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Metabolic Engineering of *Zymomonas mobilis* for Xylonic Acid Production from Lignocellulosic Hydrolysate

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Abstract: Bio-based xylonic acid produced from inexpensive lignocellulosic biomass has enormous market potential and enhances the overall economic benefits of biorefinery processes. In this study, the introduction of genes encoding xylose dehydrogenase driven by the promoter P*pdc* into *Z. mobilis* using a plasmid vector resulted in the accumulation of xylonic acid at a titer of 16.8 ± 1.6 g/L. To achieve stable xylonic acid production, a gene cassette for xylonic acid production was integrated into the genome at the chromosomal locus of *ZMO0038* and *ZMO1650* using the endogenous type I-F CRISPR-Cas system. The titer of the resulting recombinant strain XA3 reduced to 12.2 ± 0.56 g/L, which could be the copy number difference between the plasmid and chromosomal integration. Oxygen content was then identified to be the key factor for xylonic acid production. To further increase xylonic acid production capability, a recombinant strain, XA9, with five copies of a gene cassette for xylonic acid production was constructed by integrating the gene cassette into the genome at the chromosomal locus of *ZMO1094*, *ZMO1547*, and *ZMO1577* on the basis of XA3. The titer of xylonic acid increased to 51.9 ± 0.1 g/L with a maximum yield of 1.10 g/g, which is close to the theoretical yield in a pure sugar medium. In addition, the recombinant strain XA9 is genetically stable and can produce 16.2 ± 0.14 g/L of xylonic acid with a yield of 1.03 ± 0.01 g/g in the lignocellulosic hydrolysate. Our study thus constructed a recombinant strain, XA9, of *Z. mobilis* for xylonic acid production from lignocellulosic hydrolysate, demonstrating the capability of *Z. mobilis* as a biorefinery chassis for economic lignocellulosic biochemical production.

Keywords: xylonic acid; xylose dehydrogenase; *Zymomonas mobilis*; metabolic engineering; lignocellulosic hydrolysate

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1. Introduction

Xylonic acid is a five-carbon organic acid, which has been rated as one of the top 30 most valuable chemicals due to its potential as an important platform chemical by the US Department of Energy (DOE) [1,2]. It has been widely used in various fields, including industry, food, pharmaceutical, and agriculture [3,4]. Currently, the most frequently used strategy for xylonic acid production relies on chemical methods to catalyze xylose using heavy or noble metal catalysts, which is energy intensive, environmentally harmful, and expensive [5]. Compared with the chemical synthetic routes, microbial conversion offers the potential for benign processing with high specificity and reduced manufacturing costs [1]. Therefore, xylonic acid production by microorganisms from sustainable lignocellulosic

biomass materials containing abundant sugars of glucose and xylose enables the effective utilization of agricultural wastes with overall social and economic benefits [2,6].

In recent years, xylose has been widely used for the production of biofuels and biochemicals such as ethanol, xylitol, squalene, 2,3-butanediol, and lipids [7]. However, it is difficult to design and construct an efficient microbial cell factory for xylose utilization fully using lignocellulosic biomass. Consequently, the microbial conversion of xylose to xylonic acid has attracted widespread attention and is generally regarded as the most promising method because of its high efficiency [8]. The screening, development, and application of microbial chassis have greatly promoted the production of xylonic acid.

Several bacteria such as *Gluconobacter oxydans* [9,10], *Pseudomonas fragi* [11], *Enterobacter cloacae* [12], *Paraburkholderia sacchari* [5], and *Klebsiella pneumoniae* [13] possess the intrinsic ability to produce xylonic acid naturally, and the highest amount of xylonic acid was achieved at titer of 586.3 g/L using *G. oxydans* through whole-cell biocatalysis in fed-batch fermentation [14]. In addition, xylonic acid production has been achieved in non-natural microorganisms such as *Escherichia coli* [3,15], *Saccharomyces cerevisiae* [16], *Pichia kudriavzevii* [17], *Corynebacterium glutamicum* [18,19], and *Zymomonas mobilis* [20] through the co-expression of a xylose dehydrogenase (XDH) and a xylonolactonase (XL). A previous study demonstrated that the recombinant strain *Z. mobilis* ZMa BX produced 56.44 g/L of xylonic acid with a yield of 1.08 g/g and productivity of 0.99 g/L/h [20]. However, the expression of xylonic acid through a plasmid in ZMa BX poses a risk of production loss.

Z. mobilis is a natural Gram-negative ethanologenic bacterium with many excellent industrial characteristics [21], including a high specific glucose uptake rate, low biomass production, few byproducts, a low aeration cost, and high ethanol tolerance (16%) and yield (98%) [22] due to its unique Entner–Doudoroff (ED) pathway [23,24]. In addition, efficient genome-editing tools including CRISPR-Cas12a [25], endogenous Type I-F CRISPR-Cas [26], and genome-wide iterative and continuous editing system (GW-ICE) [27] have been developed and used for genetic manipulation in *Z. mobilis*. The development of genome engineering and genome-wide metabolic modeling [28,29] facilitates the efficient engineering of *Z. mobilis* for the production of other high-value biochemicals such as 2,3-butanediol, isobutanol, poly-3-hydroxybutyrate (PHB), lactic acid, succinic acid, acetaldehyde, and ethylene [30,31], which is gradually developed as an ideal lignocellulosic biorefinery chassis [32,33].

Since the production of xylonic acid through a plasmid in ZMa BX poses a risk of production loss [20], the construction of efficient and stable xylonic acid production strains was carried out in this study. Our study provided stable recombinant strains for xylonic acid production and demonstrated the potential of *Z. mobilis* as a potential platform for economic lignocellulosic biochemical production.

2. Materials and Methods

2.1. Strains, Media, and Growth Conditions

E. coli DH5α was used for plasmid construction, and *E. coli* trans110 was used as the host for plasmid demethylation. All *E. coli* strains were cultured in Luria–Bertani medium (LB, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 1.5% agar for solid) at 37 °C. *Z. mobilis* ZM4 was used as the parental strain and cultured in rich medium (RMG_{20}, 10 g/L yeast extract, 20 g/L glucose, 2 g/L KH_2PO_4, and 1.5% agar for solid) at 30 °C. Glucose was used for biomass accumulation and ethanol production. For xylonic acid production, xylose with a final concentration of 50 g/L was supplemented into the RMG_{20} medium as a fermentation medium ($RMG_{20}X_{50}$). When necessary, 100 μg/mL and 200 μg/mL spectinomycin was added for *E. coli* and *Z. mobilis*, respectively.

The lignocellulosic hydrolysate used in this study was provide by Zhejiang Huakang Pharmaceutical Co., Ltd. (Hangzhou, China). The lignocellulosic hydrolysate was diluted three times with RM- medium (10 g/L yeast extract, 2 g/L KH_2PO_4) and then used for ethanol and xylonic acid production.

2.2. Construction of Plasmid and Transformation

All plasmids and strains used in this study are listed in Table 1. The primers used in this work are listed in Table S1.

Table 1. The source and components of plasmids and strains for xylonic acid production used in *Z. mobilis* in this work.

Plasmid	Description	Source
pEZ15A	Shuttle vector containing *Z. mobilis* origin and *E. coli* origin p15A, Spe^R	[26]
PL2R	CRISPR expression plasmid used in *Zymomonas mobilis* for efficient genome engineering by repurposing the endogenous Type I-f CRISPR-Cas system, Spe^R	This work
pXA	pEZ15A carrying *XDH* genes driven by strong promoter P*pdc*, and Spe^R	This work
pL2R-ZMO0038-P*pdc-XDH*	pL2R used to knockout *ZMO0038* and replace it with P*pdc-XDH*; Spe^R	This work
pL2R-ZMO1650-P*pdc-XDH*	pL2R used to knockout *ZMO1650* and replace it with P*pdc-XDH*; Spe^R	This work
pL2R-ZMO1094-P*pdc-XDH*	pL2R used to knockout *ZMO1094* and replace it with P*pdc-XDH*; Spe^R	This work
pL2R-ZMO1547-P*pdc-XDH*	pL2R used to knockout *ZMO1547* and replace it with P*pdc-XDH*; Spe^R	This work
pL2R-ZMO1577-P*pdc-XDH*	pL2R used to knockout *ZMO1577* and replace it with P*pdc-XDH*; Spe^R	This work
pL2R-Δ*ZMO0976*	pL2R used to knockout *ZMO0976*; Spe^R	This work
pEZ15A-P*eno-xylE*	pEZ15A carrying *xylE* genes driven by strong promoter P*eno*; kana	This work
pEZ15A-P*eno-xylFGH*	pEZ15A carrying *xylFGH* genes driven by strong promoter P*eno*; kana	This work
pEZ15A-P*eno-nadK*	pEZ15A carrying *nadK* genes driven by strong promoter P*eno*; kana	This work
pEZ15A-P*eno-noxE*	pEZ15A carrying *nadK* genes driven by strong promoter P*eno*; kana	This work
pEZ15A-P*eno-ndh*	pEZ15A carrying *ndh* genes driven by strong promoter P*eno*; kana	This work
E. coli DH5α	*E. coli* strain for plasmid construction	Lab stock
E. coli Trans 110	*E. coli* strain for plasmid demethylation	Lab stock
Z. mobilis ZM4	*Z. mobilis* wild-type strain	Lab stock
EXA	*E. coli* DH5α strain containing pEZ15A-P*gap(6M)-XDH*	This work
XA1	*Z. mobilis* ZM4 strain containing pXA	This work
XA2	*ZMO0038* of ZM4 replaced with P*pdc-xdh*	This work
XA3	*ZMO1650* of XA2 replaced with P*pdc-xdh*	This work
XA4	*ZMO0976* of XA2 deleted	This work
XA5	*ZMO0976* of XA3 deleted	This work
XA6	Strain XA3 containing plasmid pXA	This work
XA7	*ZMO1094* of XA3 replaced with P*pdc-xdh*	This work
XA8	*ZMO1547* of XA7 replaced with P*pdc-xdh*	This work
XA9	*ZMO1577* of XA8 replaced with P*pdc-xdh*	This work
XA10	XA8 strain containing pEZ15A-P*eno-xylE*	This work
XA11	XA8 strain containing pEZ15A-P*eno-xylFGH*	This work
XA12	XA8 strain containing pEZ15A-P*eno-nadK*	This work
XA13	XA8 strain containing pEZ15A-P*eno-noxE*	This work
XA14	XA8 strain containing pEZ15A-P*eno-ndh*	This work

The shuttle plasmid pEZ15A was used for gene overexpression in *Z. mobilis* and *E. coli*. For the shuttle plasmid construction in this work, the linearized gene fragments were obtained by polymerase chain reaction (PCR) using primers (Sangon Biotechnology Co., Ltd., Wuhan, China) with an overlapping region of 15~21 nucleotides. After purification with the gel purification kit (TsingKe, Beijing, China), the T5 exonuclease (NEB, Ipswich, MA, USA) method was used to link the fragment to the vector. The insert size was determined by colony PCR using the universal primer pEZ15A-fwd/rev. Colonies containing the correct plasmid with the expected length of the PCR product were obtained by colony PCR screening, cultured in LB medium containing appropriate antibiotics for plasmid extraction, and further verified by Sanger sequencing (Sangon Biotechnology Co., Ltd.,

Wuhan, China). The plasmids were extracted and isolated from verified positive clones. After demethylation in *E. coli* Trans 110, the plasmids were used to transform *Z. mobilis* competent cells by Gene Pulser® (Bio-Rad, Hercules, CA, USA, 1800 V, 25 μF, 200 Ω), followed by selecting on plates containing the appropriate antibiotics [34].

2.3. Construction of Stable Xylonic Acid Producer

The plasmid pL2R was used for the integration of the *xdh* gene driven by the strong promoter P*pdc* into the chromosomal locus of *ZMO0038*, *ZMO1650*, *ZMO1547*, *ZMO1577*, and *ZMO1094* to obtain xylonic acid producing strains XA2, XA3, XA7, XA8, and XA9. The construction of the above editing plasmids was carried out according to the previous study [35]. Briefly, *Z. mobilis* ZM4 was used as the initial strain, and five rounds of genome editing were conducted. First, we replaced ZMO0038 of ZM4 with the *xdh* gene driven by the strong promoter P*pdc* to obtain strain XA2. The correct mutants were further cultivated in RMG_{20} medium without antibiotics at 30 °C for five generations to cure the editing plasmid. Then, ZMO1650 of XA2 was replaced with the xylonic acid expression cassette to obtain strain XA3. The above steps were repeated until recombinant strains XA7, XA8, and XA9 were obtained.

2.4. Batch Fermentation for Xylonic Production

For flask batch fermentation, *Z. mobilis* strains were pre-cultivated in RMG_{50} medium overnight and then incubated into the flasks containing 40 mL RMG_{50} medium in 50 mL flasks as the seed cultures. After being washed once, the seed culture was transferred into 50 mL shake flasks containing the RMGX medium and 20 g/L $CaCO_3$ with an initial OD_{600nm} value of 0.1 at 30 °C on a rotary shaker at 100 rpm. During the fermentation, cell growth in terms of the absorbance value was measured spectrobolometrically at 600 nm (UV-1800, AOE, Shanghai, China) at different time points. Samples were collected and centrifuged at 12,000 rpm for 1 min, filtered through 0.22 μm filters, and stored at −80 °C for metabolite quantification using a High-Performance Liquid Chromatograph (HPLC). Three replicates were used for each condition.

2.5. Analytical Procedures

Glucose, xylose, and ethanol in the supernatant were quantified using an HPLC system (HPLC Prominence, Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as described previously [36]. The column temperature was set at 60 °C, and a 5 mM H_2SO_4 solution was used as the mobile phase with a flow rate of 0.5 mL/min.

Since xylonic acid and xylose have similar retention times with a refractive index detector, when xylose was present, the latter was determined using a UV detector by HPLC-2030 Plus (Shimadzu, Kyoto, Japan) equipped with a Bio-Rad Aminex HPX-87H column at 60 °C, and a 5 mM H_2SO_4 solution was used as the mobile phase with a flow rate of 0.5 mL/min. When xylonic acid was present, xylose concentrations were estimated by subtraction of the xylonic acid peak from the combined xylose and xylonic acid peak [5,19].

2.6. Statistical Analysis

Data were calculated and analyzed by one-way ANOVA analysis using GraphPad Prism statistical software (version 8.0.1, GraphPad, San Diego, CA, USA) with a p-value ≤ 0.05 considered as statistically significant.

3. Results and Discussion

3.1. Expression of Xylose Dehydrogenase Using Plasmid for Xylonic Acid Production in Z. mobilis

A previous study demonstrated that the introduction of the *xdh* gene to *Z. mobilis* resulted in xylonic acid production with a productivity of 1.85 ± 0.06 g L^{-1} h^{-1} and a yield of 1.04 ± 0.04 g/g [20]. The codon-optimized *xdh* gene from *Paraburkholderia xenovorans*, which was reported to produce a high concentration of xylonic acid, was cloned into the shuttle vector pEZ15A to generate the plasmid pXA in this study, in which *xdh* is driven by the strong promoter P*pdc* [20].

Then, the plasmid was transformed into *Z. mobilis* ZM4 to generate the recombinant strain XA1 for xylonic acid production, and $CaCO_3$ was added into the media to evaluate its ability to enhance the ethanol and xylonic acid production. A total of 1.5 g/L residual glucose was still detected with 9.06 ± 0.2 g/L ethanol produced at 12 h without $CaCO_3$ supplementation (Figure 1A), while all glucose was consumed to obtain a maximum ethanol titer and yield of 9.7 ± 0.0 g/L and 0.49 ± 0.0 g/g within 12 h in the presence of $CaCO_3$ (Figure 1B). This may be due to the decrease in pH affecting glucose utilization of *Z. mobilis*. The final ethanol titer and yield after $CaCO_3$ addition was similar to that without $CaCO_3$ supplementation in the $RMG_{20}X_{50}$ medium (Figure 1A). Different from ethanol production, the titer of xylonic acid was nearly 1.84 times higher than that without $CaCO_3$ in the $RMG_{20}X_{20}$ medium (titer of 16.8 ± 1.6 g/L vs. 9.1 ± 0.3 g/L, p-value < 0.05) (Figure 1C). However, the pH of the medium decreased to 3.0 from 5.8 because of xylonic acid accumulation, which inhibited cell growth and the decrease in xylonic acid production. Thus, $CaCO_3$ was supplemented to neutralize xylonic acid during fermentation. The pH was nearly 5.0 after a 48 h fermentation with $CaCO_3$ supplementation (Figure 1D). Therefore, the solid $CaCO_3$ was added into the subsequent production of xylonic acid.

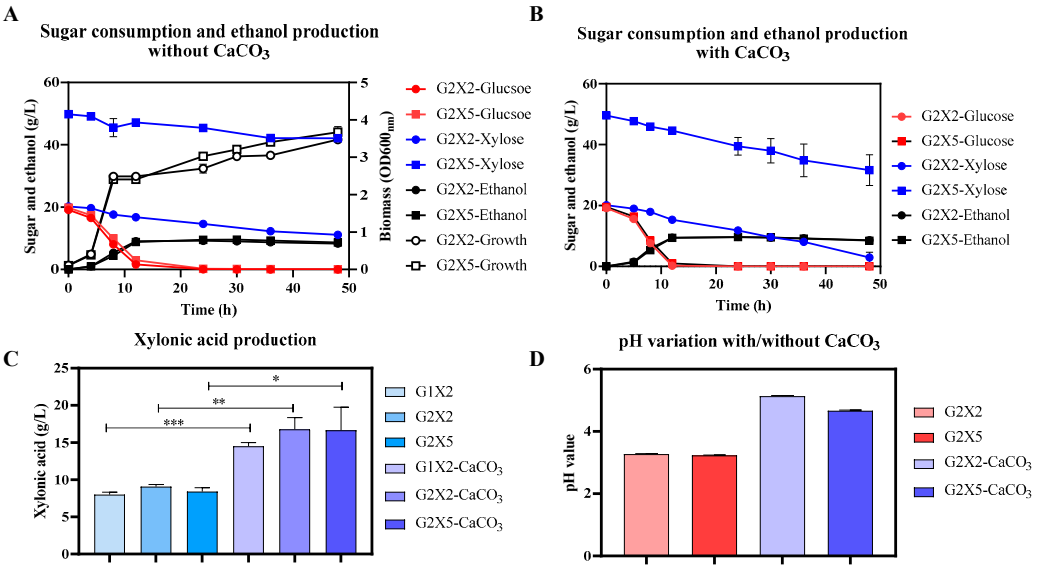


Figure 1. Sugar consumption, ethanol and xylonic acid production, and pH variation of *Z. mobilis* XA1. Fermentation performance in different sugar concentration medium without $CaCO_3$ (**A**) and with $CaCO_3$ (**B**) supplementation, as well as the titer of xylonic acid in the engineering strain XA1 (**C**) and pH variations with or without $CaCO_3$ supplementation (**D**). The results shown are at least three replicates, and the error bars represent standard deviation. * Represents a significant difference (p-value < 0.05), ** represents a p-value < 0.01, and *** represents a p-value < 0.001.

3.2. Construction of Stable Heterologous Xylonic Acid Producing Strains by CRISPR-Cas System

In order to construct stable xylonic acid-producing strains, the codon-optimized *xdh* driven by the strong promoter P*pdc* was integrated into the genome at the chromosomal

locus of *ZMO0038* using the endogenous type I-F CRISPR-Cas system [26], resulting in the recombinant strains of XA2 (Figure 2A). XA2 showed a lower titer of 10.1 ± 0.4 g/L compared with XA1 due to the copy number difference of *xdh*. Therefore, another copy number of xdh was integrated into the genome at the chromosomal locus of *ZMO1650* to obtain double copy number strain XA3 (Figure 2A). The xylonic acid production of XA3 increased to 12.2 ± 0.6 g/L at a yield of 0.97 ± 0.01 g/g (Figure 2B). These results indicated that xylonic acid production can be improved by increasing *xdh* copy numbers. In addition, a previous study demonstrated that the deletion of the *xyrA* gene encoding xylose reductase increased the production of xylonic acid [20]. Therefore, *xyrA* was deleted in strain XA2 and XA3 to obtain strain XA4 and XA5. The results showed that there were no significant differences in xylonic acid production, which may be due to insufficient copy numbers.

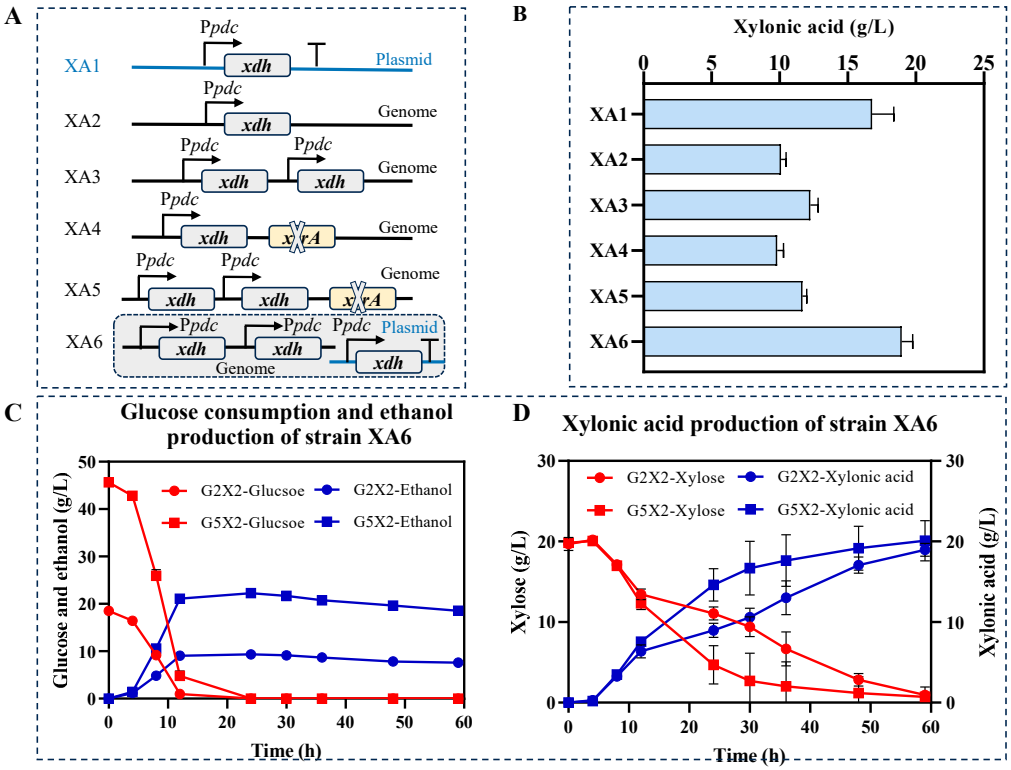


Figure 2. Fermentation performance of different recombinant strains with *xdh* gene integration. Schematic of different recombinant strains with *xdh* integration and *xyrA* deletion (**A**). Xylonic acid production of recombinant strains of XA1, XA2, XA3, XA4, XA5, and XA6 (**B**). Glucose consumption, ethanol production (**C**), and xylose consumption and xylonic acid production (**D**) of strain XA6. Error bars represent the standard deviation values obtained in triplicate experiments.

Plasmid pXA was then transformed into the recombinant strain XA3 to obtain strain XA6. Glucose, xylose consumption, ethanol production (Figure 2C), and xylonic acid accumulation (Figure 2D) of recombinant strain XA6 were then investigated. Consistent with the above results, the titer of xylonic acid increased to 19.0 ± 0.8 and 20.1 ± 2.5 g/L with the yield of 0.96 ± 0.0 and 1.01 ± 0.1 g/g in $RMG_{20}X_{20}$ and $RMG_{50}X_{20}$, respectively. These results indicated that the copy number of *xdh* can effectively increase the titer and yield of xylonic acid production.

3.3. The Effect of Oxygen and High Cell Density on Xylonic Acid and Ethanol Production

Our results demonstrated that it is difficult for the recombinant strain to fully utilize xylose greater than 50 g/L (Figure 1), which might be attributed to insufficient oxygen supply. A high xylonic acid titer and productivity can be achieved through a sealed oxygen

supply–bioreactor (SOS-BR) technology [14]; the impact of oxygen on xylonic acid and ethanol co-production was investigated using recombinant strain XA3 in shake flasks containing different medium volumes of 20, 50, or 80% liquid volume (Figure 3). As shown in Figure 3A, almost all the glucose was consumed within 12 h, and the ethanol production was inhibited at the condition of 20% liquid volume compared with 50% and 80% liquid volume with a titer of 4.43 g/L (Figure 3A, Table 2). Our results showed that ethanol production was affected by the oxygenation of the medium in the shake flask similar to our previous work for isobutanol production [36], with more carbon sources flowing into byproducts such as acetic acid, glycerol, and acetoin instead of ethanol at aerobic conditions [37].

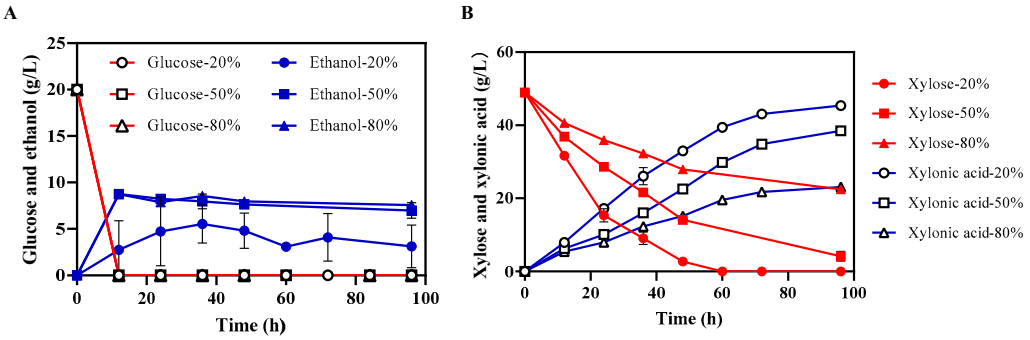


Figure 3. Glucose consumption and ethanol production (**A**) and xylose consumption and xylonic acid production (**B**) of recombinant strain XA3 in a shake flask with different medium volumes of $RMG_{20}X_{50}$ (20, 50, and 80%). Error bars represent the standard deviation values obtained in triplicate experiments.

Table 2. Fermentation performance of recombinant strain XA3 under different medium volumes in $RMG_{20}X_{50}$ after 96 h of cultivation.

Volume	Xylose Consumed (g/L)	Ethanol Concentration (g/L)	Xylonic Acid Production		
			Titer (g/L)	Yield * (g/g)	Productivity (g/L/h)
20%	48.99 ± 0.0	6.3 ± 1.1	45.4 ± 0.1	0.89	0.45
50%	43.84 ± 0.0	8.7 ± 0.1	38.5 ± 0.2	0.88	0.40
80%	26.50 ± 0.1	8.7 ± 0.1	23.3 ± 0.2	0.80	0.24

* The yield is equal to the slope obtained by performing a linear regression of the plot of the production of xylonic acid versus the cell-consumed xylose.

On the contrary, the results exhibited that the xylonic acid titer was affected by the oxygen level in the shake flasks. With the decrease in fermentation medium volume, more xylose consumption and xylonic acid production were achieved in the shake flasks. In the 20% liquid volume condition, almost all xylose was consumed, and 45.4 ± 0.1 g/L of xylonic acid was produced with a yield of 0.89 g/g. Moreover, less xylonic acid with a titer of 38.5 ± 0.2 g/L and 23.3 ± 0.2 g/L was produced in the 50% and 80% medium volume condition, respectively (Table 2). All these results suggested that aerobic fermentation is a better choice for xylonic acid production with high titer, yield, and productivity in *Z. mobilis* (Table 2). Therefore, a liquid volume of 20% was used as the subsequent fermentation condition. However, decreasing the long fermentation time is a key factor in the economic production of xylonic acid in future work.

A previous study demonstrated that high cell density was applied for sorbitol and gluconic acid production in *Z. mobilis* [38]. The seed culture of recombinant strain XA3 was transferred into shake flasks containing RMX_{50}, $RMG_{20}X_{50}$, and $RMG_{50}X_{50}$ medium and 10 g/L $CaCO_3$ with an initial OD_{600nm} value of 12 and 20, respectively (Figure S1). The

results showed that 37.8 g/L of xylonic acid was produced within 120 h in $RMG_{20}X_{50}$ with an initial OD_{600nm} value of 12, which indicates that the titer and productivity of xylose acid cannot be enhanced by simply increasing the initial inoculation concentration.

3.4. Increase in xdh Copy Numbers to Enhance Xylonic Acid Production

Since xylonic acid production can be boosted by increasing *xdh* copy numbers (Figure 2), recombinant strains with different *xdh* copy numbers were constructed to increase the xylonic acid production (Figure 4A). Another expression cassette of *xdh* under the control of P*pdc* was integrated into the genome of XA3 at the chromosomal locus of *ZMO1094* to obtain recombinant strain XA7, and the titer of xylonic acid increased to 39.5 ± 0.4 g/L from 35.2 ± 0.4 g/L in the $RMG_{20}X_{50}$ medium (Figure 4B). To further increase the xylonic acid production, recombinant strains XA8 and XA9 with four or five copies were constructed by integrating the expression cassette into the genome of XA7 at the chromosomal locus of *ZMO1547* and *ZMO1577*, respectively (Figure 4A). The xylonic acid production in XA8 and XA9 increased to 48.5 ± 1.1 g/L and 49.4 ± 0.1 g/L with the yields of 0.97 g/g and 1.04 g/g, respectively. These results indicated that the copy number of *xdh* is a key factor for xylonic acid production.

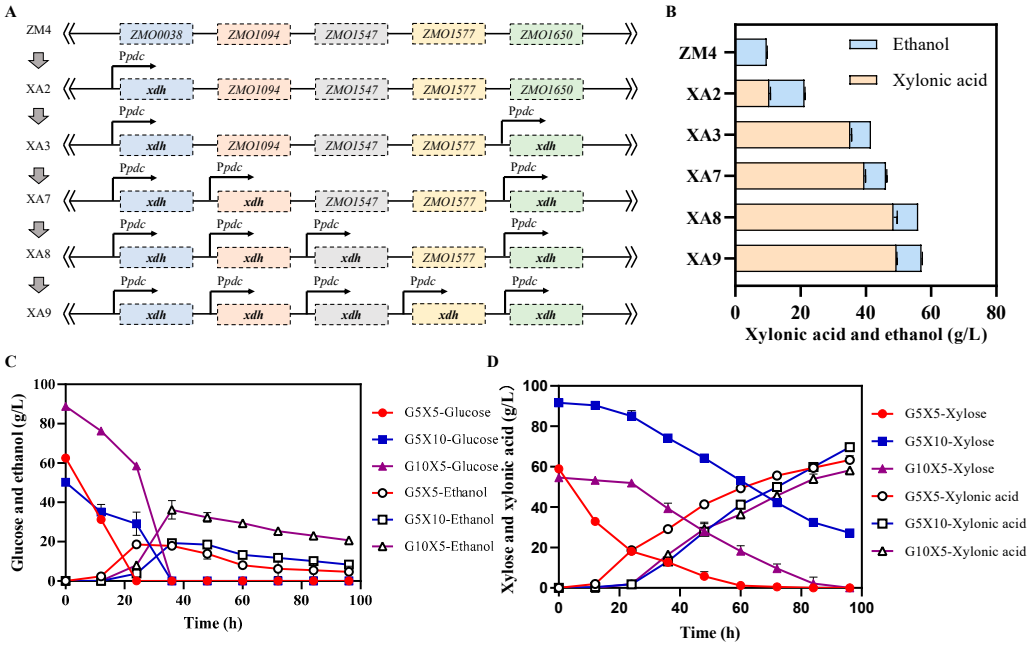


Figure 4. Improvement in xylonic acid production by increasing the copy number of *xdh*. Schematic of constructing a stable xylonic acid producer of *Z. mobilis* (**A**). Xylonic acid and ethanol production of strains with different copy numbers of *xdh* (**B**). Glucose consumption and ethanol production (**C**), as well as xylose consumption and xylonic acid production (**D**) in the medium with the different ratio of glucose and xylose using the recombinant strain XA9. Error bars represent the standard deviation values obtained in two independent experiments.

Since the initial concentration of glucose affects the xylonic acid production (Figure 2), the fermentation performance of recombinant strain XA9 in the $RMG_{50}X_{50}$ medium was further investigated (Table 3). The titer of xylonic acid increased to 51.9 ± 0.1 g/L with a maximum yield of 1.10 g/L close to the theoretical yield (Table 3). These results thus demonstrated the industrial potential of recombinant *Z. mobilis* for xylonic acid production.

Table 3. Fermentation performance of recombinant strains with different copy numbers of *xdh* after 96 h of cultivation.

Strain	Media	Glucose Consumed (g/L)	Xylose Consumed (g/L)	Ethanol Concentration (g/L)	Xylonic Acid Production		
					Titer (g/L)	Yield * (g/g)	Productivity (g/L/h)
XA3	G2X5	17.774	45.6	6.36	35.2 ± 0.4	0.77	0.37
XA7	G2X5	17.774	45.6	6.64	39.5 ± 0.4	0.86	0.41
XA8	G2X5	17.792	49.8	7.51	48.5 ± 1.1	0.97	0.51
XA9	G2X5	19.267	47.5	7.80	49.4 ± 0.1	1.04	0.51
XA9	G5X5	51.745	47.5	20.03	51.9 ± 0.1	1.10	0.54

* The yield is equal to the slope obtained by performing a linear regression of the plot of the production of xylonic acid versus the cell-consumed xylose.

Moreover, the ratios of glucose and xylose of 1:1, 1:2, and 2:1 were also investigated using strain XA9 (Figure 4C,D). The results showed that the glucose and xylose had the fastest consumption rates when the ratio was 1:1. Nearly 62.6 ± 0.0 g/L glucose and 59.0 ± 0.0 g/L were consumed, resulting in 18.7 ± 0.4 g/L of ethanol produced within 24 h and 63.34 ± 2.07 g/L of xylonic acid at a productivity of 1.05 g/L/h within 60 h produced in the $RMG_{50}X_{50}$ medium (Figure 4C,D). With the increase in the concentration of glucose or xylose, the time of glucose consumption increased to 36 h with 19.3 ± 1.0 and 36.2 ± 4.7 g/L of ethanol produced in $RMG_{50}X_{100}$ and $RMG_{100}X_{50}$, respectively. Additionally, xylonic acid production with titers of 69.7 ± 1.4 g/L and 58.2 ± 1.4 g/L and yields of 1.08 ± 0.0 g/g and 1.06 ± 0.0 g/g was achieved in the $RMG_{50}X_{100}$ and $RMG_{100}X_{50}$ medium conditions, respectively, which is the highest titer and yield of xylonic acid production achieved in *Z. mobilis*.

Since xylose transport and cofactor are important factors in enhancing xylonic acid production, two xylose transporters, *xylE* and *XylFGH*, from *E. coli* and three genes related to cofactor balance, *nadK* (encoding NAD kinase), *noxE* (NADH oxidase), and *ndh* (NADH dehydrogenase), driven by the P*pdc* promoter, were tested in recombinant strain XA9 (Figure S2). However, there was no significant increase in xylonic acid production among these strains.

3.5. Genetic and Physiologic Stability of Strain XA9

There is a risk of gene loss in multi-copy-number strain XA9 due to homologous recombination. Therefore, the genetic stability of the muti-copy recombinant strain XA9 was investigated by detecting the target gene every five generations. Three primer pairs including ChK-out-f/r, ChK-out-f/ChK-in-r, and Chk-out-r/Chk-in-f were used for genetic stability. The results showed that there was no gene loss after ten and fifteen generations (Figure 5A), suggesting the genetic stability of recombinant strain XA9 to avoid antibiotic supplementation, which can reduce production costs.

In addition, the fermentation performance of parental strain XA9 and strain XA9–15th was also investigated in $RMG_{50}X_{50}$ (Figure 5B,C). For instance, the ethanol production of XA9 and XA9–15th reached titers of 19.4 ± 1.16 and 20.7 ± 0.8 g/L, respectively, after a 24 h inoculation with all the glucose consumed (Figure 5B). Concurrently, there was no significant difference between XA9 and XA9–15th after a 96 h inoculation in the titers, which were 54.9 ± 0.5 g/L and 55.3 ± 0.3 g/L, respectively (Figure 5C). Therefore, all these results demonstrated that the recombinant strain XA9 has genetic and physiologic stability.

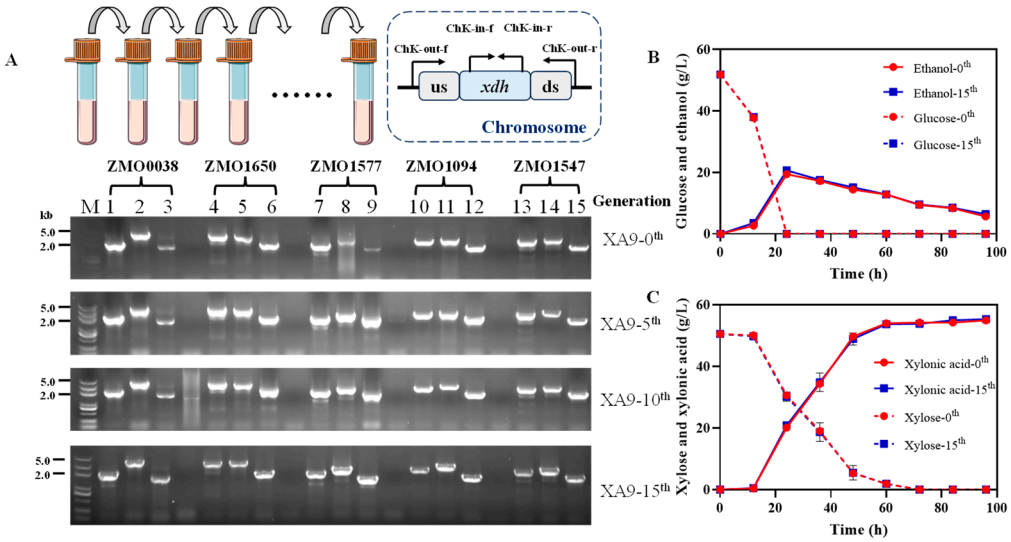


Figure 5. Genetic stability and fermentation performance of muti-copy strain XA9 during generation. Genetic stability investigation by colony PCR using the corresponding primers, including ChK-out-f/r, ChK-out-f/ChK-in-r, and Chk-out-r/Chk-in-f (**A**). Glucose consumption and ethanol production (**B**), as well as xylose utilization and xylonic acid production (**C**) after fifteen generations of muti-copy-number strain XA9 in $RMG_{50}X_{50}$ medium. Error bars represent the standard deviation values obtained in two independent experiments.

3.6. Xylonic Acid and Ethanol Co-Production Using Lignocellulosic Hydrolysate

Lignocellulosic biomass is the most abundant renewable feedstock in the world, and lignocellulosic hydrolysates after pretreatment and enzymatic hydrolysis primarily contain fermentable sugars of hexoses and pentoses such as glucose and xylose for microbial fermentation [39,40]. The hydrolysates can also be used for xylonic acid and xylitol production, which will generate ca 0.6 tons of waste xylose mother liquor per ton of xylose production during the xylose crystallization process [41].

A 30% lignocellulosic hydrolysate containing nearly 40 g/L glucose and 16 g/L xylose was used for xylonic acid and ethanol production. All glucose and xylose were utilized to produce ethanol and xylonic acid, and the titers of ethanol and xylonic acid were 4.4 ± 0.1 and 16.2 ± 0.1 g/L, respectively (Figure 6A). The yield achieved in the medium containing lignocellulosic hydrolysate was 1.03 ± 0.01 g/g, which was similar to that encountered in the pure sugar medium (Figure 6B). However, the productivity of compounds in the lignocellulosic hydrolysate were lower than that under pure sugar conditions due to the low co-utilization efficiency of glucose and xylose and the toxicity of inhibitors.

Although the xylonic acid production from xylose was achieved with a maximum yield of 1.10 g/g, further metabolic and process engineering studies are needed for glucose and xylose co-utilization to increase the titer, yield, and productivity of both ethanol and xylonic acid. For example, improving the efficient co-utilization of mixed sugars can be achieved by metabolic engineering strategies such as the introduction of the efficient sugar facilitator Glf, separation of the production process and cell growth process, reconfiguration of endogenous sugar metabolism, and adaptive laboratory evolution [42–44].

Robust microorganisms are crucial for biochemical production from lignocellulosic feedstocks. To overcome the toxicity of the hydrolysate, a systematic adaptive laboratory evolution strategy and genome minimization can be employed to develop robust strains for xylonic acid production [45]. Integration of robust bio-parts into chromosomes of *Z. mobilis* can enhance tolerance to inhibitors such as acetate, furfural, and HMF in lignocellulosic hydrolysate. In addition, there have been several reports on the use of microorganisms

such as *Aspergillus oryzae* ZN1, *Coniochaeta ligniaria* NRRL30616, *Issatchenkia occidentalis*, and *Rhodococcus aetherivorans* for the detoxification of pretreated lignocellulosic biomass [46].

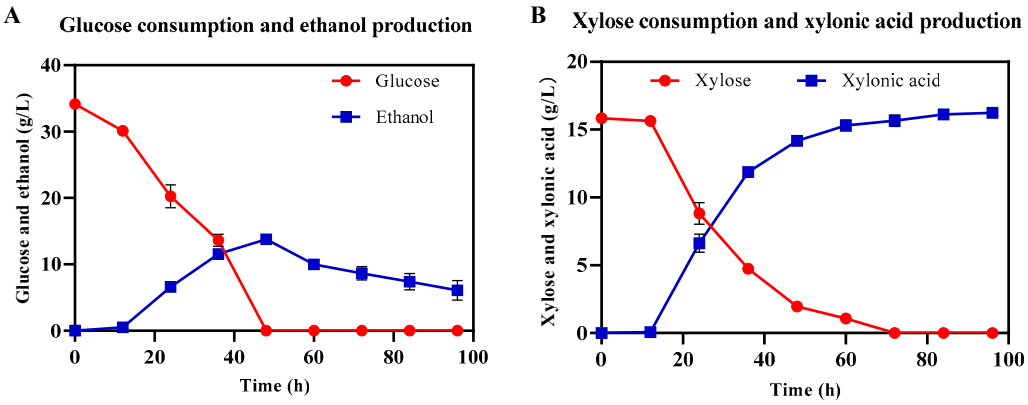


Figure 6. Fermentation performance of recombinant strain XA9 in lignocellulosic hydrolysate. Glucose consumption and ethanol production (**A**), as well as xylose consumption and xylonic acid production (**B**). The results shown are at least two, and the error bars represent standard deviation.

Xylonic acid production through chemical synthetic routes is costly due to the expensive catalysts and energy. The one step of xylose to xylonic acid in *Z. mobilis* reduces these costs and generates valuable co-products that can be economically exploited. For instance, the ethanol produced from glucose during fermentation can be purified and sold as biofuel to promote the economic benefits of lignocellulose biomass [47].

4. Conclusions

In this work, a series of recombinant strains of *Z. mobilis* were constructed using CRISPR-Cas genome engineering tools for xylonic acid production. Our results demonstrated that overexpression of *xdh* by increasing the copy numbers is crucial for xylonic acid production. A recombinant strain, XA9, with five copies exhibited the highest capacity for xylonic acid production stably at a titer of 51.9 $\pm$ 0.1 g/L and a maximum yield of 1.10 g/g in the $RMG_{50}X_{50}$ medium condition. Moreover, the fermentation condition was optimized to increase xylonic acid production in aerobic conditions. Our study thus demonstrates that *Z. mobilis* has the potential to be developed as a promising platform for xylonic acid production from lignocellulosic biomass and provides a strategy for future xylose utilization of lignocellulosic hydrolysates.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation11030141/s1, Figure S1: Effect of high cell density on xylonic acid production. Xylonic acid production with an initial OD_{600nm} value of 12 (A) and 20 (B) in RMX_{50}, $RMG_{20}X_{50}$, and $RMG_{50}X_{50}$, respectively. Figure S2: Application of metabolic strategies including xylose transporter and cofactor engineering for xylonic acid production. Xylose consumption (A) and xylonic acid production (B) of recombinant strain. Glucose utilization (C) and ethanol production (D) of recombinant strains. Table S1: Primers used in this work.

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Conflicts of Interest: S.Y. is the founder of Wuhan ZymoBiotech Inc., and a patent application is associated with this study.

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