Lowering whole cost for sugarcane-based ethanol production by engineered *Zymomonas mobilis*

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**Abstract**

Lowering cost will prompt the sustainable development of sugarcane-based ethanol industry. In this work, we developed a low-cost process for ethanol production from sugarcane by a genetically engineered *Zymomonas mobilis*. Fermentation media were first optimized, resulting in a 15.54% increase in ethanol fermentation efficiency as compared to control media. To further reduce the byproduct levan formation, a levansucrase-encoding gene of *Z. mobilis*, *sacB*, was deleted through the type I-F CRISPR-Cas system, which resulted in a further elevation of both ethanol conversion ratio and productivity comparing with the starting strain ZMS912 (87.50% vs. 76.77%, 1.95 g/L/h vs. 1.71 g/L/h). Moreover, we conducted fed-batch fermentation for ethanol production using sugarcane juice in 5 L bioreactors and employing the optimized media and engineered strain. The results showed that maximum ethanol titer of 81.59 g/L and productivity of 5.83 g/L/h were achieved. Finally, preliminary techno-economic assessment demonstrated that our efforts to modify media and strain could reduce the processing cost of ethanol production from sugarcane juice, which provides the feasibility for economic ethanol production in the future.

**KEYWORDS**

bioethanol, CRISPR-Cas, fed-batch fermentation, nitrogen source, sugarcane juice, *Zymomonas mobilis*
1 | INTRODUCTION

With the aggravation of energy shortage and environmental pollution, the replacement of fossil oil with renewable biomass fuel has aroused great interest (De Bhowmick et al., 2018; Zhang et al., 2018). Bioethanol as a clean and renewable fuel has been widely produced in many countries (Goldemberg, 2007; Mussatto et al., 2010). Currently, ethanol produced from sugar-based feedstock accounts for 40% of the total bioethanol production (Mussatto et al., 2010). Sugarcane is a major sugar crop cultivated globally. Approximately 80–120 tons of sugarcane can be produced per hectare of land (Huang et al., 2020). Sugarcane juice contains approximately 14%–22% fermentable sugars that mainly comprise of sucrose, glucose, and fructose (Goldemberg et al., 2008). Compared with other feedstocks such as starch crops and lignocellulosic biomass, the ethanol production from sugarcane juice does not require pretreatment, saccharification, and liquefaction stage, so the ethanol production process is simpler and the production cost is lower (Zhang et al., 2017).

Zymomonas mobilis, a kind of ethanologenic α-proteobacteria, has several outstanding properties making them promising for commercial bioethanol production. For example, it employs Entner–Doudoroff (ED) pathway to efficiently metabolize sugar and produce ethanol. Meanwhile, a large cell surface area allows it for fast conversion of sugar into ethanol. In addition, the high concentration of hopaloid protects its tolerant to ethanol and other environmental stimuli. Compared with classical ethanol producers S. cerevisiae that employs Embden–Meyerhof–Parnas (EMP) pathway for the sugar metabolism, using the ED pathway enables Z. mobilis to produce ethanol more efficiently with less ATP production and biomass accumulation (He et al., 2014; Xia et al., 2019; Yang et al., 2016). However, it has been reported that ethanol production of Z. mobilis was reduced when sucrose worked as carbon source, due to the generation of two main byproducts, levan and sorbitol, when the hydrolysis rate of sucrose exceeded the absorption rate of glucose and fructose (Rogers et al., 2007). Levan is a fructose polymer, formed by the catalysis of levansucrase in Z. mobilis (Silbir et al., 2014). There are three levansucrases in Z. mobilis: SacA, SacB, and SacC. Among them, the SacC sucrase activity accounts for more than 70% of the total sucrase activity, which mainly hydrolyzes sucrose into monosaccharides, but the intracellular sucrase SacA has extremely low hydrolysis efficiency of sucrose. The levansucrase SacB has sucrose-hydrolyzing and levan-generating activity (Gurunathan & Gunasekaran, 2004). Ananthalakshmy et al. overexpressed the sacB gene in Z. mobilis B14023, which increased the extracellular levansucrase activity to 187 U/ml (Ananthalakshmy & Gunasekaran, 1999). Sorbitol is formed by the conversion of fructose under the catalysis of glucose–fructose oxidoreductase (GFOR). However, previous study has shown that interference with the gfo gene in Z. mobilis resulting in poor growth and reduced ethanol fermentation capacity (Sootsuwan et al., 2013), likely because sorbitol serves as an osmoprotectant to counteract the inhibitory effects of high sugar or other products (e.g., ethanol, organic acid, etc.) on Z. mobilis (Vignoli et al., 2010). So far, there is no report on the enhancement of ethanol production by reducing levan metabolism. Recently, however, a hand of molecular techniques have been developed to modulate the metabolism and regulate the expression in Z. mobilis, including genome shuffling (Wang et al., 2019), Tn5-based transposon mutagenesis (Wang et al., 2016), clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system (Wang et al., 2021), CRISPR interference system (Banta et al., 2020), Type I-F CRISPR-Cas system (Zheng et al., 2019), CRISPR-Cas12a system (Shen et al., 2019), etc. These methods should be available to reduce byproducts, such as levan, in the ethanol production process of Z. mobilis.

Moreover, the nitrogen source in the microbial fermentation system is usually the most costly ingredient, and ethanol production will be significantly reduced due to the lack of nitrogen source (Puligundla et al., 2019; Yue et al., 2012). Z. mobilis can grow using a wide variety of nitrogen sources, of which organic nitrogen sources such as yeast extract (YE) or peptone are most conducive to cell growth (Li et al., 2020). However, the cost of YE is relatively high. Thus, replacement or partial replacement with a cheaper nitrogen source will be essential to lower the processing cost for fermentation industry, in particular for ethanol or other low value-added products.

In this study, we employed Z. mobilis for bioethanol production using sugarcane juice as substrate. First, the medium composition was investigated to minimize media costs and maximize ethanol production. Then, CRISPR-Cas technology was used to knockout a levan-synthesis gene sacB (ZMO0374) in Z. mobilis, thereby reducing its carbon flux. Simultaneously, higher ethanol concentrations were achieved through fed-batch fermentation in a 5 L fermentor. Finally, techno-economic analysis and mass balance of the entire process were performed to determine the industrial applicability of Z. mobilis in sugar-based ethanol production.

2 | MATERIALS AND METHODS

2.1 | Feedstocks, strains, and plasmid

Fresh sugarcanes were purchased from a supermarket in Chengdu city, Sichuan, China. The sugarcane was squeezed through a juicer to collect the liquid part, followed
by centrifuging at 4000 rpm for 10 min, the resulting sugar-cane juices (SCJ) were stored at −20°C for further use. For fermentation, the juice was autoclaved at 115°C for 20 min and diluted to the desired concentration in sterilized water.

Z. mobilis strain ZMS912 (GDMCC number 60583) was used as the starting strain for SCJ fermentation (Duan et al., 2019). Escherichia coli strain Trans110 (TransGen Biotech, China) was used to construct and clone plasmids for gene editing in this study. A Z. mobilis–E. coli shuttle plasmid, pMini, was the backbone to insert the spacer sequence for CRISPR-mediated gene editing in Z. mobilis (Wang et al., 2021). The plasmid encodes a spectinomycin (spe)-resistant gene for selection.

### 2.2 Media and growth conditions

A single colony of ZMS912 was inoculated into 8 ml of the rich medium (RM: 20 g/L glucose, 10 g/L YE, 2 g/L MgSO₄, 1 g/L (NH₄)₂SO₄, and 2 g/L KH₂PO₄), and cultured without shaking for 16 h at 30°C. Cell pellets were harvested by centrifuging at 4000 rpm for 5 min, and inoculated in 100 ml of the media containing SCJ (Table 1) for batch fermentation in a 250 ml flask. The batch fermentation was conducted at 30°C without shaking. All fermentations are performed in triplicate.

Escherichia coli strain Trans110 (TransGen Biotech, China) used for cloning was cultured at 37°C in Luria-Bertani (LB) media (10 g/L tryptone, 5 g/L YE, 5.0 g/L NaCl, pH 7.0).

### 2.3 Fed-batch fermentation

A fed-batch fermentation was carried out in a 5 L bioreactor (T&J Bioengineering). Z. mobilis cells were precultured in 240 ml RM and grown at 30°C for 20 h. The cultures were centrifuged at 4000 rpm for 5 min, and the cell pellet was transferred to 3 L of SCJ medium that comprised of 100 g/L (total sugar) SCJ, 1 g/L (NH₄)₂SO₄, and 2 g/L YE. The initial OD₆₀₀ was about 0.15. The fermentation was carried out at 30°C without stirring. When sugars in the fermentation broth were less than 5.0 g/L (real-time determination by HPLC), the concentrated SCJ was replenished until the ultimate fermentation volume reached about 4.5 L. The pH value of the broth was maintained at 6.0 with 2 M H₂SO₄ or 2.5 M NaOH during fermentation (based on the response surface optimization process results, the data are not shown). Cultures were sampled at 3 h intervals for analysis of the cells density, sucrose, glucose, fructose, byproducts, and ethanol concentrations. All fermentations are conducted in triplicate.

### 2.4 Plasmid construction and mutant strain selection

Primers used for plasmid construction are summarized in Table 2. In this study, the endogenous type I-F CRISPR-Cas system was used to knockout the sacB gene in ZMS912 according to previous studies (Wang et al., 2021; Zheng et al., 2019). The sgRNA fragment was ligated with a linearize pMini vector via Gibson Assembly, yielding a plasmid (pMini-SacB) possessing an artificial mini-CRISPR array. DNA fragments of 500 base pairs (bp) upstream and downstream sacB (sacB-up and sacB-down), which served as donor DNAs for gene editing, were obtained from ZMS912 cells by PCR amplification, respectively, using primer sets (sacB0374up-F and sacB0374up-R, for sacB-up; sacB0374down-F and sacB0374down-R, for sacB-down, shown in Table 2) and DNA polymerase KOD-Plus-Neo (Toyobo). The donor DNA amplicons were purified through a 1% agarose-gel electrophoresis using the Gel Extract Kit (Omega Biotek), then fused with the linearized pMini-SacB backbone via the Gibson Assembly Master Mix (NEB). Five microliters of the ligation solution was used for transformation with E. coli DH5α, and the transformation efficiency was estimated by colony colony method.

### Table 1

| Group | pH   | Total sugar (g/L) | Urea (g/L) | (NH₄)₂SO₄ (g/L) | Yeast extract (g/L) |
|-------|------|-------------------|------------|-----------------|--------------------|
| A     | 5.50 | 100               | —          | —               | —                  |
| B     | 5.50 | 100               | 0.5–2.0    | —               | —                  |
| C     | 5.50 | 100               | —          | 0.5–2.0         | —                  |
| D     | 5.50 | 100               | —          | 1.0             | 1.0–6.0            |
| E     | 5.50 | 50–150            | —          | 1.0             | 1.0                |
| F     | 4.0–6.0 | 100       | —          | 1.0             | 1.0                |

*Medium for different experimental groups. The medium for determining optimal (A) Control, (B) Initial concentration of urea, (C) Initial concentration of (NH₄)₂SO₄, (D) Initial concentration of yeast extract, (E) Initial concentration of total sugar, using sterile water for gradient dilutions. (F) pH of fermentation broth, using 2.5 M NaOH or 2 M H₂SO₄ to adjust the pH to different levels.
were introduced in *E. coli* Trans110 competent cells (TransGen) according to the manufacturer’s protocol. The transformants were selected in LB media supplemented with 100 μg/ml spe. The generating genome-editing plasmid, named pMini-TSacB (150 ng), was introduced into *Z. mobilis* S912 competent cells using a Gene Pulse Xcell (0.1 cm gap cuvettes, 1.6 kV, 200 Ω, 25 μF) (Bio-Rad). Transformants were selected at 30°C in RM supplemented with 100 mg/L spe (He et al., 2021). Microbial PCR was used to screen mutant candidates using primers sacB0374KO0374-F and sacB0374KO0374-R (Table 2). The PCR products were validated by agarose gel electrophoresis and Sanger sequencing (TsingKe).

### 2.5 | Analytical methods

#### 2.5.1 | Composition analysis of sugarcane juice

The nutrient composition of sugarcane juice is shown in Table 3. The SCJ was centrifuged at 12,000 rpm for 2 min,
and the supernatant was diluted for 20 times, the concentration of total nitrogen was measured by a TOC-TN analyzer (SHIMADZU, TOC-V). Reducing sugar was detected as described by Somogyi–Nelson (Nelson, 1944). The soluble sugar content was determined with the phenol-sulfuric acid method (Mecozzi, 2005). The total soluble solid of SCJ was determined by a hand-held refractometer (AS ONE, VBR20B). Sugars (sucrose, glucose, and fructose) of SCJ were analyzed by high-performance liquid chromatography (HPLC, see Section 2.5.2). For convenience, total sugar was presented with glucose equivalents (total sugar = glucose + fructose + 1.05 × sucrose; Rochon et al., 2019). Besides, the content of mineral elements in SCJ was determined by inductively coupled plasma (ICP, PlasmaQuant 9000).

2.5.2 | Analysis of sugars, ethanol, byproducts, and cell density

Fermentation supernatant after centrifugation was diluted by 20 folds with sterilized water and filtered with the 0.22 μm membrane. The concentrations of sucrose, glucose, fructose, ethanol, glycerol, and sorbitol were measured by HPLC (Agilent 1200 series), using an HPX-87H ion exclusion column (BioRad Aminex) heated at 35°C and a RID detector maintained at 40°C. H2SO4 (5 mM) worked as mobile phase at a flow rate of 0.6 ml/min.

The content of levan was quantified with a method described previously (Viikari, 1984). Briefly, after the broth was centrifuged at 12,000 g for 5 min to remove biomass, the supernatant was precipitated by adding three volumes of ice-cold absolute ethanol. After centrifuged at 5000 rpm for 10 min, precipitants were hydrolyzed with 0.1 M HCl at 100°C for 1 h. Then the content of levan was determined by DNS method (Nelson, 1944) with fructose as a unit.

In addition, cellular optical density was measured at a wavelength of 600 nm with a spectrophotometer (Jinko UV765). The DNA concentration was determined with a Qubit 3 fluorometer (Life Technologies).

3 | RESULTS

3.1 | Nitrogen source optimization

To check the availability for fermentation using SCJ, we measured its compounds, and found that sucrose in SCJ was abundant but nitrogen content was only 263.93 mg/L (Table 3). Nitrogen is essential for both microbial growth and metabolism. For ethanol fermentation, nitrogen is usually one of the most expensive components (Li et al., 2017). Hence, we first studied the impact of nitrogen sources such as urea, ammonium sulfate, and YE, on sugarcane ethanol fermentation by ZMS912 (Table 1).

As shown in Figure 1a,b, the fermentation remarkably decreased from 66.43% to 56.52% with the increase of urea from 0.5 to 2.0 g/L, suggesting that high urea content resulted in significant decrease of cell density and ethanol

FIGURE 1 Fermentation kinetics of Z. mobilis 912 under different nitrogen sources in SCJ fermentation medium. (a, c, f) cell growth when urea, (NH4)2SO4, yeast extract, and (NH4)2SO4 are added, respectively; (b, d, g) sugar consumption and ethanol production when urea, (NH4)2SO4, yeast extract, and (NH4)2SO4 are added, respectively. (e) control group; sugar consumption (solid line) and ethanol production (dotted line)
production. Similarly, supplementation with (NH₄)₂SO₄ also affected Z. mobilis growth and fermentation, as final ethanol fermentation efficiency and cell density (OD₆₀₀) were approximately 66.90% and 1.47, respectively, lower than those in the control medium (69.81% and 1.88). However, under all tested (NH₄)₂SO₄ concentrations, both growth and fermentation were affected without obvious differences (Figure 1c,d). This phenomenon may attribute to the excessive addition of ammonia leading to a large accumulation of byproducts, resulting in hypertonic pressure and substrate inhibition on the strain. In the control group, where no extra nitrogen was added, the fermentation showed that Z. mobilis enabled ethanol production using SCJ solely. Despite the control’s OD₆₀₀ reached 1.88, the ethanol fermentation efficiency was only 69.21% (Table 4; Figure 1e). This uncoupling between growth and fermentation performance has been reported Z. mobilis, partly due to the insufficient nitrogen source and metal ions of the fermentation medium (Li et al., 2020).

To figure out what caused the inhibition, we checked byproducts’ accumulation in the presence of urea or (NH₄)₂SO₄. Compared to the control medium, the accumulation of both sorbitol and levan increased by 85.18% and 65.20%, respectively, when 0.5 g/L urea was added (Table 4). Similarly, when (NH₄)₂SO₄ concentration is 1.0 g/L, the accumulation of sorbitol and levan also increased by 49.88% and 92.00%, respectively. Moreover, organic acids were generated in the presence of both nitrogen sources, though they were not as high as in the control (Table 4), which resulted in a significant decrease of pH in the fermentation broth, inhibiting the growth of strain and might cause a decrease in fermentation efficiency (Narendranath et al., 2001). Besides, a previous study has shown that urea can react with ethanol to produce ethyl carbamate, resulting in a lower ethanol concentration (Laopaiboon et al., 2009).

Since the combination of inorganic with organic nitrogen can significantly increase the cell growth of Z. mobilis (Veeramallu & Agrawal, 1988), we further optimized nitrogen source for SCJ fermentation, that is, a nitrogen mixture for efficient but economic fermentation. When 1 g/L (NH₄)₂SO₄ with YE of different concentrations (1.0–6.0 g/L) was added, the cell growth and fermentation were significantly enhanced (Figure 1f,g). In particular, supplementing 1 g/L YE allowed the cell density (OD₆₀₀) increased by 4.79% and 34.01%, compared to the control (i.e., SCI; 1.97 vs. 1.88) and (NH₄)₂SO₄ alone (1.97 vs. 1.47), respectively (Table 4). Moreover, ethanol fermentation efficiency of this group was also 15.54% and 20.57% higher than that of the control and (NH₄)₂SO₄ alone, respectively. This was partly attributed to the fact that YE provided sufficient nitrogen compounds, micronutrients, and other vital growth factors for the strain. On the other hand, due

### TABLE 4 Kinetic parameters of ethanol production by Z. mobilis S912 under different nitrogen sources conditions

| Strain     | Nitrogen sources                  | Consumed sugar (g/L) | Cell density (OD₆₀₀) | Parameters (mean ± SD) | Byproducts | Cell density (OD₆₀₀) | Parameters (mean ± SD) |
|------------|-----------------------------------|-----------------------|----------------------|------------------------|------------|----------------------|------------------------|
|            |                                    | P (g/L)               | Qp (g/L/h)           | Yps (g/g)              | E (%)      | Sorbitol (g/L)       | Levan (g/L)            | Acetic acid (g/L)       | Propionic acid (g/L)   |
| Z. mobilis S912 | Controlb  | 101.45 ± 0.15 | 1.88 ± 0.05 | 36.60 ± 0.59 | 2.03 ± 0.03 | 0.36 | 69.81 | 8.30 ± 0.15 | 2.50 ± 0.09 | 0.77 ± 0.01 | 3.88 ± 0.06 |
|            | 0.5 g/L Urea | 110.26 ± 0.12 | 1.50 ± 0.04 | 37.92 ± 0.68 | 1.05 ± 0.02 | 0.34 | 66.43 | 15.37 ± 0.75 | 4.13 ± 0.13 | 0.72 ± 0.03 | 3.01 ± 0.33 |
|            | 1 g/L (NH₄)₂SO₄ | 109.53 ± 0.14 | 1.47 ± 0.04 | 37.86 ± 0.99 | 1.04 ± 0.03 | 0.35 | 66.90 | 12.44 ± 0.31 | 4.80 ± 0.01 | 0.72 ± 0.04 | 2.60 ± 0.08 |
|            | 1 g/L (NH₄)₂SO₄ and 1 g/L YE | 94.80 ± 0.09 | 1.97 ± 0.04 | 39.39 ± 0.67 | 2.07 ± 0.04 | 0.42 | 80.66 | 2.27 ± 0.33 | 5.96 ± 0.26 | 0.43 ± 0.04 | 0.30 ± 0.08 |

Parameter definitions: P, ethanol concentration; Qp, volumetric ethanol productivity; YE, yeast extract; Yps, ethanol yield. The experiments were performed in triplicate.

aE, fermentation efficiency; P, ethanol concentration; Qp, volumetric ethanol productivity; YE, yeast extract; Yps, ethanol yield. The experiments were performed in triplicate.

bNo nitrogen source added, diluted with sterilized water.
to the addition of YE, the concentration of sorbitol was only 1/4 of that of the control medium (2.2 g/L vs. 8.3 g/L), the concentration of propionic acid was only 0.3 g/L (the control was 3.88 g/L). The accumulation of byproducts was decreased (Table 4), which significantly improved the cell growth environment and metabolism. Combined with the fermentation effect and low input cost, the nitrogen source condition was determined to be 1.0 g/L (NH₄)₂SO₄ and 1.0 g/L YE. This result also indicated that Z. mobilis requires less nitrogen source to grow and ferment, which laid the foundation for economic ethanol production.

3.2 | Optimization fermentation parameters in batch fermentation

3.2.1 | Initial total sugar concentration

Next, we investigated the effect of initial total sugar concentration on the ethanol fermentation. SCJ's total sugars were adjusted from 50 to 150 g/L for fermentation (Table 1, group E). The results revealed a trend that cell density and ethanol production ability increased but ethanol fermentation efficiency gradually decreased along with growing sugar concentrations (Figure 2a,b). In comparison to the fermentation under high sugar concentration (150 g/L), the ethanol fermentation efficiency under 50 g/L declined by 12.73% (Figure 2b). As shown in Figure 2b, the accumulation of sorbitol and levan is positively correlated with the increase in total sugar concentration. When 150 g/L total sugar concentration was present in the media, the sorbitol and levan amount reached 15.82 and 2.90 g/L, respectively. These results showed that higher total sugar concentration has a negative influence on fermentation efficiency. This undesirable phenomenon may attribute to the higher initial sugar concentration, the excessive accumulation of byproducts. The previous study has also shown that Z. mobilis grows on sucrose or compounds of glucose and fructose, and approximately 11% of the carbon source flows to sorbitol production to offset the harmful effects of hypertonic environment (Loos et al., 1994). Besides, levan accumulation also consumes part of sugar in SCJ and reduces ethanol output.

3.2.2 | Fermentation pH

The widespread pH spectrum for growth (from 3.5 to 7.5) and fairly strong acid tolerance are both typical features of Z. mobilis (He et al., 2014). To confirm the optimal pH for the fermentation with Z. mobilis, the pH value of SCJ medium was adjusted from 4.0 to 6.0 by 2.5 M NaOH or 2 M H₂SO₄, and fermentation lasted for 36 h (Table 1, group F). As shown in Figure 2c,d, the cell density and ethanol production stabilized at a pH range of 5.0–6.0, but the growth was obviously inhibited when the pH of broth was below 4.5. The fermentation kinetics showed that cell density, ethanol concentration, and production efficiency were 1.97, 40.78 g/L, and 78.88% at pH 5.0, respectively, far better than that at pH 4.0.

It was found that the accumulation of the two main byproducts sorbitol and levan showed different trends when pH value was shifted from 4.0 to 6.0. The concentration of levan decreased with the increase in pH from 11.19 g/L at
pH 4.0 to 2.09 g/L at pH 6.0. Whereas the accumulation of sorbitol reached 6.6–7.1 g/L in the pH range of 5.0–6.0, which was consistent with the effect of ethanol fermentation. These results indicated that lower pH (<4.5) could delay the growth and ethanol production capacity of ZMS912, but possibly promote the metabolic expression of extracellular levansucrase to generate more levan.

3.3 Ethanol fermentation by Z. mobilis S912 SacB deletion mutant

As aforementioned, the ethanol fermentation using sucrose-rich feedstock, such as SCJ, could generate byproducts such as sorbitol and levan that lead to inferior ethanol production or carbon imbalance. Albeit alleviated by optimizing nitrogen sources and fermentation parameters, the both byproducts remained in cultures. However, the deletion of gfo gene has been proven to weaken Z. mobilis robustness since sorbitol works as an osmoprotectant and seems to be essential for ethanol fermentation of Z. mobilis. Thus, to improve the ability of Z. mobilis for ethanol production with SCJ, we employed the CRISPR-Cas system to knockout the levan synthesis gene sacB. Both PCR and sequencing results revealed that sacB’s 400 bp functional domain was successfully deleted (Figure 3a). The mutant was named ZMS912/ΔSacB0374.

To verify whether the mutant can reduce the byproduct accumulation, the ethanol fermentation with SCJ was performed using the mutant ZMS912/ΔSacB0374 and its parent ZMS912 (Figure 3b,c). The cell density (OD600) of the mutant was 1.76 in the stationary phase, decreased by 4.86% compared with that of ZMS912, which means less sugar is utilized to cell growth. After 24 h of fermentation, both strains consumed all sugar, the total sugar utilization rate of ZMS912 was slightly faster than that of ZMS912/ΔSacB0374. However, compared with ZMS912, the ethanol fermentation efficiency of ZMS912/ΔSacB0374 increased by 13.98% (87.50% vs. 76.77%), and the volumetric ethanol productivity and ethanol yield were also increased by 14.04% and 12.50%, respectively (Figure 3b,c). Correspondingly, in the fermentation with the mutant, sorbitol of the mutant was reduced by 43.93%, and levan was no longer produced (Figure 3d). Besides, the deletion of sacB in ZMS912 also helped reduce significantly the formation of acetic acid and propionic acid, making fermentation more favorable and reducing the cost of downstream separation and purification.

3.4 Fed-batch fermentation of Z. mobilis using SCJ for ethanol production

To explore the availability of large-scale bioethanol production from SCJ by the mutant ZMS912/ΔSacB0374, fed-batch fermentation was conducted on bench scales (5 L). The results demonstrate that ethanol concentration and productivity were considerably improved by ZMS912/ΔSacB0374 in fed-batch fermentation (Figure 4a). After three feedings, ethanol fermentation finished after 33 h with a maximum ethanol concentration of 81.59 g/L. After the first fed-batch, the highest ethanol volume productivity was 5.83 g/L/h, which was more than 198.97%
The maximal ethanol yield and fermentation efficiencies were 0.50 g/g and 92.78% (Table 5), respectively. We also found that the fed-batch improved the cell density to a maximum (OD$_{600}$) of 2.10, which was 19.32% higher than the laboratory scale (250 ml). The better fermentation performance of scaled-up fermentation might be due to the preferable homogenization furnished by the apparatus and enhanced the mass transfer process between the culture medium and the cells (Ma et al., 2016).

Meanwhile, sorbitol increased slightly after the first two feedings but increased markedly to 8.85 g/L after the third feeding (Figure 4b). It indicated that the ethanol concentration reaching a certain threshold will promote the metabolism of $Z$. mobilis to produce more sorbitol to maintain the osmotic pressure of cells (Yang et al., 2016). Furthermore, the accumulation of glycerol, acetic acid, and propionic acid in the fermentation broth were 0.45 ± 0.01, 0.69 ± 0.23, and 1.83 ± 0.36 g/L, respectively. Such low content of organic acids and fusel contributes further to both robust fermentation and easy separation process.

### 3.5 Mass balance

The mass balance of bioethanol production by sugarcane was acquired based on the bioreactor fermentation outcome and explained in Figure 5. Based on the above research results, 1000 kg of sugarcane could produce 68.70 kg (87.07 L) ethanol, 66.31 kg CO$_2$, and 7.53 kg sorbitol under optimized conditions. These values are slightly higher than the average level of Brazilian autonomous distilleries (85.20 L/ton of sugarcane; Junqueira et al., 2017). Compared with using ZMS912, the output of ethanol increased by about 19.14 kg/ton sugarcane for engineered ZMS912/ΔSacB$_{0374}$. According to the present average selling price of ethanol in the international fuel market at 0.69 US$/kg (http://www.tradingeconomics.com/commodity/ethanol), the output of ethanol production can increase by 13.21 US$/ton sugarcane. Besides, upgrading the CO$_2$ byproduct (66.31 kg) into value-added chemicals will also increase the revenue and profitability of fuel ethanol production. Theoretically, the remaining 250 kg of bagasse was also fermented by $S$. cerevisiae or $Z$. mobilis to produce 15.25 kg (19.33 L) of ethanol (Figure 5; Santos & Cruz, 2017; Zhang & Zhu, 2017). Thereby, it
is feasible to augment ethanol production by 22.20%. The integrated first- and second-generation (1G–2G) ethanol production process will eventually produce about 106.4 L/ton sugarcane. This consequence is also close to the relevant prediction value of Junqueira et al. (108.4 L/ton of sugarcane; Junqueira et al., 2017).

**4 | DISCUSSION**

Bioethanol is the main force in future energy supply and carbon reduction, and to achieve its sustainable development, lower-cost production processes and excellent fermentation strains will play a vital role (Buchspies et al., 2020; Nie et al., 2020). This study first optimized the sugarcane-based ethanol fermentation conditions, and under the conditions of 1 g/L (NH4)2SO4 and 2 g/L YE, initial total sugar 100 g/L, pH 6.0, and no other nutrients supplement, the ethanol fermentation efficiency was increased by 15.54% compared with the control medium. Then, through CRISPR-Cas technology, the levan synthesis gene sacB in ZMS912 was knocked out, which further increased the ethanol fermentation efficiency by 13.98% (compared with ZMS912) and reduced the accumulation of byproducts. Finally, the maximum ethanol concentration of 81.59 g/L (maximum ethanol productivity up to 5.83 g/L/h) was obtained by fed-batch fermentation on a bench scale (5 L). Mass balance analysis showed that the ethanol output could reached 87.07 L/ton of sugarcane (1G).

Based on a sugarcane-based ethanol refinery with an annual capacity of 4 million tons, Longati et al. conducted a detailed techno-economic analysis, and estimated the minimum ethanol selling price (MESP) to be 471.3 US$/m^3 based on each section industrial inputs (Table S1; Longati et al., 2018). Since its output parameters are similar to the results of our study, the MESP may be applicable to our research if ZMS912/ΔSacB0374 is used in a sugarcane biorefinery. Of course, due to the complex and diversified energy balance of the practical biorefinery plant, the actual MESP should be slightly higher. Simultaneously, Z. mobilis, as a facultative anaerobe, is unnecessary to stir and control the oxygen content during the fermentation process (Yang et al., 2020). At this scale, it is estimated that approximately 28 MW of electricity can be saved every year (Pratto et al., 2020). Besides, we have not encountered any published literature reporting that the volumetric productivity of ethanol in Z. mobilis reached 5.8 g/L/h. High ethanol productivity also means that it produced in fed-batches in a shorter period, thereby adding more capacity in the same production time. For instance, comparing the current average productivity of Brazil’s ethanol refinery (about 4.5–5.0 g/L/h; Lopes et al., 2016), an ethanol plant with an annual output of 4 million tons can increase its production capacity by up to 88,000 tons per year. Meanwhile, combining relatively high ethanol levels with superior productivity also can minimize processing costs.

ZMS912/ΔSacB0374 also compared to other ethanologenic strains, including Pichia kudriavzevii, S. cerevisiae, K. marxianus, etc., which use various strategies and models from multiple sugar-based feedstocks to produce bioethanol. As shown in Table 6, ethanol titer and productivity will be higher or comparable to the consequences reported by previous authors. However, the ethanol titer we obtained was lower than that reported by Monteiro B. It was attributed to the use of a very high gravity fermentation strategy in that study, and addition of high cell biomass (7 g/100 ml), which directly offset the lag period between strain growth and ethanol fermentation (Monteiro et al., 2018). Simultaneously, the amount of nitrogen sources used in our study is lower, which can save a certain amount of production cost in large-scale ethanol production. In addition, when ZMS912/ΔSacB0374 used for fermenting sugarcane-based substrates, ethanol is the main product and no longer generates levan, which would
Overall, a process of ethanol fermentation from SCJ using engineered ZMS912/ΔSacB0374 was established. The effective combination of two nitrogen sources (NH₄)₂SO₄ and YE can shorten the fermentation time and increase ethanol concentration and yield. The fed-batch fermentation strategy further improves production efficiency. Furthermore, an effective combination of two nitrogen sources (NH₄)₂SO₄ and YE can shorten the fermentation time and increase ethanol concentration and yield. The fed-batch fermentation strategy further improves production efficiency.

### Table 6

Summary of previous results from published literature on ethanol production from SCJ or high sugar concentration substrates

| Strains       | Initial sugar | Nitrogen resources | Strategy | Substrates | Ethanol (g/L) | Productivity (g/L/h) | Reference |
|---------------|---------------|--------------------|----------|------------|---------------|-----------------------|-----------|
| *Pichia kudriavzevii* | 16.6% (w/v) | 2 g/L YE and 2 g/L peptone | Batch | SCI       | 71.90         | 4.00                  | Dhaliwal et al. (2011) |
| *S. cerevisiae* ATCC24858 | 13% (by mass) | NA                 | Batch | SSI       | 49.48         | 2.37                  | Luo et al. (2014)       |
| *S. cerevisiae* CAT-1 | 35 °Brix      | 3.2 g/L DAP        | VHG     | SCJ       | 114.80        | 4.77                  | Monteiro et al. (2018)  |
| *S. cerevisiae* DMKU 3-S087 | 200 g/L      | 1.0 g/L(NH₄)₂SO₄  | Batch   | Molasses  | 72.40         | 1.21                  | Pattanakittivorakul et al. (2019) |
| *Z. mobilis*       | 160 g/L       | 2 g/L DAP and 2 g/L urea | Batch   | Molasses  | 73.00         | 2.21                  | Khoja et al. (2015)     |
| *Z. mobilis* DSM 473 | 100 g/L       | 50 ml/min N₂       | Repeated-batch | Glucose | 47.23         | 0.68                  | Palamae et al. (2020)   |
| *Z. mobilis* AD50  | 100 g/L²      | 10 g/L YE          | Batch   | Glucose and xylose | 47.00       | 3.30                  | Sarkar et al. (2020)    |
| *Z. mobilis* S912/ΔSacB₀₇₄ | 100 g/L       | 2 g/L YE and 1 g/L(NH₄)₂SO₄ | Fed-batch | SCJ       | 81.59         | 5.83                  | This study               |

Abbreviations: DAP, diammonium phosphate; NA, not available; SSI, sweet sorghum juice; VHG, very high gravity fermentation; YE, yeast extract. a50 g/L glucose and 50 g/L xylose.

5 | CONCLUSION

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS
MXH designed the whole study and edited the entire draft manuscript. YHD participated in all experiments and data collection. BW participated in data analysis. PTL, MC, CS, QYG, RBL, and YSX participated in plasmid construction, RT-PCR and HPLC analysis. GQH participated in helpful discussions regarding the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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