Enterococcus faecium s6 Enabled Efficient Homofermentative Lactic Acid Production from Xylan-Derived Sugars

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Abstract: A thermotolerant Enterococcus faecium s6 strain exhibited homofermentative lactic acid (LA) production at high xylose concentration (≥50 g/L). In batch fermentation at 45 °C and controlled pH of 6.5, strain E. faecium s6 produced up to 72.9 g/L of LA with a yield of 0.99 g/g consumed xylose and productivity of 1.74 g/L.h from 75 g/L xylose. An increased LA concentration and productivities with high yields were obtained in repeated batch fermentation that was conducted for 24 runs. In this mode, the strain could produce LA up 81.1 g/L within 5 h fermentation at a high productivity of 13.5 g/L.h and a yield of 1.02 g/g consumed xylose. The strain was unable to consume birchwood xylan as sole carbon source. However, it could efficiently utilize xylooligosaccharides of xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose. The intracellular xylosidase activity was induced by xylose. Using xylooligosaccharide (50 g/L)/xylose (5 g/L) mixtures, the strain was able to produce maximum LA at 48.2 g/L within 120 h at a yield of 1.0 g/g consumed sugar and productivity of 0.331 g/L.h. These results indicate that E. faecium s6 is capable of directly utilizing xylan-hydrolyzate and will enable the development of a feasible and economical approach to the production of LA from hemicellulosic hydrolysate.

Keywords: xylooligosaccharides; xylose; homofermentation; lactic acid; β-xylosidase; repeated-batch fermentation

1. Introduction

Lactic acid (LA) is an important chemical mainly used in the cosmetic, food, chemical, and pharmaceutical industries. It also has the potential to replace petrochemical-derived chemicals for production of bioplastic polyactic acid materials [1]. Lactic acid can be produced by chemical synthesis or microbial fermentation routes by utilizing sugar-containing substrates. The chemical synthetic production of LA uses lactonitrile as a starting substrate and produces DL-LA racemic mixture. On the other hand, microbial fermentative LA production processes are preferred because they produce optically pure D- or L-LA that is important in the formation of bioplastic polymers with desirable mechanical properties and specific practical application. Moreover, fermentative LA production can make use of renewable feedstock, requires mild operation conditions, and has a low energy consumption [2].

Cellulosic biomasses, either lignocellulosic or algal biomass materials, are renewable materials that are considered of the most abundant feedstock in the world [3]. Such materials are mainly composed of polymeric sugars (cellulose, and hemicellulose). Direct utilization of these polymeric sugars is challenging as most LA bacteria (LAB) lack the ability to hydrolyze it [2]. Therefore, they require various pretreatments to separate and depolymerize their components into constituent monosaccharides or simple sugars, which can then be consumed by LAB. These include chemical, physical, and enzymatic hydrolysis treatments that solubilize and degrade it [4].
Cellulose fraction of cellulosic biomass is mainly composed of glucose that is available for all LAB. However, hemicellulosic fraction is mainly composed of xylan [5]. The amount of xylan is varied among different biomasses and is mainly composed of xylose that is unavailable as substrate for most microorganisms [6]. If xylose could be utilized as a substrate for LA fermentation, various forms of wood biomass can be used effectively as cheap substrate instead of expensive pure sugars [7]. Very few LAB, such as Levlac-tobacillus brevis [8], Lactococcus lactis IO-1 [9], Lactiplantibacillus pentosus [10], Leuconostoc lactis [11], E. munditii QU 25 [12], and E. faecium QU 50 [13], have been reported to ferment xylose. Except in E. munditii QU 25, and E. faecium QU 50, xylose is converted to LA by a heterofermentation pattern via phosphoketolase pathway, whereas xylose is converted to equimolar amounts of LA and acetic acid that limit the product yield to a maximum of 0.6 g/β-consumed xylose [9]. Recently, we have identified a thermotolerant agro-industrial waste-utilizing LA bacterium, E. faecium s6. Interestingly, this strain showed the capability of utilizing xylose [14], and might be efficient for utilization of various materials for LA production in a consolidated bioprocess.

The main objective of this work was to demonstrate the ability of E. faecium s6 to utilize hemicellulosic fraction in the cellulosic biomass for LA production. Firstly, the fermentation pattern of xylose conversion to LA was investigated. Then, the feasibility of using repeated-batch fermentation with increment of xylose concentration and utilization of low-cost fermentation media was explored to improve LA productivity and LA titer. Utilization of xylooligosaccharides (xylobiose, xyotriose, xylotetraose, xylopentaose, and xylohexaose) was also investigated. Utilization of mixed xylooligosaccharides and investigation of β-D-xylosidase activities were also studied. This study addresses the feasibility of LA production by E. faecium s6 from xylan-derived sugars.

2. Materials and Methods

2.1. Bacterial Strain and Fermentation Media

*Enterococcus faecium* s6, a lactic acid bacterium, previously characterized and identified by Alrefaey et al., was used in this study [14]. The modified deMan Rogosa Sharpe (mMRS) medium was used for cell growth and for inoculum preparation. It is composed of (g/L): yeast extract, 4.0; peptone, 10.0; beef extract, 8.0; K$_2$HPO$_4$, 2.0; MgSO$_4$, 0.1; MnSO$_4$, 0.05; sodium acetate, 5.0; ammonium citrate, 2.0; tween 80, 1.0 mL; and xylose, at specific concentrations as mentioned in each experiment. It was also used for main culture fermentation experiments except that xylose or xylooligosaccharides were used as substrate at specific concentrations as described in each experiment. The medium pH was adjusted to 6.5 using 10 N HCl and 10 N NaOH, and temperature was set at 45 °C. All chemicals were purchased from Sigma-Aldrich (St. Louis, MI, USA), unless mentioned otherwise.

2.2. Inoculum Preparation and Lactic Acid Production from Xylose

The strain s6 was cultivated firstly at modified mMRS medium containing xylose at 45 °C for 18 h. Stock cultures were prepared by transferring 1 mL of culture broth into 1 mL of sterile glycerol (30%, v/v) in 2 mL vials and maintained at −80 °C. For inoculum preparation, 1.0 mL of glycerol stock was transferred to a sterile tube containing 9 mL mMRS medium with 20 g/L xylose and cultivated at 45 °C for 18 h (seed culture). A pre-culture was prepared by transferring seed culture (at 10%, v/v) into the mMRS medium with 20 g/L xylose and incubated at 45 °C for 18 h.

To investigate LA production at different xylose concentrations, mMRS media were prepared at various concentrations (viz., 5, 10, 15, 50, 75, 90, and 100 g/L). Batch fermentations were performed in a one liter fermenter with a 0.4 L working volume that was inoculated at 10% from the pre-culture broth with agitation at 200 rpm. Fermentations were performed at 45 °C under controlled pH conditions (6.5) that were maintained by the addition of 10 N NaOH as a neutralizing agent. Samples were withdrawn at different time intervals to analyze biomass, xylose, and fermentation product concentrations.
To investigate LA production in repeated batch fermentation, fermentations were performed for 24 runs using 0.4 L in a 1 L jar fermentor supplemented with the specific xylose concentrations, incubated at 45 °C, and had pH controlled at 6.5 using 10 N NaOH. At the end of each run, the fermentation broth was centrifuged at 3226 × g for 10 min at 5 °C, and all the cells were resuspended in new fresh media to perform next run. Fermentation processes were initially conducted using optimized mMRS medium supplemented with 50 g/L xylose in the first 13 runs, 72 g/L xylose from run 14 to run 17, and 80 g/L xylose from run 18 to run 21. Run 22–24 were supplemented with 80 g/L xylose, but exclusion of peptone from medium composition was carried out in the 22nd run, while peptone and beef extract were excluded in the 23rd run. Peptone and beef extract were also excluded in the 24th run with supplementation of corn steep liquor at 10 g/L. Samples were withdrawn at different time intervals to analyze biomass, xylose, and fermentation product concentrations.

2.3. Lactic Acid Production from Xylan and Xylooligosaccharides

To investigate the utilization of xylan, mMRS media supplemented with 20 g/L birchwood xylan (Sigma-Aldrich) was prepared. Fermentations were performed at a 1 L fermentor at conditions mentioned above (the same as xylose fermentation experiments).

For LA production from individual xylooligosaccharides, mMRS media supplemented with around 5 g/L of xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose (Megazyme, Bray, Ireland) were prepared separately. Initial pH was adjusted at 6.5. Fermentations were conducted in test tubes with 10 mL media, inoculated with resting cells of xylose grown cells at equivalent to 10% inoculum, and incubated for 18 h at 45 °C.

Commercial XOS mixture (Wako Chemicals, Richmond, VA, USA) was used for fermentation at 50 g/L without/with 5 g/L xylose in a 1 L fermentor with 0.3 L working volume at controlled pH 6.5 by the addition of 10 N NaOH and 45 °C. Samples were collected at different time intervals to analyze biomass, sugar, lactic acid, acetic acid, formic acid, and ethanol concentrations.

2.4. β-D-Xylosidase Activity

4-Nitrophenyl β-D-xylopyranoside (PNPX) (Sigma-Aldrich, St. Louis, MI, USA) was used as a substrate for xylosidase activity. The hydrolysis of 5 mM PNPX was carried out in 50 mM phosphate buffer (pH 6.5) at 45 °C for 30 min. Assay mixture (1 mL) consisted of 0.9 mL of substrate and 0.1 mL of cell suspension (resting cells) or culture supernatant. The reaction was terminated by the addition of 1 M Na₂CO₃. Absorbance was measured spectrophotometrically at 410 nm, and the liberated p-nitrophenol concentration was inferred from p-nitrophenol standard [11]. One unit of β-D-xylosidase activity is equivalent to 1 μmol of p-nitrophenol generated per minute.

2.5. Analytical Methods

The collected samples from the fermentation broth at different intervals were analyzed for cell growth on the basis of OD₅₆₂nm measurements using a spectrophotometer. Sugars and fermentation products were analyzed using a high-performance liquid chromatography system (HPLC; Agilent 1200 series chromatograph, USA) using a refraction index detector (RID-6A) and Biorad Aminex HPX-87H column (300 × 7.8 mm) at 60 °C. Sulfuric acid (0.01 N) was used as the mobile phase at a flow rate of 0.6 mL/min. The injection volume of the sample was set at 20 μL. All analyses were conducted in triplicate. Total sugar concentrations were determined by the phenol sulfuric acid method at 490 nm [15]. Consumed sugars were calculated on the basis of the difference between initial sugar and residual sugar at specific time points. The yield based on consumed sugars (g-LA/g-consumed sugars) was defined as the ratio of the produced LA (g/L) to consumed sugar (g/L); LA productivity was defined as the ratio of LA concentration (g/L) to the fermentation time (h) and described as g/L.h; maximum productivity determined the difference between LA concentrations of two respective samples at specific time inter-
vals. Analysis of variance (ANOVA) was used to show the significant differences between treatments. The mean difference comparison between the treatments was subsequently analyzed by the Tukey HSD test at \( p < 0.05 \). Data analysis was subjected using statistical package SPSS v17 (SPSS Inc., Chicago, IL, USA). All results presented are the means of three independent replicates.

3. Results and Discussion

3.1. Lactic Acid Fermentation Pattern at Different Xylose Concentrations

Lactic acid production from xylose utilization can be achieved homofermentatively or heterofermentatively not only on the basis of the producer strain but also on the xylose concentration and fermentation conditions [9,16,17]. Therefore, this experiment was conducted to determine the LA fermentation type and by-product formation at different xylose concentrations ranging from 5.0 to 100 g/L in batch fermentation at the optimal fermentation temperature (45 °C) and controlled pH of 6.5, as shown in Figure 1.

At low xylose concentrations (5–15 g/L), cell growth was ranged from an \( \text{OD}_{562\text{nm}} \) of 7.7 to 13.0. Strain s6 showed a heterofermentation pattern with productions of LA at 2.58, 7.2, and 12.9 g/L with yields of 0.44, 0.69, and 0.81 g/g-consumed xylose at 5, 10, and 15 g/L xylose, respectively (Figure 1A). Various by-products were produced at relatively

![Figure 1](image-url)
high concentrations of acetic acid (1.44, 1.92, and 1.18 g/L), formic acid (2.51, 2.95, and 2.43 g/L) and ethanol (1.44, 2.35, and 2.09 g/L), respectively (Figure 1B).

By increasing the xylose concentration (50–100 g/L), a higher cell biomass that ranged from OD$_{562nm}$ of 11.1 to 14.4 was obtained with significantly less or without by-product formation. Sugars was completely consumed up to 75 g/L xylose (Figure 1A). At 50 and 75 g/L xylose, strain s6 achieved homofermentative LA production, with LA concentrations of 51.7 and 72.5 g/L at high yields of 1.04 and 0.99 g/g$_{\text{consumed xylose}}$, respectively, without by product formation. At 90–100 g/L xylose, a homofermentative pattern was also achieved with LA yield of 0.93–0.97 g/L; however, xylose was not completely consumed. Lactic acid concentration ranged from 65.6 to 75.5 g/L as the strain consumed only 81.1 g/L and 68.0 g/L of xylose at fermentations with 90 and 100 g/L xylose, respectively (Figure 1A). These data indicated the existence of substrate inhibition effect at concentrations higher than the initial xylose concentration of 75 g/L.

The highest LA productivity (2.16 g/L.h) and yield (1.04 g/g$_{\text{consumed xylose}}$) were obtained at 50 g/L xylose within 24 h fermentation. Lower LA productivity (1.74 g/L.h) was obtained at 75 g/L xylose due to longer fermentation time (42 h). As a conclusion, the strain *E. faecium* showed a heterofermentation pattern at low xylose concentration (<50 g/L) and a homofermentation pattern at higher concentrations; however, the substrate inhibition effect might have limited the fermentations processes at xylose concentrations equal to or greater than 90 g/L. These results indicated that a higher initial xylose concentration (50 up to less than 90 g/L) resulted in a higher concentration and yield of LA in the fermentation without by-products and high fermentation efficiency, providing that the metabolic shift by stain s6 was mainly derived from the xylose concentration [12]. This indicates that the phosphoketolase (PK) pathway and pentose phosphate (PP) pathway might be the main pathways for utilization of xylose at concentrations lower than 50 g/L, as well as the fact that the PP pathway is the only pathway for xylose metabolism at higher xylose concentrations, as has been previously reported for the *E. mundtii* QU 25 strain [12]. On the other hand, *E. faecium* QU 50 showed a homo lactic fermentation pattern at 22.0 g/L [13].

### 3.2. Improved LA Fermentation in Repeated Batch Process

Efficient sugar utilization and high LA titer, yield, and productivity are important key fermentation parameters for reducing the overall cost for industrial LA production [16,17]. For improving xylose utilization and LA fermentation efficiency, repeated-batch fermentation was conducted. This process has been previously reported to achieve several advantages in omitting the inoculum preparation step; saving labor work, energy, and time; and enhancing cell biomass and consequently reduction of fermentation time [14].

In this experiment, all cells were recycled from one batch to a subsequent batch (up to 24 runs) at 45 °C and with a controlled pH of 6.5. Initial xylose concentration was 50 g/L at the first 13 runs (optimal sugar concentration obtained in the batch fermentation experiment) and after that, xylose concentration was gradually increased up to 80 g/L (Table 1, Figure 2). In the first 13 runs, all mMR media components were used. The growth of strain s6 was gradually increased at the first 12 h, giving a value of OD$_{562nm}$ 12.2 at the first run up to OD$_{562nm}$ 57.4 at the 13th run with little residual xylose ranging from 0.88 to 3.3 g/L. This significantly reduced the fermentation time from 12 h (at the first run) to 3.5 h (at the 13th run) and resulted in a significant increase in LA productivity from 4.24 g/L.h at the 1st run to more than threefold (14.3 g/L.h) at the 13th run. Lactic acid production ranged from 47.1 to 50.8 g/L and at yield ranged from 0.984 to 1.04 g/g$_{\text{consumed xylose}}$, indicating that strain s6 produced LA by homofermentation. No acetic acid was produced, whereas little formic acid (ranging from 0.466 to 0.580 g/L) and ethanol (ranging from 1.04 to 2.89 g/L) were detected at some runs. The highest LA productivity and maximum LA productivity were obtained at the 13th run with 14.3 g/L.h and 18.3 g/L.h, respectively, being almost around about a threefold increase as compared to the first runs at 4.24 g/L.h and 5.74 g/L.h, respectively.
Figure 2. Profiles of repeated-batch fermentations for lactic acid production from xylose by *E. faecium* s6 at controlled pH 6.5 and 45 °C.
Table 1. Kinetic parameters for repeated-batch fermentation for lactic acid production from xylose by E. faecium s6 at controlled pH 6.5 and 45 °C.

| Run | Initial Xylose (g/L) | Time | OD$_{562nm}$ | Residual Xylose (g/L) | Lactic Acid (g/L) | Acetic Acid (g/L) | Formic Acid (g/L) | Ethanol (g/L) | Lactic Acid Yield (g/g) | Lactic Acid Productivity (g/L.h) | Max. Lactic Acid Productivity (g/L.h) |
|-----|----------------------|------|-------------|-----------------------|------------------|------------------|------------------|--------------|--------------------------|-------------------------------|-----------------------------------|
| 1   | 50                   | 12   | 12.2       | 3.30                  | 50.8             | 0.0              | 0.0              | 0.0          | 0.984                    | 4.24                          | 5.74                              |
| 2   | 7                    | 17.0 | 2.90       | 47.1                  | 0.0              | 0.0              | 0.0              | 0.0          | 1.00                     | 6.73                          | 7.80                              |
| 3   | 6                    | 28.0 | 1.50       | 49.5                  | 0.0              | 0.0              | 0.0              | 0.0          | 1.02                     | 8.26                          | 9.37                              |
| 4   | 5                    | 27.5 | 2.50       | 47.1                  | 0.0              | 1.04             | 0.0              | 0.993        | 9.43                     | 12.0                          |                                    |
| 5   | 4.5                  | 39.0 | 2.30       | 49.8                  | 0.0              | 0.466            | 1.18             | 1.04         | 11.0                     | 13.1                          |                                    |
| 6   | 4.5                  | 41.1 | 0.880      | 49.9                  | 0.0              | 0.564            | 2.89             | 1.01         | 11.0                     | 15.5                          |                                    |
| 7   | 4.5                  | 45.5 | 1.20       | 50.8                  | 0.0              | 0.584            | 0.0              | 1.04         | 11.2                     | 16.1                          |                                    |
| 8   | 4                    | 46.3 | 1.40       | 49.4                  | 0.0              | 0.54             | 0.0              | 1.01         | 12.3                     | 18.1                          |                                    |
| 9   | 4                    | 51.3 | 1.60       | 48.2                  | 0.0              | 0.496            | 0.0              | 0.996        | 12.0                     | 16.3                          |                                    |
| 10  | 4                    | 50.0 | 1.21       | 51.3                  | 0.0              | 0.480            | 1.35             | 1.05         | 12.8                     | 16.9                          |                                    |
| 11  | 3.5                  | 48.9 | 1.80       | 47.5                  | 0.0              | 0.0              | 0.0              | 0.987        | 13.5                     | 17.0                          |                                    |
| 12  | 3.5                  | 52.1 | 2.01       | 48.6                  | 0.0              | 0.0              | 0.0              | 1.01         | 13.8                     | 17.4                          |                                    |
| 13  | 3.5                  | 57.4 | 1.80       | 50.2                  | 0.0              | 0.540            | 1.94             | 1.04         | 14.3                     | 18.3                          |                                    |
| 14  | 72                   | 6    | 62.9       | 3.00                  | 66.8             | 0.0              | 0.0              | 5.77         | 0.956                    | 11.1                          | 11.9                             |
| 15  | 5                    | 60.4 | 5.40       | 66.8                  | 0.0              | 0.588            | 0.990            | 13.3         | 17.7                     |                               |                                    |
| 16  | 4                    | 59.9 | 3.40       | 63.3                  | 0.0              | 0.514            | 1.29             | 0.911        | 15.8                     | 20.7                          |                                    |
| 17  | 4                    | 67.4 | 4.02       | 63.6                  | 0.0              | 0.570            | 0.0              | 0.924        | 15.9                     | 21.1                          |                                    |
| 18  | 80                   | 5    | 68.6       | 5.24                  | 77.9             | 0.0              | 0.620            | 3.24         | 1.024                    | 15.5                          | 25.6                             |
| 19  | 5                    | 74.9 | 5.48       | 80.7                  | 0.0              | 0.472            | 0.0              | 1.025        | 16.5                     | 24.8                          |                                    |
| 20  | 5                    | 63.4 | 4.00       | 79.1                  | 0.0              | 0.0              | 4.2              | 1.02         | 15.8                     | 33.5                          |                                    |
| 21  | 5                    | 66.1 | 2.00       | 79.0                  | 0.0              | 0.0              | 0.0              | 0.996        | 15.8                     | 28.9                          |                                    |
| 22  | 6                    | 69.0 | 2.21       | 81.1                  | 0.4              | 0.0              | 0.0              | 1.02         | 13.5                     | 31.9                          |                                    |
| 23  | 8                    | 57.4 | 6.05       | 75.5                  | 0.1              | 0.0              | 0.0              | 1.00         | 9.4                      | 22.2                          |                                    |
| 24  | 17                   | 46.1 | 10.0       | 62.0                  | 0.0              | 0.0              | 0.0              | 0.869        | 3.64                     | 16.3                          |                                    |

In an attempt to increase the final LA concentration, the 14th–17th runs were conducted using 72.0 g/L of xylose with all mMRS media components. Although the initial sugar increased, the maximum growth was still also increased, achieving an OD$_{562nm}$ value of 67.4 with low residual xylose (3.0–5.4 g/L) and low fermentation time of 4–6 h compared to 42 h achieved in normal batch fermentation with the same sugar concentration. Increased LA concentration was obtained, ranging from 63.3 to 66.8 g/L, while the LA yield was somewhat decreased and ranged from 0.911 to 0.990 g/g$_{-consumed}$xylose with little by-product formation of formic acid (ranging from 0 to 0.570 g/L) and ethanol (ranging from 0 to 5.88 g/L), whereas no acetic acid was detected. The LA productivity increased, achieving 15.9 g/L.h at run 17 with the maximum productivity of 21.1 g/L.h.

In a further attempt to increase the LA productivity, repeated batch fermentations by strain s6 were conducted at an initial xylose concentration of 80 g/L xylose at runs 18–21 with all mMRS media components as previous runs. Cell biomass ranged from OD$_{562nm}$ of 63.4 to 74.9, while little xylose was left in the fermentation media that ranged from 2.0 to 5.48 g/L after only 5 h of fermentation. High LA titer ranging from 77.9 to 80.7 g/L was obtained at a high yield of 0.996–1.02 g/g$_{-consumed}$xylose. No acetic acid was detected, while a little formic acid (0–0.620 g/L) and ethanol (0–4.2 g/L) were produced. Comparable LA productivity was obtained (15.5–16.5 g/L.h), while maximum productivity varied from 25.6 to 33.5 g/L.h, achieving the highest maximal productivities of 33.5 g/L.h and 28.9 g/L.h at runs 20 and 21, respectively.

Peptone, yeast extract, and beef extract are the most widely used nitrogenous sources to produce LA from LAB in MRS media; however, their high costs hinder the economical production of LA [18]. For further enhancement of the LA fermentation economics by strain s6, some nitrogen sources were excluded from the fermentation media. In run 22, peptone was excluded. Interestingly, growth, LA concentration, and LA yield were almost similar to the previous run (run 21). This may be attributed to the presence of yeast extract and beef extract that may be enough for achieving cell requirements, exhibiting a stable high cell biomass at OD$_{562nm}$ of 69.0. Fermentation proceeded for a longer time (6 h) than...
5 h in the 21st run. LA concentration and yield were not affected, achieving 81.1 g/L and 1.02 g/g consumed xylose, respectively. No formic acid or ethanol were detected, while a little amount of acetic acid (0.4 g/L) was produced. Under these conditions, the strain achieved LA productivity at 13.5 g/L.h and maximal productivity of 31.9 g/L.h. By excluding peptone and beef extract from the fermentation medium at run 23 (medium contained only 4 g/L of YE as the sole nitrogen source), a decrease in biomass was obtained with OD$_{562nm}$ of 57.4, and the fermentation time was elongated to 8 h. Residual xylose was 6.0 g/L with LA titer of 75.2 g/L at a yield of 1.0 g/g consumed xylose. No byproducts were detected. The LA productivity was decreased, achieving 9.4 g/L.h, and the maximal productivity was 22.2 g/L.h.

In a trial to enhance fermentation, the 24th run was conducted with the same components of the previous run but with supplementation with 10 g/L of corn steep liquor as a cheap nitrogen source. Unexpectedly, further decline in the fermentation efficiency was obtained. Cell biomass was decreased to OD$_{562nm}$ of 46.1 with 10 g/L residual xylose after prolonged fermentation of 17 h. Only 62.0 g/L of LA was produced at a decreased yield of 0.869 g/g consumed xylose, although no by-products were detected in the fermentation media. The LA productivity was significantly decreased to 3.64 g/L.h, and also maximal LA productivity was decreased to 16.3 g/L.h.

The comparison between the impacts of fermentation modes (repeated batch and normal batch fermentation) on xylose utilization by strain s6 indicated that the high increase in cell biomass is the main reason for increment of LA fermentation efficiency as there is no significant increase in the LA/biomass yield coefficient. This indicates that high cell density culture would greatly enhance LA fermentation by *E. faecium* s6. Our results highlighted that the substrate inhibition may be alleviated in repeated batch fermentation as the *E. faecium* s6 exhibited a short initial lag phase followed by a rapid growth phase at all batches of different xylose concentrations, and the growth was not decreased, except with the exclusion of high amount of nitrogen source. Further studies to find the relationship between cell concentration and substrate concentration/inhibition might lead to further enhancement, not only for LA productivity but also LA concentration. The relatively high temperature (45 °C) integrated with the high cell density may be the reason for avoiding contamination risk during the fermentation processes that provides quick lactate formation, as previously reported [14,19]. The cell adaptability due to repeated batch might contribute to the enhancement of the LA production with increasing xylose concentration in repeated runs. The stability of *E. faecium* s6 for elongated fermentation at least for 21 runs might be attributed to its adaptability to various chemical, biological, or physical conditions of thermostolerance, pH, osmotic stress, product concentration, and even the inhibitory byproduct compounds [14]. This clearly appeared when changing the medium composition in the last three runs (from the 22nd to the 24th run), affecting fermentation efficiency. Therefore, we assume that increasing the adaptability under the new condition might further improve the economics of LA production. The strain s6 previously showed improved LA production in repeated runs, achieving 64.7 g/L of LA with productivity of 2.16 g/L.h, yield of 0.94 g/g consumed xylose, and maximum productivity of 4.91 g/L.h by utilization of beet molasses [14]. Repeated batch fermentation by *E. faecium* s6 coupled with increased xylose concentration achieved a gradual increase in cell growth and greatly improved LA productivity as compared to fermentation in batch modes using respective sugar concentrations. Therefore, in this study, we succeeded in establishing a highly productive biosystem for obtaining LA with a high productivity in the semi-continuous system (repeated batch fermentation) for at least 24 runs within 135 h that would greatly reduce the production cost of LA.

To the best of our knowledge, this is the first study to achieve long-term homofermentative LA production from LAB by repeated batches using xylose. However, the utilization of glucose in repeated batch fermentation was previously reported by *E. mundtii* QU 25, achieving LA productivity of 16.7 g/L.h [19]. Moreover, *E. faecalis* RKY1 achieved LA productivity of 6.20 g/L.h with 100 g/L glucose [20], and LA productivity of 4.0 g/L.h
from wood hydrolyzate containing 50 g/L glucose [21]. *E. hirae* BoM1-2 achieved LA productivity of 39.9 g/L.h using a low glucose concentration of 40 g/L. The improved LA productivity by *Enterococcus hirae* BoM1-2 with the gradual increase of glucose concentrations in repeated runs was attributed to cell adaptability to the fermentation conditions [22]. Similarly, the adaption and mutation of *Lactobacillus paracasei* NRRL B-4564 by the gradual increase of beet molasses sugar had improved LA production in repeated batch fermentation [23].

### 3.3. Utilization of Xylan and Xylooligosaccharides (XOS)

As *E. faecium* s6 exhibited efficient xylose utilization, it was of interest to further investigate whether this strain is able to ferment other hemicellulose-derived sugars. Firstly, in a trial to investigate xylan utilization by *E. faecium* s6, batch fermentations were conducted with 20 g/L birchwood xylan at 45 °C and pH controlled at 6.5. Unfortunately, the strain showed very weak growth with an OD<sub>562nm</sub> of 2.0 and could not utilize xylan as a sole carbon source, achieving maximum LA production at 0.739 g/L after 12 h (data not shown). This indicates the lack of xylanase activity by *E. faecium* s6, and, therefore, suitable treatment should be performed for efficient utilization of cellulosic biomass by this strain. Previously, Hu et al. introduced a xylanase gene to *Levilactobacillus brevis* that could consume xylan directly, producing 1.7 g/L lactic acid in hetero-fermentation after 4 days [5].

On the other hand, LA fermentation of xylan-derived XOS (xylotriose, xylotetraose, xylopentaose, and xylohexaose) was investigated separately, as shown in Figure 3. As indicated, the strain was able to efficiently utilize all tested XOS. Cell biomass ranged from an OD<sub>562nm</sub> of 4.16 to 5.29, with higher cell biomass obtained at large xylooligosaccharides (cellopetose and cellohexose) at OD<sub>562nm</sub> of 5.12 and 5.29, respectively. Consequently, high LA concentration was obtained by utilization of higher XOS, achieving maximum values of 2.54, 2.45, and 2.03 g/L for xylohexaose, xylopentaose, and xylotetraose utilization, respectively. However, lower LA were obtained at 1.05 g/L and 1.61 g/L by xylobiose and xylotriose utilization, respectively. Formic acid and acetic acid were detected for all tested XOS that ranged from 0.595 to 0.865 g/L, and 0.180 to 0.650 g/L, respectively; however, no ethanol was produced at all tested sugars. The LA yield ranged from 0.619 to 0.844 g/g<sub>-consumed sugar</sub>. This low yield might be in accordance with the low initial sugar used, as this strain showed a heterofermentative pattern at low xylose concentration [24,25]. As the yield exceeded the maximum theoretical yield (0.6 g/g) for pentose utilization by PK pathway, *E. faecium* s6 might also utilize both PK and PP pathways for utilization of XOS at low concentration, as expected for xylose. A previous study showed that *Leuconostoc lactis* SHO-47 and *Le. lactis* SHO-54 could consume XOS from xylobiose to xylohexaose [11].

Utilization of high XOS concentrations (50 g/L) was conducted under controlled pH fermentation conditions at 6.5 and 45 °C, as shown in Figure 4A. The strain grew rapidly and achieved a maximum biomass of OD<sub>562nm</sub> 13.1 after 12 h. Most XOS were consumed as indicated from HPLC profiles at zero time and after 144 h (Figure 5A,B). Strain *E. faecium* s6 metabolized 36.8 g/L of XOS homofermentative within 144 h and produced 36.2 g/L of LA at a yield of 0.983 g/g<sub>-consumed sugar</sub> and productivity of 0.269 g/L.h. Few by-products were produced of acetic acid (1.57 g/L), formic acid (1.31 g/L), and ethanol (1.37 g/L). The maximum LA productivity was 1.18 g/L.h. The low achieved cell biomass might be attributed to the lack of rapidly consumed monosugars (xylose) in the sugar mixture, as shown from HPLC analysis at zero time.
Figure 3. Lactic acid fermentation of xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose by *E. faecium* s6 at initial pH 6.5 and 45 °C.

Utilization of high XOS concentrations (50 g/L) was conducted under controlled pH fermentation conditions at 6.5 and 45 °C, as shown in Figure 4A. The strain grew rapidly and achieved a maximum biomass of OD 562 nm 13.1 after 12 h. Most XOS were consumed as indicated from HPLC profiles at zero time and after 144 h (Figure 5A,B). Strain *E. faecium* s6 metabolized 36.8 g/L of XOS homofermentatively within 144 h and produced 36.2 g/L of LA at a yield of 0.983 g/g -consumed sugar and productivity of 0.269 g/L.h. Few by-products were produced of acetic acid (1.57 g/L), formic acid (1.31 g/L), and ethanol (1.37 g/L). The maximum LA productivity was 1.18 g/L.h. The low achieved cell biomass might be attributed to the lack of rapidly consumed monosugars (xylose) in the sugar mixture, as shown from HPLC analysis at zero time.

Figure 4. Lactic acid fermentation of xylooligosaccharides mixture (A), and xylose/xylooligosaccharides mixture (B) by *E. faecium* s6 at controlled pH 6.5 and 45 °C.
Figure 5. HPLC profiles of lactic acid fermentation by E. faecium s6 in mMR broth supplemented with XOS (A,B) and xylose/XOs mixture (C,D) as carbon source. Fermentation times: (A,C) 0 h, (B,D) 144 h.

In order to enhance XOS utilization, fermentation of 50 g/L of XOS plus 5 g/L xylose was investigated. As expected, higher biomass was obtained with OD$_{562\text{nm}}$ at 16.6 after 24 h of fermentation. Consequently, high sugar concentration was consumed, achieving a maximum LA production of 48.2 g/L within 120 h at a yield of 1.0 g/g-consumed sugar and productivity of 0.331 g/L.h. The maximum sugar utilization rate was enhanced, achieving a maximum LA productivity at 3.02 g/L.h as compared to 1.18 g/L.h in XOS without xylose supplementation. Few by-products were also produced of acetic acid (1.85 g/L), formic acid (1.18 g/L), and ethanol (1.41 g/L). To the best of our knowledge, the high concentration of LA from XOS obtained in the present study is the first to be reported by LAB. The residual un consumed sugars might indicate that strain s6 was unable to utilize XOS larger than xylohexaose (Figure 5C,D). Leuconostoc lactis SHO-47, Le. lactis SHO-54, and Le lactis IO-1 produced only 2.3 g/L, 2.2 g/L, and 1.3 g/L lactic acid, respectively, from 8.5 g/L hydrolyzed xylan [11].

The microbial utilization of XOSs requires the action β-xylosidase, exo-oligoxylanase, and α-L-arabinofuranosidase [26]. By investigating β-D-xylosidase of culture supernatant and resting cells grown in XOS and XOS/xylose media, we were unable to detect any β-D-xylosidase activity in the culture supernatant, indicating that these enzymes are localized in the cell membrane or intracellularly. This also indicate that XOS might be firstly imported into the cells by oligosaccharide transporters, followed by further degradation to monosugars, as has been reported by Leuilotbacillus brevis S27, Lactiplantibacillus plantarum S26, and Latilactobacillus sakei S16 [26]. On the other hand, resting cells of cells grown in a mixture of xylose and XOSs showed more than a threefold increase in xylosidase activities at 0.838 U/mg-DCW at 16.6 after 24 h of fermentation. Consequently, high sugar concentration was consumed, achieving a maximum LA production of 48.2 g/L within 120 h at a yield of 1.0 g/g-consumed sugar and productivity of 0.331 g/L.h. The maximum sugar utilization rate was enhanced, achieving a maximum LA productivity at 3.02 g/L.h as compared to 1.18 g/L.h in XOS without xylose supplementation. Few by-products were also produced of acetic acid (1.85 g/L), formic acid (1.18 g/L), and ethanol (1.41 g/L). To the best of our knowledge, the high concentration of LA from XOS obtained in the present study is the first to be reported by LAB. The residual un consumed sugars might indicate that strain s6 was unable to utilize XOS larger than xylohexaose (Figure 5C,D). Leuconostoc lactis SHO-47, Le. lactis SHO-54, and Le lactis IO-1 produced only 2.3 g/L, 2.2 g/L, and 1.3 g/L lactic acid, respectively, from 8.5 g/L hydrolyzed xylan [11].

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density culture might be better for enhanced LA production by xylan-derived sugars by Enterococcus faecium s6.

4. Conclusions

A potential thermotolerant Enterococcus faecium s6 showed powerful and effective utilization of pentose sugars (xylose and xylooligosaccharides) into homo lactic acid production at 45 °C and controlled pH of 6.5. The strain could be utilized for 24 subsequent fermentation runs (repeated batch fermentation) within 135 h, achieving high LA titer and productivity from xylose. Besides this, it could efficiently utilize xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose and produced up to 48.2 g/L of LA at a yield of 1.0 g/\text{g-consumed sugar} from xylooligosaccharide mixtures after 120 h. Our results indicate that Enterococcus faecium s6 is a potent strain for utilization of cellulosic biomass hydrolysate into homofermentative lactic acid production without carbon loss.

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