# Cellulosic Ethanol Production by Engineered Consortia of *Scheffersomyces Stipitis* and *Zymomonas Mobilis*

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**Research**

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# Abstract

**Background:** As one of the clean and sustainable energies, lignocellulosic ethanol has achieved much attention around the world. The production of lignocellulosic ethanol does not compete with people for food, while the consumption of ethanol could contribute to the carbon dioxide emission reduction. Two of the conditions that are needed to attain cost-efficient lignocellulosic ethanol production at an industrial scale are the simultaneous transformation of glucose and xylose to ethanol and a highly efficient ethanol fermentation process.

**Results:** In this study, the consortia consisting of suspended *Scheffersomyces stipitis* CICC1960 and *Zymomonas mobilis* 8b were cultivated to successfully depress carbon catabolite repression (CCR) in 80G40XRM. With this strategy, a 5.52% more xylose consumption and a 6.52% higher ethanol titer were achieved by the consortium, in which the inoculation ratio between *S. stipitis* and *Z. mobilis* was 1:3, at the end of fermentation compared with the *Z. mobilis* 8b mono-fermentation. Subsequently, one copy of the xylose metabolic genes was inserted into the *Z. mobilis* 8b genome to construct *Z. mobilis* FR2, leading to the xylose final-consumption amount and ethanol titer improvement by 15.36% and 6.81%, respectively. Finally, various concentrations of corn stover hydrolysates, in which the sum of glucose and xylose concentrations in the hydrolysates were 60, 90, and 120 g/L respectively, were used to evaluate the fermentation performance of the consortium consisting of *S. stipitis* CICC1960 and *Z. mobilis* FR2. Fermentation results showed that a 1.56% - 4.59% higher ethanol titer was achieved by the consortium compared with the *Z. mobilis* FR2 mono-fermentation, and a 46.12% - 102.14% higher ethanol titer was observed in the consortium fermentation when compared with the *S. stipitis* CICC1960 mono-fermentation.

**Conclusions:** The fermentation strategy used in this study, i.e., using a genetically modified consortium, had a superior performance in ethanol production, as compared with the *S. stipitis* CICC1960 mono-fermentation and the *Z. mobilis* FR2 mono-fermentation alone. Thus, this strategy has potential for future lignocellulosic ethanol production.


# 1. Background

Lignocellulosic biomass, like hardwood, softwood, and grasses, is produced by plant photosynthesis from solar energy and is the most abundant renewable feedstock in the world. The bio-conversion of lignocellulosic biomass into ethanol by microbial fermentation is viewed as one of the most promising ways to partially replace traditional fossil fuels, since the combustion of ethanol produces less particulate matter, carbon monoxide, and hydrocarbons than fossil fuels (1). Additionally, as bioethanol has a high octane number, mixing bioethanol with gasoline could improve the anti-detonating quality of transportation fuel.

Lignocellulosic biomass is primarily degraded into glucose and xylose after pretreatment and enzymatic hydrolysis. The simultaneous and efficient bioconversion of the two sugars into ethanol are two of the

prerequisites for large-scale production of cellulosic ethanol. However, due to carbon catabolite repression (CCR), a considerable amount of wild microbes and engineered microbes having exogenous xylose-metabolic pathways, like *Zymomonas mobilis*, *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus amyloliquefaciens*, prefer to use glucose, and therefore their xylose utilization generally lags behind glucose utilization (2–5). This greatly hampers the large-scale application of cellulosic ethanol in industry.

Extensive studies have been attempted to relax CCR. For example, as all known xylose transporters are suppressed by glucose, many researchers have tried to engineer glucose-insensitive xylose transporters by evolutionary engineering, error-prone PCR, and site-directed mutagenesis. By this way, researchers have successfully built Gal2-N376F, CiGXS1 FIVFH497* and AN25-R4.18 (6–8). In addition, adaptive evolution, computation simulation, and rational design have been used to find appropriate intracellular targets to alleviate CCR, such as the phosphoenolpyruvate transferase system (PTS), the cyclic AMP receptor protein (CRP), and the xylose operon regulatory protein (9, 10). However, the complex nature of CCR makes it difficult for us to entirely unveil how CCR functions. Moreover, those engineered strains developed by the abovementioned methods typically cannot co-utilize glucose and xylose with high efficiency (9).

An alternative method is to build artificial consortia to co-ferment glucose and xylose. A common practice is to build a consortium consisting of a xylose-specific strain due to the deficiency in the PTS system and a wild strain that utilizes glucose because of CCR (9). In this way, for instance, 20.82 g/L butanol with a yield of 0.35 g/g was produced from glucose and xylose using two *E. coli* strains. These results were comparable with butanol titers and yields produced in previous studies from glucose alone (11). Another strategy is to use two wild species to ferment the glucose and xylose mixture. For example, when *Scheffersomyces stipitis* and *S. cerevisiae* were co-fermented together, the xylose removal efficiency and ethanol production showed remarkable improvement than in their mono-fermentation alone (12). In comparison with two-stage fermentation (glucose and xylose are consumed in a separate fashion), the consortium fermentation was advantageous in terms of assimilating glucose and xylose concomitantly and shortening fermentation time.

*Z. mobilis* is an excellent ethanol-producing species whose ethanol production efficiency can reach as high as 98%, which is higher than *S. cerevisiae* (13). However, the wild *Z. mobilis* cannot utilize xylose unless it has been transformed by the exogenous xylose metabolic pathway, like *Z. mobilis* 8b (14). Additionally, *S. stipitis* is recognized as one of the best microbes in nature in terms of its xylose assimilation ability, but it has a severe CCR phenomenon. In recent studies, the consortium of *S. stipitis* and *Z. mobilis* has been studied for the co-fermentation of glucose and xylose. However, these studies generally involved two-stage fermentation, or the total sugar concentrations in lignocellulosic hydrolysate medium were low, which is unrealistic in large-scale fermentation (15–18). Even for synthetic medium containing pure sugars, the xylose removal efficiency still requires improvement (19).

The present work focuses on investigating the potential of consortium fermentation consisting of *S. stipitis* and *Z. mobilis* in a synthetic medium and corn stover hydrolysate, with a great emphasis on alleviating CCR, increasing the sugar removal efficiency, and increasing the bioethanol production. The corn stover was chosen since it is one of the most common annual agricultural wastes produced in China (20). Instead of wasting the natural resource by burning it, it is better to transform it into useful products, like ethanol, and contribute to global warming mitigation.

## 2. Results And Discussion

## 2.1 Co-culture of *S. stipitis* CICC1960 and *Z. mobilis* 8b in 80G40XRM

Artificially synthesized 80G40XRM (80 g/L glucose + 40 g/L xylose) was first used to explore the appropriate mode of this consortium fermentation with the aim of improving the glucose-and-xylose co-utilization efficiency. The ratio between glucose and xylose in this medium was 2:1, aiming to simulate the real ratio in lignocellulosic hydrolysates.

In a previous study (21), when *S. stiptis* was pre-cultured in the medium with glucose as the sole carbon source (glucose medium), its xylose-metabolic gene expression, such as D-xylose reductase and xylitol dehydrogenase expression, was inhibited. Hence, when *S. stiptis* was inoculated in the xylose medium (xylose was the sole carbon source in the fermentation medium) later, its xylose-metabolic genes needed to be synthesized from scratch. Therefore, *S. stiptis* exhibited an apparent lag in fermentation in the xylose medium. In contrast, when *S. stiptis* was precultured in the xylose medium, the two xylose-metabolic genes were fully expressed and the aforementioned problem of lag in the xylose fermentation was alleviated, especially if *S. stiptis* was inoculated with high initial density, such as $A_{620}$ = 40. To this end, we decided to preculture *S. stiptis* CICC1960 in YP120X (120 g/L xylose) and then inoculate it to the 80G40XRM (fermentation medium) with a 100% (v/v) inoculum size, hoping to alleviate the CCR phenomenon in *S. stiptis*. However, as shown in Fig. 1a and 1b, *S. stipitis* CICC1960 still could not assimilate glucose and xylose at the same time. This might have been a result of the glucose repression on xylose transportation into the cells (6), or the inoculum size used in this study (the initial $OD_{600}$ in fermentation was about 1.8) was not high enough. For economic reasons, the inoculum size was not further increased in this study.

For *Z. mobilis* 8b, 80G40XRM was used as its seed culture medium, since *Z. mobilis* 8b cannot grow well in xylose medium (data not shown). The results of the *Z. mobilis* 8b mono-fermentation are shown in Fig. 1 and Additional file 1: Table S1. During fermentation, *Z. mobilis* 8b showed a strong ability to utilize glucose and xylose simultaneously with ethanol productivity reaching 5.10 g/L/h. More specially, the CCR in *Z. mobilis* 8b was largely alleviated, which may be attributed to the high inoculum size used in this study (the initial $OD_{600}$ was approximately 1.8). In 2004, Ali Mohagheghi et al. found that *Z. mobilis* 8b (the initial inoculum density was 0.2 at 600 nm) exhibited an apparent lag of xylose utilization, while

glucose could be assimilated immediately (22). Since the xylose-metabolic genes were inserted to the genome of *Z. mobilis* 8b, their expression was generally low in individual cells. When *Z. mobilis* 8b were inoculated with high density, activities of those enzymes encoding xylose-metabolic genes would be higher than the ones under a low initial density inoculation, and this may contribute to the alleviation of CCR in this study (14). Glucose was completely removed within six hours by *Z. mobilis* 8b, while xylose still remained at 6.16 g/L at 21 h. The ethanol yield of the *Z. mobilis* 8b mono-fermentation was 0.48 g/g (the theoretical ethanol yield is 0.51 g/g sugars).

When *S. stipitis* CICC1960 and *Z. mobilis* 8b were co-cultured together with different inoculation ratios (3:1–1:3), the glucose consumption profiles of the consortia did not differ considerably from the *Z. mobilis* 8b mono-fermentation (Fig. 1a). Glucose was completely removed within nine hours by the consortia. On top of this, no apparent CCR was shown since xylose was rapidly assimilated at the very early stage (Fig. 1b). Although xylose consumption rates by the consortia were lower than that in the *Z. mobilis* 8b mono-fermentation. It was shown that, in general, the higher the ratio of *Z. mobilis* 8b applied, the higher the rates of xylose assimilation achieved. In special, when *S. stipitis* CICC1960 : *Z. mobilis* 8b = 3:1 (initial inoculum size proportion), the xylose assimilation rate was 0.92 g/L/h; when *S. stipitis* CICC1960 : *Z. mobilis* 8b = 1:3, the xylose assimilation rate improved to 2.38 g/L/h. At 21 h, the consortia fermentation reached the endpoint of fermentation. Interestingly, when *S. stipitis* CICC1960 : *Z. mobilis* 8b = 1:3, the xylose consumption reached 36.73 g/L, which was significantly ($P < 0.01$) higher than that in the *S. stipitis* CICC1960 mono-fermentation and that in the *Z. mobilis* 8b mono-fermentation (Additional file 1: Table S1). Correspondingly, the ethanol titer of this consortium reached 57.21 g/L, which was 48.37% higher than that of the *S. stipitis* CICC1960 mono-fermentation and 6.52% higher than that of the *Z. mobilis* 8b mono-fermentation. These results (*S. stipitis* CICC1960 : *Z. mobilis* 8b = 1:3) were comparable with or better than other consortia fermentations listed in Table 1, in terms of xylose removal efficiency, ethanol yield, and ethanol productivity.

Table 1
Comparison of various consortia fermentation profiles in synthetic medium

| [a] **Fermentation mode** | **Initial sugar concentration (g/L)** | **Xylose removal efficiency (%)** | **Ethanol yield (g/g)** | **Ethanol productivity (g/L/h)** | **Reference** |
|---|---|---|---|---|---|
| Two-stage fermentation | | | | | |
| Suspended *Z. mobilis* + suspended *S. stipitis* | 80 g/L glucose + 40 g/L xylose | 62.5 | - | 1.56 | (15) |
| [b] Suspended *S. stipitis* + suspended *Z. mobilis* | 60g/L xylose + 100 g/L glucose | - | 0.474 | 1.416 | (16) |
| Suspended *Z. mobilis* + suspended *S. stipitis* | 80 g/L glucose + 40 g/L xylose | 0.67 | 0.36 | 0.41 | (19) |
| One-stage fermentation | | | | | |
| Suspended *S. cerevisiae* + suspended *S. stipitis* | 75 g/L glucose + 30 g/L xylose | 79.6 | 0.4 | 1.26 | (37) |
| Suspended *S. cerevisiae* + suspended *S. stipitis* | 20 g/L glucose + 10 g/L xylose | - | 0.416 | 0.608 | (38) |
| [c] Immobilized *Z. mobilis* + immobilized *S. stipitis* | 80 g/L glucose + 40 g/L xylose | 72.5 | 0.37 | 0.87 | (19) |
| **[d] Suspended** Z. mobilis **+ suspended** S. stipitis | **80 g/L glucose + 40 g/L xylose** | **84.95** | **0.50** | **4.99** | **This study** |

[a] Two-stage fermentation means the two species were inoculated into the medium sequentially, while one-stage fermentation means the two species were inoculated into the medium simultaneously

[b] Xylose was first depleted by *S. stipitis*. Then, glucose medium and *Z. mobilis* were added to the same system to initiate the glucose fermentation

[c] *Z. mobilis* and *S. stipitis* were immobilized separately

[d] *S. stipitis* CICC1960 : *Z. mobilis* 8b = 1:3

-, not available

Due to the outstanding fermentation ability of this consortium (*S. stipitis* CICC1960 : *Z. mobilis* 8b = 1:3), the ratio between *S. stipitis* and *Z. mobilis* of 1:3 was later employed in the fermentation of corn stover hydrolysate.

## 2.2 Genetic engineering of *Z. mobilis* 8b

In *Z. mobilis*, ZMO0256 encoding D-lactate dehydrogenase is involved in the production of lactate as a byproduct; ZMO0689 encoding glucose-fructose oxidoreductase participates in xylitol and sorbitol production (23). It was demonstrated that disruption of ZMO0689 could improve xylose fermentation performance of *Z. mobilis* (24). In order to introduce one copy of xylose metabolic genes (*xylA*, *xylB*, *tktA*, *talB*) into the *Z. mobilis* 8b genome and improve its xylose assimilation performance, the engineered strains *Z. mobilis* FR1 (ZMO0256::$P_{pdc}$-*talB*-*tktA*) and *Z. mobilis* FR2 (ZMO0256::$P_{pdc}$-*talB*-*tktA*; ZMO0689::$P_{pdc}$-*xylA*-*xylB*) were sequentially constructed.

The fermentation performances of *Z. mobilis* FR1 and *Z. mobilis* FR2 were evaluated in 80G40XRM and were compared with their parental stain *Z. mobilis* 8b. As shown in Fig. 2a, the three strains did not differ in their glucose assimilation profiles, and glucose was depleted within 8.5 h. For the xylose consumption and ethanol production profiles (Fig. 2b and 2c), *Z. mobilis* FR1 did not show much difference with *Z. mobilis* 8b. However, *Z. mobilis* FR2 accelerated its xylose assimilation rate in the mid-to-late fermentation period (Fig. 2b): the xylose consumption and ethanol production achieved by *Z. mobilis* FR2 were increased by 15.36% and 6.81% respectively at 20.5 h as compared with *Z. mobilis* 8b (Additional file 1: Table S2). Besides, the ethanol productivity of *Z. mobilis* FR2 was 5.08 g/L/h, which was significantly ($P < 0.01$) higher than *Z. mobilis* 8b (4.84 g/L/h). The ethanol yield of *Z. mobilis* FR2 was 95.47%, which was comparable with A3 (96.6%) and AD50 (96%), the two best strains developed so far by adaptive laboratory evolution (23, 25).

However, as shown in Fig. 2a and 2b, though CCR was alleviated in *Z. mobilis* FR2 fermentation, its xylose utilization rate was still lower than its glucose utilization rate, and there was still a slight amount of xylose that remained (approximately 2.5 g/L) at the endpoint. These results agreed with other *Z. mobilis* strains, including C25, 39676/pZB4L (26), ZM4/$Ac^R$ (pZB5, pJX1) (27), A3 (23), and AD50 (25), yet no exact reason of the incomplete xylose utilization has been identified thus far. In *Z. mobilis*, xylose is transported through a glucose facilitated diffusion protein (25), which is a native glucose transporter and has low affinity to xylose. This low affinity xylose transport might be the burden behind the above-mentioned problems. Further investigation into this field would greatly promote the commercialization of cellulosic ethanol.

## 2.3 Effect of oxygen on *Z. mobilis* FR2 fermentation

In the above experiments, as *S. stipitis* could not grow under static cultivation, 150 rpm was applied in the *S. stipitis* mono-fermentation, the *Z. mobilis* mono-fermentation, and the consortia fermentation that consisted of the two species. However, as *Z. mobilis* is a facultative anaerobe, it can ferment under both static and aerobic conditions. Therefore, a further study was conducted to check whether there was any difference in *Z. mobilis* FR2 fermentation profiles under static and agitated (150 rpm) conditions in 80G40XRM. As shown in Fig. 3, oxygen significantly boosted *Z. mobilis* FR2's glucose ($P < 0.05$) and xylose ($P < 0.01$) consumption rates and improved ethanol productivity by 54.51% ($P < 0.01$). However, at

fermentation endpoint (27 h), the static fermentation of *Z. mobilis* FR2 showed an increase in xylose consumption and ethanol production by 1.65 g/L and 3.45 g/L, respectively compared with that in agitated fermentation. To achieve high ethanol productivity, ethanol fermentation under 150 rpm was kept in this study.

In theory, the 1.65 g/L more xylose consumed by *Z. mobilis* FR2 in static condition should be transformed into 0.84 g/L ethanol (the theoretical ethanol yield is 0.51 g/g xylose). However, the real difference in ethanol titers between the static fermentation and agitated fermentation was as high as 3.45 g/L. This suggests oxygen had a negative effect on ethanol production by *Z. mobilis* FR2. In 1990, Tanaka et al. found that *Z. mobilis* produced a little more acetaldehyde (0.28–4.49 g/L) when oxygen was supplied (28). Acetaldehyde was primarily produced by NADH dehydrogenase, one of the key components in the *Z. mobilis* respiratory chain. This enzyme has the same cofactor (NADH) as ethanol dehydrogenase. During aerobic fermentation, the activity of NADH dehydrogenase was higher than that of ethanol dehydrogenase. Therefore, a large amount of NADH was used to reduce the dissolved oxygen concentration in the medium. Due to the lack of sufficient NADH, the transformation from acetaldehyde to ethanol by ethanol dehydrogenase was inhibited, and thus negatively affected the ethanol production of *Z. mobilis* under agitated cultivation (29). One evidence for this hypothesis is that for *Z. mobilis* mutant strains, whose NADH dehydrogenase is defective, this negative effect of oxygen on *Z. mobilis* ethanol fermentation could be alleviated (30–32).

### 2.4 Assimilation of corn stover hydrolysates by consortium composed of S. stipitis CICC1960 and Z. mobilis FR2

First, it was evaluated whether *Z. mobilis* FR2 could outcompete *Z. mobilis* 8b in corn stover hydrolysate fermentation. As shown in Fig. 4a, a difference was not observed regarding glucose assimilation ability of the two strains: glucose assimilation rates were 2.54 g/L/h and 2.51 g/L/h for *Z. mobilis* FR2 and *Z. mobilis* 8b, respectively, and glucose was depleted within 30 h. As for xylose assimilation (Fig. 4b), though the two strains did not exhibit a difference during the first 24 hours, the assimilation rate of *Z. mobilis* 8b gradually decreased, while the rate for *Z. mobilis* FR2 remained stable. At 60 h, *Z. mobilis* FR2 assimilated 14.60 g/L xylose, which was significantly higher ($P < 0.05$) than that of *Z. mobilis* 8b (10.60 g/L). Additionally, *Z. mobilis* FR2 produced 37.13 g/L ethanol in 120 g/L corn stover hydrolysate (concentration here refers to the total amount of glucose and xylose in corn stover hydrolysate before sterilization), while *Z. mobilis* 8b produced 34.93 g/L ethanol (Fig. 4c). These results agreed with the fermentation results in 80G40XRM (Fig. 2). Due to the better xylose assimilation ability of *Z. mobilis* FR2, *Z. mobilis* FR2 was used to replace *Z. mobilis* 8b in the next consortium fermentation with *S. stipitis* CICC1960 in corn stover hydrolysates.

As shown in Fig. 5, while consortium fermentation (*S. stipitis* CICC1960 : *Z. mobilis* FR2 = 1:3) and *Z. mobilis* FR2 mono-fermentation did not show any difference in glucose and xylose consumption rates and amounts in the 60 and 90 g/L corn stover hydrolysates fermentation, the consortium produced slightly more ethanol (~ 0.86 g/L) than the *Z. mobilis* FR2 mono-fermentation (Table 2). Additionally, the

consortium fermentation in the two cases was better than the *S. stipitis* CICC1960 mono-fermentation in terms of glucose assimilation, xylose assimilation, and ethanol production rates and quantities, and did not exhibit strong CCR which was evident in the *S. stipitis* CICC1960 mono-fermentation (Fig. 5). For the 120 g/L corn stover hydrolysate fermentation, the glucose assimilation rate of the consortium (2.83 g/L/h) was slightly slower than *Z. mobilis* FR2 (3.24 g/L/h), while xylose assimilation rates were nearly the same prior to 36 h. However, the consortium finally produced 33.05 g/L ethanol at endpoint, which was 1.02 g/L higher than the *Z. mobilis* FR2 mono-fermentation and 16.7 g/L higher than the *S. stipitis* CICC1960 mono-fermentation (Table 2).

Table 2
Fermentation profiles in the corn stover hydrolysate

| [a] Corn stover hydrolysate concentration (g/L) | *S. stipitis* CICC1960 : *Z. mobilis* FR2 | [b] Glucose consumed (g/L) | [b] Xylose consumed (g/L) | [b] Ethanol | | |
|---|---|---|---|---|---|---|
| | | | | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) |
| 60 | 1:0 | 38.27 ± 0.53 | 8.64 ± 0.49 | 13.40 ± 0.75 | 0.29 ± 0.01 | 0.73 ± 0.04 |
| | 0:1 | 38.27 ± 0.53 | 13.50 ± 0.00 | 18.72 ± 0.46 | 0.36 ± 0.01 | 2.08 ± 0.05 |
| | 1:3 | 38.27 ± 0.53 | 13.50 ± 0.00 | 19.58 ± 0.32 | 0.38 ± 0.01 | 2.18 ± 0.04 |
| 90 | 1:0 | 57.30 ± 1.55 | 7.74 ± 0.25 | 18.45 ± 0.35 | 0.28 ± 0.01 | 0.53 ± 0.01 |
| | 0:1 | 57.30 ± 1.55 | 18.62 ± 0.15 | 26.96 ± 0.24 | 0.36 ± 0.00 | 1.99 ± 0.08 |
| | 1:3 | 57.30 ± 1.55 | 18.87 ± 0.04 | 27.38 ± 0.58 | 0.36 ± 0.01 | 2.01 ± 0.05 |
| 120 | 1:0 | 67.35 ± 1.52 | 4.88 ± 0.19 | 16.35 ± 2.06 | 0.23 ± 0.03 | 0.19 ± 0.02 |
| | 0:1 | 80.61 ± 4.72 | 12.56 ± 0.74 | 32.03 ± 1.14 | 0.34 ± 0.01 | 1.32 ± 0.03 |
| | 1:3 | 80.61 ± 4.72 | 12.69 ± 0.60 | 33.05 ± 0.79 | 0.35 ± 0.01 | 1.11 ± 0.02 |

Data are mean ± standard error from three replicates

[a] The corn stover hydrolysate concentration refers to the total concentrations of glucose and xylose in the hydrolysate before autoclave sterilization

[b] All data were calculated based on the real sugar concentrations

In 2014, Lalit K. Singh et al. separated kans grass hydrolysate into a xylose-rich portion and a glucose-rich portion through organic solvent extraction and then used *S. stipitis* and *Z. mobilis* to ferment each sugar (54 g/L xylose and 100 g/L glucose) sequentially. The ethanol productivity in their study was 0.723 g/L/h, which was lower than that in our study (1.11 ~ 2.18 g/L/h) (16). This was because the two-stage

fermentation Lalit K. Singh et al. employed led to an increase in the fermentation time. In 2020, Ferdian Wirawan et al. used immobilized *Z. mobilis* and *S. stipitis* to sequentially ferment 50 g/L sugarcane bagasse (17). Though the productivity in their study was high (1.868 g/L/h), the actual sugar concentration in the hydrolysate was only 11 g/L glucose, 4 g/L xylose, and 4 g/L cellobiose, which is impractical in real applications. These comparisons demonstrated that the consortium fermentation mode utilized in this study has an edge in ethanol production compared with other existed modes of *S. stipitis* and *Z. mobilis* co-fermentation.

We note that the ethanol titer and productivity values in the 120 g/L corn stover hydrolysate were lower than the values in 80G40XRM (Table 2, Additional file 1: Tables S1 and S2). This was because many of the inhibitors, like phenols, were presented in the corn stover hydrolysate (33). These inhibitors negatively affected the microbial fitness in the lignocellulosic hydrolysate and thus reduced the ethanol yield and productivity. Although it has been shown that the immobilization of microbes could alleviate the negative effect to some degree, the exact mechanisms are not clear (34).

Duong Thi Thuy Nguyen et al. found that the presence of living *Z. mobilis* cells negatively affected the xylose assimilation performance of *S. stipitis*, suggesting that there might be an amensalism relationship between the two species (19, 35). Similarly, in our study, when the initial inoculum $OD_{600}$ was controlled to 0.1 in the 80G40XRM fermentation, no improvement was observed in the consortium fermentation profiles compared with the *Z. mobilis* mono-fermentation profiles (data not shown). In contrast, when a high inoculum size (an initial $OD_{600}$ of approximately 1.8) was used, the consortium of *S. stipitis* and *Z. mobilis* assimilated more xylose and produced more ethanol in both the 80G40XRM and 120 g/L corn stover hydrolysate. This implied a commensalism or cooperation relationship between the two species during fermentation (35). Perhaps this positive relationship could only be exhibited under specific conditions, such as under a high cell density.

Exploring the interactions between *S. stipitis* and *Z. mobilis* under various cell densities, as well as the ethanol fermentation performance in corn stover hydrolysate by co-immobilized cells of the two species, are our future research goals. Indeed, a deep understanding of the interactions between *S. stipitis* and *Z. mobilis* and the mechanisms behind the protective role of co-immobilization on cells will pave the way to further enhance the consortium fermentation performance while lowering the inoculum size, and will help promoting the industrial progression of cellulosic ethanol production.

# 3. Conclusions

This study focused on evaluating a fermentation method to efficiently transform glucose and xylose to ethanol via artificial consortia composed of suspended *S. stipitis* and suspended *Z. mobilis*. By fermentation process optimization and genetic engineering, the consortium built here exhibited enhanced xylose assimilation ability and ethanol production performance in both 80G40XRM and corn stover hydrolysate, and did not experience evident CCR phenomenon. Hence, this study proved that *S. stipitis*

and *Z. mobilis* could be co-cultured together in suspension in cellulosic ethanol production and provided a novel strategy for further application.

# 4. Materials And Methods

## 4.1 Strains, plasmids and primers

*S. stipitis* CICC1960 was purchased from the China Center of Industrial Culture Collection. *Z. mobilis* 8b was kindly given by Shihui Yang, Hubei University, and was used as the starting strain for further strains constructions. Plasmid Pmini was kindly given by Nan Peng, Huazhong Agricultural University. All primers were synthesized by Tsingke Biotechnology Co., Ltd. (Chengdu, China) and purified via polyacrylamide gel electrophoresis.

All strains, plasmids and sgRNAs are listed in Additional file 1: Table S3. Primers are listed in Additional file 1: Table S4.

## 4.2 Genetic engineering and strain development

The CRISPR-Cas system was used to engineer *Z. mobilis* FR1 and *Z. mobilis* FR2 in this study. Specifically, the plasmids Pmini-$P_{pdc}$-*talB-tktA* and Pmini-$P_{pdc}$-*xylA-xylB* were constructed using Gibson Assembly® Protocol (New England BioLabs, Ipswich, US) and were transformed into *E. coli* trans110 (TransGen Biotech, Beijing, China). After verification of the plasmid sequences by sequencing, the two plasmids were transformed into *Z. mobilis* 8b sequentially to construct *Z. mobilis* FR1 (ZMO0256::$P_{pdc}$-*talB-tktA*) and *Z. mobilis* FR2 (ZMO0256::$P_{pdc}$-*talB-tktA*; ZMO0689::$P_{pdc}$-*xylA-xylB*). ZMO0256up500, *pdc* promoter, *talB*, *tktA*, ZMO0256down500, ZMO0689up540, *xylA*, *xylB*, and ZMO0689down500 were all amplified from *Z. mobilis* 8b itself, using the corresponding primers listed in Additional file 1: Table S4.

## 4.3 Preparation of the corn stover hydrolysates

### 4.3.1 Alkaline pretreatment (36)

Corn stover was collected from Jianyang, Sichuan, China. After air-drying, milling and passing through a sieve with an aperture size of 380 μm, the corn stover was mixed with 1.34% (w/v) NaOH solution (The solid loading was 10% (w/v)), loaded into reaction kettles, and transferred into a drying oven sequentially. The treatment parameters were 140°C and 6 h.

The pretreated corn stover was washed intensively with water or diluted $HNO_3$ until the pH of the washing water turned neutral. The washed corn stover was oven dried and stored in sealed bags at room temperature.

### 4.3.2 Enzymatic hydrolysis

Pretreated corn stover (10 g) was mixed with 100 mL citric acid buffer (8.823 g/L tri-sodium citrate dihydrate, 3.843 g/L citric acid, pH 4.8) and 5 mL cellulase (Sigma-Aldrich, Saint Louis, US). Afterwards,

the mixture was placed in an incubator at 50°C and 150 rpm for 72 h. When the enzymatic hydrolysis was finished, the hydrolysate was centrifuged twice at 3000 g for 25 min each time. The hydrolysate was then centrifuged again at 10000 g for 5 min for the further removal of the remaining solids. Sugars in the lignocellulosic hydrolysate were concentrated using a rotary evaporator to attain 60 g/L, 90 g/L, and 120 g/L hydrolysates with regard to the concentrations of total glucose and xylose. Subsequently, 10 g/L yeast extract, 2 g/L $KH_2PO_4$, 1 g/L $(NH_4)_2SO_4$, and 2 g/L $MgSO_4 \cdot 7H_2O$ were added to the hydrolysates before adjusting the pH of the hydrolysates to 5.6 by 5 M NaOH.

# 4.4 Growth and fermentation conditions

## 4.4.1 Preparation of the seed cultures of *S. stipitis* and *Z. mobilis*

*S. stipitis* was streaked in a YPD plate (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone, and 15 g/L agar) and cultured at 30°C for approximately one day. A single colony was transferred from the YPD plate into 5 mL of YP120X medium (120 g/L xylose, 10 g/L yeast extract, and 20 g/L peptone) and cultivated at 30°C and 150 rpm for 19–36 h. Afterwards, the entire pre-seed culture was poured into a 100 mL of fresh YP120X medium and cultivated at 30°C and 150 rpm for 22 h. At this point, the $OD_{600}$ value of the seed culture was approximately 1.8.

Similarly, *Z. mobilis* was streaked in a RM plate (20 g/L glucose, 10 g/L yeast extract, 2 g/L $KH_2PO_4$, 1 g/L $(NH_4)_2SO_4$, 2 g/L $MgSO_4 \cdot 7H_2O$, and 15 g/L agar) and cultivated at 30°C for two days. A single colony was transferred from the RM plate into 5 mL of 80G40XRM (80 g/L glucose, 40 g/L xylose, 10 g/L yeast extract, 2 g/L $KH_2PO_4$, 1 g/L $(NH_4)_2SO_4$, and 2 g/L $MgSO_4 \cdot 7H_2O$) and cultivated statically at 30°C for 19–36 h until the culture was turbid. The entire culture was then poured into 100 mL of the fresh 80G40XRM and cultivated statically at 30°C for 22 h. The $OD_{600}$ value of the seed culture was approximately 1.8 at this point.

## 4.4.2 Fermentation conditions

The seed cultures of *S. stipitis* and *Z. mobilis* were centrifuged at 4°C and 4000 rpm for 5 min, washed once with sterile $ddH_2O$, and suspended with 1/25 (v/v) new fermentation medium, respectively (for 80G40XRM fermentation, 80G40XRM was used to suspend cells, while sterile $ddH_2O$ was used for corn stover hydrolysates fermentation). The concentrated cells were then inoculated into the corresponding fermentation medium with different ratios of *S. stipitis* and *Z. mobilis*. The total inoculum size was 100% (v/v, the ratio of seed culture volume/ fermentation culture volume).

The total fermentation volume was 50 mL for 80G40XRM fermentation and 10 mL for corn stover hydrolysates fermentation. The fermentation conditions were 30°C and 150 rpm, unless there was further indication in the portion for exploring the effect of oxygen on *Z. mobilis* fermentation (as seen in 2.3).

Four replicates were performed for the 80G40XRM fermentation, while three replicates were performed for the corn stover hydrolysates fermentation.

# 4.5 Analytical methods

## 4.5.1 Determination of glucose, xylose and ethanol concentrations during fermentation

The glucose, xylose, and ethanol titers in the fermentation samples were analyzed by an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, US) equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Richmond, US). The mobile phase was 5 mM $H_2SO_4$. The operating parameters were 20 µL injection volume, 0.6 mL/min rate, and 35°C.

## 4.5.2 Calculation of ethanol productivity and yield

Ethanol productivity = Ethanol titer / fermentation time

Ethanol yield = Ethanol titer / glucose and xylose consumed

The theoretical ethanol yield is 0.51 g/g sugars consumed.

## 4.5.3 Statistical analysis

Data are presented as mean ± standard error. All figures were prepared using Prism 8 (GraphPad Software, LLC).

Significant differences were statistically analyzed using IBM® SPSS® Statistics (Version 22, US). If $P > 0.05$ in the homogeneity of variance test, a one-way ANOVA followed by Turkey test was used. Otherwise, a nonparametric test (Kruskal-Wallis H) was used.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

Not applicable.

### Competing interests

The authors declare that they have no competing interests.


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### Author's contributions

Experimental design was carried by LS, BW, PL, QZ, MH, and FT. Data acquisition was performed by LS. Data was analyzed by LS, JY. Manuscript was written by LS and ZZ, and was revised by PL, CS, SS, QZ, SY, NP and FT. All authors read and approved the final manuscript.


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# Figures

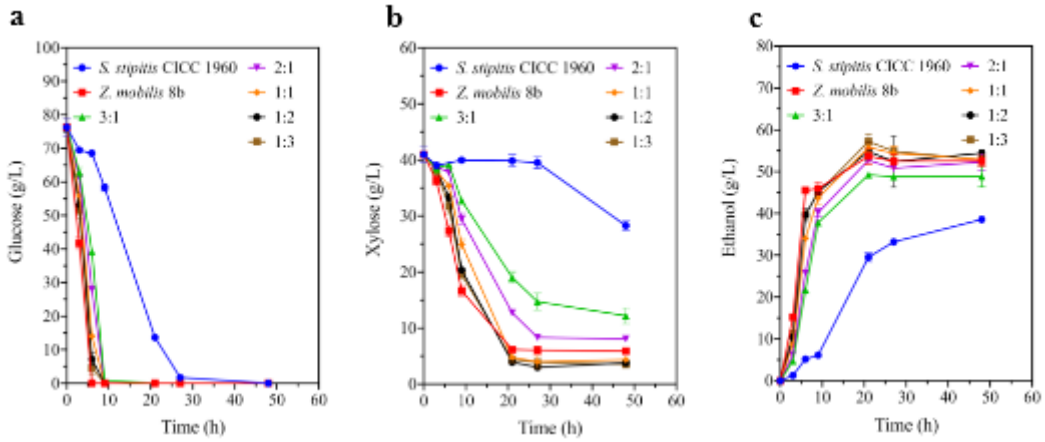



**Figure 1**

Fermentation profiles of consortia consisting of S. stipitis CICC1960 and Z. mobilis 8b in 80G40XRM. a Glucose assimilation profiles. b Xylose assimilation profiles. c Ethanol production profiles. Data are mean ± standard error from four replicates

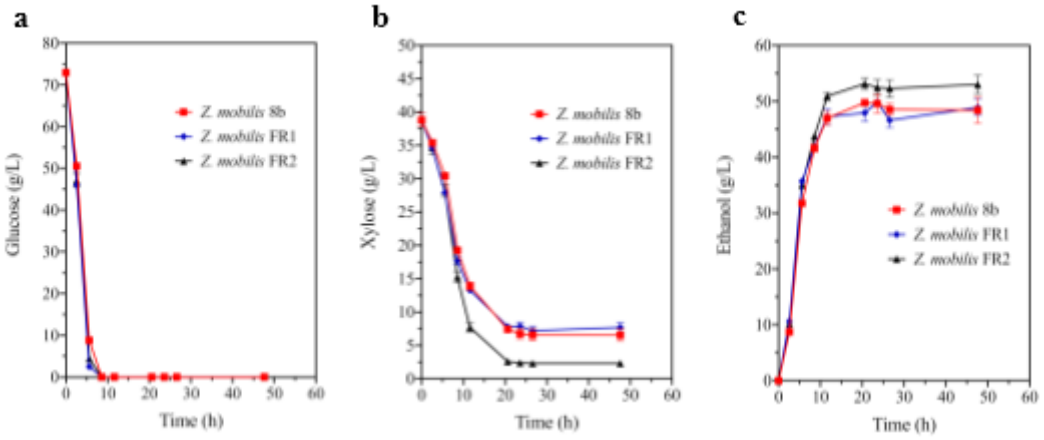
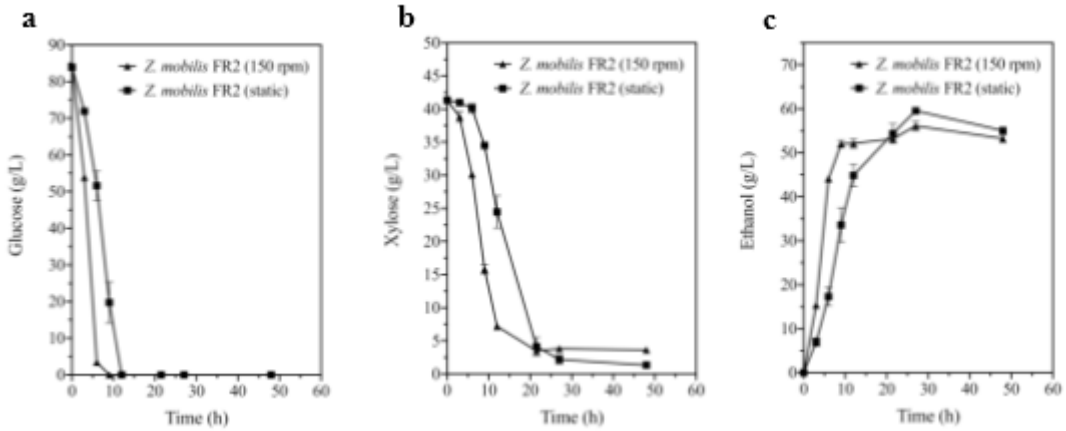


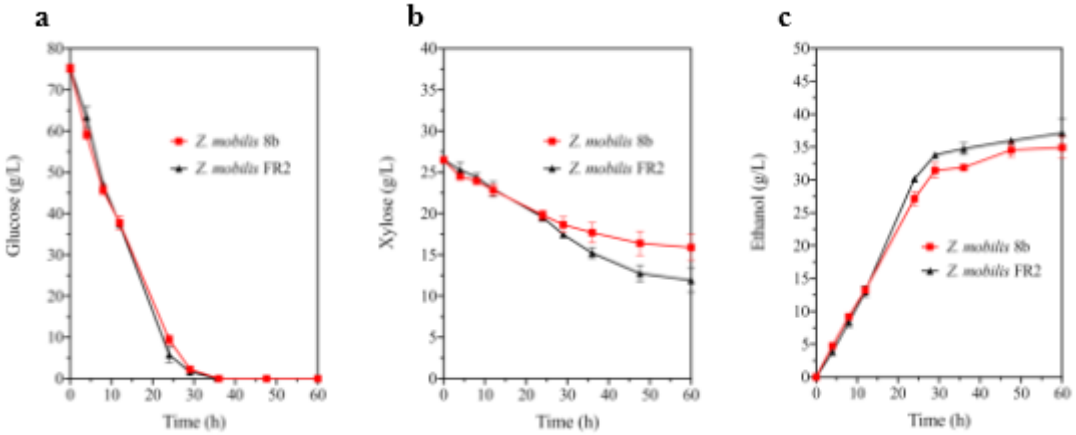

**Figure 2**

Fermentation profiles of Z. mobilis 8b, Z. mobilis FR1, and Z. mobilis FR2 in 80G40XRM. a Glucose assimilation profiles. b Xylose assimilation profiles. c Ethanol production profiles. Data are mean ± standard error from four replicates



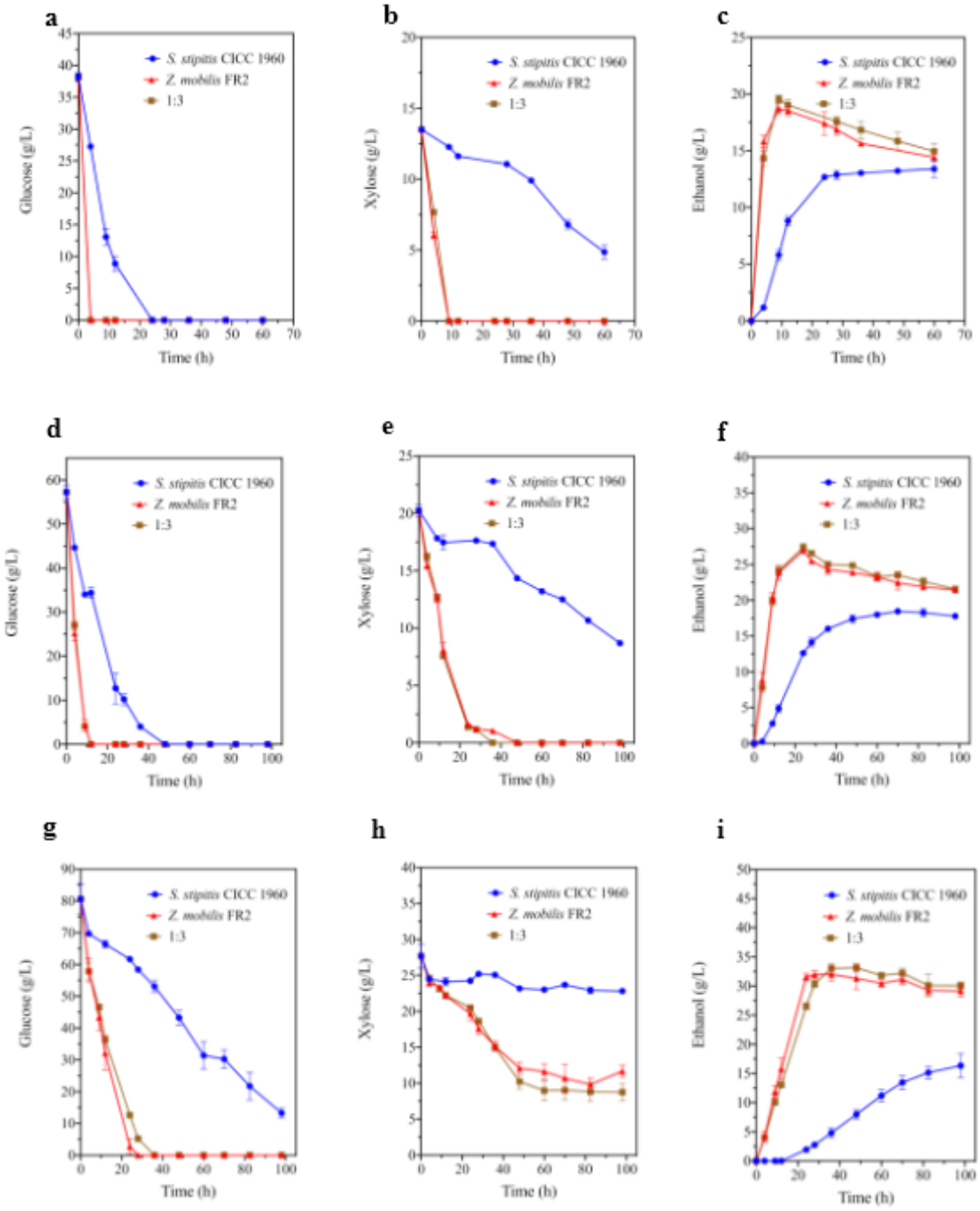

## Figure 3

Fermentation profiles of Z. mobilis FR2 in 80G40XRM with different rotation speeds. a Glucose assimilation profiles. b Xylose assimilation profiles. c Ethanol production profiles. Data are mean ± standard error from four replicates




## Figure 4

Fermentation profiles of Z. mobilis 8b and Z. mobilis FR2 in corn stover hydrolysate. a Glucose assimilation profiles. b Xylose assimilation profiles. c Ethanol production profiles. The total concentrations of glucose and xylose in the hydrolysate was 120 g/L before autoclave sterilization. Data are mean ± standard error from three replicates




## Figure 5

Consortium fermentation profiles in various concentrations of corn stover hydrolysates. a, b, c Glucose, xylose and ethanol fermentation profiles in 60 g/L of corn stover hydrolysate. d, e, f Glucose, xylose and ethanol fermentation profiles in 90 g/L of corn stover hydrolysate. g, h, i Glucose, xylose and ethanol fermentation profiles in 120 g/L of corn stover hydrolysate. The concentration of corn stover hydrolysates represents the total amount of glucose and xylose in the hydrolysates before autoclave sterilization. For consortium fermentation, the inoculation ratio between S. stipitis CICC1960 and Z. mobilis FR2 was 1:3. Data are mean ± standard error from three replicates

# Supplementary Files

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- SupplementaryInformation.docx