.

For fermentation experiments, sugar mixtures containing 100 g L^{-1} glucose and 60 g L^{-1} xylose (G100X60) or 100 g L^{-1} cellobiose and 60 g L^{-1} xylose (C100X60) were used in mMRS medium. Batch fermentation was carried out at agitation of 200 rpm with an automatic pH control at 7.0 by 10 M NaOH addition.

To investigate the effects of nitrogen sources, C100X60-containing mMRS medium was supplemented with yeast extract, peptone, ammonium sulphate, or urea (all at 6 g L^{-1}). Batch fermentation was performed in test tubes containing 15 mL mMRS medium supplemented with 100 g L^{-1} $CaCO_3$ as a neutralizing agent.

To test lactic acid production using simulated energy cane hydrolysates,[13] mMRS medium containing 10 g L^{-1} glucose, 80 g L^{-1} cellobiose, and 40 g L^{-1} xylose (G10C80X40) was supplemented with 6 g L^{-1} yeast extract. Batch fermentation was carried out at agitation of 200 rpm and the pH was maintained at 7.0 by addition of 10 M NaOH or NH_4OH.

To test lactic acid production in the fed-batch fermentation, mMRS media (G10C80X40) was supplemented with 6 g L^{-1} yeast extract. The fed-batch fermentation was performed with agitation at 200 rpm and the pH was maintained at 7.0 with 10 M NH_4OH. A mixture of 5 g L^{-1} glucose, 40 g L^{-1} cellobiose, and 20 g L^{-1} xylose (G5C40X20) with 1 g L^{-1} yeast extract was added to the fermentation broth after 36 h cultivation.

Fermentation parameters

The fermentation parameters evaluated in this study were as follows: maximum sugar consumption rate (g L^{-1} h^{-1}) was calculated as the ratio of consumed sugar concentration (g L^{-1}) to each sampling period (h). The yield of lactic acid based on substrate consumed (g g^{-1}) was defined as the ratio of lactic acid produced (g L^{-1}) to amount of sugar consumed (g L^{-1}). Lactic acid productivity (g L^{-1} h^{-1}) was calculated as the ratio of the highest lactic acid concentration (g L^{-1}) to the indicated fermentation time (h). Maximum lactic acid productivity (g L^{-1} h^{-1}) was calculated between each sampling period within exponential growth phase.

Analytical methods

Cell growth was monitored by optical density at 562 nm (OD_{562}) using an ultraviolet (UV)-1600 visible spectrophotometer (BioSpec; Shimadzu; Tokyo, Japan). One unit of OD_{562} corresponded to 0.218 g dry cell weight (DCW) per L.[12] Cellobiose, xylose, glucose, and fermentation products were detected by high-performance liquid chromatography (HPLC; US HPLC-1210; Jasco, Tokyo, Japan) equipped with a SUGAR SH-1011 column

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(Shodex; Tokyo, Japan). Samples taken during fermentation were centrifuged at 2000 × *g* at 4 °C for 10 min to remove solids, and supernatants were filtered through a membrane filter (Dismic-13HP, 0.45 μm; Advantec; Tokyo, Japan). HPLC analysis was performed at the column temperature of 50 °C with 3 mM $HClO_4$ as a mobile phase at a flow rate of 1.0 mL min^{-1} using an injection volume of 20 μL. Protein concentration in crude enzyme extracts was determined by using the BCA Protein Assay Kit™ (Thermo Scientific; Rockford, IL, USA).

Preparation of crude enzyme extracts

For the preparation of crude enzyme extracts to measure the activity of xylose isomerase and xylulokinase, cells were grown in mMRS medium with G100X60 and C100X60 for 14 h when glucose and cellobiose were still present in the fermentation broth and collected by centrifugation at 8000 × *g* for 15 min at 4 °C. The harvested cells were washed twice with 0.85% (w/v) NaCl, suspended in 10 mL of 50 mM potassium phosphate buffer (pH 7.0), pretreated with 15 g L^{-1} lysozyme, and disrupted by using a French press. Cell debris was removed by centrifugation at 7190 × *g* for 10 min at 4 °C, and the clarified supernatants were used as crude extracts for the enzymatic assays described below.

To investigate the localisation of β-glucosidase, cell supernatants were ultracentrifuged at 75 000 × *g* for 40 min at 4 °C, and the enzymatic activity was measured in the intracellular and cell membrane-bound fractions.

Enzyme assays

Assays were performed using a Shimadzu UV-160A recording spectrophotometer and 1 mL quartz cuvettes at 43 °C. All chemicals were of analytical grade. Enzymes and other biochemical reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All experiments of each enzyme were carried out in triplicate.

Xylose isomerase (EC 5.3.1.5) activity was determined in a reaction mixture containing Tris–HCl buffer (pH 7.0), 100 mM; $MgCl_2$, 10 mM; NADH, 0.15 mM; sorbitol dehydrogenase, 2 U; and crude enzyme extracts. The reaction was initiated by addition of 500 mM xylose as described by Kuyper *et al.*[14] The decrease in the absorbance at 340 nm, indicative of NADH oxidation, was monitored for 5 min. One unit of the enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μmol xylulose per min.

Xylulokinase (EC 2.7.1.17) activity was measured as described previously[15] in a reaction mixture containing glycylglycine, 250 mM (pH 7.0); $MgSO_4$, 5 mM; NADH, 0.15 mM; pyruvate kinase, 1 U; lactate dehydrogenase, 3 U; phosphoenolpyruvate, 1.5 mM; D-xylulose, 5 mM; and ATP, 1 mM. The reaction was initiated by adding crude enzyme extracts, and the decrease in the absorbance at 340 nm during the reaction was monitored for 5 min. One unit of activity was defined as the amount of the enzyme phosphorylating 1 μmol of xylulose per min.

β-Glucosidase (EC 3.2.1.21) activity was estimated according to a previously described method[12,16] using *p*-nitrophenyl-β-D-glucopyranoside as substrate. The assay mixture (1 mL) consisting of 0.9 mL of substrate and 0.1 mL of diluted whole cell suspension was incubated at 43 °C for 30 min. The release of *p*-nitrophenol was measured at 410 nm and compared with the *p*-nitrophenol standard. One unit of the enzyme activity was equivalent to 1 μmol of *p*-nitrophenol generated per min.

Transketolase (EC 2.2. 1.1) activity in the pentose phosphate/glycolytic pathway was measured according to the method of Tanaka *et al.*[17] and Kochetov.[18] The reaction mixture contained citrate-phosphate buffer (pH 7.0), 100 mM; $MgCl_2$, 1.5 mM; triose phosphate isomerase, 0.5 U; α-glycerophosphate dehydrogenase, 0.5 U; thiamine pyrophosphate, 0.1 mM; NADH, 0.15 mM; potassium ribose 5-phosphate, 1.2 mM; potassium xylulose 5-phosphate, 0.9 mM; and crude enzyme extract. One unit of the enzyme activity was defined as the amount of the enzyme catalysing the formation of 1 μmol glyceraldehyde 3-phosphate per min.

Results and discussion

Batch fermentation using mixtures of glucose–xylose and cellobiose–xylose

In our previous study, it was shown that *E. mundtii* QU 25 efficiently produced lactic acid using high initial concentrations of glucose (100 g L^{-1}),[19] xylose (103 g L^{-1}),[11] and cellobiose (151 g L^{-1})[12] as the sole carbon sources. However, there were no reports on the fermentative production of lactic acid using high concentration of mixed sugars derived from lignocellulosic biomass. In this study, we initially performed batch fermentations with *E. mundtii* QU 25 using a mixture of glucose (100 g L^{-1}) and xylose (60 g L^{-1}) (G100X60). During the early phase (up to 24 h), *E. mundtii* QU 25 grew rapidly, reaching the maximum DCW of 3.19 g L^{-1} mainly by using glucose at a much higher maximum consumption rate (6.19 g L^{-1} h^{-1}) than xylose (0.748 g L^{-1} h^{-1}) (Fig. 1A and Table 1). Xylose consumption was not stimulated even after 24 h of cultivation when glucose concentration decreased to less than 20 g L^{-1}, and the amount of utilised xylose was only 25.6 g L^{-1} with a residual concentration of 36.7 g L^{-1} after 96 h. As a result, *E. mundtii* QU 25 growing on G100X60 produced 71.2 g L^{-1} lactic acid with a yield of 0.603 g g^{-1}. Thus, *E. mundtii* QU 25 growing on this sugar mixture apparently exhibited CCR of xylose utilisation, resulting in an insignificant decrease of xylose even after almost complete consumption of glucose.

On the other hand, when glucose was replaced by cellobiose (C100X60), the DCW was increased to 3.54 g L^{-1} (Table 1). C100X60-grown cultures also exhibited simultaneous consumption of both cellobiose and xylose significantly increasing the maximal consumption rate of xylose from 0.748 g L^{-1} h^{-1} in G100X60 to 1.78 g L^{-1} h^{-1} in C100X60, and demonstrating high total consumption (56.2 g L^{-1}) and low residual concentration (3.78 g L^{-1}) of xylose (Fig. 1B and Table 1). These results suggest that replacement of glucose by cellobiose could counteract CCR of xylose consumption. Furthermore, C100X60-grown cultures demonstrated a much higher lactic acid concentration of 90.2 g L^{-1} and yield of 0.756 g g^{-1} compared to those in G100X60 cultures (Table 1). Interestingly, only small amounts of by-product (0.369 g L^{-1} acetic acid) were detected. This study

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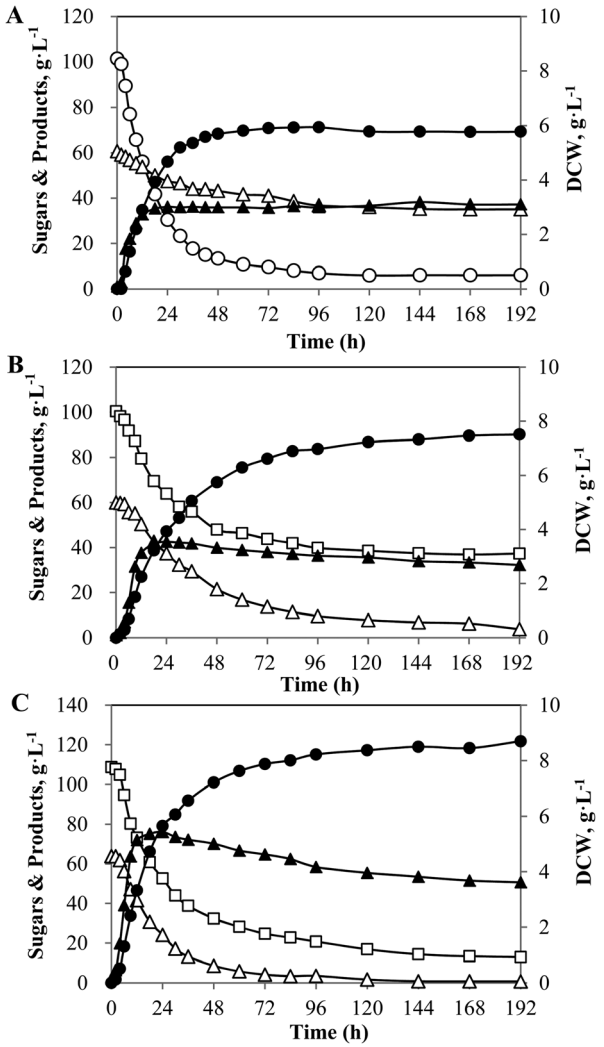

Fig. 1 Profile of lactic acid production from sugar mixtures G100X60 (A), C100X60 (B), and C100X60 supplemented with yeast extract (C). *E. mundtii* QU 25 was cultured in a 1 L jar fermenter containing 0.4 L mMRS medium at 43 °C with agitation at 200 rpm and pH 7.0 controlled with 10 M NaOH. Symbols: open circles, glucose concentration; closed circles, lactate concentration; open triangles, xylose concentration; closed triangles, dry cell weight; open squares, cellobiose concentration. Data points represent the mean values from three independent experiments.

succeeded in establishing conditions for homolactic fermentation of sugar mixtures by wild type LAB without CCR, which have not been previously reported. Other fermentation studies also showed that replacement of glucose by cellobiose prevented CCR of xylose consumption in production of ethanol and butanol using genetically engineered *Saccharomyces cerevisiae*[13] and wild type *Clostridium saccharoperbutylacetonicum*,[10] respectively.

Activity of enzymes involved in xylose metabolism in cultures with and without CCR

Here, we described a unique strategy to co-ferment hexose and pentose sugars simultaneously for production of lactic acid without glucose-induced CCR. Although CCR mechanisms in LAB have been poorly characterised, the activity of enzymes involved in sugar metabolism has been considered to be responsible for CCR in some LAB such as *Lactobacillus brevis*[20] and *Lactobacillus plantarum*.[8] However, knowledge of the related mechanisms in the *Enterococcus* genus is quite limited. In order to clarify CCR of xylose consumption by glucose and to understand the mechanisms underlying CCR absence in *E. mundtii* QU 25 grown on cellobiose and xylose mixtures, the activity of key enzymes in xylose metabolism and cellobiose hydrolysis were analysed in cells grown in C100X60 and G100X60 for 14 h (Table 2).

In the C100X60-grown cells, the enzymes initiating xylose catabolism, xylose isomerase, and xylulokinase, showed specific activities of more than three-fold higher (0.511 ± 0.057 and 0.633 ± 0.088 U per mg protein, respectively) than those in G100X60-grown cells (0.153 ± 0.011 and 0.183 ± 0.015 U per mg protein, respectively), which led to high xylose consumption in cellobiose-cultured cells. Therefore, in *E. mundtii* QU 25, glucose-induced CCR of xylose consumption might result from a low activity of xylose isomerase and xylulokinase, as observed in *L. brevis*[20] and *L. plantarum*.[8] On the other hand, the activity of transketolase, an intermediate enzyme in xylose catabolic pathway, was similar in C100X60 and G100X60 cultures (0.598 ± 0.160 and 0.530 ± 0.087 U per mg protein, respectively). Furthermore, the activity of β-glucosidase, which catalyses the hydrolysis of cellobiose to glucose, was much higher in C100X60 than in G100X60 whole cells suspensions (24.2 ± 1.8 and 0.266 ± 0.024 U per mg DCW, respectively) (Table 2). This value was almost equal to 25.7 U per mg DCW exhibited by *E. mundtii* QU 25 grown on cellobiose as a sole carbon source.[12] Furthermore, β-glucosidase was localised in the cytoplasmic membrane fraction (Table 2). Consequently, in *E. mundtii* QU 25 using the cellobiose and xylose mixture for lactic acid fermentation, CCR of xylose consumption could be avoided because of the high activity of the enzymes initiating xylose catabolism and simultaneous cellobiose consumption from the high activity of β-glucosidase.

In *Firmicutes* bacteria, several mechanisms of CCR have been considered, including genetic repression of metabolic enzymes.[4,21] In general, two proteins are thought to regulate genetic expression, phospho-carrier protein (HPr) and catabolite control protein A (CcpA). Serine-phosphorylated HPr couples with CcpA in the presence of the high intracellular concentration of glucose 6-phosphate and fructose 1,6-bisphosphate derived from glucose (favourable sugar), thereby repressing transcription of the genes or operons encoding metabolic enzymes for non-favourable sugars. However, we did not detect glucose in the fermentation broth of C100X60-grown *E. mundtii* QU 25 (Fig. 1B), and the cellobiose consumption rate (2.58 g L^{-1} h^{-1}) with C100X60 was much lower than the glucose consumption rate (6.19 g L^{-1} h^{-1}) with G100X60. Although it is not known whether glucose originated from the extracellular or intracellular hydrolysis of cellobiose, these results indicate that the hydrolysis of cellobiose by β-glucosidase is a rate-limiting step in lactic acid production, and that the intracellular concentration of glucose, glucose 6-phosphate, and fructose 1,6-bisphosphate in C100X60-grown cells is too low to cause

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Table 1 Kinetic parameters of lactic acid fermentation using sugar mixtures including glucose, xylose, and cellobiose by *E. mudtii* QU 25 in several fermentation modes

Mixed sugars[a]	Fermentation mode	Additional nutrient	pH neutraliser	Time[b] (h)	DCW_{max} (g L^{-1})	C_{Glc}[c] (g L^{-1})	C_{Cel}[d] (g L^{-1})	C_{Xyl}[e] (g L^{-1})	Max. r_{Cel}[f] (g L^{-1} h^{-1})	Max. r_{Xyl}[g] (g L^{-1} h^{-1})	Max. C_{LA}[h] (g L^{-1})	C_{AA}[i] (g L^{-1})	Y_{LA}[j] (g g^{-1})	Max. P_{LA}[k] (g L^{-1} h^{-1})
G100X60	Batch	—	NaOH	96	3.19	95.2	—	25.6	—	0.748 [0–2 h]	71.2	0	0.603	3.29 [6–9 h]
C100X60	Batch	—	NaOH	192	3.54	—	63.1	56.2	2.58 [9–12 h]	1.78 [4–6 h]	90.2	0.369	0.756	3.23 [6–9 h]
C100X60	Batch	Yeast extract	NaOH	192	5.43	—	95.8	63.2	5.03 [4–6 h]	3.05 [6–9 h]	122	0.382	0.766	5.58 [4–6 h]
G10C80X40	Batch	Yeast extract	NaOH	60	5.33	11.8	79.9	40.8	5.45 [4–6 h]	2.38 [4–6 h]	106	0	0.798	5.96 [4–6 h]
G10C80X40	Batch	Yeast extract	NH_4OH	60	6.67	10.9	83.7	39.1	4.23 [4–6 h]	2.44 [2–4 h]	115	0.148	0.863	7.33 [2–4 h]
G10C80X40	Fed-batch	Yeast extract	NH_4OH	240	6.41	14.8	113	58.7	4.51 [6–9 h]	2.50 [6–9 h]	163	0.723	0.870	7.21 [4–6 h]

[a] G100X60, glucose 100 g L^{-1} and xylose 60 g L^{-1}; C100X60, cellobiose 100 g L^{-1} and xylose 60 g L^{-1}; G10C80X40, glucose 10 g L^{-1}, cellobiose 80 g L^{-1} and xylose 40 g L^{-1}. [b] Fermentation time at maximum lactic acid concentration. [c] Glucose consumption. [d] Cellobiose consumption. [e] Xylose consumption. [f] Maximum cellobiose consumption rate at the indicated time. [g] Maximum xylose consumption rate at the indicated time. [h] Maximum lactic acid concentration. [i] Acetic acid concentration. [j] Yield of lactic acid at maximum lactic acid concentration. [k] Maximum productivity of lactic acid at the indicated time.

CCR. These considerations and the enzymatic activity results suggest that fermentation with C100X60 should not repress transcription of the genes encoding xylose isomerase and xylulokinase. More studies are needed to further confirm our hypothesis such as metabolome and transcriptome analyses and investigation of the transport systems for cellobiose and xylose.

Effects of nitrogen sources on lactic acid production

The addition of nitrogen sources to bacteria growth media has been shown to stimulate not only sugar utilisation but also lactic acid production.[22] In normal MRS media, the C : N ratio was extremely high because of the high concentration of carbon sources in C100X60, which led to 37.3 g L^{-1} residual cellobiose even after 192 h fermentation (Fig. 1B). In this study, the effects of several organic and inorganic nitrogen sources on sugar utilisation and lactic acid production were investigated in order to improve sugar consumption by *E. mundtii* QU 25. In a preliminary experiment, batch fermentations in C100X60-containing mMRS medium supplemented with 6 g L^{-1} of yeast extract, peptone, ammonium sulphate, or urea was performed. Among the nitrogen sources, yeast extract showed the best improvement in the consumption of both cellobiose and xylose as well as in the production of lactic acid (concentration and yield) (data not shown).

The consumption of cellobiose and xylose was enhanced by the addition of yeast extract (Fig 1B and C and Table 1). The maximal DCW was also significantly increased from 3.54 to 5.43 g L^{-1} following the addition of yeast extract (Table 1). Furthermore, simultaneous utilisation of cellobiose and xylose was achieved (Fig. 1C). As a result, the addition of yeast extract could improve lactic acid production (concentration, 122 g L^{-1}, maximum productivity, 5.58 g L^{-1} h^{-1}, and yield, 0.766 g g^{-1}) compared to the corresponding parameters (90.2 g L^{-1}, 3.23 g L^{-1} h^{-1}, and 0.756 g g^{-1}, respectively) in the culture without any additions. Increases in sugar utilisation, cell growth, and lactic acid production by supplementation with yeast extract have been also reported for other LAB including *L. delbrueckii*[23] and *L. coryniformis* subsp. *torquens*.[24] In summary, the addition of yeast extract significantly improved both the utilisation of mixed sugars and the production of lactic acid by *E. mundtii* QU 25.

Lactic acid production from simulated energy cane hydrolysate

Energy cane is a hybrid of commercial and wild sugarcanes and a source of inedible biomass, which has a much higher fibre content (including cellulose and hemicellulose) than commercial sugarcane. There is growing interest in using energy cane for production of valuable chemicals and fuel.[25] Recently, ethanol production from simulated energy cane hydrolysates with simultaneous utilisation of mixed sugars by genetically engineered *S. cerevisiae* (10 g L^{-1} glucose, 80 g L^{-1} cellobiose, and 40 g L^{-1} xylose) has been reported.[13] Therefore, to investigate the feasibility of lactic acid production from energy cane hydrolysates, batch fermentations were carried out in G10C80X40-containing mMRS medium supplemented with 6 g

Table 2 Enzymatic activities for cells grown in media containing glucose–xylose or cellobiose–xylose mixture[a]

	Xylose isomerase	Xylulokinase	Transketolase	β-Glucosidase			
Carbon source[b]	(U per mg protein)	(U per mg protein)	(U per mg protein)	(U per mg of DCW) [whole cell]	(U per mg protein) [cell-bound]	(U per mg protein) [intracellular]	(U per mg protein) [extracellular]
G100X60	0.153 ± 0.011	0.183 ± 0.015	0.530 ± 0.087	0.266 ± 0.024	ND[c]	ND[c]	ND[c]
C100X60	0.511 ± 0.057	0.633 ± 0.088	0.598 ± 0.160	24.2 ± 1.8	2.39 ± 0.62	ND[c]	ND[c]

[a] Averages with standard deviations are based on three independent fermentations. [b] G100X60, glucose 100 g L^{-1} and xylose 60 g L^{-1}; C100X60, cellobiose 100 g L^{-1} and xylose 60 g L^{-1}. [c] Not detected.

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L^{-1} yeast extract with pH controlled at 7.0 by 10 M NaOH or NH_4OH as neutralisers.

The batch fermentation with G10C80X40 using NaOH resulted not only in simultaneous sugar utilisation without CCR but also in a high DCW (5.33 g L^{-1}) (Fig. 2A) similar to that obtained with C100X60 (5.43 g L^{-1}) (Fig. 1C). This suggested that low glucose concentration in hydrolysates derived from lignocellulosic biomass should not affect the ability of *E. mundtii* QU 25 to simultaneously ferment hexose and pentose sugars into lactic acid. Although the 106 g L^{-1} lactic acid concentration obtained with G10C80X40 was lower than the 122 g L^{-1} obtained with C100X60 (due to a lower initial sugar concentration), the lactic acid yield (0.798 g g^{-1}) and the maximal lactic acid productivity (5.96 g L^{-1} h^{-1}) obtained with G10C80X40 were comparable or higher than those obtained with C100X60 (0.766 g g^{-1} and 5.58 g L^{-1} h^{-1}, respectively). Thus, these results support the feasibility of efficient lactic acid production from energy cane hydrolysates by *E. mundtii* QU 25 without CCR.

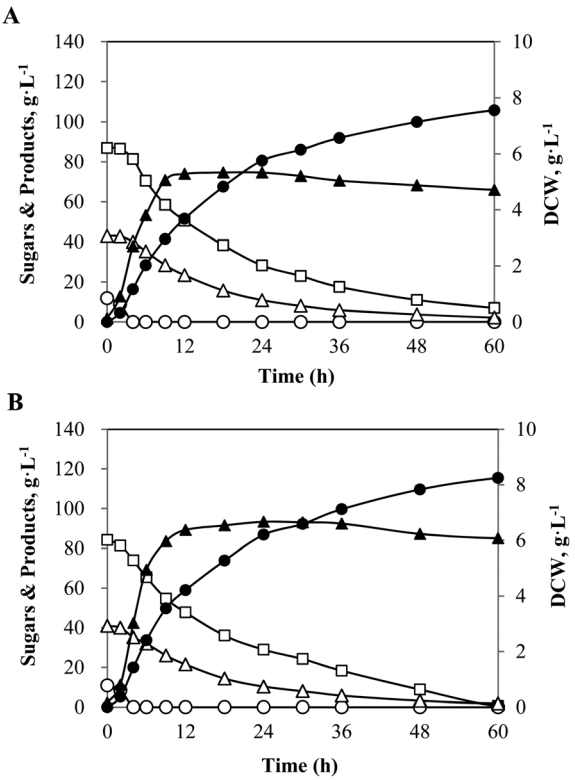

Fig. 2 Production of lactic acid by *E. mundtii* QU 25 from simulated energy cane hydrolysate (G10C80X40) in the batch fermentation using different neutralising agents, (A) NaOH and (B) NH_4OH. Symbols: open circles, glucose concentration; closed circles, lactate concentration; open triangles, xylose concentration; closed triangles, dry cell weight (DCW); open squares, cellobiose concentration. Data points represent the mean values from three independent experiments.

To investigate the effect of pH neutraliser on lactic acid production from mixed sugars, the batch fermentation was performed in G10C80X40-grown cultures at pH 7.0 controlled with 10 M NH_4OH. As shown in Fig. 2B, in NaH_4OH-controlled cultures, the maximal DCW increased from 5.33 to 6.67 g L^{-1}. Furthermore, sugar concentration and consumption were quite similar between NH_4OH- and NaOH-controlled cultures (Table 1 and Fig. 2). Surprisingly, despite the similar sugar consumption, lactic acid production (concentration, 115 g L^{-1}; maximum productivity, 7.33 g L^{-1} h^{-1}; yield, 0.863 g g^{-1}) was markedly improved by using NH_4OH compared to NaOH (106 g L^{-1}, 5.96 g L^{-1} h^{-1}, and 0.798 g g^{-1}, respectively). Inhibition of Na^+ on LAB growth has been reported.[26] In addition, using NH_4OH as a neutraliser enhanced cell growth of several LAB, including *L. delbrueckii* subsp. *lactis*, *Lactobacillus pentosus*, *L. brevis*, and *Leuconostoc* sp.,[27,28] because NH_4^+ is considered to serve as a nitrogen source for LAB,[29] and lactic acid production by LAB is known to be associated with cell growth.[30] These results indicate that NH_4OH is a better neutraliser for pH control instead of NaOH, which can also serve as a nitrogen source to stimulate growth of *E. mundtii* QU 25, thereby preventing inhibition by Na^+.

To achieve homolactic acid production process without CCR from various lignocellulosic biomasses, the sugar mixture compositions of hydrolysates suggested to be significant, in particular concentrations of xylose and glucose. While initial xylose concentration of more than 40 g L^{-1} exhibited homolactic acid production (Table 1 and Fig. 2), heterolactic acid production was observed from low initial xylose concentration (<*ca.* 10 g L^{-1}) using glucose–xylose mixture (unpublished data). Although CCR of xylose consumption was considered to occur under high glucose concentration of 25 g L^{-1} and over in glucose–xylose mixture (unpublished data), even high concentration of cellobiose (80 g L^{-1}) in sugar mixture with low glucose (10 g L^{-1}) should not result in CCR (Fig. 2). These considerations suggested the accomplishment of homolactic acid fermentation without CCR by *E. mundtii* QU 25 from

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lignocellulosic biomass-derived hydrolysates containing mainly xylose (≥10 g L^{-1}) and cellobiose with less glucose (<*ca.* 25 g L^{-1}). Therefore, it is desirable for hydrolysates to be obtained from lignocellulosic biomasses containing high proportion of hemicellulose such as leaves[2] and grasses[2] by β-glucosidase-free cellulases to suppress the formation of glucose.[31]

Improved lactic acid production in fed-batch fermentation mode

All sugars in the mixture were completely consumed when the simulated energy cane hydrolysate was used in the batch fermentation (Fig. 2B). In an attempt to improve lactic acid production, a single-pulse fed-batch fermentation was performed: the culture started in G10C80X40-containing mMRS medium supplemented with 6 g L^{-1} yeast extract and was additionally fed with G5C40X20 and 1 g L^{-1} yeast extract after 36 h (pH was controlled at 7.0 with 10 M NH_4OH) (Fig. 3 and Table 1). After additional feeding, *E. mundtii* QU 25 fermented all the sugars (glucose, cellobiose, and xylose) simultaneously to lactic acid (Fig. 3). As a result, the fed-batch fermentation significantly improved sugar consumption (cellobiose, 113 g L^{-1}; xylose, 58.7 g L^{-1}) and L-lactic acid production (163 g L^{-1}) at an optical purity of ≥99.7% compared to those values observed in the batch fermentation (83.7 g L^{-1}, 39.1 g L^{-1}, and 115 g L^{-1}, respectively) (Table 1). In addition, the maximum consumption rates of cellobiose (4.51 g L^{-1} h^{-1}) and xylose (2.50 g L^{-1} h^{-1}), and the maximum lactic acid productivity (7.21 g L^{-1} h^{-1}) and yield (0.870 g g^{-1}) in the fed-batch fermentation were quite comparable to those in the batch fermentation (4.23 g L^{-1} h^{-1}, 2.44 g L^{-1} h^{-1}, 7.33 g L^{-1} h^{-1}, and 0.863 g g^{-1}, respectively). These results indicate that the fed-batch fermentation method is a better approach than batch fermentation in regard to improvement of mixed sugar consumption and lactic acid production without CCR.

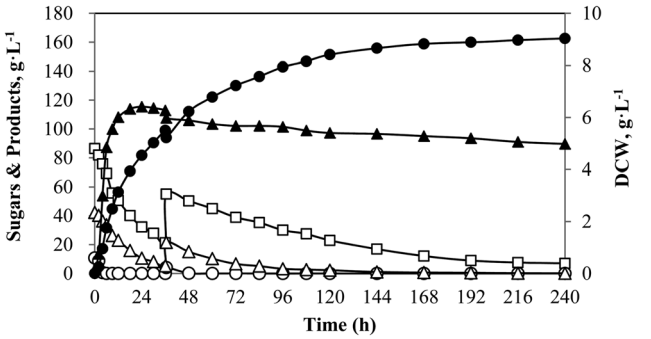

Fig. 3 Production of lactic acid by *E. mundtii* QU 25 from simulated energy cane hydrolysate by single-pulse fed-batch fermentation using NH_4OH as a neutralising agent. Fermentation was conducted in a 1 L jar fermenter containing 0.4 L of mMRS medium with initial G10C80X40 sugar mixture and 6 g L^{-1} yeast extract. G5C40X20 mixture and 1 g L^{-1} yeast extract were added at 36 h of cultivation. Symbols: open circles, glucose concentration; closed circles, lactate concentration; open triangles, xylose concentration; closed triangles, dry cell weight (DCW); open squares, cellobiose concentration. Data points represent the mean values from three independent experiments.

There is currently great interest in efficiently simultaneous utilizations of pentoses and hexoses in hydrolysates derived from lignocellulosic biomasses in co-fermentation for lactic acid production.[2] Table 3 shows the results of recent studies on lactic acid co-fermentation by several microorganisms such as fungi,[32] genetically engineered bacteria (*Escherichia coli*[33] and *L. plantarum*[8]), and *L. brevis*[20] using several sugar mixtures derived from lignocellulosic biomass. Basically, most of these studies mainly investigated mixtures of glucose and xylose in the batch fermentations. A few authors reported production of lactic acid without CCR by using genetically engineered strains.[8,33] The

Table 3 Comparison on recent data with present work using various sugar mixtures derived from lignocellulosic biomass

Substrate[a]	Microorganism	Fermentation mode	C_{LA}[b] (g L^{-1})	Y_{LA}[c] (g g^{-1})	P_{LA}[d] (g L^{-1} h^{-1})	Max. P_{LA}[e] (g L^{-1} h^{-1})	Isomer (optical purity, %)	pH neutraliser	Ref.
G75X25	*Rhizopus oryzae* RQ4015	Batch	83	0.83	1.38	2.1	L (−)	Na_2CO_3	32
G40X40	*Escherichia coli* FBR19 *ΔptsG*	Batch	64.3	0.77	0.45	—	L (−)	2.5 M KOH + 2.5 M NaOH	33
G50X25A5	*Lactobacillus plantarum ΔldhL1::PxylAB-xpk1::tkt-Δxpk2::PxylAB*	Batch	61.2	0.80	1.7	4.9	D (99.5)	10 M NH_4OH	8
G75X25	*Lactobacillus plantarum ΔldhL1::PxylAB-xpk1::tkt-Δxpk2::PxylAB*	Batch	74.2	0.78	2.85	5.6	D (99.5)	10 M NH_4OH	8
G10X10	*Lactobacillus brevis* ATCC 14869	Batch	12.5	0.579	0.568	—	—	No control	20
C100X60	*Enterococcus mundtii* QU 25	Batch	122	0.766	0.635	5.58	L (99.7)	10 M NaOH	This work
G10C80X40	*Enterococcus mundtii* QU 25	Fed-batch	163	0.870	0.679	7.21	L (99.7)	10 M NH_4OH	This work

[a] G75X25, glucose 75 g L^{-1} and xylose 25 g L^{-1}; G40X40, glucose 40 g L^{-1} and xylose 40 g L^{-1}; G50X25A5, glucose 50 g L^{-1}, xylose 25 g L^{-1} and arabinose 5 g L^{-1}; G10X10, glucose 10 g L^{-1} and xylose 10 g L^{-1}; C100X60, cellobiose 100 g L^{-1} and xylose 60 g L^{-1}; G10C80X40, glucose 10 g L^{-1}, cellobiose 80 g L^{-1} and xylose 40 g L^{-1}. [b] C_{LA}, maximum lactic acid concentration. [c] Y_{LA}, lactic acid yield. [d] P_{LA}, overall lactic acid productivity. [e] Max. P_{LA}, maximum lactic acid productivity. —, not described.

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present study is the first to investigate lactic acid fermentation from glucose–cellobiose–xylose mixture and to overcome CCR of xylose consumption even in the presence of low glucose by using cellobiose, which resulted in a high yield of lactic acid and fewer by-products. In addition, we first establish a fed-batch fermentation process for lactic acid production using sugar mixtures and could produce approximately 2 times higher lactic acid (163 g L^{-1}) than the maximum reported value (83 g L^{-1}) previously.[32] Compared with low lactic acid concentrations in the literatures, the obtained high concentration of lactic acid should be separated and purified from the fermentation broth more easily by several downstream technologies such as electrodialysis[34] and crystallization.[35] Moreover, because a high concentration of β-glucosidase is always required for complete the hydrolysis of cellulose to glucose during the pretreatment of lignocellulosic biomass,[36] our fermentation approach should reduce the cost of this process. Furthermore, to the best of our knowledge, we achieved the highest L-lactic acid concentration (163 g L^{-1}), maximum lactic acid productivity (7.21 g L^{-1} h^{-1}), and yield (0.870 g g^{-1}) to date at ≥99.7% of optical purity in the fed-batch fermentation. Therefore, this study demonstrates potential of using low-cost feedstock of lignocellulosic biomass for the production of valuable compounds.

Conclusion

We first demonstrated a unique strategy for co-fermentation of hexose and pentose sugars derived from lignocellulosic biomass by *E. mundtii* QU 25 and for the production of L-lactic acid by using cellobiose as an alternative substrate to glucose. We found that CCR of xylose consumption by glucose may be caused by low activity of the enzymes initiating the catabolism of xylose, and that fermentation of cellobiose did not negatively affect the activity of these enzymes. Furthermore, supplementation with yeast extract and the use of NH_4OH as a pH neutraliser improved the sugar utilisation and production of high purity lactic acid. Finally, an efficient system of L-lactic acid production based on the fed-batch fermentation of mixed sugars was successfully established.

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