Biochar-mediated enhanced ethanol fermentation (BMEEF) in *Zymomonas mobilis* under furfural and acetic acid stress

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**Abstract**

**Background:** Pretreatment of lignocellulosic biomass generates different types of inhibitors (e.g., furfural and acetic acid), which could remarkably inhibit subsequent ethanol fermentation. Here, biochar as an additive in the fermentation broth was first applied to enhance ethanol production by *Z. mobilis* wild-type strain ZM4 in the presence of typical inhibitors.

**Results:** This study showed that the biochar-mediated tolerance to furfural and acetic acid for the strain *Z. mobilis* ZM4 was the highest reported level, resulting in much higher ethanol productivity under stress conditions than that in non-treated conditions. Further analysis showed that adsorptive detoxification was not the controlling factor for enhanced ethanol production under stress conditions, attributed to its low removal of furfural (< 20%) and incapability of acetic acid removal. When biochar was filtered from the biochar-treated inhibitor-containing broth, it still showed enhanced ethanol production. Furthermore, *Z. mobilis* immobilized on biochar was also observed. Thus, biochar extracts in the fermentation broth and cell immobilization on biochar might be the controlling factors for enhanced ethanol production under stress conditions.

**Conclusions:** These results indicate that biochar-mediated enhanced ethanol fermentation (BMEEF) might be a promising strategy for ethanol production from lignocellulosic biomass.

**Keywords:** Lignocellulosic hydrolysate, Furfural, Acetic acid, Biochar, *Zymomonas mobilis*
the major drawback when applied to ethanol fermentation using lignocellulose feedstock containing furfural and acetic acid generated from pretreatment [13]. Apart from removing inhibitors before fermentation, creating mutants capable of tolerating furfural and acetic acid is efficient for the economic production of cellulosic biofuels [14, 15]. However, although several efforts have been applied to improve these inhibitors’ tolerance in Z. mobilis, including forward and reverse genetics, so far, the reported highest concentrations of furfural and acetic acid that Z. mobilis could tolerate were 3.0 g/L and 8.0 g/L [10, 16], respectively. But further efforts are still needed to meet the requirement of practical lignocellulose biorefinery with high inhibitor contents in the pretreated feedstock, and due to the complex mechanism of furfural and acetic acid stress in Z. mobilis, developing a robust strain will also be a difficult challenge [13–15].

Biochar, attributed to its special characteristics, such as high porosity, rich functional groups, abundant nutrients, is an emerging versatile material for various applications [17–23], such as soil amendment/remediation [19], crop production promotion [20], water pollution control [17, 18], and even anaerobic digestion [21, 22] and composting [23]. For example, in anaerobic digestion, biochar has shown its capacity in improving the fermentation of easy-acidification substrates by promoting buffering capacity [22]. However, little is known about the effects of biochar as a fermentation additive to improve ethanol fermentation by Z. mobilis in the presence of a high concentration of inhibitors in the hydrolysate [24, 25]. Biochar has shown its capacity in adsorptive detoxification of phenols, furfural, and 5-HMF [26–28]. However, biochar was not efficient in acetic acid adsorption. Moreover, adsorptive detoxification requires a high dose of biochar to achieve high removal of adsorbable inhibitors. A large amount of biochar for detoxification would impede its practical application. For example, the application of 4% biogas digestate-derived biochar in synthetic medium removed more than 94% of 5-HMF and 99% of furfural after 24 h of contact time [28].

Here, biochar-mediated enhanced ethanol fermentation (BMEEF) was developed by applying biochar as an additive in fermentation broth to promote ethanol production by Z. mobilis wild-type strain ZM4 in the presence of typical inhibitors. Adsorbable furfural and non-adsorbable acetic acid were chosen as typical inhibitors in this study. Biochar for BMEEF was not applied as a detoxification agent, so its dose was <1/10 of the dose used for adsorptive detoxification [28], which was more suitable for practical application due to its much lower dose. Ethanol production under stress from furfural or acetic acid and co-stress was observed to confirm the effects of adsorptive detoxification, biochar extracts in the fermentation broth and immobilized Z. mobilis cells on biochar-enhanced ethanol production under various stress conditions. The results of this study could provide novel insights into the effects of biochar on ethanol fermentation under stress conditions.

Results and discussion
Biochar enhanced ethanol production under acetic acid stress

Through forward and reverse genetics, the reported highest concentration of acetic acid that Z. mobilis could tolerate was 8.0 g/L [10]. Therefore, in this study, four different gradient concentrations (3.0, 6.0, 9.0, and 12.0 g/L) of acetic acid were employed. As shown in Fig. 1a, b, and Table 1, wild-type strain ZM4 was dramatically suppressed by 6.0 g/L acetic acid and could hardly survive under 9.0 g/L acetic acid. Actually, when a supplement of 6.0 g/L acetic acid was added, strain ZM4 consumed 97.86% glucose within 48 h, and when 9.0 g/L acetic acid was added, ZM4 could hardly survive, only consumed 6.24 ± 2% glucose within 96 h. However, with 3.5% biochar addition, strain ZM4 consumed 97.93% glucose and produced 25.10 ± 0.12 g/L ethanol within 16 h under 6.0 g/L acetic acid stress condition and consumed 92.0% glucose and produced 23.58 ± 1.03 g/L ethanol within 48 h with 9.0 g/L acetic acid addition. As reported, the highest concentration of acetic acid generated during the pretreatment step of biomass was nearly 10.0 g/L [29]. With 3.5% biochar addition, the ZM4 strain could consume 81.51% of the initial glucose after fermentation for 84 h in the presence of 12.0 g/L acetic acid. Considering that biochar was not efficient in acetic adsorption (Additional file 1: Figure S1), adsorptive detoxification might be not the controlling factor for enhanced ethanol production under acetic acid stress conditions. In anaerobic digestion conditions, biochar has also shown its capacity in improving the fermentation of easy-acidification substrates through its buffering capacity [22].

The BMEEF is an efficient and convenient method to promote the production of ethanol by Z. mobilis wild-type strain ZM4 under the high concentration of acetic acid. Compared with the reported acetic acid-tolerant Z. mobilis strains [4, 10, 30], biochar addition remarkably shortened the fermentation time and enhanced ethanol productivity. For example, a mutant ZMA7-2 (tolerant to 7.0 g/L acetic acid) was obtained via three rounds of adaptive laboratory evolution (ALE) [4], which consumed 96% glucose within 48 h. Besides, a flocculent mutant ZM401 and mutants ZMAQ8-1 and ZMAC8-9 with high tolerance to acetic acid were obtained by nitrosoguanidine (NTG) and ARTP mutagenesis, respectively [10, 30]. For ethanol fermentation by the acetic acid-tolerable
mutant (ZM401), pH was maintained at 6.0 by KOH to promote ethanol production under 6.0 g/L acetic acid. Actually, when RM was supplemented with 6.0 g/L acetic acid, the pH value was 3.92. However, the pH value was uncontrolled for BMEEF. Moreover, although acetic acid-tolerable mutants (ZMAQ8-1 and ZMAC8-9) could produce ethanol with a high productivity at a certain stress condition, they were still not efficient in ethanol fermentation under high concentration of acetic acid (like in the presence of 8.0 g/L acetic acid), with the productivities of 0.32 and 0.3 g/L/h, respectively (Table 1) [10]. While in the presence of 9.0 g/L acetic acid, BMEEF by strain ZM4 could produce ethanol at a productivity of 0.39 ± 0.02 g/L/h. Thus, BMEEF was more efficient and convenient than the genetic engineering of strains.

Biochar facilitated ethanol production under furfural stress

Furfural is another key inhibitor in the cellulosic hydrolysate. The highest concentration of furfural tolerated by Z. mobilis was 3.0 g/L in previous studies [16, 31]. Therefore, four different gradient concentrations (1.0, 2.0, 3.0, and 4.0 g/L) of furfural were employed in this study. As shown in Fig. 1c, d, and Table 1, Z. mobilis ZM4 was dramatically suppressed by over 2.0 g/L furfural. Actually, when a supplement of 3.0 g/L furfural was added, strain ZM4 consumed 99.64% glucose within 72 h. When 4.0 g/L furfural was added, strain ZM4 consumed 99.76% glucose within 84 h with a longer lag phase. However, with 3.5‰ biochar addition, strain ZM4 consumed 99.71% glucose and produced 23.99 ± 0.88 g/L ethanol within 24 h in the presence of 3.0 g/L furfural and consumed 99.72% glucose and produced 22.82 ± 0.85 g/L ethanol within 48 h under 4.0 g/L furfural stress. Thus, these results indicated that the addition of biochar remarkably reduced fermentation time under furfural stress conditions.

Various furfural-tolerant mutants were developed for ethanol production under furfural conditions. For example, mutant ZMF3-3 that is tolerant to 3.0 g/L furfural was screened through three rounds of ALE and consumed...
80% glucose within 48 h [4]. In addition, mutant ZM4-MF2 was obtained by error-prone PCR of the global transcription sigma factor RpoD in Z. mobilis ZM4, which consumed 92.8% glucose and produced 9.8 g/L ethanol within 54 h in the presence of 3.0 g/L furfural under 20.0 g/L glucose [31]. However, with 3.5‰ biochar addition, strain ZM4 could consume 99.71% glucose and produce 23.99±0.88 g/L ethanol within 24 h in the presence of 3.0 g/L furfural under 50.0 g/L glucose, which was more efficient in ethanol production under furfural stress condition than some of the previously reported inhibition-tolerant mutant. Thus, BMEEF was more efficient and convenient than the genetic engineering of strains.

### Biochar improved ethanol production under acetic acid and furfural stress

Lignocellulosic hydrolysate is a complex system, and the high abundance of furfural can form synergistic inhibition with acetic acid [6]. Therefore, the BMEEF under co-stress by acetic acid and furfural was investigated.

#### Table 1 Conversion of glucose to ethanol by Z. mobilis under acetic acid and/or furfural stresses

| Strain | Fermentation time (h) | Glucose consumed (g/L) | Ethanol Titer (g/L) | Ethanol Yield (g/g glucose) | Ethanol Productivity (g/L/h) | Theoretical yield (%) | References |
|--------|-----------------------|------------------------|---------------------|-----------------------------|-----------------------------|------------------------|------------|
|        |                       |                        |                     |                             |                             |                        |            |
| 50.0 g/L glucose + 3.0 g/L acetic acid | | | | | | | |
| ZM4 + C | 12 | 51.60±0.37 | 26.39±0.18* | 0.50±0.00*** | 2.20±0.02*** | 97.85 | This study |
| ZM4 | 20 | 51.79±0.58 | 24.83±0.59 | 0.48±0.01 | 1.24±0.03 | 93.93 |            |
| 50.0 g/L glucose + 6.0 g/L acetic acid | | | | | | | |
| ZM4 + C | 16 | 51.47±0.01*** | 25.10±0.12*** | 0.49±0.00*** | 1.57±0.01*** | 95.89 |            |
| ZM4 | 48 | 50.61±0.05 | 22.69±0.08 | 0.45±0.00 | 0.47±0.00 | 88.06 |            |
| 50.0 g/L glucose + 9.0 g/L acetic acid | | | | | | | |
| ZM4 + C | 60 | 51.26±0.00 | 23.58±1.03*** | 0.46±0.02*** | 0.39±0.02 | 90.02 |            |
| ZM4 | – | 51.35±0.18 | 0.18±0.01 | 0.00±0.00 | – | – |            |
| 50.0 g/L glucose + 12.0 g/L acetic acid | | | | | | | |
| ZM4 + C | 108 | 51.17±0.10 | 21.79±0.17*** | 0.43±0.00*** | 0.20±0.00 | 84.15 |            |
| ZM4 | – | 51.27±0.04 | 0.06±0.04 | 0.00±0.00 | – | – |            |
| 100.0 g/L glucose + 6.3 g/L acetic acid, pH 6.0 | | | | | | | |
| ZM401 | 24 | 99.9 | 48.9 | 0.49 | 2.04 | 95.89 | [30] |
| 50.0 g/L glucose + 8.0 g/L acetic acid | | | | | | | |
| AQ8-1 | 64 | 49.59 | 20.76 | 0.42 | 0.32 | 82.19 | [10] |
| AC8-9 | 72 | 41.85 | 21.46 | 0.43 | 0.30 | 84.15 |            |
| 50.0 g/L glucose + 1.0 g/L furfural | | | | | | | |
| ZM4 + C | 12 | 51.39±0.02 | 23.77±0.14** | 0.46±0.00** | 1.98±0.01*** | 90.02 |            |
| ZM4 | 28 | 51.39±0.04 | 22.57±0.23 | 0.44±0.00 | 0.81±0.01 | 86.11 |            |
| 50.0 g/L glucose + 2.0 g/L furfural | | | | | | | |
| ZM4 + C | 16 | 51.04±0.47 | 23.57±0.53 | 0.46±0.01* | 1.47±0.03*** | 90.02 |            |
| ZM4 | 32 | 51.28±0.56 | 22.21±0.79 | 0.43±0.01 | 0.69±0.02 | 84.15 |            |
| 50.0 g/L glucose + 3.0 g/L furfural | | | | | | | |
| ZM4 + C | 24 | 51.42±0.06 | 23.99±0.88 | 0.47±0.02 | 1.00±0.04*** | 91.98 |            |
| ZM4 | 72 | 51.28±0.19 | 22.74±0.62 | 0.44±0.01 | 0.32±0.01 | 86.11 |            |
| 50.0 g/L glucose + 4.0 g/L furfural | | | | | | | |
| ZM4 + C | 48 | 51.18±0.12** | 22.82±0.85* | 0.45±0.02* | 0.48±0.02*** | 88.06 |            |
| ZM4 | 84 | 51.64±0.09 | 20.35±1.09 | 0.39±0.02 | 0.24±0.01 | 76.32 |            |
| 200 g/L glucose + 3.0 g/L furfural | | | | | | | |
| F211 | 22 | N/A | N/A | 0.46 | N/A | 90.02 | [16] |
| F27 | 28 | N/A | N/A | 0.47 | N/A | 91.98 |            |
| 50.0 g/L glucose + 3.0 g/L furfural | | | | | | | |
| ZM4-MF2 | 54 | 9.8 | 22.74±0.62 | 0.44±0.01 | 0.32±0.01 | 86.11 |            |

*P values calculated by one-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001

Three repeats were performed for each fermentation.
concentrations of two inhibitors in a mixture were 6.0 and 3.0 g/L, respectively. As shown in Fig. 2a, b, strain ZM4 was dramatically suppressed by a mixture of these two inhibitors. While with 3.5‰ biochar addition, strain ZM4 consumed 97.15% of the initial glucose and produced 22.05 ± 0.58 g/L ethanol after fermentation 48 h, and in less than 60 h, strain ZM4 in untreated fermentation broth consumed 51.17% of the glucose. So far, there are few studies on multi-resistant Z. mobilis strains. The Z. mobilis mutant AcRIM0347 obtained by hfq gene insertion of Z. mobilis AcR was resistant to 0.75 g/L HMF, 1 g/L furfural or 1 g/L vanillin for 16, 19 or 21 h, respectively [9]. Although the mutant AcRIM0347 is resistant to a variety of inhibitors, the assay did not investigate a mixture of inhibitors. Thus, BMEEF might be a promising strategy to improve ethanol production under practical complex conditions with a variety of inhibitors.

**Mechanisms for BMEEF**

**Cell immobilization on biochar**

The SEM images presented in Fig. 3 (biochar with Z. mobilis in RM medium, with Z. mobilis in RM medium supplemented with 6.0 g/L acetic acid, and with Z. mobilis in RM medium supplemented with 3.0 g/L furfural) highlighted the potential of biochar to provide a suitable habitat for microbial colonization. Cell immobilization on biochar in the fermentation broth and subsequent remarkable promotion of biofuel production were also observed by the ethanol fermentation by S. cerevisiae and K. marxianus in the presence of biowaste-derived biochar [25, 32]. However, the previous results about cell immobilization were obtained under conditions without inhibitors [33]. Here, the cell immobilization of Z. mobilis was observed under inhibition conditions, and, more importantly, immobilized Z. mobilis on biochar showed unexpected resistance to inhibitions. Thus, immobilizing Z. mobilis densely and homogeneously to the surface of biochar could be a novel strategy for bioethanol production, especially for bioethanol production under the practical condition with multi-inhibitors.

Two stages might be involved in the immobilization of microbes on biochar. The initial stage might be the adsorption of microbes onto biochar, which could be interpreted by the theory of colloid stability, and the second stage might be the biofilm formation [34, 35]. Cell immobilization on the surface of the biochar is a result of either physical adsorption by electrostatic force or due to natural cell attachment into the porous or covalent binding between the membrane and the support [36]. Further studies are needed to elucidate the formation of cell immobilization on biochar and the effects of immobilized ethanologenic strains on inhibition mitigation.

**Unexpected effects of biochar extracts**

Biochar in RM medium with inhibitor (8.0 g/L acetic acid or 4.0 g/L furfural) was filtered before strain inoculation to confirm the effects of biochar extracts on ethanol fermentation. As shown in Fig. 4, in the presence of 8.0 g/L acetic acid, strain ZM4 in the filtered biochar-treated medium showed a faster fermentation rate and consumed 93.98% glucose within 48 h, while strain ZM4 in biochar-untreated medium only consumed 30.44% glucose in less than 24 h. Moreover, in the presence of 4.0 g/L furfural, the fermentation time of ZM4 in the filtered biochar-treated medium was reduced by 24 h, compared to the biochar-untreated system. Thus, these results suggested that biochar extract promoted ethanol fermentation under stress conditions.
Fig. 3  SEM of *Z. mobilis* interaction with biochar. **a** Biochar in RM medium with *Z. mobilis*. **b** Biochar in RM medium supplemented with 6.0 g/L acetic acid with *Z. mobilis*. **c, d** Biochar in RM medium supplemented with 3.0 g/L furfural with *Z. mobilis*.

Fig. 4  Effects of filtered medium of biochar on ethanol fermentation by *Z. mobilis* ZM4. "A8" and "F4" indicate *Z. mobilis* ZM4 fermented in the presence of 8.0 g/L acetic acid and 4.0 g/L furfural, respectively. "A8-C" and "F4-C" indicate *Z. mobilis* ZM4 co-cultured with filtered biochar-treated medium in the presence of 8.0 g/L acetic acid and 4.0 g/L furfural, respectively.
To further elucidate the effects of biochar extract, the nutrients in biochar-treated medium were investigated. Actually, 719.5 mg/L TN, 806 mg/L TOC, 674.2 mg/L TP, 193.2 mg/L K, and 12.03 mg/L Mg were detected in biochar-filtered medium, which were improved compared to raw RM medium. As previously reported, the glucose utilization of Z. mobilis is controlled by coupled phosphorylation; the addition of inorganic phosphate will lead to an improvement in the utilization of glucose by Z. mobilis, and Mg$^{2+}$ ions, which has been confirmed to protect the viability of Z. mobilis via preventing the breakdown of RNA under starvation [37]. Besides, K$^+$ ions are a cofactor of the most intracellular enzyme, and this property may benefit the growth of Z. mobilis and resistance to inhibitors. What’s more, some small molecules in biochar might act as allosteric regulators of a specific enzyme to facilitate resistance to inhibitors in the strain, and this needs further study. Thus, these results suggested that biochar could provide extra nutrients for promoting strain ZM4 resistant to acetic acid and furfural, facilitating ethanol production under stress conditions.

**Beyond adsorptive detoxification**

Inhibitors such as furfural and HMF were prone to be adsorbed. However, a little adsorbent is highly efficient for acetic acid adsorption. Thus, adsorbable furfural and non-adsorbable acetic acid were chosen as two types of inhibitors in this study. As shown in Fig. 5, biochar adsorbed 15.25% and 18.75% of furfural in the presence of 3.0 and 4.0 g/L furfural within 12 h, respectively, indicating that the furfural concentration in the fermentation broth was still very high. In addition, the concentration of acetic acid was nearly invariable during the entire fermentation process (Additional file 1: Figure S1). Thus, these results suggested that adsorptive removal of inhibitors might not be the dominant mechanism for enhanced ethanol production under stress conditions.

**Biochar-mediated enhanced ethanol fermentation (BMEEF)**

In this study, BMEEF was developed using 3.5‰ biochar as an additive to promote ethanol production, especially under stress conditions, not just as an adsorbent to remove inhibitors. Adsorptive removal of inhibitors by various adsorbents, e.g., biochar, activated carbon, has been developed for inhibition mitigation or detoxification. However, these adsorbents are not used as an additive in the fermentation broth, but used as a detoxification agent and removed out before ethanol fermentation [28]. When biochar was applied as a detoxification agent, it focused on the high removal of inhibitors. For example, previous results showed that more than 94% of 5-HMF and 99% of furfural were removed in the synthetic medium after 24 h of contact time by 4% biochar [28]. However, for BMEEF, the amount of biochar was as low as 3.5‰, which was much lower than the amount used in detoxification. Moreover, the removal of furfural by 3.5‰ biochar was <20%, and no obvious decrease of acetic acid in the fermentation broth was observed. Thus, adsorptive detoxification was not critical for BMEEF, and strains were allowed to exist with a low dose of biochar and a certain amount of inhibitors.

Even under non-stress conditions, BMEEF could remarkably promote ethanol fermentation by Z. mobilis. As shown in Additional file 1: Figure S2, ethanol production in biochar-added RM medium without any inhibitors was investigated to confirm the effects of biochar on ethanol production. Results showed that, with the addition of 3.5‰ biochar in RM medium without any
inhibitors, strain ZM4 consumed nearly 100% glucose and produced 27.44 g/L ethanol within 7.5 h, and in less than 4.5 h, strain ZM4 consumed 55.16% glucose and produced 15.09 g/L ethanol, indicating that biochar facilitated strain growth and subsequent ethanol production under non-stress conditions.

Mechanism analysis further suggested that BMEEF was not dependent on adsorptive detoxification due to its low removal for furfural and incapability in acetic acid removal in the fermentation broth, but dependent on the cell immobilization on biochar surface and biochar extract to supply nutrients (or some possible small organic molecules) to the broth. Further studies are needed to elucidate the formation process of cell immobilization on biochar, functional components in biochar extract, and their effects on strain growth and ethanol production under stress conditions.

Furthermore, attributed to the inhibition mitigation effects of BMEEF, it might be competent for the fermentation in concentrated hydrolysate with a high concentration of glucose and thus for the production of high concentration of ethanol in the fermentation broth, which needs further study. The immobilized strain on biochar might facilitate the recovery of strains in the fermentation broth, which could be beneficial for repeated batch fermentation [25, 32].

**Conclusion**

In this study, the performance of BMEEF under various stress conditions (furfural and acetic acid) was first investigated. Results showed that with 3.5% (w/v) biochar in the fermentation broth, ethanol production was remarkably enhanced in the presence of high concentrations of furfural and acetic acid, especially under co-stress conditions. Further analysis showed that BMEEF was not dependent on adsorptive removal of inhibitors due to its low removal for furfural and incapability in acetic acid removal in the fermentation broth, but dependent on the cell immobilization on biochar surface and biochar extract to supply nutrients (or some possible small organic molecules) to the broth. Thus, BMEEF might be a promising strategy for ethanol production from lignocellulosic biomass.

**Materials and methods**

**Treatment of media and bacterial preparation**

*Zymomonas mobilis* wild-type strain ZM4 was grown in rich medium (20.0 g/L glucose, 10.0 g/L yeast extract and 2.0 g/L KH₂PO₄) for the overnight stock culture. Biochar was prepared via the pyrolysis of wheat straw at 600 °C for 2 h at a heating rate of 10 °C/min in a vacuum tube furnace (MXQ1200-30, China) and then sieved to a particulate size of 80 mm [38].

Cultures were inoculated from a fresh plate of RM. The overnight stock culture was cultured for 12 h at 30 °C without shaking. The final optical density (OD) (Jingke UV765, Shanghai) of the overnight stock culture at 600 nm was 1.8. The OD₆₀₀ was measured to assess the rate of bacterial growth. To 50 mL of RM medium, 3.5‰ (by weight, 0.175 g) biochar was added. Cell pellets were harvested from 2 mL of the overnight stock culture by centrifuging at 3000 rpm for 4 min at 4 °C. The harvested cell pellets were then inoculated into the two different media (RM and biochar-treated medium) to begin bacterial growth. In this set of experiments, bacteria grew in the presence of biochar or untreated RM medium throughout the time course (12 h) of each experiment. All growth and fermentations were carried out in triplicate.

An additional set of experiments was performed to investigate the influence of the biochar on typical inhibitors (adsorbable furfural and non-adsorbable acetic acid) generated during lignocellulose pretreatment. For these experiments, 3.5‰ (by weight, 0.175 g) biochar was added to 50 mL RM media (in 100 mL flask) with different acetic acid concentrations (3.0, 6.0, 9.0, and 12.0 g/L), or different furfural concentrations (1.0, 2.0, 3.0, and 4.0), or 6.0 g/L acetic acid plus 3.0 g/L furfural. Meanwhile, biochar-untreated RM medium was also supplemented with the corresponding concentrations of acetic acid or/and furfural as a control. 2 mL of the overnight stock culture was harvested and inoculated into each flask and placed in the incubator at 30 °C without shaking. All growth and fermentations were carried out in triplicate.

In addition, the third set of experiments was performed where biochar was removed from the medium before inoculation. For these experiments, the same 3.5‰ (by weight, 0.175 g) biochar was added to 50 mL RM media with 8.0 g/L acetic acid and 4.0 g/L furfural, respectively. Then the media was first filtered through a 0.45-µm sterile syringe filter and then passed through a 0.22-µm sterile syringe filter. Meanwhile, as a comparison, biochar-untreated RM medium was also supplemented with 8.0 g/L acetic acid and 4.0 g/L furfural, respectively. Cultivation methods were the same as the first and second sets of experiments. All cell growth and ethanol fermentation were carried out in triplicate.

**Characteristics of biochar**

The mineral elements (e.g., K, Mg) present in the biochar were determined by ICP (PlasmaQuant PQ9000, Germany). The N content in the biochar was determined by a TN analyzer (SHIMADZU, Japan). The total carbon (TC) content and total organic carbon (TOC) contents were determined by a TOC-V CPH analyzer (SHIMADZU, Japan).
**SEM analysis**

Biochar inoculated with *Z. mobilis* ZM4 cells was imaged using scanning electron microscope (SEM). Samples were prepared by chemical fixation and freeze-drying. Briefly, two milliliters of the co-cultures was first fixed with 2.5% glutaraldehyde at 4 °C for 12 h, rinsed by 0.1 M PBS buffer (pH 7.0) twice, dehydrated with graded ethanol, treated with isooamyl acetate (for replacement of ethanol), and then the samples were observed by scanning electron microscope (Phenom Pro, Netherlands Phenom) after freeze-drying.

**Analytical methods**

After filtration with a 0.22-μm membrane and ten-time dilution, the concentrations of glucose, ethanol, acetic acid, and furfural in fermentation medium were analyzed by high-performance liquid chromatography (HPLC; Agilent 1200), equipped with refractive index detector. An HPX-87H ion exclusion column (Bio-Rad Aminex) was used at 65 °C with 5 mM H2SO4 as the mobile phase and run at a flow rate of 0.6 mL/min. The injection volume was set to 20.0 μL. One-way ANOVA was used to test all experimental data and the correlation was analyzed with the Pearson test (two tailed) using Statistical Product and Service Solutions Software (SPSS, version 19.0).

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13068-020-1666-6.

**Additional file 1: Figure S1.** Biochar-facilitated ethanol fermentation in acetic acid stress conditions. No acetic acid removal (a), and little pH changes (b). **Figure S2.** Effects of biochar on ethanol fermentation by *Z. mobilis* ZM4 in the presence of RM medium. "RM" indicates *Z. mobilis* ZM4 fermented in RM medium. "RMC" indicates *Z. mobilis* ZM4 co-cultured with 3.5‰ biochar fermented in RM medium.

**Abbreviations**

BMEEF: Biochar-mediated enhanced ethanol fermentation; ARTP: Atmospheric and room temperature plasma; ALE: Adaptive laboratory evolution; NTG: N-Methyl-N-nitro-N-nitrosoguanidine; RM: Rich medium; OD: Optical density; HPLC: High-performance liquid chromatography; SEM: Scanning electron microscope.

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**Authors’ contributions**

MXH designed the whole study and wrote the final manuscript, WTW carried out all experiments, data collection, and manuscript editing, LCD prepared he biochar sample, designed the experiment, and revised the manuscript, BW participated in data analysis, BFQ helped in the use of TN, TOC-V CPH and ICP analyzer, TFH helped in the use of SEM. GQH participated in helpful discussions regarding the manuscript and helped in its editing. All authors read and approved the final manuscript.

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**Availability of data and materials**

*Z. mobilis* AC8-1 and AC8-9 have been deposited at Guangdong Microbial Culture Center (GDMCC) under the Accession number GDMCC60258 and 60259.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**References**

1. Harmsen P, Huijgen W, Bermudez L, Bakker R. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR-Food Biobased Research; 2010.
2. He MX, Wu B, Shui ZX, Hu QC, Wang WS, Tan FR, Tang XY, Zhu QL, Pan K, Li Q. Transcriptome profiling of *Zymomonas* mobilis under furfural stress. Appl Microbiol Biotechnol. 2012;95(1):189–99.
3. Lawford HG, Rousseau JD. Cellulosic fuel ethanol. In: Biotechnology for fuels and chemicals; 2003. p. 457–69.
4. Shui ZX, Qin H, Wu B, Ruan ZY, Wang LS, Tan FR, Wang JL, Tang XY, Dai LC, Hu GQ. Adaptive laboratory evolution of ethanologenic *Zymomonas* mobilis strain tolerant to furfural and acetic acid inhibitors. Appl Microbiol Biotechnol. 2015;99(13):5739–48.
5. Almeida JR, Modig T, Peterson A, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Z. mobilis*. J Chem Technol Biotechnol Int Res Process Environ Clean Technol. 2007;82(4):340–9.
6. Almeida JR, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl Microbiol Biotechnol. 2009;82(4):625.
7. Dong H, Bao J. Metabolism: biofuel via biodetoxification. Nat Chem Biol. 2010;6(5):316.
8. Musiattato SI, Roberto IC. Alternatives for detoxification of diluted-acid lignocellulosic hydrolysates for use in fermentative processes: a review. Bioresour Technol. 2004;93(1):1–10.
9. Yang S, Pelletier DA, Lu T-YS, Brown SD. The Zymomonas mobilis regulator hfq contributes to tolerance against multiple lignocellulosic pretreatment inhibitors. BMC Microbiol. 2010;10(1):135.

10. Wu B, Qin H, Yang Y, Duan G, Yang S, Xin F, Zhao C, Shao H, Wang Y, Zhu Q, et al. Engineered Zymomonas mobilis tolerant to acetic acid and low pH via multiplex atmospheric and room temperature plasma mutagenesis. Biotechnol Biofuels. 2019;12(1):10.

11. Yang S, Fei Q, Zhang Y, Contreras LM, Utturkar SM, Brown SD, Himmel ME, Zhang M. Zymomonas mobilis as a model system for production of biofuels and biochemicals. Microb Biotechnol. 2016;9(6):699–717.

12. He RX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, Shui ZX, Dai LC, Zhu QL, Pan K. Zymomonas mobilis: a novel platform for future biorefineries. Biotechnol Biofuels. 2014;7(1):101.

13. Franden MA, Pilath HM, Mohagheghi A, Pienkos PT, Zhang M. Inhibition of growth of Zymomonas mobilis by model compounds found in lignocellulosic hydrolysates. Biotechnol Biofuels. 2013;6(1):99.

14. Xia J, Yang Y, Liu C-G, Yang S, Bai F-W. Engineering Zymomonas mobilis for robust cellulose ethanol production. Trends Biotechnol. 2019. https://doi.org/10.1016/j.tibtech.2019.02.002.

15. Wang X, He Q, Yang Y, Wang J, Haning K, Hu Y, Wu B, He M, Zhang Y, Bao J, et al. Advances and prospects in metabolic engineering of Zymomonas mobilis. Metab Eng. 2018;35:57–73.

16. Huang S, Xue T, Wang Z, Ma Y, He X, Hong J, Zou S, Song H, Zhang M. Furfural-tolerant Zymomonas mobilis derived from error-prone PCR-based whole genome shuffling and their tolerant mechanism. Appl Microbiol Biotechnol. 2018;102(7):3337–47.

17. Dai L, Tan F, Li H, Zhu N, He M, Zhu Q, Hu G, Wang L, Zhao J. Calcium-rich biochar from the pyrolysis of crab shell for phosphorus sorption removal. J Environ Manag. 2017;198(Part 1):70–4.

18. Dai L, Zhu W, He L, Tan F, Zhu N, Zhou Q, He M, Hu G. Calcium-rich biochar from crab shell: an unexpected super adsorbent for dye removal. Bioresour Technol. 2018;267:510–6.

19. O’Connor D, Peng T, Zhang J, Tsang DC, Alessi DS, Shen Z, Bolan NS, Hou D. Biochar application for the remediation of heavy metal polluted land: a review of in situ field trials. Sci Total Environ. 2018;619:815–26.

20. Biederman LA, Harpole WS. Biochar and its effects on plant productivity and nutrient cycling: a meta-analysis. GCB Bioenergy. 2013;5(2):202–14.

21. Liu F, Liu Y, Shao L, He P. Powdered biochar doubled microbial growth in anaerobic digestion of oil. Appl Energy. 2019;247:605–14.

22. Wang D. Improving anaerobic digestion of easy-acidification substrates by promoting buffering capacity using biochar derived from vermicompost. Bioresour Technol. 2017;227:286–96.

23. Novák J, Moore E, Skopas K, Hall K, Williams A. Future biochar research directions. Biochar from biomass and waste. Amsterdam: Elsevier; 2019. p. 423–35.

24. Sambusiti C, Monlau F, Antoniou N, Zabaniotou A, Barakat A. Simultaneous detoxification and bioethanol fermentation of furans-rich synthetic hydrolysate by digestate-based pyrochar. J Environ Manag. 2016;183:1526–31.

25. Kyrtsou M, Chatzizisis V, Costa CN, Kallis M, Koutsokeras L, Constantinides G, Koutinas M. Biowaste-based biochar: a new strategy for fermentative bioethanol overproduction via whole-cell immobilization. Appl Energy. 2019;242:480–91.

26. Richard D, Núñez MDLD, Schweich D. Adsorption of complex phenolic compounds on active charcoal: adsorption capacity and isotherms. Chem Eng J. 2001;88(1):1–7.

27. Li Y, Shao J, Wang X, Deng Y, Yang H, Chen H. Characterization of modified biochars derived from bamboo pyrolysis and their utilization for target component (furfural) adsorption. Energy Fuels. 2014;28(8):5119–27.

28. Monlau F, Sambusiti C, Antoniou N, Zabaniotou A, Solhý A, Barakat A. Biochars from bioenergy residue as novel bio-adsorbents for lignocellulosic hydrolysate detoxification. Bioresour Technol. 2015;187:379–86.

29. Mills TY, Sandoval NR, Gill RT. Cellulosic hydrolysate toxicity and tolerance mechanisms in Eschenichia coli. Biotechnol Biofuels. 2009;2(1):26.

30. Zhao N, Bai Y, Liu CG, Zhao XQ, Xu JF, Bai FW. Flocculating Zymomonas mobilis is a promising host to be engineered for fuel ethanol production from lignocellulosic biomass. Biotechnol J. 2014;9(3):562–71.

31. Tan F-R, Dai L-C, Wu B, Qin H, Shui Z-X, Wang J-L, Zhu Q-L, Hu Q-C, Ruan Z-Y, He M-X. Improving furfural tolerance of Zymomonas mobilis by rewiring a sigma factor RpoD protein. Appl Microbiol Biotechnol. 2015;99(12):5363–71.

32. Diao O. Effect of poultry litter biochar on Saccharomyces cerevisiae growth and ethanol production from steam-exploded poplar and corn stover; 2014.

33. Hill RA, Hunt J, Sanders E, Tran M, Burk GA, Mlsna TE, Fitzkee NC. Effect of biochar on microbial growth: a metabolomics and bacteriological investigation in E. coli. Environ Sci Technol. 2019;53(5):2635–46.

34. Morales CFL, Strathmann M, Fleming H-C. Influence of biofilms on the movement of colloids in porous media: Implications for colloid facilitated transport in subsurface environments. Water Res. 2007;41(10):2059–68.

35. Yallop M, Paterson D, Wellburn P. Interrelationships between rates of microbial production, exopolymer production, microbial biomass, and sediment stability in biofilms of intertidal sediments. Microb Ecol. 2000;39(2):116–27.

36. Rivera-Utrilla J, Bautista-Toledo I, Ferro-Garcia MA, Moreno-Castilla C. Activated carbon surface modifications by adsorption of bacteria and their effect on aqueous lead adsorption. J Chem Technol Biotechnol. 2001;76(12):1209–15.

37. Dawes EA, Large PJ. Effect of starvation on the viability and cellular constituents of Zymomonas anserina and Zymomonas mobilis. J Gen Appl Microbiol. 1970;60(1):31–42.

38. Dai L, Bo W, Tan F, He M, Wang W, Han Q, Tang X, Zhu Q, Ke P, Hu Q. Engineered hydrochar composites for phosphorus removal/recovery: lanthanum doped hydrochar prepared by hydrothermal carbonization of lanthanum pretreated rice straw. Bioresour Technol. 2014;161(3):327–32.

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